

Proceedings of the 10th International Barley Genetics Symposium

Editors

Salvatore Ceccarelli and Stefania Grando



International Center for Agricultural Research in the Dry Areas

Proceedings of the 10th International Barley Genetics Symposium

5-10 April 2008, Alexandria, Egypt

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International Center for Agricultural Research in the Dry Areas

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Foreword

The Barley Genetics Symposium, first held in 1963, continues to be the most important forum for barley scientists and the industry worldwide, where research results and production trends are discussed, and future strategies decided. The 10th International Barley Genetics Symposium was held at the Bibliotheca Alexandrina, Egypt, from 5 to 10 April 2009. This was the first time the Symposium was held in Africa or the Middle East region – the cradle of agriculture and of barley domestication.

The Symposium was jointly organized by the International Center for Agricultural Research in the Dry Areas (ICARDA) and the Bibliotheca Alexandrina, with support from the Food and Agriculture Organization of the United Nations and the OPEC Fund for International Development. It was attended by nearly 200 scientists from 47 countries and by 13 ICARDA scientists including its Director General. The symposium was opened by Dr J. Spunar, Chairman of the International Barley Genetics Symposium, followed by inaugural addresses by Dr Mahmoud Solh, ICARDA Director General, and Dr Ismail Serageldin, Director, Bibliotheca Alexandrina.

“Barley can help people survive climate change and global warming. It is the crop of the poor people,” said Dr Stefania Grando, ICARDA’s Principal Barley Breeder and Chair of the local organizing committee of the Symposium.

The proceedings contain the papers presented at the opening session and the twelve scientific sessions, namely Germplasm and genetic resources, Molecular breeding, Barley genomics, Abiotic stresses, Biotic stresses, Barley uses (three sessions: malt, food and feed), Biochemistry, cytogenetics and transformation, Functional genomics, Barley development, and Success stories. The proceedings also summarize the conclusions and recommendations of two technical workshops held as part of the Symposium: the Generation Challenge Program workshop, and the workshop on ‘Barley genetic linkage groups, barley genome, genes and genetic stocks’.

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Opening Session

Barley development in Egypt

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Abstract

Barley (*Hordeum vulgare* L.) is the main crop and widely grown in the rainfed areas of the north coastal region and in the newly reclaimed lands with saline soils in Egypt. The total harvested area averaged 57 000 ha during the 1980s, increasing to 135 000 ha in 2005/06. Barley yield has gradually increased over the past few decades, from 2.92 t/ha in the 1980s to about 3.63 t/ha in 2005/06. Under rainfed conditions, barley productivity increased from 0.44 to 1.90 t/ha in the same period, which is considered high compared with other rainfed areas in the world that exceed 200 mm/yr; Egypt receives about 130 mm/yr. The main objectives of the barley program in Egypt are to develop new cultivars with high yielding capacity and tolerance to various stresses (drought, salinity, poor soil fertility, diseases, insects, etc.), along with suitable cultural packages, and also to improve and maintain high-quality seed as well as a technology transfer program through extension agencies. To achieve these objectives, the barley program developed several breeding strategies to address different constraints to barley production. Breeding activities included screening of local and exotic materials, crossing blocks, and yield test trials. Exotic germplasm and nurseries are annually provided by several international and regional organizations, such as ICARDA, CIMMYT and ACSAD, to support breeding objectives. The present paper also includes other barley activities and topics, such as Farmer Field Schools, Farmers' Participatory Breeding, and malting quality. The paper briefly includes the last five years' results under rainfed conditions.

Introduction

In Egypt, only about 2.5% of the total area of the country is cultivated, with about 30% of the cultivated lands affected by salinity. In addition, about one million acres [400 000 ha] suffer from waterlogging. Egypt has about 120 000 ha in the North West Coast (NWC) region and about 40 000 ha in North Sinai. The long-term average annual precipitation is about 135 mm in NWC, and slightly higher in North Sinai. Barley is the main crop grown on a large scale in NWC, being well adapted to the short growing season, relatively tolerant to drought stress, and a key element in the feeding regime of small ruminants. It is mainly used as animal feed, and recently as human food because of its nutritional and health properties. Most of the rainfed areas are planted with local barley varieties, which are low yielding and are more vulnerable to diseases and insect pests than the improved, recommended cultivars. Therefore, using drought-tolerant barley cultivars is of great importance, especially if they are resistant to diseases and insects and have the potential to produce high yields under drought stress conditions. In addition, improving water use efficiency through cultural practices is also imperative, since water supply is the main limiting factor.

The coastal plain is the center of the main agricultural activities in the coastal region. It has been mostly cultivated with barley since Roman times, and for this reason it is locally known as the 'barley plain'. Barley is an ideal crop for integrated farming systems based on crop+livestock production. It is used either for grazing as green fodder or harvested as grain and straw at maturity to be fed as

supplements, with the stubble left in the field for grazing.

Table 1 shows barley area, productivity and production in the rainfed lands and old lands during the period 1980 to 2007. The barley area in the Nile Valley has declined gradually, especially where land and irrigation are available and other strategic crops such as wheat can be grown. At the same time, the barley area increased in the new, reclaimed lands under various irrigation systems. The total harvested area was 57 000 ha in the 1980s, increasing to 135 000 ha by 2006/07. Barley yields have tended to increase gradually over the past few decades, from 2.92 t/ha in the 1980s to 3.63 t/ha in 2005/2006. Under rainfed conditions, barley yield increased from 0.44 t/ha during the period 1986–1991 to 1.97 t/ha in 2006/07. In Egypt, where the average rainfall is about 130 mm with very erratic distribution during and

among growing seasons, barley productivity is considered high compared with other rainfed areas in the world receiving more than 200 mm/yr.

The main objective of the Barley Research Department in the Agricultural Research Centre, is to focus on the sustainability of barley production and productivity by developing barley genotypes that are suitable for the harsh environments of the marginal areas of Egypt, along with cultural practices that include the best management methods for other biotic and abiotic stresses.

Description of barley-targeted areas on the northwest coast

The NWC region forms a coastal belt about 20 km wide that extends from west to east for about 500 km. The area is classified as a dry

Table 1. Changes in barley area, yield, and total production in the rainfed areas, New Reclaimed Lands and Old Lands in the period from 1980 to 2006/07.

Year	Production Area ('000 ha)				Productivity (t/ha)				Total Production (t)
	Rainfed Areas	New Lands	Old Lands	Total Area	Rainfed Areas	New Lands	Old Lands	Mean	
1981–86	NA*	NA	57.02	57.02	NA	NA	2.92	2.92	NA
1986–91	32.74	2.37	43.22	78.33	0.44	1.36	3.03	1.61	126 111
1991–96	53.06	13.10	24.06	90.22	0.97	2.42	3.56	2.40	216 528
1996–97	27.85	12.19	17.66	57.71	1.04	2.66	3.64	2.18	125 807
1997–98	31.51	9.96	18.53	59.98	1.52	2.74	3.50	2.47	148 150
1998–99	27.43	12.09	17.22	56.74	1.43	2.88	3.74	2.01	114 047
1999–00	24.81	6.13	17.94	48.88	1.70	2.69	3.77	2.98	145 662
2000–01	76.31	9.25	13.81	99.36	1.65	3.07	3.92	3.88	385 516
2001–02	71.26	11.61	24.98	107.87	1.71	3.12	3.97	2.94	317 300
2002–03	53.01	33.56	15.99	84.56	1.57	4.01	4.23	3.27	276 511
2003–04	45.00	50.00	20.00	115.00	1.70	4.30	4.40	3.46	397 900
2004–05	60.30	55.10	20.40	135.80	1.80	4.50	4.60	3.63	492 954
2005–06	58.21	35.17	9.47	102.85	1.47	3.77	4.12	3.56	366 147
2006–07	61.43	25.26	9.85	96.54	1.29	3.88	3.99	3.05	294 447

* NA = Data not available.

arid climate zone. It is characterized by warm summers (20 to 35°C), mild winters (10 to 20°C), and $P/E+P < 0.03$ (where P is annual precipitation and E is annual evaporation). Rainfall ranges between 100 and 200 mm/yr. Most of the rainfall (70% or more) occurs within the winter months (November to February), with a maximum during December and January. The prevailing rainfall gradient falls from north to south and from west to east. The basic traditional production system is a mixed, rainfed farming system, which includes animal (mainly sheep and goats) husbandry, cereal production (mainly barley), olives and figs, and to a limited extent almonds and grapes. More than 85% of the population of the region is Bedouin. They have lived in this area for centuries and have developed values, a social system and techniques in sheep breeding and agriculture that have allowed them to survive in harsh natural surroundings. The economic basis of their existence has remained the husbandry of sheep and goats.

Barley utilization

The major use of barley in Egypt is for animal feed and malt production. The grain is used in blends with other feed materials for most farm animals. Barley malt is used in making malted milk, barley foods and in brewing beer. Attempts to improve nutritional value of the barley grains include increasing protein content and improving the amino acid balance in the protein through higher lysine content. Barley is nutritious and it has the dietary fiber that is important for intestinal function and lowering cholesterol content in the blood. It has an exciting taste and texture. It can be used in many forms, such as barley flour, barley grits, barley flakes and pearled barley.

Food uses of malt are numerous, but the quantities used are small. A number of types of malt syrup, malted-milk concentrate, enzyme supplementation of wheat flour and breakfast foods are typical examples. As malt

is used for flavor or enzymatic reactions in most cases, relatively low concentrations are employed.

Constraints and threats facing barley sustainability in the rainfed areas

The NWC is a coastal desert ecosystem with fragile natural resources. The management of these resources and their productivity face several constraints. Many of these constraints have strong bearings on the sustainable development of the region, and are considered below.

Physical constraints

- Amount, distribution and reliability of rainfall in relation to the distribution of cultivable soils and the technical and economic feasibility of increasing the efficiency of water use. However, it is clear that considerable amounts of rainfall remain unutilized each year and are lost through a combination of evaporation, seepage and run-off into uncultivable wadis. The increased installation of water harvesting and storage structures has undoubtedly raised total production of cereal and horticultural crops and led to a continuous expansion of the cultivated area.
- Soils are characterized by low fertility. Very low organic matter, low supplying power for most macronutrients (N and P) and micronutrients (Mn and Zn) are common features for calcareous and coarse-textured non-calcareous soils. Fertilizer use is very limited under rainfed conditions. Appropriate means and techniques are needed to correct the low soil fertility status. The soils in the region face water and wind erosion. Land degradation and resource depletion (e.g. loss of biodiversity) through overgrazing are major environmental problems confronting the region.

Technical constraints

Technical factors limiting crop production include the low yield potential of crop varieties currently cultivated, the husbandry practices adopted for specific crops in the context of the mixed farming systems, and the methods of harvesting and post-harvest handling with respect to optimization of both crop quantity and quality. When improved cultivars have been identified and introduced to the region, there has been no or limited formal follow-up by the extension service to monitor subsequent spread and impact on productivity or overall production.

Socio-economic and institutional constraints

Bedouin society is governed by long-established traditions, which continue to have a major impact on the adoption of unfamiliar crop and animal production practices. There is little organized marketing of agricultural commodities produced in the region and private traders are able to exploit the situation to their advantage. Agricultural development has been constrained due to insufficient financial resources.

Other social and institutional constraints

These include:

- A mobile nomadic community;
- Only 50% of farmers in the medium and large farm groups are likely to be prosperous enough to have adequate surplus for investment;
- A very low rate of enrollment of boys and girls in primary schools;
- Adaptive research, extension and training facilities exist in the area, but with only limited infrastructure and very low staffing levels; and
- Interaction between the institutions and the communities to be served has not been systematic and has lacked an organized framework in which to operate.

Management and productivity of barley

The Barley Research Department of the Agricultural Research Centre (ARC) focuses on developing drought-tolerant and high-yielding barley cultivars to satisfy both Bedouin and environmental needs. Cultural practices (tillage, seeding rate, fertilizer application, etc.) along with varietal development are also emphasized to increase barley productivity in the region. As a result of these efforts, four highly drought-tolerant, high-yielding barley cultivars have been recently released, namely Giza 125, Giza 126, Giza 2000 and Giza 132 (Noaman *et al.*, 1995a, b, c, 2003, 2007a, b). They have been registered and published in Crop Science under PI nos. 583827, 583828, 642787 and 642786, respectively.

The breeding methodology planned for the coming seasons is to develop promising genotypes using the 'Single Seed Descent' (SSD) technique, which has proved highly efficient and time saving in breeding programs in many other research centers both in the region and globally. We also recommend the use of manure fertilizer to improve soil characteristics, and the application of small amounts of N and P in order to maintain a high level of productivity in these risky areas. This is not an easy task, because Bedouin in those areas apply nothing and just do the minimum. As noted above, it is a very high-risk area and Bedouin apply the lowest possible input just in case. We are also considering using both the Doubled-Haploid technique and a Participatory Breeding approach, especially in the marginal areas under rainfed conditions.

Major goals of the barley program in Egypt

The major current goals can be summarized as:

- Developing new cultivars with high yielding capacity and tolerance to different

kinds of stresses (drought, salinity, poor soil fertility, diseases, insects, etc.);

- Developing a complete recommended agronomic package that interacts with the newly developed cultivars to achieve higher yield under different stress conditions;
- Improving and maintaining high quality seed; and
- Transferring new technology to farmers' fields through strengthening the linkage between the researchers, extensionists and farmers.

Major activities

Breeding activities focus on screening of barley landraces and genotypes for drought and salinity tolerance, improving barley cultivars, and breeding for disease and aphid resistance as well as heat tolerance.

- Entomology and pathology research includes field survey and assessment of yield losses due to aphid infestation and infection with major barley diseases, especially powdery mildew, net blotch and leaf rust.
- Research on agronomy and physiology focuses on assessing development of barley plants grown under drought and salt stresses, suitable fertilization and seeding rates, and rotations involving barley and leguminous crops.
- Verification and on-farm field trials are conducted on stations and on farmers' fields as well at various localities and under various conditions.
- Demonstration fields and extension training are also provided annually.

Major achievements

Major achievements include:

- Release of four drought-tolerant barley cultivars suitable for the rainfed areas in the NWC, namely Giza 125, Giza 126, Giza 132 and Giza 2000;

- Release of two two-row barley cultivars for the malting and brewery industries, namely Giza 127 and Giza 128, with high malting and yield potential (El-Sayed *et al.*, 1995a, b);
- Release of three hulless barleys for human food consumption, namely Giza 129, Giza 130 and Giza 131 (El-Sayed *et al.*, 2003). These cultivars have good tolerance to drought stress;
- Establishing recommended agronomic packages for the new cultivars on rainfed and new reclaimed lands as well as on the old irrigated lands; and
- As a result of the technology transfer and training programs for extensionists and through conducting on-farm demonstration and verification trials, yield gains have been recorded of more than 20% from irrigated fields and more than 40% on the rainfed and newly reclaimed lands.

During the last five seasons, the Barley Research Department has successfully achieved numerous objectives, as summarized in the following sections.

Breeding program

Several breeding procedures, such as introductions, single seed descent, backcrossing and modified pedigree methods, were followed to solve different problems and constraints facing barley production areas, including drought, salinity, low soil fertility, and disease, insect and heat stresses. Breeding activities included screening of local and exotic materials, crossing blocks and yield testing trials. These activities are summarized below.

Exotic germplasm

More than 1200 accessions introduced from various international sources (through ICARDA and ACSAD) were in trials during the past five years under different environmental conditions, testing against drought, salinity, poor soil fertility, heat stress, insects and diseases.

Local breeding materials

More than 500 crosses are made every year at Giza and Sakha Research Stations, from which the resulting populations ($F_2 - F_5$) are tested in the target areas of barley production, such as in the NWC and Sinai and new reclaimed lands at Nubaria, Ismailia, and the west bank of the River Nile in Beni Suef and Menya Governorates (Middle Egypt) to select the most suitable genotypes for local areas and conditions.

Evaluation trials

National Yield Trials (classified as A, B, D, and On-farm Verification Trials) for the Irrigated Program are conducted at different barley production areas that include saline soils, new reclaimed areas and areas with high temperature, aiming to identify the highest yielding genotypes under such conditions. In 2006/07, 56 yield trials were conducted at 15 locations representing different environmental stresses to evaluate about 120 genotypes in the irrigated fresh and saline areas, as well as on new lands. At the same time, 33 yield trials to evaluate 96 genotypes were conducted at 8 locations in the NWC and North Sinai, representing different rainfed areas (Table 2).

Pathology

Cereal pathologists work together with barley breeders looking for sources of resistance to the major barley diseases (powdery mildew, net blotch and leaf rust). Screening and evaluation of breeding materials for their reaction to those major diseases at seedling and adult stages under natural field conditions are conducted every year. Selections are tested in the greenhouse under severe artificial infection and resistant genotypes are promoted to yield evaluation trials under natural infection to be evaluated for yield performance and disease resistance.

Entomology

Aphid infestation is one of the most important problems affecting barley production areas under both irrigated and rainfed conditions in Egypt. Aphids cause yield losses of 30–50% due to both their direct feeding on plants and by transmitting some viral diseases, such as BYDV. Screening nurseries for aphid resistance are evaluated every year at hot spots for aphid infestation, in which the genotypes resistant at different degrees are promoted to yield evaluation trials for confirmation. Those proved resistant are planted in the field under natural infestation, especially at Giza and Mallawy (Middle Egypt), where infestation is high.

Agronomy

The agronomy program concentrates mainly on management practices, especially under rainfed conditions (i.e. sowing methods and fertilization), required for the new promising barley genotypes. The recommended cultural practices—seeding rate, fertilizer application, row spacing, sowing dates, seeding depth, etc.—that came out of the agronomy experiments are transferred to the farmers' fields through a Technology Transfer Program in collaboration with extension agents in the governorates.

Seed production

Individual plants are selected from each new cultivar. Seeds from such plants are multiplied to increase Breeder seed. Breeder seed is sown to produce Basic seed (Foundation seed) at Sakha and Gemmeiza Research Stations. Basic seed is then sent to the Central Authority for Seed Production (CASP) to produce the Registered and then Certified seed. Barley growers are provided every year with new Certified seed of the new released varieties for seed multiplication (commercial use).

Table 2. Number of genotypes and yield trials under different environmental conditions during the 2006/07 growing season.

	A-yield trial	B-yield trial	D-yield trial	On-farm verification trial	Total
No. of genotypes	64	32	16	8	120
Plot size	1.6 m ²	2.4 m ²	10.5 m ²	20 m ²	
Number of trials					
Fresh water irrigation	4	6	8	10	28
Saline soil	2	2	3	4	11
New land	3	4	5	5	17
Total	9	12	16	19	56

	Screening yield trial	Local barley yield trial (LBYT)	Advanced barley yield trial (ABYT)	Verification trial	Total
No. of genotypes	60	24	8	4	96
Plot size	0.8 m ²	3.6 m ²	10.5 m ²	20 m ²	
Number of trials					
North West Coast	5	5	4	6	20
North Sinai	3	3	3	4	13
Total	8	8	7	10	33

A-trials (64 genotypes; plot size: 4-row, 2.5 m long and 20 cm apart, with 3 replications) are grown annually at 4–6 locations at Sakha, Gemmeiza (North Delta), Ismailia, Nubaria (New Reclaimed areas), New Valley (South Egypt), and Mallawy (Middle Egypt). Numbers of selected superior genotypes differ according to different areas. The promising lines selected from A-trials that significantly exceed the national check varieties for irrigated areas (Giza 123 and Giza 132) (Ahmed *et al.*, 1998; Noaman *et al.*, 2007b) are promoted to B-yield trials.

B-trials (32 genotypes; plot size: 6-row, 2.5 m long and 20 cm apart, with 3 replications) are grown annually at 6–8 different locations. The selected genotypes that are significantly higher in yield and other agronomic characteristics than the national check(s) are promoted to D-yield trials next season.

D-trials (16 genotypes; plot size: 3 × 3.5 m, with 3 replications) are usually grown in most of the barley production areas under irrigation (8–10 locations). The lines superior to the check cultivars are further tested in large-scale trials (the verification yield trials or E-yield trials) in the following season across all the country).

On-Farm Verification trials (also termed E-trials) (8 genotypes; plot size 20 m² with three replications) are grown at 8–10 locations across all the country. Those lines that show superiority and stability across all the locations are selected and given to farmers to be grown in their fields in final farmers' demonstration trials, after which they are registered and certified, and are released to be disseminated to the farmers.

Regarding the rainfed areas, 33 evaluation yield trials were conducted at 8 locations in the rainfed areas in NWC and North Sinai in four different types of yield trials (Screening trials with 60 genotypes; local barley yield trials with 24 genotypes; advanced barley yield trials with 8 genotypes; and verification trials with 4 genotypes).

Technology transfer program

The main objectives of the technology transfer program are:

- Use of new cultivars of barley in farmers' fields; and
- Use of new recommended production packages for barley in farmers' fields.

About 55 demonstration fields were conducted in 2006/07 in different governorates and in old land, new reclaimed land and rainfed areas. About 5 harvest days and traveling workshops were conducted in some locations to show farmers the effects of applying these new technologies in barley fields, leading to increased grain and straw yields of barley, especially under rainfed and new land conditions by using the new cultivars, such as Giza 126 and Giza 132, that are suitable for the rainfed areas, and Giza 123 and Giza 2000 that are suitable for the new reclaimed lands.

Training activities

Regular visits by all barley disciplines, including breeders, agronomists, pathologists and entomologists, have been made to the experimental and farmers demonstration plots. During these visits, fruitful discussions have been held with the extension staff and farmers about the major problems facing barley production and different solutions and alternatives to solve such problems.

Hulless barley

The objectives of the hulless barley project supported by French Food Aid are:

- To develop high yielding, disease resistant and stable hulless barley genotypes adapted to drought stress in rainfed areas and in the new reclaimed lands of Egypt.
- To identify hulless barley genotypes having better human nutritional value with high lysine, high protein and high beta-glucan content for human consumption.
- To determine the proper crop management package to be used in rainfed areas and new reclaimed lands.

- To disseminate the varietal and management packages to the farmers through establishing on-farm verification and demonstration trials, and initiating a strong extension program in order to transfer hulless barley production technology to the extension specialists and farmers.
- To establish in- and out-country training activities for the extension specialists and scientists to acquire new experience in breeding and production of hulless barley.
- To produce breeder and basic seed of hulless barley.

The main achievements during six seasons of the project (1998/99 – 2003/04) are summarized below.

On-farm activities

Three promising hulless barley genotypes with the three newly developed hulless barleys used as checks (Giza 129, Giza 130 and Giza 131) were grown at seven sites in rainfed areas in NCW and North Sinai, and in irrigated lands, including new reclaimed and old lands. Two of these promising lines exhibited high yielding potential and stability under different barley disease-challenge situations across the barley production areas. They were then promoted to the on-farm verification trials for further testing under farmers' field conditions.

Back-up breeding research

More than 1000 exotic lines introduced from different institutions, including ICARDA (Syria), CIMMYT (Mexico) and INRA (France), along with some local barley genotypes were tested and evaluated by the barley breeding program, and some of them were used in the crossing block for different purposes. Plant breeders, pathologists and food technologists worked together to develop drought tolerant, stable and disease resistant hulless barley genotypes with high food quality.

As a result of these efforts, three new hulless barley cultivars having high yield potential with a high degree of resistance to barley disease as well as good quality (high protein, lysine, beta-glucan and soluble dietary fiber content) for human consumption were developed. They have been registered and listed as cvs. Giza 129, Giza 130 and Giza 131. They are also adapted and grow fairly well under different environmental conditions. In addition, the proper percentage of barley flour in bread making mixed with wheat flour was tested. Healthy flat loaf bread was prepared using 20% hulless barley flour mixed with 80% wheat flour (82% extraction). The flat loaf bread produced had good quality, i.e. morphological and technological traits.

Evaluation of crackers and cookies made of hulless barley showed that using 20% wheat flour (72% extraction) plus 80% whole meal hulless barley gave the highest total score and acceptance. It was strongly recommended to use hulless barley as a healthy ingredient in bakery products such as crackers and cookies. This could help save wheat flour for bread making to reduce the wheat shortage in Egypt.

Agronomy

The application of biofertilizer (Azotein) in the presence of mineral fertilizers (N and P) could enhance plant growth and grain yield of hulless barley in rainfed and new reclaimed sandy soils in Egypt.

Increasing N levels from 70 to 140 kg/ha and from 140 to 210 kg/ha caused significant increase in days to heading, plant height, spike length, number of spikes per m², number of grains per spike, spike grain weight and 1000-grain weight).

- Planting on 20 November and fertilizing with 190 kg/ha N and 140 kg/ha P₂O₅ could be recommended for hulless barley to be cultivated in poor sandy soils, such as in Ismailia.
- The highest grain yield was obtained when

irrigating hulless barley with 2800 m³/ha. LHB93/4 hulless barley genotype gave the highest grain yield when an N rate of 190 kg/ha was applied.

Extension activities

Certified seed of the new released hulless barley cultivars Giza 129, Giza 130 and Giza 131 were given to the farmers along with a cultural package, to be grown side by side with their own varieties using the traditional cultural practices in each locality. These three cultivars in the extension fields significantly out-yielded farmers' usual varieties at all sites.

Economic assessment of recommended barley cultivars in the rainfed and saline soil areas

Although barley has less importance in the old land of Egypt than other cereal crops, it remains one of the most important cereal crops in rainfed areas. The main research objective was to compare the new technologies with the traditional ones, and to investigate the influence of the improved cultivars along with their recommended agronomic packages and technology on yields, costs and gross benefits for selected farmer groups. Analytical sampling was designed and selected from the governorates that were identified by barley scientists and socio-economists. A sample of 24 farmers was selected from two locations, Kafr El Sheikh (saline soils) and North Sinai (rainfed areas), as shown in Tables 3 and 4.

Partial budget analysis indicated that there were significant differences in favor of the participants' yields, with an average increase ranging from 22.1% to 74.4%. Results also indicated that the variable costs of participants were higher than those of the non-participants by about 98.3% in North Sinai and 26.6% in Kafr El-Sheikh. The increase in the Gross Benefits of the participants ranged from 25.7% to 112%. Marginal Rate of Return

Table 3. Partial budget of recommended package of barley cultivars under saline soil conditions at Kafr El-Sheikh.

Item	Yield (t/ha)	Total return (LE)	Total variable cost (LE)	Gross benefit (LE)	Variable costs (LE)	B/C ¹ ratio	MRR ² %
Giza 123							
Participants	4.28	3976	1402	2574	457	2.84	
Non-participants	3.50	3390	1343	2047	361	2.52	
Difference	0.78	586	59	527	96		549
%	22.3	17.3	4.4	25.7	26.6		
Giza 125							
Participants	4.89	4428	1402	3026	457	3.16	
Non-participants	3.50	3390	1343	2047	361	2.52	
Difference	1.39	1038	59	979	96		1020
%	39.7	30.6	4.4	47.8	26.6		
Giza 126							
Participants	5.07	4517	1402	3115	457	3.22	
Non-participants	3.50	3390	1343	2047	361	2.52	
Difference	1.57	1127	59	1068	96		11 125
%	44.9	33.2	4.4	52.2	26.6		
Giza 2000							
Participants	4.90	4414	1402	3012	457	3.15	
Non-participants	3.50	3390	1343	2047	361	2.52	
Difference	1.40	1024	59	965	96		1005
%	40.0	30.2	4.4	47.1	26.6		

¹ B/C = Benefit to Cost Ratio; ² MRR = Marginal Rate of Return. SOURCE: Survey Data.

(MRR) was greater than 50%. It could be concluded that barley growers are expected to adopt the recommended package in the governorates in this study.

Kafr El-Sheikh Governorate

The average yield in participant's fields was greater than the average of non-participant fields, with 22.3, 39.7, 44.9 and 40% rates of increase for Giza 123, Giza 125, Giza 126 and Giza 2000, respectively (Table 3). The Gross Benefit for participants ranged from Egyptian Lira (LE) 2574/ha for Giza 123 to LE 3115/ha for Giza 126, exceeding that for the non-participants by 25.7 and 52.2%, respectively. The variable costs of the participants amounted to about LE 457/ha, exceeding that of the non-participants by 26.6%, while Benefit/Cost ratio of the participant's plots

was higher than that of the non-participants. MRRs were greater than 50%, satisfying the requirements for the recommended package to be adopted by the barley growers in these areas.

North Sinai Governorate

The average yield in participant's fields was 1.19 t/ha for Giza 123, 1.05 t/ha for Giza 125, 1.48 t/ha for Giza 126 and 1.50 t/ha for Giza 2000, compared with an average of 0.86 t/ha in non-participant fields (Table 4). The Gross Benefits for participants exceeded those for the non-participants by 66.8, 48.4, 112 and 112% for Giza 123, Giza 125, Giza 126 and Giza 2000, respectively. The variable costs of the participants amounted to about LE 119/ha, exceeding that for the non-participants by 98.3%. Benefit/Cost ratio of the participant's

Table 4. Partial budget of recommended package for barley cultivars under rainfed conditions in North Sinai.

Item	Yield (t/ha)	Total return (LE)	Total variable cost (LE)	Gross benefit (LE)	Variable costs (LE)	B/C ¹ ratio	MRR ² %
Giza 123							
Participants	1.19	1082	323	759	119	3.30	
Non-participants	0.86	675	220	455	60	3.07	
Difference	0.33	407	103	304	59		
%	38.4	60.3	46.8	66.8	98.3		
Giza 125							
Participants	1.05	998	323	675	119	3.10	
Non-participants	0.86	675	220	455	60	3.07	
Difference	0.19	323	103	220	59		356
%	22.1	47.9	46.8	48.4	98.3		
Giza 126							
Participants	1.48	1290	323	967	119	4.00	
Non-participants	0.86	675	220	455	60	3.07	
Difference	0.62	615	103	512	59		868
%	72.1	91.1	46.8	112	98.3		
Giza 2000							
Participants	1.50	1287	323	964	119	3.98	
Non-participants	0.86	675	220	455	60	3.07	
Difference	0.64	612	103	509	59		863
%	74.4	90.7	46.8	112	98.3		

¹ B/C = Benefit to Cost Ratio; ² MRR = Marginal Rate of Return. SOURCE: Survey Data.

plots was higher than that of the non-participant plots, while MRRs were greater than 50%, satisfying the requirements for the recommended package to be adopted by the barley growers in this area.

The role of gender in barley production

Results showed that the role of women in the production of barley was clear in North Sinai and Matrouh governorates, where women participation in labor cost represented about 64 and 18%, respectively. The average wage per day of a woman was LE 7–8 against LE 10–15 for a man and LE 5–7 for a child. Men only were involved in the production of barley in Kafr El-Sheikh governorate.

Future perspectives

Higher productivity per unit area and unit water

- Increase barley productivity through maximizing production per unit area and unit water to bridge the increasing gap between actual or average national production and the potential yield achieved by the research program.
- Develop new cultivars with high yielding capacity and tolerant to different stresses (drought, salinity, poor soil fertility, diseases, insects, etc.).
- Develop high-yielding barley cultivars suitable for the newly reclaimed lands and rainfed areas in the north coastal region of Egypt, including North Sinai and NWC.

- Develop complete recommended agronomic packages that interact with the new cultivars to achieve higher yield under various stress conditions.
- Improve and maintain high quality seed.
- Transfer new technology to farmers' fields through strengthening the linkage between the researchers, extensionists and farmers.
- Use of the recommended barley cultivars and trying to increase percent coverage of certified seed of the newly developed cultivars in targeted barley areas according to the varietal policies regularly established.
- Use of modern and new technologies in crop improvement and use of mechanization to increase yield and minimize yield losses due to old-fashioned agriculture, especially during harvest and threshing, and also post-harvest storage.
- Enhance the use of biodiversity and maintain barley genetic germplasm through cooperation with national and international organizations and genebanks.
- Horizontal expansion of barley cultivated area
- In the old lands, expand barley production in the marginal areas with poor drainage systems and those located in the tails of irrigation canals; and in those areas with high salinity and waterlogging problems.
- Expand in newly reclaimed lands with sandy and calcareous soils, such as Nubaria and Ismailia areas, and also along the west bank of the Nile River in Middle and Upper Egypt.
- In rainfed areas, expand across the north coast east and west in Sinai and Matrouh governorates at Arish and Rafah.

New barley products

- Making barley bread, especially in Matrouh, and North Sinai and New Valley.
- Special bread for diabetics and patients with high blood pressure.
- Baby foods and diets for aging people
- Use of two-row barley in malting and

brewery industries for local consumption and export. This will require expanding the area under malting barley.

Use of barley as forage and mixed with forage crops

- Develop dual-purpose barley for food and feed, and for admixture to winter forage crops to overcome livestock production gap problems.
- It can be intercropped with leguminous crops.
- It can be used as green forage for animal feeding, e.g. livestock and small ruminants such as sheep and goats, where it can be used as a multiple cut crop.
- It should be used as a constituent of the animal diet for milk production.
- It can be added to alfalfa silage or other forage crops such as maize.

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Plant breeding and climate change

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Abstract

The paper discusses the contribution of plant breeding to the adaptation of crops to future climate.

Climate change is now unequivocal, particularly in terms of increasing temperature, increasing CO₂ concentration, widespread melting of snow and ice, and rising global average sea level, while the increase in the frequency of drought is very likely but not as certain.

Yet, climate changes are not new and some of them have had a dramatic impact, such as the appearance of leaves about 400 million years ago as a response to a drastic decrease of CO₂ concentration, the birth of agriculture due to the end of the last ice age about 11 000 years ago, and the collapse of civilizations due to the late Holocene droughts between 5000 and 1000 years ago.

The climate change occurring now will have—and is already having—an adverse effect on food production and food quality, with the poorest farmers and the poorest countries most at risk. The adverse effect is a consequence of the expected or likely increased frequency of some abiotic stresses such as heat and drought, and of the increased frequency of biotic stresses (pests and diseases). In addition, climate change is also expected to cause losses of biodiversity, mainly in more marginal environments.

Plant breeding has always addressed both abiotic and biotic stresses, and strategies of adaptation to climate changes may include

a more accurate matching of phenology to moisture availability using photoperiod-temperature response; increased access to a suite of varieties with different duration to escape or avoid predictable occurrences of stress at critical periods in crop life cycles; improved water use efficiency; and re-emphasis on population breeding to provide a buffer against increased unpredictability. These measures must go hand in hand with breeding for resistance to biotic stresses, and with an efficient system of variety delivery to farmers.

As a crop that has been always considered as the most resilient amongst the winter cereals and that has a range of possible uses still largely unexplored, barley is an ideal crop that farmers in a number of countries are already using as a response to climate changes.

Climatic changes today

Today nobody questions whether climate changes are occurring or not, and the discussion has shifted from whether they are happening to what to do about them.

The most recent evidence from the Fourth Assessment Report on Climate Change of the Intergovernmental Panel on Climate Change (IPCC, 2007) indicates that the warming of the climate system is unequivocal, as is now evident from observations of increases in global average air and ocean temperatures, widespread melting of snow and ice, and rising global average sea level.

Quoting from the report:

- “Eleven of the last twelve years (1995-2006) rank among the twelve warmest years in the instrumental record of global surface temperature (since 1850)”
- “The temperature increase is widespread over the globe, and is greater at higher northern latitudes. Land regions have warmed faster than the oceans”.
- Rising sea level is consistent with warming. Global average sea level has risen since 1961 at an average rate of 1.8 mm/yr and since 1993 at 3.1 mm/yr, with contributions from thermal expansion, melting glaciers and ice caps, and the polar ice sheets.
- Observed decreases in snow and ice extent are also consistent with warming. Satellite data since 1978 show that annual average Arctic sea ice extent has shrunk by 2.7% per decade, with larger decreases in summer of 7.4% per decade. Mountain glaciers and snow cover on average have declined in both hemispheres.

It is also very likely that over the past 50 years cold days, cold nights and frosts have become less frequent over most land areas, and hot days and hot nights have become more frequent, and it is likely that heat waves have become more frequent over most land areas, the frequency of heavy precipitation events has increased over most areas, and

since 1975 the incidence of extreme high sea level has increased worldwide. There is also observational evidence of an increase in intense tropical cyclone activity in the North Atlantic since about 1970, with limited evidence of increases elsewhere. There is no clear trend in the annual numbers of tropical cyclones, but there is evidence of increased intensity.

Changes in snow, ice and frozen ground have with high confidence increased the number and size of glacial lakes, increased ground instability in mountain and other permafrost regions, and led to changes in some Arctic and Antarctic ecosystems (Walker, 2007).

The projections to year 2100 for concentration indicate that CO₂ emission is expected to increase by 400% and CO₂ atmospheric concentration is expected to increase by 100% (Figure 1).

Some studies have predicted increasingly severe future impacts, with potentially high extinction rates in natural systems around the world (Williams *et al.*, 2003; Thomas *et al.*, 2004).

Climatic changes in history

Even though climate changes are one of the major current global concerns, they are not new. Several climate changes occurred before, with dramatic consequences. Among

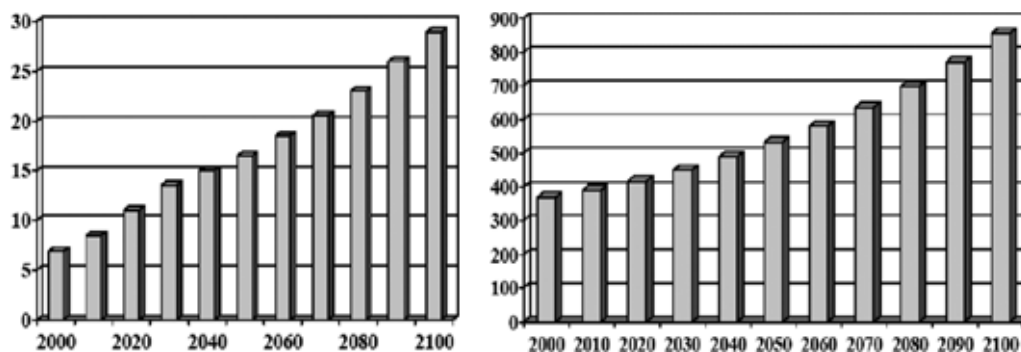


Figure 1. Projected CO₂ emission in billion tonne carbon equivalent (left) and atmospheric CO₂ concentration in parts per million (right).

these is the decrease in CO₂ content which took place 350 million years ago and which is considered to be responsible for the appearance of leaves—the first plants were leafless and it took about 40–50 million years for the leaves to appear (Beerling *et al.*, 2001).

A second climatic change was that induced by perhaps the most massive volcanic eruption in Earth's history, which occurred in Siberia when up to 4 million cubic kilometres of lava erupted onto the Earth's surface. The remnants of that eruption cover today an area of 5 million square kilometers. This massive eruption caused, directly or indirectly, though the formation of organohalogens, a worldwide depletion of the ozone layer. The consequent burst of ultraviolet radiation explains why the peak eruptions phase coincides with the timing of the mass extinction that wiped out 95% of all species.

The third major climate change was the end of the last Ice Age (between 13 000 and 11 500 BC), with the main consequence that much of the earth became subject to long dry seasons. This created favorable conditions

for annual plants, which can survive the dry seasons either as dormant seeds or as tubers, and eventually agriculture started, as we know today, in that area of the Near East known as the Fertile Crescent, around 9 000 BC, and soon spread to other areas.

The fourth climatic change was the so called Holocene flooding, which took place about 9 000 years ago and is now believed to be associated with the final collapse of the Ice Sheet, resulting in a global sea level rise of up to 1.4 m (Turney and Brown, 2007). Land lost from rising sea levels drove mass migration to the north west and this could explain how domesticated plants and animals, which by then had already reached modern Greece, started moving towards the Balkans and eventually into Europe.

During the last 5 000 years, drought, or more generally limited water availability, has historically been the main factor limiting

crop production. Water availability has been associated with the rise of multiple civilizations, while drought has caused the collapse of empires and societies, such as the Akkadian Empire (Mesopotamia, ca. 4200 calendar yr B.P.), the Classic Maya (Yucatan Peninsula, ca. 1200 calendar yr B.P.), the Moche IV–V Transformation (coastal Peru, ca. 1500 calendar yr B.P.) (de Menocal, 2001) and the early bronze society in the southern part of the Fertile Crescent (Rosen, 1990).

How do people respond to climatic changes?

Although the debate about climate change is relatively recent, people, for example in Africa, have been adapting to climate changes for thousands of years. In general, people seem to have adapted best when working as a community rather than as individuals. The four main strategies of adaptation have been (a) changes in agricultural practices; (b) formation of social networks; (c) embarking on commercial projects, such as investing in livestock; and (d) seeking work in distant areas. The first three of these strategies rely on people working together to improve their community (Giles, 2007).

In continuous coping with extreme weather events and climatic variability, farmers living in harsh environments in Africa, Asia and Latin America have developed or inherited complex farming systems that have the potential to bring solutions to many uncertainties facing humanity in an era of climate change (Altieri and Koohafkan, 2003). These systems have been managed in ingenious ways, allowing small farming families to meet their subsistence needs in the midst of environmental variability without depending much on modern agricultural technologies (Denevan, 1995). They can still be found throughout the world, covering some 5 million hectares, are of global importance to food and agriculture, and are based on the cultivation of a diversity of crops

and varieties in time and space that have allowed traditional farmers to avert risks and maximize harvest security in uncertain and marginal environments, under low levels of technology and with limited environmental impact (Altieri and Koohafkan, 2003). One of the salient features of the traditional farming systems is their high degree of biodiversity, in particular the plant diversity in the form of polycultures and/or agroforestry patterns. This strategy of minimizing risk by planting several species and varieties of crops is more resilient to weather events, climate variability and change, and resistant to adverse effects of pests and diseases, and at the same time stabilizes yields over the long term, promotes dietary diversity and maximizes returns, even with low levels of technology and limited resources (Altieri and Koohafkan, 2003).

The term autonomous adaptation is used to define responses that will be implemented by individual farmers, rural communities or farmers' organizations, or a combination, depending on perceived or real climate change in the coming decades, and without intervention or coordination by regional and national governments and international agreements. To this end, pressure to cultivate marginal land, or to adopt unsustainable cultivation practices as yields drop, may increase land degradation and endanger the biodiversity of both wild and domestic species, possibly jeopardizing future ability to respond to increasing climate risk later in the century.

One of the options for autonomous adaptation includes the adoption of varieties

and species with increased resistance to heat shock and drought (Bates *et al.*, 2008).

Climatic changes, food and agriculture

Using the results from formal economic models, it is estimated (Stern, 2005) that in the absence of effective counteraction, the overall costs and risks of climate change will be equivalent to losing at least 5% of global gross domestic product (GDP) each year. If a wider range of risks and impacts is taken into account, the estimates of damage could rise to 20% of GDP or more, with a disproportionate burden on and increased risk of famine for the poorest countries (Altieri and Koohafkan, 2003).

The majority of the world's rural poor, about 370 million of the poorest, live in areas that are resource poor, highly heterogeneous and risk prone. The worst poverty is often located in arid or semi-arid zones, and in mountains and hills that are ecologically vulnerable (Conway, 1997). In many countries, more people, particularly those at lower income levels, are now forced to live in marginal areas (i.e. floodplains, exposed hillsides, arid or semi-arid lands), putting them at risk from the negative impacts of climate variability and change.

Climatic changes are predicted to have adverse impacts on food production, food quality and food security. One of the most recent predictions (Tubiello and Fischer, 2007; Table 1) is that by the year 2080 the number of undernourished people will

Table 1. Expected number of undernourished in millions, incorporating the effect of climate.

	1990	2020	2050	2080	Ratio 2080/1990
Developing countries	885	772	579	554	0.6
Asia, Developing	659	390	123	73	0.1
Sub-Saharan Africa	138	273	359	410	3.0
Latin America	54	53	40	23	0.4
Near East and North Africa	33	55	56	48	1.5

SOURCE: Tubiello and Fischer, 2007

increase by 1.5 times in the Near East and North Africa and by 3 times in sub-Saharan Africa compared with 1990.

Agriculture is extremely vulnerable to climate change. Higher temperatures eventually reduce crop yields while encouraging weed, disease and pest proliferation. Changes in precipitation patterns increase the likelihood of short-term crop failures and long-term production declines. Although there will be gains in some crops in some regions of the world, the overall impacts of climate change on agriculture are expected to be negative, threatening global food security (Nelson *et al.*, 2009).

Food insecurity is likely to increase under climate change, unless early warning systems and development programs are used more effectively (Brown and Funk, 2008). Today, millions of hungry people subsist on what they produce. If climate change reduces production while populations increase, there is likely to be more hunger. Lobell *et al.* (2008) showed that increasing temperatures and declining precipitation over semi-arid regions are likely to reduce yields for maize, wheat, rice, and other primary crops in the next two decades. These changes could have a substantial negative impact on global food security.

Climate change increases child malnutrition and reduces food energy consumption dramatically. Thus, aggressive agricultural productivity investments are needed to raise food energy consumption enough to offset the negative impacts of climate-change on the health and well-being of children (Nelson *et al.*, 2009).

How do crops respond to climatic changes?

Adapting crops to climatic changes has become an urgent challenge that requires some knowledge of how crops respond to those changes. In fact, plants have responded to increasing CO₂ concentration

from pre-industrial to modern times by decreasing stomatal density—reversing the change described earlier which led to the appearance of leaves—as shown by the analysis of specimens collected from herbaria over the past 200 years (Woodward, 1987). In *Arabidopsis thaliana* the gene HIC (High Carbon Dioxide) prevents changes in the number of stomata in response to increasing CO₂ concentration (Gray *et al.*, 2000); mutant hic plants exhibit up to 42% increase in stomatal density in response to a doubling of CO₂. The implication is that the response of the stomatal density to increasing CO₂ concentration in many plant species is now close to saturation (Serna and Fenoll, 2000). Stomatal density varies widely within species: for example, in barley, stomatal density varies from 39 to 98 stomata/mm² (Miskin and Rasmusson, 1970), suggesting that the crop has a fairly good possibility of adaptation.

Today it is fairly well known how plants respond to increased CO₂ concentration, which has both direct and indirect effects on crops. Direct effects (also known as CO₂-fertilization effects) are those affecting the crops by the presence of CO₂ in ambient air, which is currently sub-optimal for C₃ type plants like wheat and barley. In fact, in C₃ plants, mesophyll cells containing ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) are in direct contact with the intercellular air space that is connected to the atmosphere via stomatal pores in the epidermis. Hence, in C₃ crops, rising CO₂ increases net photosynthetic CO₂ uptake because RuBisCO is not CO₂-saturated in today's atmosphere and because CO₂ inhibits the competing oxygenation reaction, leading to photorespiration. CO₂-fertilization effects include increased photosynthetic rate, reduced transpiration rate through decreased stomatal conductance, higher water use efficiency (WUE), and lower probability of water stress occurrence. As a consequence, crop growth and biomass

production should increase by up to 30% for C_3 plants at doubled ambient CO_2 ; other experiments show 10–20% biomass increase under double CO_2 conditions. In theory, at 25°C, an increase in CO_2 from the current 380 ppm to that of 550 ppm, projected for the year 2050, would increase photosynthesis by 38% in C_3 plants. In C_4 plants, such as maize and sorghum, RuBisCO is localized in the bundle sheath cells in which CO_2 concentration is three to six times higher than atmospheric CO_2 . This concentration is sufficient to saturate RuBisCO and in theory would prevent any increase in CO_2 uptake with rising CO_2 . However, even in C_4 plants, an increase in water use efficiency via a reduction in stomatal conductance cause by increased CO_2 may still increase yield (Long *et al.*, 2006).

However, the estimates of the CO_2 -fertilization have been derived from enclosure studies conducted in the 1980s (Kimball, 1983; Cure and Acock, 1986; Allen *et al.*, 1987), and today they appear to be overestimated (Long *et al.*, 2006).

In fact, free-air concentration enrichment (FACE) experiments, representing the best simulation of the future elevated CO_2

concentration, gives much lower (about 50% lower) estimates of increased yields due to CO_2 -fertilization (Table 2).

Indirect effects (also known as weather effects) are the result of solar radiation, precipitation and air temperature, and, keeping management the same, above a certain temperature threshold, cereals yields typically decrease with increasing temperatures and increase with increased solar radiation. If water is limiting, yields eventually decrease because of higher evapotranspiration. Precipitation will obviously have a positive effect when it reduces water stress, but can also have a negative effect such as by causing waterlogging.

Therefore, the most likely scenario for plant breeding is the following:

- higher temperatures, which will reduce crop productivity, are certain;
- increased CO_2 concentration is certain, with both direct and indirect effects;
- increased frequency of drought is highly probable;
- increases in the areas affected by salinity is highly probable; and
- increased frequency of biotic stress is also highly probable.

Table 2. Percentage increases in yield, biomass and photosynthesis of crops grown at elevated CO_2 (550 ppm) in enclosure studies versus FACE (Free-Air Concentration Enrichment) experiments (Long *et al.*, 2006).

Source	Rice	Wheat	Soybean	C_4 crops
Yield				
Kimball (1983)	19	28	21	–
Cure and Acock (1986)	11	19	22	27
Allen <i>et al.</i> (1987)	–	–	26	–
Enclosure studies	–	31	32	18
FACE studies	12	13	14	0 ¹
Biomass				
Cure and Acock (1986)	21	24	30	8
Allen <i>et al.</i> (1987)	–	–	35	–
FACE studies	13	10	25	0 ¹
Photosynthesis				
Cure and Acock (1986)	35	21	32	4
FACE studies	9	13	19	6

¹ Data from only one year (Leahey *et al.*, 2006)

Given this scenario, biotechnology and conventional breeding may help by developing new cultivars with enhanced traits better suited to adapt to climate change conditions. These include drought and temperature stress resistance; resistance to pests and disease; and tolerance of salinity and waterlogging. Breeding for drought resistance has historically been one of the most important and common objectives of several breeding programs for all the major food crops in most countries (Ceccarelli *et al.*, 2007; Ceccarelli, 2010). Opportunities for new cultivars with increased drought tolerance include changes in phenology or enhanced responses to elevated CO₂. With respect to water, a number of studies have documented genetic modifications to major crop species (e.g. maize and soybean) that increased their water-deficit tolerance (Drennan *et al.*, 1993; Kishor *et al.*, 1995; Pilon-Smits *et al.*, 1995; Cheikh *et al.*, 2000), although this may not extend to a wide range of crops. In general, too little is currently known about how the desired traits achieved by genetic modification perform in real farming and forestry applications (Sinclair and Purcell, 2005).

Thermal tolerances of many organisms have been shown to be proportional to the magnitude of temperature variation they experience: lower thermal limits differ more among species than upper thermal limits (Addo-Bediako *et al.*, 2000). Therefore a crop like barley, which has colonized a huge diversity of thermal climates, may harbor enough genetic diversity to breed successfully for enhanced thermal tolerance.

Soil moisture reduction due to precipitation changes could affect natural systems in several ways. There are projections of significant extinctions in both plant and animals species. Over 5 000 plant species could be affected by climate change, mainly due to the loss of suitable habitats. By 2050, the Fynbos Biome (Ericaceae-dominated ecosystem of South Africa, which is an International Union for

the Conservation of Nature and Natural Resources (IUCN) 'hotspot') is projected to lose 51–61% of its extent due to decreased winter precipitation. The succulent Karoo Biome, which includes 2 800 plant species at increased risk of extinction, is projected to expand south-eastwards, and about 2% of the family Proteaceae is projected to become extinct. These plants are closely associated with birds that have specialized in feeding on them. Some mammal species, such as the zebra and nyala, which have been shown to be vulnerable to drought-induced changes in food availability, are widely projected to suffer losses. In some wildlife management areas, such as the Kruger and Hwange National Parks, wildlife populations are already dependant on water supplies supplemented by borehole water (Bates *et al.*, 2008).

With the gradual reduction in rainfall during the growing season for grass, aridity in central and west Asia has increased in recent years, reducing the growth of grasslands and increasing the bareness of the ground surface (Bou-Zeid and El-Fadel, 2002). Increasing bareness has led to increased reflection of solar radiation, such that more soil moisture evaporates and the ground becomes increasingly drier in a feedback process, thus adding to the acceleration of grassland degradation (Zhang *et al.*, 2003). Recently it has been reported that the Yangtze river basin has become hotter and it is expected that the temperature will increase by up to 2°C by 2050 relative to 1950 (Ming *et al.*, 2009). This increase will reduce rice production by up to 41% by the end of the century and maize production by up to 50% by 2080.

The negative impact of climatic changes on agriculture and therefore on food production is aggravated by the greater uniformity that exists now, particularly in the crops of developed-country agriculture compared to 150–200 years ago. The decline in agricultural biodiversity can be quantified as follows: while it is estimated that there

are approximately 250 000 plant species, of which about 50 000 are edible, we actually use no more than 250—out of which 15 crops give 90% of the calories in the human diet, and 3 of them, namely wheat, rice and maize, give 60%. In these three crops, modern plant breeding has been particularly successful, and the process towards genetic uniformity has been rapid: the most widely grown varieties of these three crops are closely related and genetically uniform (pure lines in wheat and rice and hybrids in maize). The major consequence is that our main sources of food are more genetically vulnerable than ever before, i.e. food security is potentially in danger. The danger has become real with the rapid spreading of diseases such as UG99, but applies equally well to climatic changes, as the predominant uniformity does not allow the crops to evolve and adapt to changing environmental conditions. The expected increase in biofuel monoculture production may lead to increased rates of biodiversity loss and genetic erosion. Another serious consequence of the loss of biodiversity has been the displacement of locally adapted varieties, which might hold the secret of adaptation to the future climate (Ceccarelli and Grando, 2000; Rodriguez *et al.*, 2008; Abay and Bjørnstad, 2009).

Combining participation and evolution: Participatory-Evolutionary plant breeding

One of the fundamental breeding strategies to cope with the challenge posed by the climate changes is to improve adaptation to a likely shorter crop season length by matching phenology to moisture availability. This should not pose major problems as photoperiod-temperature response is highly heritable. Other strategies include increasing access to a suite of varieties with different duration to escape or avoid predictable occurrences of stress at critical periods

in crop life cycles, shifting temperature optima for crop growth, and re-emphasizing population breeding.

In all cases, the emphasis will be on identifying and using sources of genetic variation for tolerance or resistance to a higher level of abiotic stresses, and the two most obvious sources of novel genetic variation are the genebanks (ICARDA has one of the largest genebanks, with more than 100 000 accessions of several species, including important food and feed crops such as barley, wheat, lentil, chickpea and vetch) and farmers' fields. Currently there are several international projects aiming at the identification of genes associated with superior adaptation to higher temperatures and drought; at ICARDA, but also elsewhere, it has been found that landraces, and when available wild relatives, harbor a large amount of genetic variation, some of which is of immediate use in breeding for drought and high-temperature tolerance.

The major difference between the two sources of genetic variation is that the first is static, in the sense that it represents the genetic variation available at the collection sites at the time the collection was made, while the second is dynamic, because landraces and wild relatives are heterogeneous populations and as such they evolve and can generate continuously novel genetic variation.

Adaptive capacity in its broadest sense includes both evolutionary changes and plastic ecological responses; in the climate change literature, it also refers to the capacity of humans to manage, adapt and minimize impacts (Williams *et al.*, 2008). All organisms are expected to have some intrinsic capacity to adapt to changing conditions; this may be via ecological (i.e. physiological and/or behavioral plasticity) or evolutionary adaptation (i.e. through natural selection acting on quantitative traits). There is now evidence in the scientific literature that evolutionary adaptation has occurred in a variety of species in response to climate

change, both in the long term, as seen earlier in the case of stomata (Woodward, 1987) or over a relatively short time, e.g. five to 30 years (Bradshaw and Holzapfel, 2006). However, this is unlikely to be the case for the majority of species and, additionally, the capacity for evolutionary adaptation is probably the most difficult trait to quantify across many species (Williams *et al.*, 2008).

Recently Morran *et al.* (2009) have used experimental evolution to test the hypothesis that outcrossing populations are able to adapt more rapidly to environmental changes than self-fertilizing organisms, as suggested by Stebbins (1957), Maynard Smith (1978) and Crow (1992), explaining why the majority of plants and animals reproduce by outcrossing as opposed to selfing. The advantage of outcrossing is to provide a more effective means of recombination and thereby generating the genetic variation necessary to adapt to a novel environment (Crow, 1992). The experiment of Morran *et al.* (2009) suggest that even outcrossing rates lower than 5%, therefore comparable with those observed in self-pollinated crops such as barley, wheat and rice, allowed adaptation to a stress environments as indicated by a greater fitness accompanied by an increase in the outcrossing rates. This experiment, even though conducted on a nematode, is relevant for both self- and cross-pollinated crops and provides the expectations for evolutionary plant breeding, a breeding method introduced by Suneson more than 50 years ago working with barley (Suneson, 1956). Its 'core features are a broadly diversified germplasm, and a prolonged subjection of the mass of the progeny to competitive natural selection in the area of contemplated use'. Its results showed that traits relating to reproductive capacity, such as higher seed yields, larger numbers of seeds per plant, and greater spike weight, increase in populations due to natural selection over time.

At ICARDA we are combining evolutionary plant breeding with participatory

plant breeding (PPB), which is seen by several scientists as a way to overcome the limitations of conventional breeding, by offering farmers the possibility to choose, in their own environment, which varieties better suit their needs and conditions. PPB exploits the potential gains of breeding for specific adaptation through decentralized selection, defined as selection in the target environment (Ceccarelli and Grando, 2007).

The evolutionary breeding that ICARDA is combining with the participatory programs implemented in Syria, Jordan, Iran, Eritrea and Algeria aims at increasing the probability of recombination within a population that is deliberately constituted to harbor a very large amount of genetic variation. Such a population consists of a large mixture of nearly 1600 F_2 lines of barley and is planted in 19 locations in the 5 countries where it is left evolving under the pressure of the changing climatic conditions. The breeder and the farmers will superimpose artificial selection with criteria that may change from location to location and with time. While the population is evolving, lines can be derived and tested as pure lines in the participatory breeding program, or a sub-sample of the population can be used for cultivation. The key aspect of the method is that the population is left evolving for an indefinite amount of time, thus becoming a unique source of continuously better adapted genetic material directly in the hands of the farmers. The latter guarantees that the improved material will be readily available to farmers without the bureaucratic and often inefficient systems of variety release and formal seed production.

Conclusions

The major danger when discussing the adaptation of crops to climate changes is that these discussions usually take place in comfortable offices isolated both from the outside climate and from the people who will be most affected by its changes.

By bringing back the analysis of the problems and the search for solutions to the thousands of small-scale and traditional family farming communities and indigenous peoples in the developing world that will be affected by climatic changes, by combining the indigenous agricultural knowledge systems with scientific knowledge, and by making use of the lessons from the past, we may be able to provide better adapted varieties that, together with appropriate agronomic techniques, could help millions of rural people to reduce their vulnerability to the impact of climate change.

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Session 1

Germplasm and genetic resources

New approaches to analysis of germplasm and genetic resources

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Introduction

Extensive genetic resources and germplasm are available for use in barley improvement. Effective use of these resources in barley breeding requires the availability and application of tools for germplasm characterization (Table 1). Recent developments in molecular approaches allow more efficient discovery and analysis of genetic polymorphism.

DNA Banking

Germplasm characterization and utilization is facilitated by the availability of DNA collections that can be screened for genetic research and breeding (Rice *et al.*, 2006). We have established the Australian Plant DNA Bank (www.biobank.com) for this purpose. The collection currently contains wild barley, barley landraces and commercial barley cultivars.

Genotyping technologies

Plant genotyping has been widely conducted by microsatellite (SSR) analysis (Henry, 2001), and this technique continues to be very useful for characterization of barley germplasm (Jilal *et al.*, 2008). However single nucleotide polymorphism (SNP) analysis (Henry, 2008) has been replacing SSR analysis as more DNA sequence data becomes available.

SSR

SSR analysis has been widely applied to barley germplasm characterization and in barley breeding. Early work on SSR loci derived from genomic libraries has been supplemented by sourcing of SSR from expressed sequences. These genic SSRs have proven very useful in germplasm characterization (Chabane *et al.*, 2005). Recently, very large numbers of SSRs have been derived from analysis of expressed sequence tag (EST) sequences, and these are now available for use in barley.

SNP discovery

The discovery of SNP in barley has largely been achieved by analysis of EST sequences or by re-sequencing using di-deoxy sequencing. Recent developments in DNA sequencing technology provide greatly improved technology for cost-effective discovery of SNPs.

TILLING

The TILLING (Targeting Induced Local Lesions in Genomes) method allows the targeted identification of mutants (SNPs) in any gene of interest following chemical mutation of the seed. When used to explore variation in wild populations or germplasm, the technique is called ecoTILLING.

Table 1. Some recent developments in tools for the characterization of genetic resources for barley improvement.

Technology	Applications	References
DNA banking	gene discovery	Rice <i>et al.</i> , 2006
TILLING/ecoTILLING	targeted mutagenesis/diversity analysis	Till <i>et al.</i> , 2006
EMAIL	rare mutant discovery	Cross <i>et al.</i> , 2007
EST/SAGE	transcript identification	White <i>et al.</i> , 2006
Micro-arrays	transcript profiling	White <i>et al.</i> , 2006
Mass array	SNP genotyping/transcript profiling	Pattemore and Henry (unpublished)
Next-generation sequencing	gene and SNP discovery	Wicker <i>et al.</i> , 2006

EMAIL

We have modified the TILLING protocol, first by adapting the technique for analysis using capillary electrophoresis (Cordeiro *et al.*, 2006) and then by employing internal rather than end labeling (Cross *et al.*, 2008). The improved technique, endonucleolytic mutation analysis by internal labeling (EMAIL), has greater sensitivity for detection of rare alleles in larger bulks.

SNP analysis

SNP analysis in barley has been achieved using both widely available technologies and more high volume automated instruments.

Simple PCR methods

Allele-specific PCR can be used to detect barley SNPs in simple and robust assays (Bundock *et al.*, 2006). Quantitative SNP analysis can be achieved routinely by modified real-time PCR protocols (Kennedy *et al.*, 2006). This allows analysis of heterozygotes or pooled samples.

Mass Array

We have adopted the Sequenom Mass Array system that allows analysis of up to 40 SNP alleles in 384 samples per hour for use in barley variety identification.

Transcript profiling tools

Transcriptome analysis is an important tool for gene discovery. We have applied serial analysis of gene expression (SAGE) to identify the genes expressed in germinating barley (White *et al.*, 2006) and to compare the transcriptomes of barley genotypes. The expressed genes identified by SAGE have been used to construct micro-arrays to allow the more cost-effective analysis of the barley transcriptome for larger numbers of genotypes.

Impact of next generation sequencing

The development of new sequencing technologies has generated capacity to undertake barley gene sequencing on a very much larger scale (Wicker *et al.*, 2006). These developments are likely to have a large impact on barley germplasm analysis and barley improvement.

Gene diversity in wild barley populations

The diversity of individual genes in wild barley populations has been studied using conventional DNA sequencing (Bundock

and Henry, 2004). Genomic approaches to analysis of gene diversity in barley germplasm are now likely using the newly available technologies.

Applications to analysis of global barley diversity

SSR analysis has been applied to the analysis of a large set of barley landraces representing global diversity (Jilal *et al.*, 2008). This work is now being extended to analysis of SNP in candidate genes for food quality.

Diversity of wild barley in relation to climate

The impact of climate change on biodiversity is of specific concern in relation to the potential loss of diversity in the wild relatives of major crop species. Barley was probably the first crop to be domesticated and was derived from the wild barley, *Hordeum spontaneum*. Barley seeds contain an abundant protein, the bifunctional amylase/subtilisin inhibitor (BASI) that provides apparent defense of the seed against fungi. We recently found that diversity of the *Isa* locus was related to water availability in the environment of wild barley populations (Cronin *et al.*, 2007). More recently, we have undertaken analysis of diversity of other genes in these populations. Proline accumulation has been widely linked to abiotic stress tolerance in plants. Two genes encoding betaine aldehyde dehydrogenase (BAD) associated with proline metabolism are found in plants. These proteins act as aldehyde dehydrogenases and may have separate or multiple functions in the pathway of proline metabolism (Bradbury *et al.*, 2005). BAD expression has been shown to provide salt tolerance in barley. Abiotic stress tolerance has also been associated with alcohol dehydrogenase (*adh1*). The diversity of these genes and of other biotic stress genes, *rpg1* and a homologue of this gene, revealed considerable variation in diversity.

Analysis of diversity at loci determining grain quality

The genetics of traits important for both feed and malting quality of barley are being defined and the variation of these traits in barley germplasm has traditionally been assessed by phenotypic analysis (Fox *et al.*, 2006a, b). The variation in starch properties (Waters and Henry, 2007; Shapter *et al.*, 2007) and the genes that control them in barley are not well characterized in relation to end uses, especially for human food. The rapid developments in molecular technologies are likely to greatly advance our understanding of these traits in the near future. A genome sequence would be a key tool for barley breeding, as illustrated by the use of the rice genome sequence to easily identify desirable alleles of important genes for breeding.

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Genetic diversity and population structure of WANA barley

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Abstract

Genetic diversity and relationships within and among 185 accessions of wild and cultivated barley from six countries in West Asia and North Africa (WANA) was studied with 36 Simple Sequence Repeats (SSR) markers. All SSR markers used in this study were highly polymorphic and in all subspecies/origin (S/O-) groups. Analysis of molecular variance revealed a general high level of genetic variation in wild as well as landraces of barley. Wild barley S/O-groups showed higher genetic diversity and allelic richness than the cultivated barley. Population structure analysis for all accessions identified eight main groups, which was partly in agreement with subspecies and geographical distributions. Two groups were predominantly cultivated barley, one composed mainly of Near Eastern barley accessions and the second containing mostly North African barley. The remaining six groups comprised primarily wild barley accessions. Three of these six clusters consisted purely of wild barley accessions. Hybridization and introgression between wild and cultivated barley were identified in both directions. Structure analysis, together with Nei's genetic distance and Multi Dimensional Scaling, revealed that WANA cultivated barley, especially that from the Fertile Crescent, did not undergo a strong bottleneck during domestication.

Introduction

In order to breed and improve barley cultivars for future needs, it is very important to search for new sources of useful variation and to understand the relationship among cultivated and wild barley populations, focusing on the centers of barley domestication and diversification. Centers of domestication have an enormous value for providing new genetic resources that can be very useful for existing or upcoming biotic or abiotic threats due to climate changes. The Fertile Crescent in West Asia, with its arid and semi-arid climate and high agro-ecological diversity, is a center of domestication and diversification for many important crop plants. Consequently, in the Fertile Crescent, wild relatives of crop plants such as the progenitor of cultivated barley, *Hordeum vulgare* subsp. *spontaneum* (hereafter referred to as *H. spontaneum*) are widely distributed (Harlan and Zohary, 1966). It is widely assumed that cultivated barley was domesticated in the Fertile Crescent about 8 000–12 000 years Before Present (BP) from brittle two-row barley similar to the present-day *H. spontaneum* (Badr *et al.*, 2000; Salamini *et al.*, 2002; Turpeinen *et al.*, 2003; Zohary, 1999; Zohary and Hopf, 2000). However, further centers of domestication for barley, mainly the Himalaya region, Morocco and the Horn of Africa, have been postulated (Bekele, 1984; Molina-Cano *et al.*, 1987; Orabi *et al.*, 2007).

Table 1. Country of origin, subspecies, S/O-group, sample size and the sources of barley accessions.

Country of origin	Species	S/O-group	No. of lines	Source
Palestine	<i>H. spontaneum</i>	HsPa	98	Copenhagen University
Syria	<i>H. spontaneum</i>	HsSy	10	ICARDA genebank
Jordan	<i>H. spontaneum</i>	HsJo	10	ICARDA genebank
Turkey	<i>H. spontaneum</i>	HsTk	9	ICARDA genebank
Morocco	<i>H. spontaneum</i>	HvMo	8	Dr Molina-Cano, IRTA, Lleida, Spain
Syria	<i>H. vulgare</i>	HvSy	10	ICARDA genebank
Jordan	<i>H. vulgare</i>	HvJo	10	ICARDA genebank
Turkey	<i>H. vulgare</i>	HsTk	10	ICARDA genebank
Tunisia	<i>H. vulgare</i>	HvTn	10	ICARDA genebank
Morocco	<i>H. vulgare</i>	HsMo	10	ICARDA genebank

The aim of this investigation was to study genetic variation between and among WANA barley S/O-groups. A further aim was to detect gene flow between wild and cultivated barley.

Material and methods

Plant materials

In total, 50 landraces of barley belonging to *H. vulgare* and 135 accessions of its progenitor *H. spontaneum* from different countries in WANA were included in this investigation. We grouped the accessions according to their respective species (*H. vulgare* or *H. spontaneum*) and their country of origin into subspecies/origin (S/O) groups (Table1).

DNA extraction, PCR and genotyping

DNA was extracted from barley seedlings by CTBA according to Saghai-Marroof *et al.* (1984), with minor modifications. PCR reactions were carried out for all lines with 34 Simple Sequence Repeats (SSR) primers labeled with different fluorescent dyes. The PCR products were separated and visualized using a semi-automated ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Statistical analysis

The gene diversity (GD) of each SSR was calculated using GENALEX 6 software (Peakall and Smouse, 2006) according to the formula developed by Nei (1973). The molecular variation (AMOVA) among individuals within and among groups of accessions was also calculated by GENALEX 6. Differentiation among S/O-groups was conducted according to Nei's genetic identity for each pair-wise combination of S/O-groups. STRUCTURE 2.2 (Pritchard *et al.*, 2007) was used to identify the number of groups ($K = 1$ to 10) over 20 runs and with a length of burn-in and Markov chain Monte Carlo (MCMC) of 10 000 each. The best appropriate number of groups (K) was calculated based on Evanno *et al.* (2005) using an ad hoc statistic ΔK . To confirm whether WANA barley clustering was based on special criteria such as geographical distribution or classification, Multi Dimensional Scaling (MDS) was calculated by NTSYS-pc 2.1 software (Rohlf, 2000).

Results and discussion

AMOVA among and within groups was calculated based on seven setups starting from a general setup including all S/O-groups to a setup analyzing each S/O-group

separately (Table 2). The division of all accessions based on the species explained the lowest percentage of the molecular variance. Partitioning the wild barley according to origin explained a higher percentage of molecular variance (12%) than doing the same for landraces. Dividing the Moroccan barley into landraces and wild barley resulted in the highest percentage of explained variation (16%) of all AMOVA in this investigation.

MDS of WANA barley showed a clear separation between *H. spontaneum* and *H. vulgare* (landrace) accessions (Figure 1). The geographical origin did not seem to influence the clustering. Almost all wild barley lines clustered on the right half of the MDS while the majority of the landraces were distributed on the left half and closer to the center of the MDS. Three landraces from Jordan, Syria and Turkey spread at the right half or the center of the MDS, together with wild barley accessions (Figure 1).

Structure analysis of the WANA barley resulted in eight groups. Two groups, 7 and 8, are composed exclusively of 25 and 23 HsPa lines, respectively. Group number 4 is predominantly composed of wild barley (22 line accessions out of 25). The three other accessions are landraces from Turkey and Syria that show multilocus structures similar

to the wild barley. Both groups 2 and 3 include one single landrace from Jordan, apart from wild barley accessions. Cluster 5 and 6 contain the majority (45 out of 50) of landraces. Interestingly, the majority of the Fertile Crescent landraces were grouped in group 6, while North African landraces were grouped in group 5.

A clear separation between wild and cultivated barley was found based on MDS (Figure 1) as well as based on the structure analysis. Structure analysis grouping showed two pure *H. spontaneum* groups (7 and 8) and two predominantly *H. vulgare* groups (5 and 6) (Figure 2). A similar strong separation between *H. vulgare* and *H. spontaneum* has been also seen by Badr *et al.* (2000). Such clear separation between *H. spontaneum* and *H. vulgare* led Wei *et al.* (2005) in a study of a global population composed of 506 cultivated and wild barley accessions to conclude that *H. vulgare* and *H. spontaneum* are two different species. However, crosses between *H. spontaneum* and *H. vulgare* are easily made, and hybrids are not uncommon in natural habitats (Giles and von Bothmer, 1985; Harlan and Zohary, 1966). Thus, the high inter-fertility between *H. vulgare* and *H. spontaneum* together with the relatively high out-crossing rate (0–1.8%) (Abdel-Ghani *et al.*, 2004) and the coexistence

Table 2. Analysis of molecular variance (AMOVA).

Accessions	Grouping criterion	Among Groups			Within Groups		
		DF	SS	%	DF	SS	%
All	S/O-groups	9	342.3	10	175	2524.3	90
All	subspecies	1	94.1	6	183	2885.0	94
All without HsPa	subspecies	1	40.9	4	85	1287.6	96
Landraces	S/O-groups	4	97.2	8	45	602.2	92
Wild barley without HsPa	S/O-groups	3	98.4	12	33	470.3	88
All	countries	3	105.0	7	73	1074.8	93
Syrian	S/O-groups	1	28.9	9	18	263.3	91
Jordanian	S/O-groups	1	27.8	7	18	277.4	93
Turkish	S/O-groups	1	28.1	9	17	249.3	91
Moroccan	S/O-groups	1	28.7	16	16	168.2	84

DF = Degrees of freedom; SS = sum of squares.

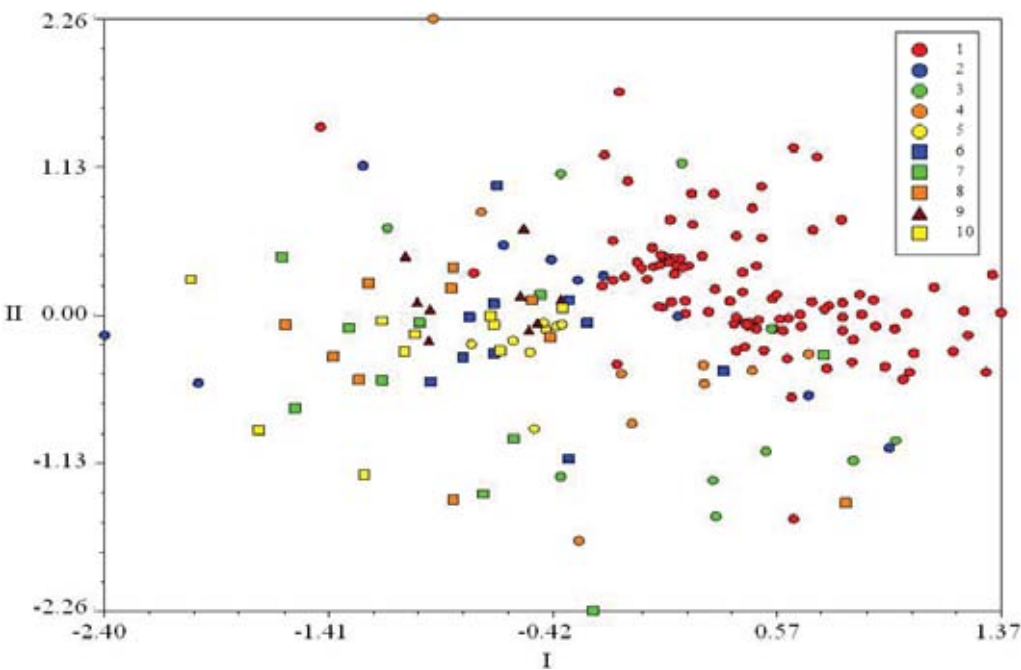


Figure 1. Multi-dimensional scaling (MDS) of WANA barley.
Key: 1 = HsPa, 2 = HsSy, 3 = HsJo, 4 = HsTk, 5 = HsMo, 6 = HvSy, 7 = HvJo, 8 = HvTk, 9 = HvTu, 10 = HvMo.

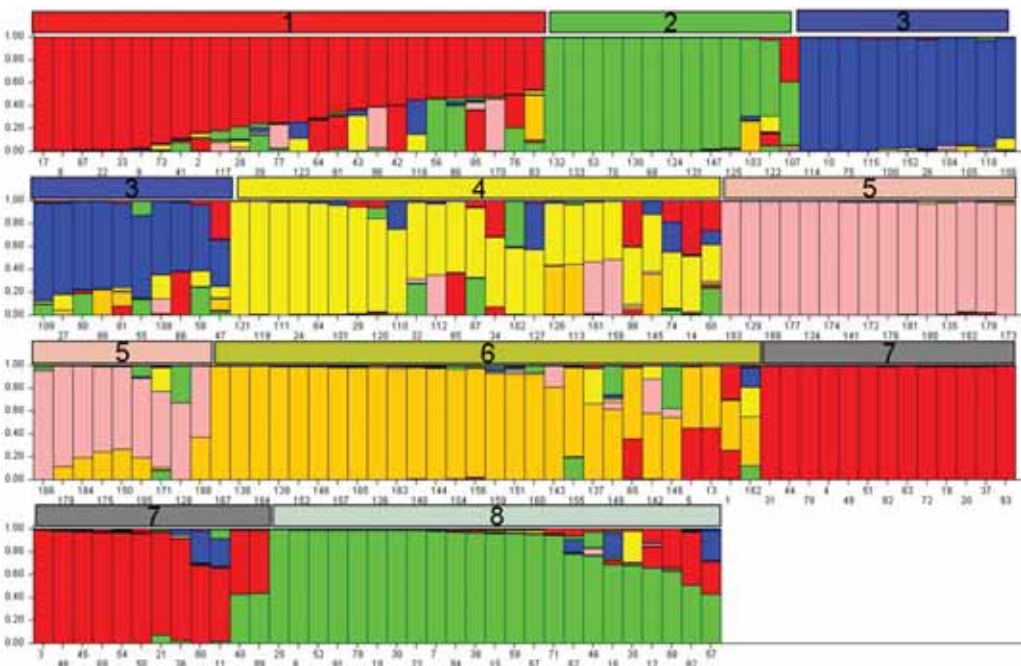


Figure 2. Structure analysis of WANA barley showing 8 groups (Number in the boxes above the bar indicate the group number).

of both species give the WANA region additional value as a source of valuable genetic diversity. The WANA region presents a vast in situ conservation region for barley, allowing dynamic evolutionary processes to continuously interact and produce useful variation. This dynamic evolutionary process can be seen at the species level or in the interaction between *H. spontaneum* and *H. vulgare* as gene-exchange between wild and cultivated barley. The adaptation is the most important trait for surviving in harsh climates. Harlan and Zohary (1966) reported an introgression between wild barley and cultivated barley mainly when the Bedouins cultivate a little barley in the wadi bottoms in good years, where hybrid swarms between wild and cultivated barley can be found. Thus, introgression is possible and may lead under relatively high outcrossing rates to genotypes in the cultivated barley that have a multilocus structure similar to the wild barley, as has been found in lines from Jordan in groups 2 and 3, or lines from Syria and Turkey in group 4 (Figures 1 and 2), or vice versa, with wild barley lines similar to cultivated barley lines in group 6. Moreover, three *H. vulgare* lines were grouped with the *H. spontaneum*, sharing the same multilocus structure with *H. spontaneum*. These three lines might be a result of recent domestication or a result of intercrosses between wild and cultivated barley. Interaction enriches the genetic diversity of the barley population, allowing genes to exchange as required between *H. spontaneum* and *H. vulgare* and provides continuously new sources of genetic diversity that can be used to improve yield under traditional low-input agricultural systems. Consequently, WANA barley was and still is a very useful source of genes to overcome actual or coming challenges for biotic and abiotic threats.

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Variation of agronomic traits in Nordic and Baltic spring barley

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Abstract

The variation of agronomic traits in barley from Nordic and Baltic countries was studied, aiming at investigating differences in agronomic traits and trait stability of modern Nordic and Baltic barley cultivars compared to those grown at the beginning and in the middle of the 20th century. The material studied represented barley from different breeding periods, including landraces and cultivars from the 1890s up to modern varieties and breeding lines. The variation in harvest index, plant height, thousand-kernel weight, volumetric weight, heading and maturity date was recorded for 128 two-row and 68 six-row accessions across six environments (3 locations and 2 years). The agronomic data distinguished very well the two-row and six-row types, as well as the cultivars representing different breeding periods.

Introduction

Plant breeding in the Nordic and Baltic countries has, to a large extent, been focused on crosses between a few original lines, supplemented by the use of exotic material

mainly as donors of disease resistance genes (Fischbeck, 1992). This causes concern about the increasing narrowing of the elite gene pool (Melchinger *et al.*, 1994), which could have negative implications for maintaining rates of gain by selection. The material included in this study has been previously analyzed with DNA markers (ISSR and SSR) (Kolodinska-Brantestam *et al.*, 2004; 2007). All marker systems indicated changes in variability and also some changes in allele distribution and composition. In some groups of material these changes were more pronounced compared with others, e.g. depending on country of origin, row type and geographical region.

Many agronomic traits are economically important and the knowledge of their variation is of great value to the breeder, and this is why it is important to study changes in these traits (Atanassov *et al.*, 2001). Earlier studies have shown that there are changes in agronomic characters that have occurred due to the breeding process in barley, such as reduced plant height and lodging susceptibility, and increase in harvest index. Since quantitative traits are influenced by environmental factors, evaluations need

to be carried out over several years (Wych and Rasmusson, 1983; Bulman *et al.*, 1993; Jedel and Helm, 1994; Ortiz *et al.*, 2002; Nurminiemi *et al.*, 2002).

The aim of this study was to analyze differences in agronomic traits and trait stability of modern Nordic and Baltic barley cultivars compared with barley material grown at the beginning and in the middle of the 20th century.

Materials and methods

In total, 128 two-row and 68 six-row accessions representing different breeding periods were included in the experiment (Table 1). The plant material was provided by the Nordic Gene Bank (effective 1 January 2008: the Nordic Genetic Resources Center), genebanks in the Baltic countries and plant breeding companies in Nordic and Baltic countries (Boreal Plant Breeding Ltd. in Finland; Planteforsk Kvithamar Research Centre in Norway; Svalöf Weibull AB in Sweden; The Abed Foundation and Sejet Plant Breeding in Denmark; Priekuli and Stende Plant Breeding Stations in Latvia; Jõgeva Plant Breeding Institute in Estonia; Lithuanian Institute of Agriculture in Lithuania). The size of each plot was ~1.5 m²

with ~200 seeds per plot (6 rows). The trials were performed over two years, 2002 and 2003, at three locations: Bjørke (southern Norway, 60°47' N, 11°13' E), Landskrona (southern Sweden, 55°52' N, 12°51' E) and Priekuļi (Latvia, 57°19' N, 24°20' E) (Table 2).

Agronomic traits

Six agronomic traits were scored: days to heading, days to maturity, plant height, harvest index, volumetric weight and thousand-kernel weight. At all sites, days to heading was measured as the number of days from 1st of June until 50% of inflorescence emergence in each plot. Similarly, days to maturity at all sites was measured as the number of days from 1st of July until 50% of the spikes were mature in each plot. Plant height was measured in centimeters from the soil surface to the tip of the inflorescence of the most typical sample per plot (excluding awns). Harvest index was calculated as the ratio between the total grain yield from the plot (excluding border plants) and the above ground biomass yield after plants had dried. Volumetric weight was measured as the weight of grain contained in a 0.25 L cylinder and converted to kilograms per hectolitre.

Table 1. Number of two- and six-row spring barley cultivars from different breeding periods and countries of origin.

Origin	Period and type						Total
	Landraces and cultivars before 1930		Cultivars 1931-1970		Cultivars after 1971 and breeding lines		
	six-row	two-row	six-row	two-row	six-row	two-row	
Denmark	5	6	0	8	0	10	29
Estonia	0	0	1	1	2	16	20
Finland	5	4	5	4	11	3	32
Latvia	1	1	1	1	1	19	24
Lithuania	1	1	0	2	0	16	20
Norway	8	0	6	3	13	0	30
Sweden	5	7	3	9	0	17	41
Nordic and Baltic	25	19	16	28	27	81	196

Table 2. Meteorological characteristics of Baltic and Nordic environments included in the experiment, and time of sowing.

Location	Year	Sowing week	Rainfall (mm)				Average temperature (°C)			
			May	June	July	August	May	June	July	August
Bjørke	2002	17	82.9	67.4	128.5	136.5	11.7	15.2	16.0	18.4
	2003	21	75.1	85.7	67.1	53.2	9.1	14.9	17.6	15.1
Landskrona	2002	13	53.5	75.3	76.4	68.9	13.1	16.0	17.8	20.0
	2003	15	70.7	40.4	76.0	45.7	12.2	16.3	18.6	18.0
Priekuļi	2002	17	38.2	111.1	67.0	11.3	13.5	16.3	19.5	18.9
	2003	19	88.0	59.2	87.8	175.8	11.9	14.0	19.7	15.7

The moisture content of grain was on average 8% (2002) and 9% (2003) in Bjørke, 10% in Priekuļi, and 12% in Landskrona. The moisture content of the straw was 5% in Bjørke and 10% in Priekuļi and Landskrona. Statistical analysis

To reduce the experimental error (Yau, 1997; Sarker *et al.*, 2001) within each trial, a 14×14 square lattice design (Cochran and Cox, 1957) with two replications was chosen as experimental layout. The adjusted means for the lattice design were calculated (Kotz and Johnson, 1983) using MATLAB (version 5.3, MATHWORKS Inc. 1999) and the adjusted treatment means were used in further calculations. The cultivars were classified using principal component analysis (MINITAB Release14 Statistical Software). Two-row and six-row cultivars were analyzed separately since the inflorescence type bears a relationship to multiple agronomic traits in barley (Kjær and Jensen, 1996; Marquez-Cedillo *et al.*, 2001). The environmental stability of the accessions was calculated using regression analysis (Eberhart and Russel, 1966).

Results

Changes of trait values

When modern material was compared with material from earlier breeding periods, different trends were observed for the six traits (Figure 1). Harvest index was higher

in modern material compared with material released before 1930, and with landraces in both six-row and two-row Nordic and Baltic barley; plant height, as expected, significantly decreased during the course of plant breeding. Thousand-kernel weight, volumetric weight, days to heading and days to maturity did not change significantly during the breeding period studied (Figure 1).

Harvest index of six-row and two-row materials showed no significant differences within the same breeding period. The same was also true for plant height (Figure 1). Two-row cultivars from all breeding periods had, on average, higher thousand-kernel weight and volumetric weight, and later heading and maturity than six-row, and the differences were significant.

Principal component analysis was conducted for each trial separately (Figure 2). In each trial the first component separated fairly well into six-row and two-row, explaining 41% to 59% of the variation, depending on the trial. The second component explained 23% to 29% of the variation and distinguished the old cultivars from the modern ones in all trials.

Environmental stability

In modern material, the most common types of cultivar are stable, whereas in the cultivars before 1930 and in landraces, the most common are responsive types for harvest

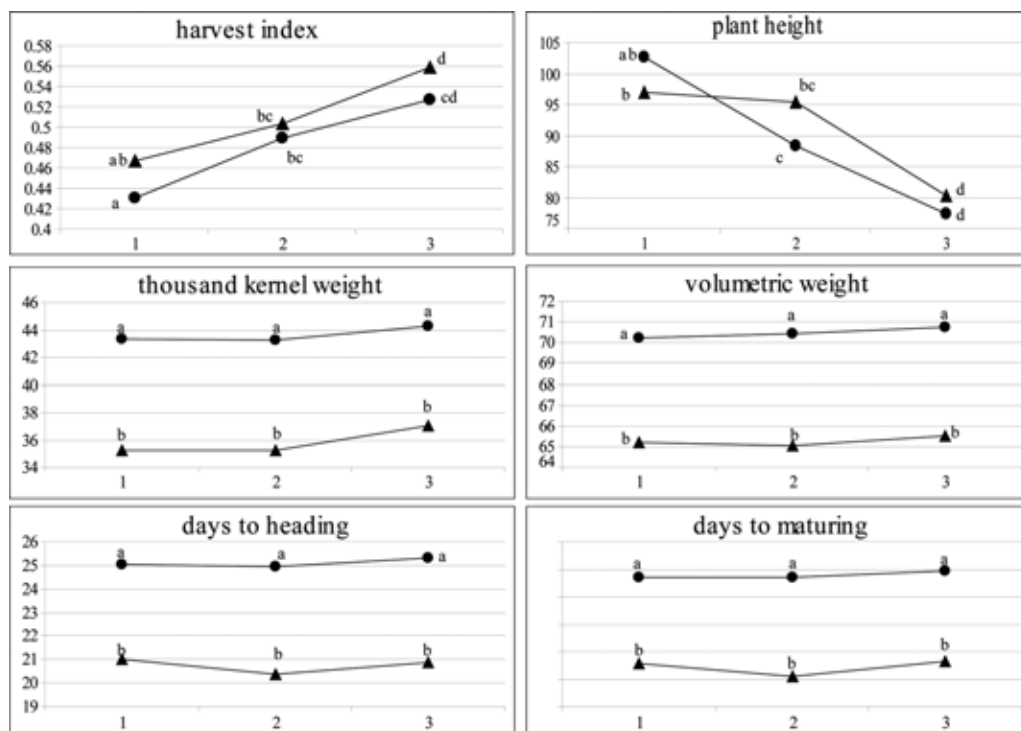


Figure 1 Thousand-kernel weight, volumetric weight, days to heading and days to maturity in cultivars from different breeding periods: landraces and cultivars before 1930 (1), cultivars 1931-1970 (2), cultivars after 1971 and breeding lines (3); ▲ - six-row barley, ● - two-row barley; comparisons between all groups are made at $p < 0.05$, values designated by the same letter did not differ significantly.

Table 3. Percentage of cultivars in different breeding periods with different types of response to environmental changes based on regression analysis: stable ($bi < 1$) or responsive ($bi > 1$) cultivars to environmental changes ($p < 0.05$).

Trait	Type	Landraces and cultivars released before 1930		Cultivars released 1930–1970		Cultivars released after 1971 and breeding lines	
		Stable	Responsive	Stable	Responsive	Stable	Responsive
Harvest index	Two-row	10	63	36	43	72	13
	Six-row	20	72	6	75	52	30
Plant height	Two-row	63	10	14	14	41	38
	Six-row	40	12	50	19	7	59
Volumetric weight	Two-row	68	10	36	36	57	22
	Six-row	20	64	12	75	26	37
Thousand-kernel weight	Two-row	16	42	4	57	18	46
	Six-row	84	4	69	13	70	4
Days to heading	Two-row	26	5	21	0	17	3
	Six-row	8	24	0	56	0	37
Days to maturity	Two-row	26	5	21	0	17	3
	Six-row	8	24	0	56	0	37

index. This is true for both two-row and six-row cultivars. For plant height, the proportion of cultivars expressing stable environmental response has decreased over time. In modern six-row barley material, the proportion of responsive cultivars reaches 59%.

Significant differences in the environmental stability of harvest index

were found between two-row and six-row cultivars within breeding periods. Six-row cultivars showed a higher proportion of stable cultivars.

A higher proportion of cultivars with stable response to the environment for volumetric weight was found in two-row barley than in six-row barley. For thousand-kernel weight,

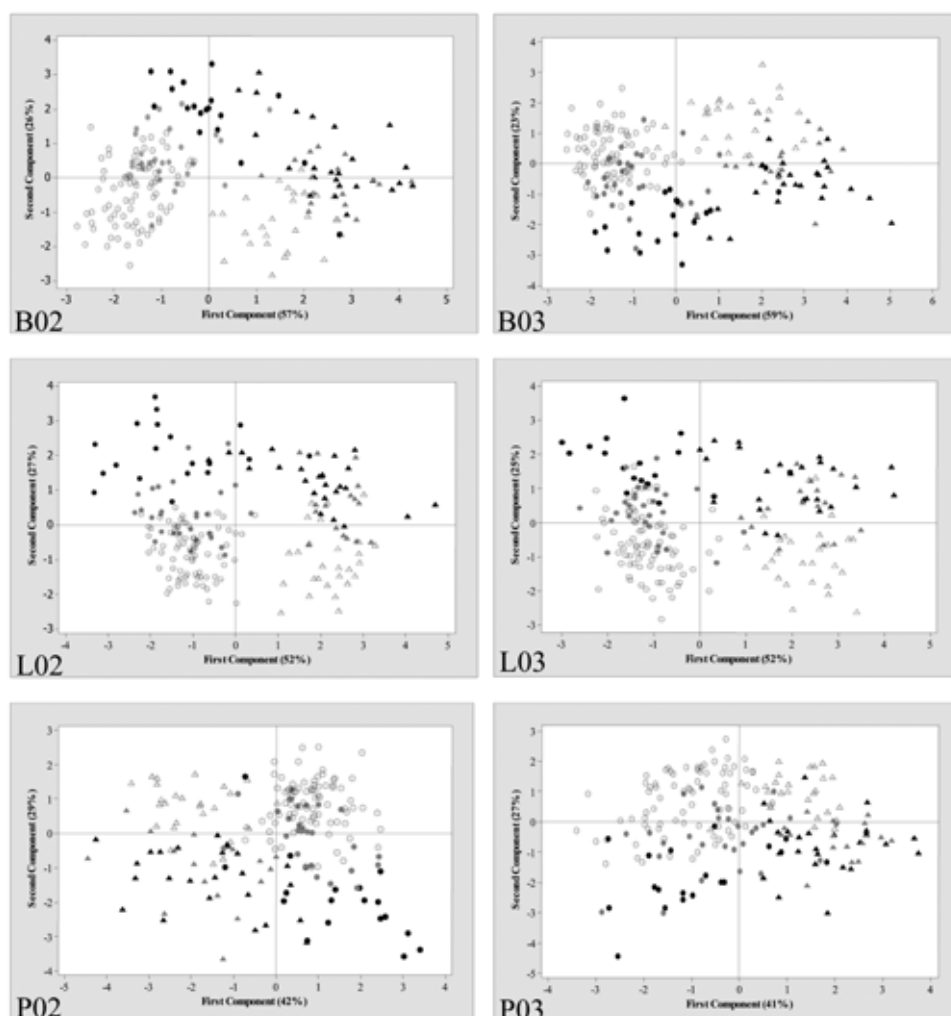


Figure 2. Biplots of first two principal components. Plots B02 – Bjørke 2002; B03 – Bjørke 2003; L02 – Landskrona 2002; L03 – Landskrona 2003; P02 – Priekuļi 2002; P03 – Priekuļi 2003. Symbols for accessions are ● – two-row landraces and cultivars before 1930; ● – two-row cultivars 1931-1970; ○ – two-row cultivars after 1971 and breeding lines; ▲ – six-row landraces and cultivars before 1930; ▲ – six-row cultivars 1931-1970; △ – six-row cultivars after 1971 and breeding lines.

the opposite was true, as the majority of six-row cultivars expressed a stable response. For days to heading and days to maturity, more cultivars showing a responsive behavior could be found in six-row barley compared with the two-row barley material.

Discussion

The partitioning of material according to the breeding periods could not be shown using pooled molecular data, only when countries were analyzed separately (Kolodinska-Brantestam *et al.*, 2004, 2007). Using pooled agronomical data, this partitioning was evident (Figure 2). Modern and old cultivars are mainly distinguished by plant height and harvest index (principal component analysis). This is expected since the harvest index and plant height have changed significantly during the breeding process, as shown in this study and elsewhere (Boukerrou and Rasmusson, 1990; Ortiz *et al.*, 2002). For the modern Nordic barley, genetic gains also have been detected (Ortiz *et al.*, 2002; Öfversten *et al.*, 2004).

A change in stability of harvest index and plant height has also taken place between different breeding periods. Modern cultivars express a more stable harvest index parameter, but vary more in plant height response to environment compared with old cultivars and landraces. The modern short-straw cultivars were easily detected, with stable and high harvest index values and stable plant height parameters. These cultivars have a potential for future plant growing and breeding in a changing climate and also for ecological farming.

The differentiation between two-row and six-row barley cultivars shown in this study (Figure 1) is in agreement with our previous studies of molecular markers on the same material (Kolodinska-Brantestam *et al.*, 2004; 2007). Others have also reported similar differentiation, not only in phenotypic traits (Lasa *et al.*, 2001) but also

in molecular parameters (Ordon *et al.*, 2004). The distinction between two-row and six-row cultivars shown in this study (Figure 2) is mainly explained by differences in days to heading, days to maturity, volumetric weight and thousand-kernel weight (principal component analysis). This is not surprising, as volumetric weight and thousand-kernel weight are strongly influenced by row type.

According to Marquez-Cedillo *et al.* (2001), the *vrs1* locus on chromosome 2 (2H), which determines row type, coincides with the largest-effect QTL determining volumetric weight. Hori *et al.* (2003) found QTLs for thousand-kernel weight in the marker interval *vrs1* and microsatellite Bmag125. Six-row cultivars are mainly grown in the northern parts of the region. Here, typically early maturing cultivars are grown. Days to maturity and days to heading are highly correlated characters ($p < 0.001$). The differences between two-row and six-row cultivars were also seen with respect to environmental stability. Differences between two-row and six-row barleys in their reaction to the growing conditions were also been reported by Nurminiemi *et al.* (1996).

Nowadays, six-row cultivars are mostly grown in the northern part of the Nordic countries, whereas in the southern part and in the Baltic countries almost only two-row barleys are found. This suggests that photosensitivity could have played a role in the responsive reaction of days to heading and days to maturity of six-row barley cultivars, making them a better choice for barley growing in the north.

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Developmental mutants as a guide to the barley phytomer

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Abstract

Morphological mutants expose variations in the phytomeric system (basic structural units) of barley (*Hordeum vulgare* L.) to observation and analysis. To detect phytomeric mutants, visual assessment of mutant families in the 'Optic' barley mutation grid population was conducted at various growth stages using laboratory, glasshouse and field screens. Barley mutants present in other collections, those described in literature reports, and descriptions of grass ontology (development of plant structures) were evaluated also. The origins of vegetative and generative structures of the barley plant can be explained by a single repeating phytomer. Each phytomer can consist of a half-node with bud and root initials on opposite sides, an internodal region, and a second half-node with side arms attached. The side arms can be divided into two classes, single or paired, with some paired side arms fused. The combination of developmentally distinct phytomers present in axillary buds leads to a 2 + 1 association of side arms or bracts. The underlying structure is a phytomeric triad composed of three phytomers and is basic to barley ontology and to understanding the architecture of the inflorescence, flower, endosperm and embryo.

Introduction

Rutishauser and Sattler (1985), in their review of previous studies, describe the plant as formed from repetitive units with a stem and leaf model being most widely accepted. Development of the phytomer concept was reviewed and extended by Bossinger *et al.* (1992). The repeating unit in higher plants is called a phytomer and has a specific spatial arrangement of meristematic regions that give rise to an ordered development of organs. Different organs such as floral organs arise as developmental variants from the same phytomer. Therefore, the phytomer concept is basic to understanding plant development and systematics.

The barley phytomer

Barley (*Hordeum vulgare* L.) was chosen by Bossinger *et al.* (1992) to expand the phytomer concept in monocots because it has a relatively simple, distichous arrangement of leaves and spikelets produced on opposite and alternating sides of vegetative stems and floral axes, respectively. For a detailed description of barley morphology and anatomy see Reid (1985). Barley also has an advantage in that it is a diploid ($2n = 14$) and many developmental mutants are available for study. Bossinger *et al.* (1992)

presented an elaborate model explaining development of the phytomeric system for vegetative and generative structures. Two phytomer types were proposed: type 1, which are produced in series (one on top of the other), and type 2, which develops as a branch from an axillary bud and produces a sheath-like structure with two prominent veins. Bossinger *et al.* (1992) generated a model in which the architectural units of the stem, tiller (stem branch), rachis, rachilla and floret are formed by various combinations of these phytomers. The phytomeric structure of barley published in the model of Bossinger *et al.* (1992) has a number of weaknesses; the embryonic structure was not considered, suppression of phytomeric regions was not explained clearly, and origins of floral units and reproductive organs were not examined in detail. Phenotyping of new induced mutants has identified additional and novel morphological mutants that can shed more light on the structure of the barley phytomer (Forster *et al.*, 2007). The results of this study were the basis for development of a single phytomer model (Figure 1).

The nodal structure of barley (and other grasses) is critical with respect to grass architecture as all structures arise from nodes. They link phytomers together to form axes of vegetative and reproductive structures (Bossinger *et al.*, 1992). The node is composed of two segments or half-nodes. Phytomers of the stem (culm), rachis and rachilla are connected together by a half-

node at each end. Individual phytomers are oriented at about 180° with respect to the one below it. Elongation of internodes determines the stature of the plant and the spatial arrangement of buds and branches. Bud and root initials can arise only from the half-node at the base of the phytomer, the upper half of a node. As described by Bossinger *et al.* (1992), the half-node at the top of the type 1 phytomer, the lower half of a node, gives rise to a single side arm. Contributions of two phytomers are arranged so that the sheath of the lower phytomer protects the developing organs of the next one.

Bud and branch phytomers

The other link between phytomers is bud or branch formation from nodes. Buds can potentially develop only from the nodal segment at the base of each phytomer. The first phytomer of the bud is highly modified and was called a type 2 phytomer by Bossinger *et al.* (1992). The first phytomer of the bud is composed of only an internodal type of segment and the upper half-node from which two side arms can arise (Figure 2). It has no potential for formation of root or axillary bud initials. Another phytomer can form only from the apical meristem of the half-node. The second phytomer is structurally complete, type 1 according to Bossinger *et al.* (1992), and has the possibility of developing bud and root initials from its lower half-node and a single side arm from its upper half-node. The bracts attached to this association

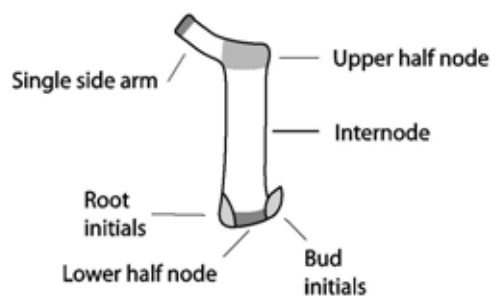


Figure 1. The basic phytomer of the barley plant.

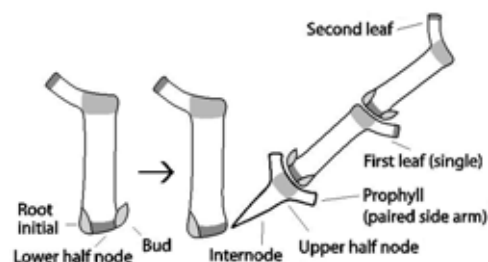


Figure 2. A diagram of branch of tiller formation from a bud at the base of the phytomer.

of two phytomers gives rise to a 2 + 1 pattern of side branches, which is described as being responsible for the basic floral structural of barley (Forster *et al.*, 2007).

If this broadened model of the phytomer concept is correct, the plant architecture and many structural mutants of barley should be explained by either normal processes operating during the development of individual phytomers or aberrant control of phytomer development caused by genetic changes. This paper aims at providing additional insights about genetic and structural control of barley ontology.

Materials and methods

A two-row, semi-dwarf, spring barley cultivar Optic was chosen for study as it was a top quality malting cultivar adapted to northwest European conditions. Grain of Optic was mutagenized with ethyl methanesulphonate (EMS). The treated grain is referred to as the M_0 generation and on germination produces M_1 plants. No more than two M_2 grains per M_1 plant were grown on to produce a structured population of over 21 000 M_3 families, as described by Caldwell *et al.* (2004). The M_1 generation was grown under glasshouse conditions. M_2 individual plants, M_3 rows and plots of subsequent generations were generally grown in fields near Dundee, Scotland. The M_3 and subsequent generations were inspected for phenotypic variation during the 2003, 2004 and 2005 seasons. Inspections were conducted at all stages of plant development. Harvested seed and ears were labeled and stored.

When parts of the Optic mutation grid population were grown under laboratory, glasshouse and field plots for visual assessment at various growth stages, notes were taken on mutant types and frequencies. Mutants showing morphological abnormalities were classified as possible phytomeric mutants. Barley morphological mutants present in other collections and those

described in literature reports were evaluated based on field observations and literature descriptions. The 'Bowman' backcross-derived lines (Lundqvist and Franckowiak, 2003) were grown at Dundee, Scotland, in 2005 to determine phenotypic expression of various mutants in this environment. Descriptions of grass anatomy were consulted to aid in the construction of an architecture model of the barley plant.

Results and discussion

The phytomer model has been progressively clarified based on novel morphological mutants detected in Optic barley (see <http://germinate.scri.sari.ac.uk/barley/mutants/>; also see Caldwell *et al.*, 2004), as well as mutants described in the Barley Genetics Stocks Database (BGSD, <http://ace.untamo.net>). To guide refinement of our view of the phytomer, we adopted the simplest explanation for each new organ development pattern observed in mutants. Models that did not agree with the observed phenotypes and literature descriptions of grass anatomy were rejected.

Presence of paired bracts

Tillers and other branches of the barley plant arise as buds that contain a highly specialized first phytomer. This phytomer has an internode segment, two side arms (bracts), and a nodal segment containing a meristematic region at its tip where another phytomer can develop. Conservation of the underlying genetic system suggests that most if not all buds should have a similar highly modified first phytomer. The paired nature of some side arms (e.g. glumes) is obvious, but others were detected only in mutants. An Optic mutant having a split palea was among the first identified, which brought into question the ontology of the palea (Forster *et al.*, 2007). Von Bothmer and Jacobsen (1985) describe the palea of *Hordeum* species as a bifid structure that, along with the lemma,

protects floral parts and, after pollination, the developing caryopsis (a one seeded fruit). The explanation for a split palea is that the normal palea represents two (paired) structures that are fused together at a common edge. The normal fusion mechanism is dysfunctional in this mutant. This assumption is supported by the fact that the normal palea has two keels and awned palea (*adp1*) mutants possess two awns (see Table 1 for references to all quoted phytomer mutants of barley). Also, split and divided paleas are well known in other members of the Triticeae and are botanical descriptors of species such as *Triticum timopheevii* (Zhuk.) Zhuk. and *T. turgidum* L. (Peterson, 1965). Based on this information, Forster *et al.* (2007) concluded that the palea originates from paired side arms (bracts) of the first phytomer of a bud or branch.

If a consistent pattern is followed during vegetative development, other paired structures of the barley plant that originate from buds (e.g. coleoptile, prophyll and glumes) should occur as fused or unfused pairs (Table 2). The second split organ mutant observed in seedlings of the Optic grid population was a split coleoptile (Forster *et al.*, 2007). The coleoptile is a sheath that protects the terminal bud of the embryo (plumule or epicotyl) as it grows during germination. The coleoptile has two mid-ribs and two vascular bundles opposite each other (Arber, 1934) and is thought to originate from two fused structures (Bossinger *et al.*, 1992). This conclusion is supported by the split coleoptile seedling. The semi-dwarf mutant *uzul* also produces two notches at the tip of the coleoptile and may represent a partial split of the coleoptile into two parts. The coleoptile is also regarded as having a similar structure to that of the prophyll (Gould and Shaw, 1983; Guignard, 1962; Jacques-Felix, 1957). The prophyll is the organ that protects the developing axillary shoots (tillers), but no split prophyll mutants have been reported. The auricles and glumes are additional examples of paired bracts that

presumably develop as the side arms of a branch phytomer.

Paired bracts in reproductive structures

Extension of the concept of paired side arms into reproductive structure is more difficult to conceive. The spikelet is subtended by a pair of glumes (an unfused pair) and the floret is subtended by a palea (a fused pair). The next structural pair of side arms is the lodicules. Barley does not have a second pair of lodicules, but several grass species are known to possess two pairs of lodicules, dorsal and anterior pairs, with the dorsal pair being absent in some species (Guedes and Dupuy, 1976; Dahlgren *et al.*, 1985). Thus, the assumption is that formation of the second pair of lodicules is suppressed in barley (Forster *et al.*, 2007). Likewise, development of paired structures below the ovary is probably suppressed. The next structural pair is the styles and their attached stigmas. If the developmental pattern observed in vegetative parts of the plant is reproduced in the floral organs, all of these paired bracts arise from the first phytomer of a branch.

The origin of certain floral organs as side arms of a branch phytomer should be reflected in floral structure mutants. These mutants can cause a conversion of one floral part into another, but the spatial arrangement of floral components is retained generally. The glumes can range in size from normal to lemma-like as determined by alleles of the elongated outer glume 1 (*eog1*) locus. The lodicules of the multi-ovary 1 (*mov1*) mutant become somewhat leafy or sepal-like. Conversion of the lodicules into anthers occurs in the laxatum-a (*lax-a*) mutants, but the extra anthers have two rather than four microsporangia (Bossinger *et al.*, 1992). In the multi-ovary (*mov*) mutants, stamens are partially or completely converted into pistils. Failure of a functional ovary to develop occurs in ovaryless (*ovl*) mutants, while anther development fails in some male sterile genetic (*msg*) mutants.

Table 1. List of barley mutants that alter phytomer development and reference information.

Gene symbol ¹	Gene or locus name/description	Type specimen (where available)
	Aborted seedling	Optic mutant
abr1	Accordion basal rachis internode 1	BGS 472
acr1	Accordion rachis 1	BGS 097
adp1	Awned palea 1	BGS 593
als1	Absent lower laterals 1	BGS 101
Ari	Breviaristatum/short awn and short plants	BGS 132 and others
ari.256	Breviaristatum. 256/short awn, compact spike tip	
ari-k	Breviaristatum-k	BGS 553
asp1	Aborted spike 1	BGS 649
Bra	Bracteatum/leafy bracts on rachis	BGS 586, 619
brc1	Branched 1/branched spike	BGS 613
Brh	Brachytic/dwarf	BGS 001 and others
brh13	Brachytic 13/short, compact spike tip	BGS 656
brh16	Brachytic 16/short, compact spike tip	BGS 044
Btr	Non-brittle rachis	BGS 115 and 116
Cal	Calcaroides/hooded lemma	BGS 146 and others
Com	Compositum/branched spikelets	BGS 071 and 473
cr11	Curly lateral 1	BGS 325
cst1	Corn stalk 1/reduced tillering and height	
cul2	Uniculum 2	BGS 253
cul4	Uniculum 4	BGS 617
dub1	Double seeds 1	BGS 661
Eam	Early maturity/photoperiod responses	BGS 065 and others
eli-a	Eligulum-a/liguleless and small auricle	BGS 623
eog1	Elongated outer glume 1	BGS 057
Flo	Extra floret	BGS 074, 182 and 327
Gra-a	Granum-a/numerous tillers with thin leaves	BGS 131
int-c	Intermedium spike-c/six-row spike modifier	BGS 178
int-h	Intermedium spike-h	BGS 544
int-i	Intermedium spike-i	BGS 545
int-m	Intermedium spike-m	BGS 547
Kap1	Hooded lemma 1/deformed floret on lemma	BGS 152
lax-a	Laxatum-a	BGS 474
lax-c	Laxatum-c	BGS 475
Lbi	Long basal rachis internode	BGS 308 and others
le11	Leafy lemma 1	BGS 235
Lfb1	Leafy bract 1/leafy collar	BGS 343
Lfs	Leafless/variable leaf blade development	
lig1	Liguleless 1	BGS 060
lin1	Lesser internode number 1	BGS 099
Lks	Short awn	BGS 010 and 172
Lks1	Awnless 1	BGS 075
lnt1	Low number of tillers 1	BGS 118
Mnd	Many-noded dwarf	BGS 633 and others
mov1	Multiovary 1	BGS 043
mov2	Multiovary 2	BGS 147
Msg	Male sterile genetic	BGS 357 and others
mul1	Multiflorus 1/supernumerary florets = vrs4	BGS 124
ops1	Opposite spikelets 1/shortened rachis internodes	BGS 624
Ovl	Ovaryless	BGS 176 and 646
Raw	Smooth awn	BGS 312 and others
rtt1	Rattail spike 1	BGS 051
sbk1	Subjacent hood 1/Calcaroides	BGS 062
sca1	Short crooked awn 1	BGS 128
sdw2	Semidwarf 2/collar leaf	BGS 133
seg8	Shrunk endosperm 8	BGS 455
Sil1	Subcrown internode length 1	BGS 228
snb1	Subnodal bract/additional bract on spikelet 1	BGS 026
srh1	Short rachilla hair 1	BGS 321
	Split palea	Optic mutant
tfm1	Thick filament 1	
trd1	Third outer glume 1/additional bract on spikelet	BGS 202
trp1	Triple awned lemma 1	BGS 061
Tst	Tip sterile/smaller spikelets toward spike tip	BGS 636, 647
ubs4	Unbranched style 4	BGS 011
uzu1	Uzu 1/semibrachytic	BGS 102
viv1	Viviparoides 1/plantlets on the spike	BGS 627
vrs1	Six-row spike 1	BGS 006 and others
vrs3	Six-row spike 3	BGS 315
vrs4	Six-row spike 4 = Multiflorus 1	BGS 124
Vrs1.t	Deficiens 1/extremely reduced lateral spikelet	BGS 067

¹ These mutants are described in greater detail in the Barley Genetics Stocks AceDB Database, in Barley Genetics Newsletter Vol. 37, and by personal communication. Gene nomenclature is based on rules given at the 9th International Barley Genetics Symposium (Franckowiak and Lundqvist, 2005).

Table 2. Barley organs classified as developing from single or paired side arms and listed by their nodal origin.

Side arms associated with phytomeric triads of the main axes		Origin of the bud (triad)
Paired side arms	Single side arm	
None	Scutellum (cotyledon)	Unknown (suspensor)
Coleoptile (fused)	First leaf sheath	Cotyledonary node
Prophyll (fused)	Tiller leaf sheath	Stem node
Glumes (2)	Lemma	Rachis node
Palea (fused)	Filament of first stamen	Lemma node, upper half
Lodicules (2)	Filament of second stamen	Anther node, upper half
None, commonly	Filament of third stamen	Anther node, upper half
None	Ovary	Anther node, upper half
Styles (2)	Ovule stalk	Ovary node, upper half
Lateral endosperm lobes	Central endosperm lobe	Unknown
Side arms associated with phytomeric triads of side arms		
Auricles (2)	Leaf blade	Tip of the leaf sheath
None, except <i>Kap1</i> mutants	Lemma awn	Tip of the lemma
Anther lobes (2)	None	Tip of the filament
None	Stigma	Tip of the style
Outer and inner integuments	Nucellus	Tip of the ovule stalk

Phytomeric units based on the 2 + 1 pattern

The concept of a pair of leaf-like organs in vegetative buds was developed by Bossinger *et al.* (1992) and extended by Forster *et al.* (2007) to a 2 + 1 model of side arms. Although paired bracts are observed frequently in the barley plant, paired bracts of vegetative organs are always associated with a single structured bract, e.g. the coleoptile (paired) and the first seedling leaf (single); auricles (paired) and leaf blade (single); and the prophyll (paired) and the first tiller leaf (single). The paired bracts of reproductive organs are often paired with a single bract, e.g. glumes (paired) and lemma (single), the palea (paired) and the first anther (single), lodicules (paired) and the second anther, and vestigial lodicules (paired) and the third anther (Forster *et al.*, 2007). The ovary (single) does not appear to be subtended by a paired structure. Based on drawings of the vascular structure of the wheat ovary by

Batygina (see Lersten, 1987), a pair of styles arise below the ovule.

The bracts in the 2 + 1 association of the prophyll and the first tiller leaf are orientated at 180° to protect the bud initials and expose the root initials (Bossinger *et al.*, 1992). The orientation observed in most other 2 + 1 bract associations in barley seems similar. The single bract is generally larger than the paired bracts in the 2 + 1 association, which probably arises from at least two linked phytomers (Forster *et al.*, 2007).

The 2 + 1 association of the phytomeric side arms in buds arises from a specific combination of three developmentally different phytomers (Figure 3). The first phytomer in this phytomeric triad has only an internode and two side arms attached to the upper half-node. The second phytomer has a lower half-node from which bud and root initials can form, an internode, and an upper half-node with a single side arm attached. The third phytomer of the triad has only a lower

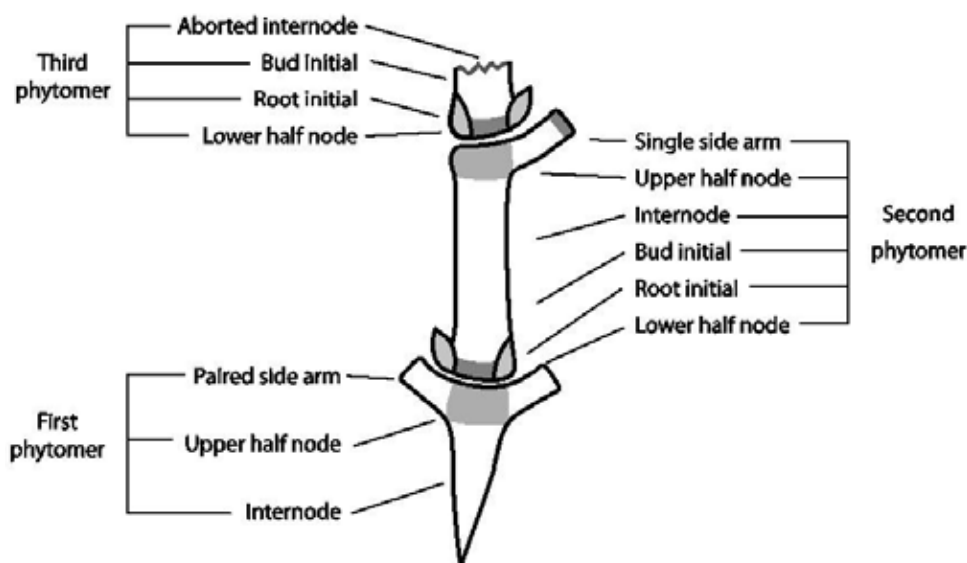


Figure 3. The basic phytomeric triad of the barley plant.

half-node from which a bud can develop. Theoretically, the bud at the lower half-node of the second phytomer can expand, but rarely does. Repeated bud formation from the lower half-node of the third phytomer of the phytomeric triad causes a stacking of triads. Stacked phytomeric triads account for the basic architecture of reproductive organs in barley (Figure 4). This stacking pattern for phytomeric triads is preserved in vegetative parts of the barley plant, with one exception. The culm and the rachis are formed when the third phytomer of the triad does not abort and phytomers are added on top of each other by the activity of the apical meristem.

Endosperm and embryo development

If the 2 + 1 arrangement of side arms is basic to the architecture of vegetative and reproductive structures, a similar pattern could also occur in the endosperm and the embryo. The simplest genetic control system probably involves side arm modifications associated with retention of the underlying phytomeric triad configuration. Forster *et al.*

(2007) noted a 2 + 1 association of endosperm lobes in a previously identified mutant. Felker *et al.* (1985) reported that the central lobe in shrunken endosperm genetic 8 (*seg8*) mutant fails to enlarge and kernels are very thin with a distinct dorsal crease in the caryopsis. Since only the lateral lobes enlarge, the *seg8* mutant probably only affects the endosperm lobe that forms slightly later. If these assumptions are correct, the endosperm is constructed from a highly modified phytomeric triad.

Application of the phytomeric triad concept to embryonic structure is more difficult because most embryo mutants are not viable. Low germination percentages were noted in a number of lines in the Optic grid population. The description of the grass embryo by Esau (1977) was used as a guide in relating embryonic development to the phytomeric triad. A club-shaped embryo is observed at five days after pollination with the suspensor or internode of the branch phytomer attached to the vascular system. At day 10, the enlarged upper part starts to form the scutellum or cotyledon that is appressed to the endosperm. Hence, the

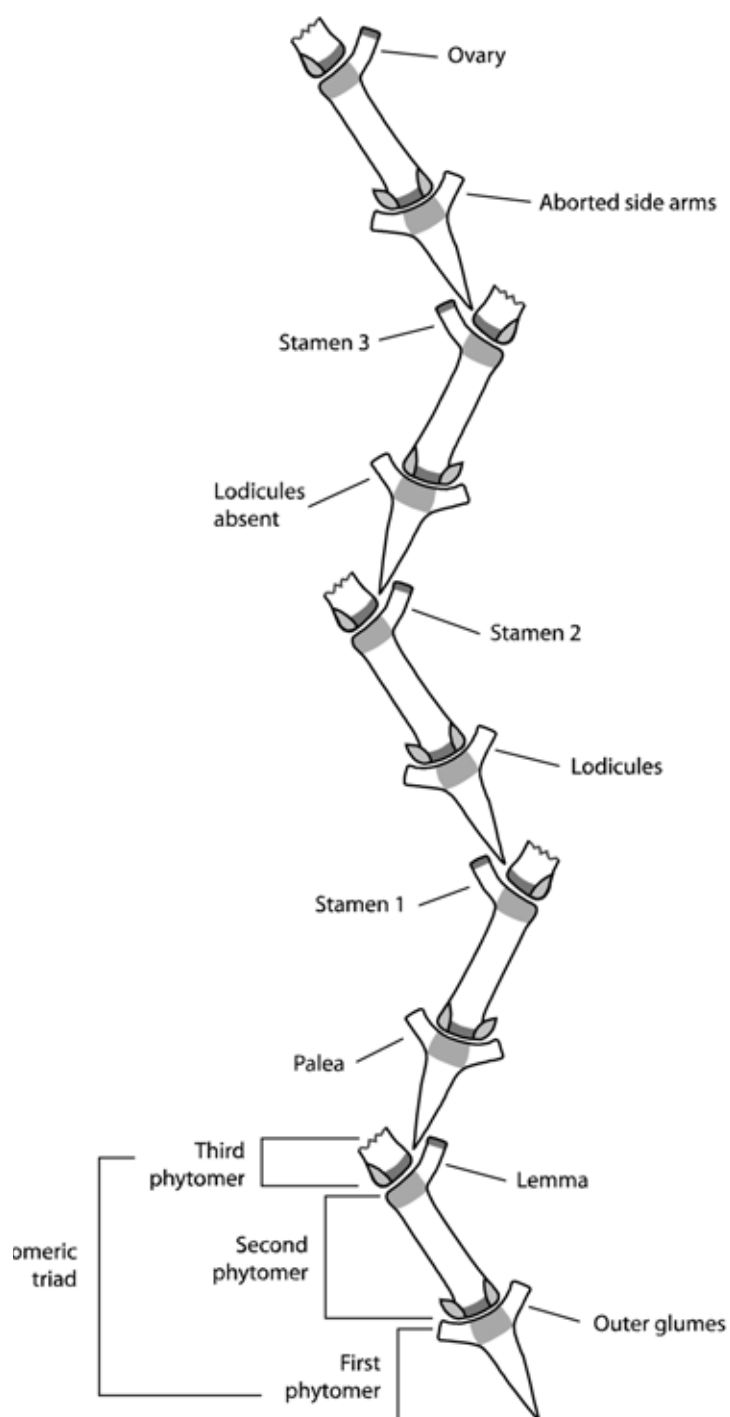


Figure 4. A diagram of stacked phytomeric triads present in floral organs of barley.

scutellum is positionally similar to the single side arm of the second phytomer of a triad. Esau (1977) described the embryo axis as laterally attached to the scutellum. Thus, the epicotyl or plumule corresponds to the bud developing from the third phytomer of the initial phytomeric triad. In this view of embryonic structures, the scutellum is also a single side arm that protects a developing bud. The seminal roots are adventitious and arise below the scutellar or cotyledonary node (Bossinger *et al.*, 1992). The only node from which the radical, and the coleorhiza that protects it, can arise is the lower half-node of the second phytomer of the first embryonic triad (Figure 5). This hypothesis assumes that the embryo develops as a phytomeric triad.

The epicotyl is formed from the second phytomeric triad in the embryo. Its first phytomer produces the mesocotyl (Guignard, 1962; Jacques-Felix, 1957) and the coleoptile, which protects the first seedling leaf (single) and the apical meristem (Figure 5). The internode above the coleoptile forms the sub-crown internode, which elongates in barley to place the crown above the seed position (Bossinger *et al.*, 1992). If the seed is planted very deep, the internode of the next phytomer may elongate also. The other extreme is suppression of the sub-crown internode elongation by the *Sill* gene in some winter barley cultivars. The phytomeric triad model of embryo structures requires a number of regulatory events to

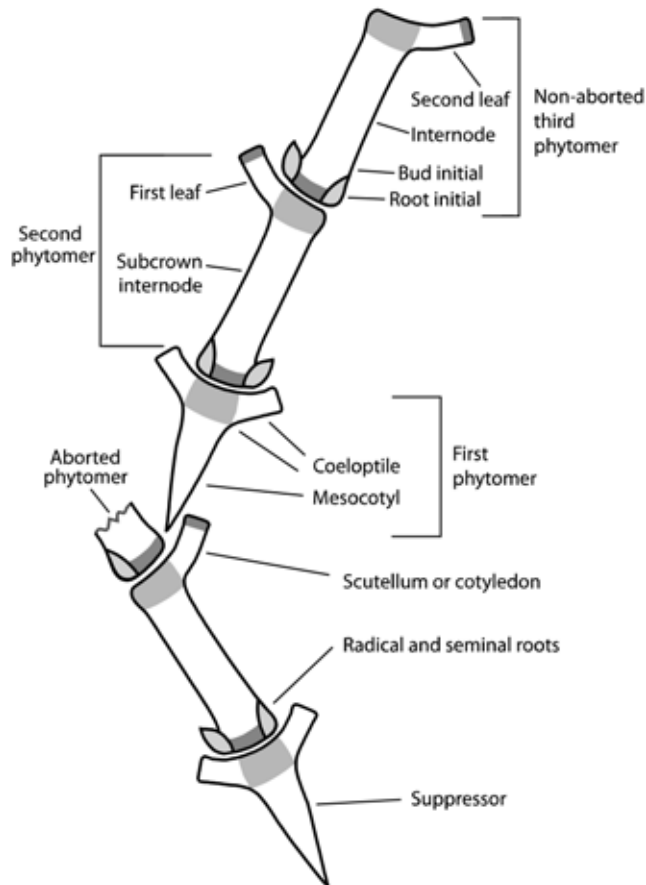


Figure 5. A diagram of embryonic structures developed as phytomeric triads in barley.

occur in the epicotyl: (1) the third phytomer of the second triad completes development to establish a repeating phytomer pattern of the primary tiller, and (2) buds formed at the lower half-nodes in the primary tiller are temporarily suppressed until after the seedling is established. If the third phytomer does not complete development, seedlings should have only one normal leaf. Such mutants were observed in Optic mutation grid population.

If two phytomeric triads can account for the architecture embryo structures observed in barley seedlings, the model should explain also phenomena observed in normal seedlings, e.g. development of only one set of seminal roots and tiller formation near the seed node. Removal of the radicle before or after germination causes seedling death. The embryo apparently does not have the capacity to develop a second root system. When seedlings are growing vigorously, a tiller can form near the cotyledonary node, which presumably arises by re-activation of the nodal bud below the sub-crown internode on the second phytomer of the second phytomeric triad.

Bud numbers and their activation

Generally only one axillary bud forms from the half-node at the base of the phytomer; however, there are exceptions. Three buds normally develop from each rachis node and produce three spikelets (Bonnett, 1966a). In wild barley and two-row cultivars, only the central spikelet develops fully and sets seed. Three fertile spikelets develop at each rachis node in certain mutants at the six-row spike 1 (*vrs1*) locus. Komatsuda *et al.* (2007) demonstrated that the evolution of cultivated six-row barley involved at least three independent mutations at the *vrs1* locus. Restrictions on the number of spikelets at each rachis node are lost in the *vrs4* mutants, but their positional arrangement is not. Additional spikelets arise in pairs

on opposite sides of previously formed spikelets. Thus, one fertile lateral spikelet is formed on each side of the central spikelet of two-row barley and converts it to six-row barley. The *vrs4* mutants can form four additional spikelets, one on each side of the lateral spikelet. In vigorous *vrs4* plants, a few additional spikelets can form near the four additional spikelets; thus, the potential numbers of floral buds that could develop at rachis nodes are 1, 3, 7, and probably 15 (Forster *et al.*, 2007). A single adventitious floral bud (spikelet) arises occasionally below the central bud (spikelet) in the case of extra floret (*flo*) mutants, probably from the bud of the second phytomer of the phytomeric triad. Lateral spikelets can have a short pedicel in some six-row mutants at the *int-c*, *vrs3*, and *vrs4* loci. These mutants indicate that the internode below the lemma has the capacity to elongate.

Extending multiple bud formation to other nodal meristems is more difficult. Seminal root numbers were not studied, but they may be produced from nodes in the same 2n-1 series as are floral buds. Multiple bud formation in vegetative parts of the plant seems possible because the unculm 4 (*cul4*) mutants show a proliferation of tiller buds in leaf axes where a single tiller develops normally. Suppression of root and bud formation and growth is a normal part of the plant ontology. Roots develop from lower phytomers only when conditions are favorable for their anchorage in the soil. The formation of branches on the culm is normally suppressed, except in a few mutants (Bossinger *et al.*, 1992). Mutants showing different degrees of reduced tillering have been identified in barley (Forster *et al.*, 2007). Unculm 2 (*cul2*) mutants have only a primary tiller. Low tiller number (*lnt1* or *int-1*) mutants produce only 2 to 4 well formed tillers. A few more tillers can develop in the absent lower lateral 1 (*als1*) and corn stock 1 (*cst1*) mutants. Other cul mutants produce a primary tiller plus a few weak tillers in which development is delayed. The opposite

spikelets 1 (*ops1*) mutant reduces tillering to a lesser degree. Spike malformations are associated with the drastically reduced tillering of the *cul*, *als1* and *lnt1* mutants.

The leaf blade is apparently involved in suppression of axillary buds in developing seedlings because mutants that greatly reduce the size of the leaf blade, leafless 1 (*lfs1*), granum-a (*gra-a*), and many-noded dwarf (*mnd*) mutants, show large increases in tillering. Some mutants with a reduced number of fertile rachis nodes also show increased tiller numbers; thus, an energy balance is probably involved in the control of tiller development and survival (Wych *et al.*, 1985).

Mutations of phytomeric units

If floral structures are formed by stacking of phytomeric triads, most floral mutants should retain their structural integrity. The Optic population contained completely sterile mutants where normal spikelets formed, but the lemma enclosed numerous bracts (Forster *et al.*, 2007). Closer examination of the spikelets revealed that a palea-lemma association was repeated numerous times. Other floral organs were not present. Since this change was caused by single recessive mutation, potential explanations of the observed phenotype are highly restricted. The 2 + 1 association of bracts was retained, but further differentiation of floral organs was inhibited. A similar phenotypic expression was observed in the multi-ovary 2 (*mov2*) mutant, where extra carpel-like structures form, presumably from repeating triads.

More common are mutants in which floral buds on the rachis fail to establish a phytomeric triad by partial abortion of its third phytomer. Unrestricted formation of new phytomers, proliferation of spike branches and floral buds in the inflorescence produces the rattail-like spike (*rtt*) mutants (Reid, 1985). Spikelets fail to form at the lower rachis nodes in the branched spike (*brc*) mutants and, instead, secondary

spikes of various sizes develop. Bonnett (1966b) observed that some barley varieties develop supernumerary spikes when they grow under certain glasshouse conditions. Supernumerary spikes often form from the first rachis node in certain many-noded dwarf (*mnd*) mutants and in the accordion basal rachis internode (*abr1*) mutant. Formation of plantlets instead of spikelets occurs at a variable frequency in the viviparoides (*viv*) mutants.

Transition to reproductive structures

Before reproductive structures can form, transition from phytomers of the culm to those of the rachis must occur. Spike development is preceded by a specialized culm phytomer or peduncle in which the sheath is reduced to a collar. Timing of this transition in terminal buds is determined by a number of photoperiod response genes (Forster *et al.*, 2007). Physical transition to the collar and spike is delayed in the many noded dwarf (*mnd*) mutants and nearly stopped in some tillers of the viviparoides (*viv*) mutants. An extreme example of transition failure is the aborted spike 1 (*asp1*) mutant in which spike formation is greatly delayed or never occurs. The collar forms a small leaf-like protrusion in the leafy bract (*Lfb1*) and semi-dwarf 2 (*sdw2*) mutants. A small leaf develops at the collar and its size gradually diminishes for several rachis nodes in the third outer glume 1 (*trd1*) and bracteatum (*bra*) mutants. The formation of a collar ridge at a few nodes above the collar was reported in normal barley by Bonnett (1966a). He also observed that one or two rachis nodes at the base of the spike fail to produce spikelets.

Elongation of the first rachis internode is not adequately suppressed in the long basal internode (*lbi*) mutants and in some intermedium spike (*int-h* and *int-i*) mutants (Forster *et al.*, 2007). In the accordion basal rachis internode (*abr1*) mutant, several basal rachis internodes are elongated. The rachis

internodes remain unusually long in the accordion rachis (*acr*) mutants. The number of barren rachis nodes at the base of the spike is increased in some *acr* and *lax* mutants. The lesser internode number 1 (*lin1*), short crooked awn 1 (*sca1*), and tip sterile (*tst*) mutants show a reduction in spike length and in the number of fertile rachis nodes formed.

Phytomers in leaves and awns

Auricles and a ligule are formed in the transition region between the leaf sheath and its blade or the leaf collar region. Since the auricles and the leaf blade show a 2 + 1 association, they are probably formed from a meristematic region near the tip of the sheath (Forster *et al.*, 2007). If this association of side arms is similar to that found in a phytomeric triad, these organs arise from a modified bud that developed in the leaf collar

region located at the tip of the sheath. The auricles would be the side arms of the first phytomer of the triad and the leaf blade the side arm of the second (Figure 6). The ligule may represent an extension of the internode of the third (aborted) phytomer. The aberrant phenotype in some unicum (*cul*) mutants with several partly formed auricles along the sides of the sheath is difficult to explain. The transition region between the sheath and blade is nearly lost and no auricles or ligules are formed in the liguleless 1 (*lig1*) mutants.

Forster *et al.* (2007) considered the lemma and its awn as parallel organs to the leaf sheath and its blade and reported that several mutant phenotypes can be explained by activation of a meristematic zone between the lemma and the awn. The leafy lemma 1 (*lell*) mutant converts the lemma and its awn into a leaf-like structure that is divided

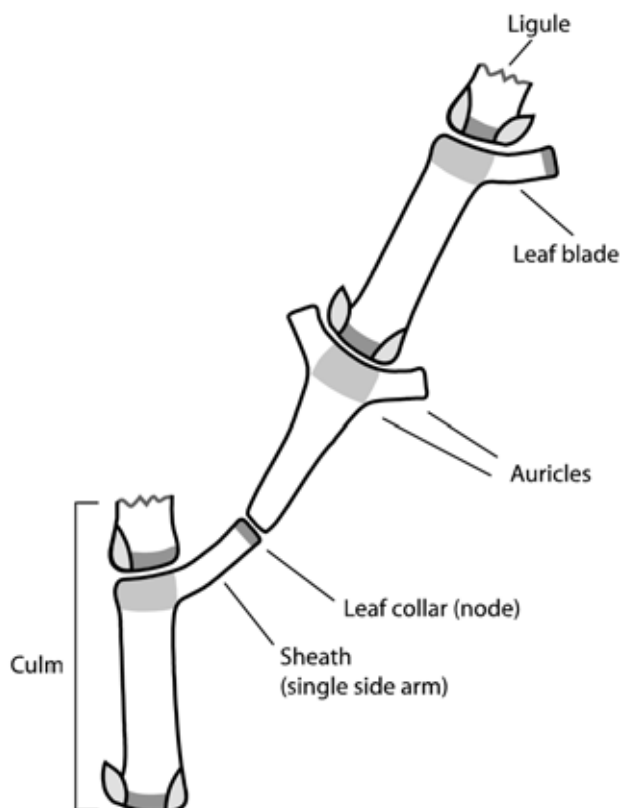


Figure 6. A diagram of leaf blade phytomer of barley formed on a sheath meristem.

into distinct sheath and blade segments. The hooded lemma (*Kap1*) mutant produces floral-like structures near the tip of the lemma instead of an awn (Bonnett, 1966b). These reproductive structures are produced presumably by phytomeric triads activated by altered biochemical signal levels (Müller *et al.*, 1995). Several other mutants are expressed phenotypically as variants of the meristem region near the tip of the lemma. The positioning of hood in the subjacent hood 1 (*sbk1*) or calcaroides (*cal*) mutants is lower on the lemma. Development of the awn fails in the awnless 1 (*Lks1*) mutant. Two awn branches at the base of the awn can develop in the triple-awned lemma 1 (*trp1*) mutants. The base of the lemma awn is malformed in the curly lateral 1 (*crl1*) mutant and is easily broken in *ari-k* mutants. The base of the awn is wider than normal in the laxatum-c (*lax-c*) mutants. It is interesting that the leafless 1 (*lfs1*) gene and other small leaf mutants affect only development of the leaf blade and not the sheath. The parallel mutants of the awn are placed in the short awn (*lks*) and brevistaratum (*ari*) groups of mutants. Mutants reducing both leaf blade and awn length are placed in both the brachytic (*brh*) and *ari* mutant groups.

Developmental parallels are observed in mutants affecting both the awns and the stigma. Plants with both *eog1* and *Kap1* genes form glumes that are lemma-like and have hoods. The lemma awns of smooth awn (*raw*) mutants have fewer and smaller barbs and their stigmas have fewer stigma hairs. The unbranched style (*ubs4*) mutants reduce the length of both the awn and stigma.

The development of ovules and stamens follow this same pattern as leaf blades and awns: a single side arm supports a modified nodal region from which an axillary bud can develop. Thus, the stamen is composed of a filament, the single side arm, and an anther formed from a fused pair of side arms, which should arise from the first phytomer of a bud. The anther has two lobes and four

lodicules, which form by folding halves of each side arm. Esau (1977) described the filament as having a single vascular bundle. The thick filament 1 (*tfm1*) mutant is mostly male sterile because the filament is converted into a sheath-like bract and fails to elongate at anthesis. The ovule may be structurally similar to the stamen. A stalk or funiculus supports a nucellus surrounded by the outer and inner integuments (Lersten, 1987). Organ conversion in the *mov* mutants suggest that the stalk is similar to the filament, while the paired integuments could be similar to the anther lobes.

Multifloreted spikelets

An indeterminate flowering mutant was found in the Optic mutation grid population, which produced two or more florets within a spikelet (Forster *et al.*, 2007). The alternating florets face each other and the multifloreted structure is contained within a pair of glumes. The phytomeric structure of this mutant is similar to that of the wheat (*Triticum* spp.) spikelet and can be created simply by the production of fertile (rather than infertile) rachilla phytomers (Forster *et al.*, 2007). Florets enclosed within the spikelet appeared to arise from the same attachment point because internodes of the rachilla failed to elongate. Since paired side arms (glumes) are not associated with individual florets, each floret is probably formed as a branch from a phytomer instead of a phytomeric triad that normally develops following formation of the glumes.

This mutant aided in discerning ontology of other multiple floret variants and mutants in barley. Early heading barley mutants can occasionally produce a double kernel in the central spikelet; however, the lemmas of the two florets are often fused. The male-sterile genetic 3 (*msg3*) mutant is characterized by development of two or three ovaries enclosed in an enlarged lemma-like bract. The anthers abort but up to three kernels can develop following hand pollination. A series of

mutants have closely placed or fused multiple spikelets. The compositum (com) mutants form small branches or multiple spikelets at rachis nodes in the lower half of the spike. Instead of the rudimentary spikelets at the tip of the spike (Bonnett, 1966a), several mutants placed in different gene symbol groups (*ari.256*, *brh13*, *brh16*, *dub1*, *int-i* and *int-m*) produce shortened spikes with densely packed or multiple spikelets at their tips (Forster *et al.*, 2007).

Nodal structure

The phytomer model assumes that half-nodes of two phytomers are fused together and elements of the vascular system to pass through them. In wild barley, the strong association between half-nodes of the rachis is not retained after physiological maturity. Disarticulation at the rachis nodes is one of the mechanisms that facilitate dissemination and self-planting of spikelets in wild barley. The brittle rachis (*btr*) mutants prevent formation of the abscission layer in the rachis nodes and shattering of the spike. Additional support for a complex nodal structure comes from the eligulum-a (*eli-a*) mutants, where the ligules fail to form, auricle development is poor, and culms break easily at the junction between half-nodes.

Conclusions

The concept that plants are composed of repeating structural units has gradually evolved over time and expanded to a stem and leaf model of the phytomer in which half-node elements are separated by an internode (reviewed by Bossinger *et al.*, 1992). More details were added to the barley phytomer model and the 2 + 1 association of side arms or bracts in axillary buds was described by Forster *et al.* (2007). The term phytomeric triad is introduced in this paper to describe the combination of three phytomers underlying the 2 + 1 association of side arms. The single side arm from the middle phytomer is often larger than the two side arms from the first

phytomer. In the vegetative parts of the barley plant, the paired side arms take on largely a role of protecting the single side arm and the bud formed on the third phytomer of the triad. If the third phytomer fails to abort, the repeating phytomers that compose most of the vegetative structures of the plant and core structure of the spike develop. In reproductive structures, developmental suppression of the third phytomer is re-established following formation of the lemma. Subsequent organs develop in a 2 + 1 pattern of side arms from an underlying stack of phytomeric triads. The role of the paired side arms gradually diminishes until the ovary is formed. However, the single side arm retains a prominent role during floral development. A phytomeric triad forms at the end of the single side arm (sheath) in the vegetative parts of the plant to form the auricles and leaf blade. In the case of floral organs, the meristematic region of a single side arm forms the awn on the lemma, anthers on filaments, and the integuments of the ovule. The embryo develops apparently as a phytomeric triad and a bud from its third phytomer develops into the epicotyl or plumule.

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Progress of the hulless barley breeding program

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Abstract

The hulless barley (HB) breeding program was started at Priekuli Plant Breeding Institute, Latvia, in 2000 with the purpose of introducing the hulless gene into Latvian breeding material, and producing varieties adapted to local conditions with valuable grain quality features for feed and healthy food uses. This study summarizes data obtained from yield trials of 22 F_5 to F_7 advanced HB breeding lines. One covered and two HB check varieties were used. Important traits for HB, including beta-glucan, starch and protein content, germination ability, resistance to sprouting, threshability and grain color, were analyzed, along with agronomic traits. The average yield of HB lines during three years was 3.71 t/ha (ranging from 69 to 92% of the yield of the covered check cv. Ansis). The yield of three lines did not significantly differ from the covered check ($p < 0.05$). The highest yield level was obtained in 2007 (average 4.04 t/ha), but the highest yield relative to the covered check was in 2006 (max. 111%). Average beta-glucan content in 2005–2006 ranged between 5.4 and 7.5%, which makes it possible to select varieties suitable for feed and food purposes. Average starch content (63.8%) exceeded that of the covered check by 2.4%, and the average volume weight (801 g/L) was 16% higher than that of covered check. A wide range in sprouting resistance was observed. Registration of HB varieties will be considered in the very near future.

Introduction

Hulless barley (HB) is a traditionally grown cereal in several Asian, African and Latin American countries, and modern HB breeding is well developed in Canada, USA and Australia (Newman and Newman, 1998; Bhatti, 1999; Rosnagel, 2000). There are several breeding programs dealing with HB in European countries (e.g. the Czech Republic, Sweden, Germany and Italy). A hulless barley breeding program was started at Priekuli Plant Breeding Institute in 2000 with the purpose of introducing the hulless gene into Latvian breeding material and to produce varieties adapted to local conditions, with valuable grain quality features for feed and healthy food uses. Large-scale testing of various HB genotypes in Latvian growing conditions was performed and more suitable breeding source material was selected (Belicka and Legzdina, 2000; Legzdina, 2001, 2002).

Significant traits of particular interest in HB breeding are threshability, resistance to embryo damage during harvesting, resistance to sprouting, and grain chemical composition including beta-glucan, vitamins and essential amino acids. Threshability is important, because HB grain with undetached hulls loses its superiority over covered barley. Notable threshability improvement has been achieved by Canadian breeders (Rosnagel, 2000). Ram and Singh (1996) reported that HB threshability is controlled by two non-allelic gene pairs with additive effect.

Sprouting can be a problem for HB if there is significant precipitation during grain maturation. The majority of HB genotypes have a short dormancy period and the grain has high water absorbing ability. Breeding for sprouting resistance has been tried in Australia (Box and Barr, 1997). Poor germination ability and emergence of HB seed can be observed, mostly due to embryo damage during harvesting. This problem can be reduced by selection of genotypes with higher germination ability (Rossnagel, 2000). Higher germination ability has been observed in round kernels in contrast with elongated kernels (Box and Barr, 1997).

Compared to other cereals, barley endosperm has relatively high beta-glucan content. Beta-glucan in barley grain usually varies between 2 and 7% (Grausgruber *et al.*, 2004). Beta-glucan has positive roles in human nutrition and health, such as lowering blood cholesterol levels, increasing mineral and vitamin bio-availability, and controlling colon cancer (Klopfenstein, 1988), whereas it has negative effect in chick feeding (Bergh *et al.*, 1999). In several studies, 3.3–20.0% cholesterol reduction due to consumption of barley products has been demonstrated. Lipid-associated barley components (vitamin E and tocotrienols) may also be responsible for reduction in blood cholesterol; they also function as antioxidants. Barley food products have a very low glycaemic index and are recommended in diets for type II diabetics (Newman and Newman, 2004). Viscosity is often used to predict the total and soluble beta-glucan content in grain (Bhatti *et al.*, 1991), with higher viscosity associated with health promoting ability of HB. At the same time, barley with low extract viscosity provides significantly better feed conversion and weight gain than high viscosity barley (Campbell *et al.*, 1989).

The aim of this paper is to summarize the preliminary results of the first yield testing of HB breeding lines from the program. Specific traits for HB differing from covered

barley and specific traits for food barley were analyzed, along with common agronomic features.

Material and methods

This study summarizes data obtained from yield trials conducted during 2005–2007 with 22 F_5 to F_7 advanced HB breeding lines. Three of the lines are doubled haploids. Pedigrees of the lines include both HB and covered genotypes (except three lines); genotypes with Latvian origin are included in most of them. Covered check cv. Ansis and two HB checks (the Czech line KM-2084 and the German cv. Taiga) were used.

In 2005 and 2006, the lines were tested in various nurseries with 1 to 4 replications, and with a plot size varying from 2.3 to 12.3 m² depending on availability of seed. Since the yield level in different nurseries differed, the relative yield as a percentage of the covered check Ansis was calculated and used in evaluation. In 2007, all the 22 lines were tested in 4 replications in 12.3 m² plots. Seed rate was 400 germinating seeds per m². Soil characteristics were as follows: sod-podzolic (2005–2006) and sod-podzolic gley (2007) soil, loamy sand and sandy loam, organic matter 1.2–2.7%, pHKCl 5.2–5.5 (2005, 2007) and 6.0 (2006), P205 133–189 mg/kg, K2O 132–184 mg/kg, MgO 104–132 mg/kg. Fertilizer application included: N 81–86, P 40.5–43, K 43–67.5 kg/ha. The herbicides Granstar and Primus and insecticide Fastac were applied.

The average air temperature and precipitation differed between years (Figure 1).

Beta-glucan, starch and protein content were determined by Infratec grain analyzer. Results are shown as percentage of dry matter. Waxy starch type was determined by the iodine colorimetric test. Germination ability was determined in 2006–2007 using 100 seeds in two replications in Petri dishes between moist filter paper at 20°C for 7 days. Infection with powdery mildew

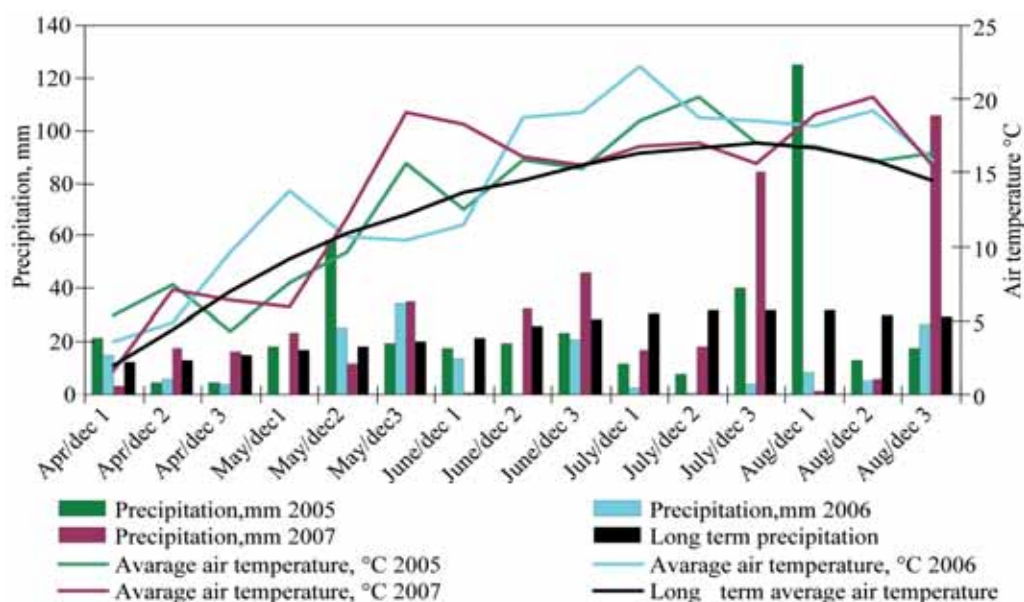


Figure 1. Meteorological conditions in 2005–2007 and long-term average data

(*Blumeria graminis* f.sp. *hordei*) and net blotch (*Drechslera teres* (Sacc.) Shoem) was assessed according to a scale of 0–4 (0 = no infection, 4 = very high infection). Spikes infected with loose smut (*Ustilago nuda* (Jens.) Rostr.) and plants infected with leaf stripe (*Drechslera graminea* Ito) were counted. Resistance to sprouting was scored visually on the field in 2005, with a scale of 0–4 (0 = no sprouting, 4 = all grains sprouted). In 2007, sprouting resistance was analyzed in the laboratory for 3 spikes per line in Petri dishes between moist filter paper at 20°C; sprouted grains after 10 days were counted and sprouting percentage calculated. Each spike was considered one replication. Threshability was assessed visually in grain samples according to a scale of 0–4 (0 = no grain with undetached hulls, 4 = all grains with undetached hulls). A scale from 0 to 3 was used for grain color (0 = yellow or very light brown, 3 = dark brown). Viscosity was determined in 2006 and 2007 (for check varieties in 2007 only) by Rapid Visco analyzer and expressed in RVUs (Rapid Visco units). Analysis of variation and

correlation was applied. Vitamin E content in grain was determined only in 2007, using AOAC Official Method 971.30.

Results and discussion

The average yield of HB lines during three years was 3.71 t/ha, ranging from 69 to 92% of the yield of the covered check cv. Ansis. The highest average yield level was obtained in 2007 (4.04 t/ha), but all the lines yielded significantly less than the covered check variety. The highest yield relative to the covered check was recorded in 2006 (mean 87% and maximum 111% of the check). It may be due to higher resistance to drought of HB compared to covered barley because 2006 was an extremely dry year. Both hullless checks yielded on average significantly less than cv. Ansis. The average yield of the highest yielding lines is shown in Figure 2. The average yield of three breeding lines (K170, PR-3528 and PR-3527) did not differ significantly from the covered check ($p < 0.05$). Nine lines had mean relative yield of between 88 and 92% of cv. Ansis,

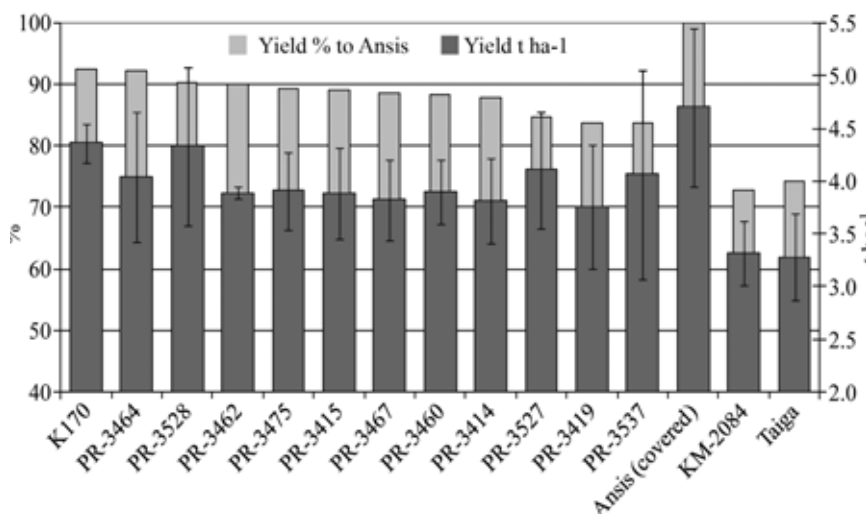


Figure 2. Absolute and relative yield of best yielding breeding lines (2005–2007 mean).

which can be considered as equal to covered barley, considering that the hull content is 10–12%. The yield of nine breeding lines was significantly higher than the yield of the HB check cv. Taiga. Line PR-3462 had the highest yield stability between years ($CV = 1.2\%$); the yield of the best yielding line K170 ($CV = 4.4\%$) was also relatively stable. High yield differences between years (CV up to 24% for PR-3537) were recorded, mostly due to the reduced yield in the very dry conditions of 2006.

Differences between the average germination ability of genotypes were not significant (Table 1), but the influence of

the year was highly significant ($P < 0.001$) because of increased embryo damage in the dry harvest conditions of 2006. In 2006, the mean HB germination ability was 87% and only five lines had less than 85% seeds able to germinate, while in 2007 it was 67% and only two lines surpassed 85% and are considered as most resistant to germ damage.

Days to maturity differed significantly among the lines. Most of the HB lines were earlier maturing than the covered check variety, except two lines that included the high yielding line K170. There was considerable variation in plant height. Five lines were shorter than Ansis, including the

Table 1. Agronomic traits of HB lines and check varieties (2005–2007 mean).

Genotypes		Grain yield (t/ha)	Grain yield as % of cv. Ansis	Germination ability (%)	Days to maturity	Plant height (cm)
HB lines (n = 22)	average	3.71	84	77	98	73
	range	3.17–4.36	69–92	62–90	93–104	58–94
cv. Ansis (covered check)		4.70	100	95	100	66
KM-2084 (HB check)		3.32	73	88	98	61
cv. Taiga (HB check)		3.28	74	82	100	71
LSD _{0.05}		0.35	–	n.s.	2.9	11.7
P		0.001	–	0.1	<0.001	0.002

good yielding lines K170, PR-3527 and PR-3537, whereas mean plant height of two lines was > 90 cm. No remarkable lodging was registered during the testing period.

There was a significant difference among the lines in disease reactions (Table 2), with the exception of leaf stripe, which was observed only in 2007. Loose smut is considered a greater problem for HB than for covered barley. It is encouraging that infection with loose smut was less than that of HB checks for all breeding lines. Practically no loose smut infection was found for two breeding lines, and 13 lines had fewer infected spikes than the covered check. Two lines were resistant to powdery mildew and 4 had weak infection levels (< 0.5). Only three lines had greater infection with net blotch than the covered check, and 8 lines had a weak infection level (< 0.5). Four lines were not infected with leaf stripe whereas four lines had a high infection level.

About one week of continuous rain just before the harvest of 2005 made it possible to score sprouting in the field. The data were compared with the results obtained in the laboratory in 2007. Sprouting in the field and in laboratory tests were significantly correlated ($r = 0.52$, $r_{0.05} = 0.36$), although discrepancies were found for some lines. Influence of genotype on sprouting in laboratory tests was highly significant and genotypic differences explained 75% of

variability. Two lines proved to be most resistant (<2% germination in 7 days and score 0.5 on the field), whereas four lines proved to be susceptible to sprouting (>20% germination in 7 days).

The highest mean crude protein content was found in the check KM-2084 (Table 3). Fourteen of the breeding lines had significantly higher protein content than the covered check. High protein content up to 17.9% was found in the dry conditions of 2006. Starch content of all HB lines was higher than that of cv. Ansis and the differences were significant for 20 lines. Highest starch content was found in the line K170, while 14 breeding lines had significantly higher average beta-glucan content than the covered check. The highest beta-glucan content (average values 6.5–6.9%) was found for 3 waxy starch lines with the American variety Merlin in their pedigree, whereas the non-waxy line K170 with Merlin in the pedigree had the lowest beta-glucan content in 2006 and 2007. In the dry and warm year 2006, beta-glucan content was higher (mean 6.9%). Therefore, from this material it is possible to select varieties suitable for feed and food purposes with low and high beta-glucan, respectively. There was significant correlation between beta-glucan content and grain color ($r = -0.48$, $r_{0.05} = 0.42$), which shows that high beta-glucan HB tends to be associated with lighter colored grain. Viscosity differed significantly between the

Table 2. Infection with diseases (2005–2007 mean) and sprouting resistance of HB lines and check varieties (2005 and 2007).

Genotypes		Loose smut, spikes per 10 m ²	Powdery mildew, 0–4	Netblotch, 0–4	Leaf stripe, plants per 10 m ²	Sprouting in the field 0–4	Sprouting in laboratory %
HB lines	Average	5	1.0	0.7	6	1.3	14
(n = 22)	Range	0–17	0–2.6	0.2–2.6	0–55	0–3	076–
cv. Ansis (covered check)		4	0.4	0.9	0	0	–
KM-2084 (HB check)		19	1.0	0.9	0	1.1	4.1
cv. Taiga (HB check)		20	0.6	1.4	4	0.8	–
LSD0.05		11.3	1.05	0.79	n.s.	–	20.3
P		0.01	<0.001	<0.001	0.2	–	<0.001

Table 3. Grain quality of HB lines (2005–2007 mean).

Genotypes		Protein %	Starch %	beta- glucan %	Viscosity RVU	TGW g	Volume weight g/L	Thresh- ability 0–4	Grain color 0–3
HB lines (n = 22)	Average	13.3	64.1	5.8	359	41.9	797	0.8	1.2
	Range	12.1– 14.6	62.5– 66	5.1–6.9	227–500	38–48	779–812	0.4–1.3	0.52.3–
cv. Ansis (covered check)		12.2	62.3	4.7	288 ^a	48.2	695	4	–
KM-2084 (HB check)		15.0	61.8	6.1	451 ^a	44.6	792	1.1	1.2
cv. Taiga (HB check)		14.1	62.7	6.1	239 ^a	43.8	799	0.6	2.3
LSD0.05		0.94	1.2	0.88	n.s.	4.73	24.7	n.s.	0.83
P		<0.001	<0.001	0.003	0.14	0.008	<0.001	0.7	<0.001

^a Measured in 2007 only.

years 2006 and 2007 ($p < 0.001$) with mean values of 456 and 261, respectively, and there was no significant influence of genotype. This is in agreement with Campbell *et al.* (1989), who reported lower viscosity in cooler and moister conditions. Acid extract viscosity correlates negatively with the amount of precipitation (Perez-Vendrell *et al.*, 1996). Relatively stable and high viscosity in both years was found for two lines (mean 463 and 500 RVU, CV = 2.3 and 7.2%), whereas one line had a relatively stable low viscosity (290 RVU, CV = 21%). Lines with high viscosity tended to be resistant to sprouting, and vice versa, but the correlation was not significant. Lines with waxy starch tended to have very large viscosity differences between the years (CV = 70–92%); it was high in 2006 and very low in 2007. The viscosity of waxy barleys decreases rapidly in wet weather conditions; it might be caused by genetic linkage between waxy character and sprouting resistance (K.J. Mueller, pers. comm.). There was significant negative correlation between viscosity and b-glucan content in 2007 ($r = -0.52$, $r_{0.05} = 0.44$), which disagrees with other findings (Campbell *et al.*, 1989; Bhatti *et al.*, 1991). The correlation in 2006 was positive, but weak and not significant. Viscosity is influenced not only by beta-glucan content, but also by other factors, one of which is the presence of beta-glucanase in the sample (Izydorczyk *et al.*, 2000). Vitamin E content

in 2007 (data not shown) ranged from 31.8 to 42.5 mg/kg; four lines contained more than 40 mg/kg vitamin E. The covered check cv. Ansis contained high amounts of vitamin E (41.3 mg/kg), while the HB check KM-2084 had only 36.3 mg/kg.

TGW of HB lines was significantly lower than that of the covered check except for three lines. The volume weight of all lines significantly surpassed the covered check; three lines had a maximum volume weight of 812 g/L. There was a significant and negative correlation between volume weight and threshability ($r = -0.46$, $r_{0.05} = 0.42$), which supports the possibility of using volume weight as a selection criterion for improved threshability (Rosnagel, 2000). Five lines had mean threshability scores of 0.4–0.5, but no significant differences between threshability scores of genotypes were found. The mean grain color score of four lines was 0.5 (between very light and light brown), which should be an advantage for food uses.

Registration of HB varieties is considered in the very near future. Since the lines are coming from the first HB crosses made in the breeding program, the majority of them need more improvement and are included in further crosses. Some advantages and some disadvantages were found for all the lines. None of the lines could be registered as a food variety; all lines with elevated beta-glucan content were susceptible to powdery

mildew. Several lines could be acceptable as HB varieties for feed (PR-3528, K170, PR-3527 and PR-3475). The main advantages are high volume weight, good threshability, high starch content, lower beta-glucan content and lower viscosity.

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Investigation and utilization of spring barley germplasm for breeding programs

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Abstract

It is important to know the genetic variability within genetic resources before they are utilized in breeding. During 2004–2007 a set of 50 barley genotypes from the collection of spring barley genetic resources was analyzed at the Lithuanian Institute of Agriculture. We assessed the variation of grain yield, 1000-kernel weight, hectolitre weight, grain grading, protein and starch content, nitrogen, starch and carbon (calculated from starch data) content, and ratio of Cstarch:N per single grain. The genotypes showed high variation in coarse grain output and medium variation in grain yield. The variation in the other quality parameters was low. The meteorological conditions had a considerable impact on protein and starch synthesis and accumulation per grain. The differences in grain quality between the varieties could be a result of different numbers of grain per plot area. The average ratio of C content calculated from starch and N content in single grain was 11.09 in dry seasonal meteorological conditions. The ratio of C content calculated from starch data, as well as N content per single grain, could be an additional indicator for evaluation of spring barley suitability for malt.

Introduction

The grain quality characters of spring barley can vary substantially due to genetic characteristics, growing technologies and environmental conditions (Bertholdsson, 1999; Tamm and Tamm, 2002; Nurminiemi

et al., 2002). Moderate supply of mineral nitrogen (N) has the most important impact on malting barley yield, especially on grain quality (Qi *et al.*, 2006). Supplying plants with plenty of mineral nitrogen induces accumulation of protein; however, protein content has a negative relation with malt extract content (Moral *et al.*, 1998). Reduction in ear size increased grain N concentration under all environmental conditions (Dreccer *et al.*, 1997). Therefore, N content per single grain can be related to the grain number per plot area.

Over 30 characters are used for evaluation of malting grain, malt, wort and beer quality (Analytica-EBC, 1998). The main observed quality characters, which are in close relation to extract yield, are protein content and grain fraction >2.5 mm (Qi *et al.*, 2006; Bertholdsson, 2004; Zhang *et al.*, 2001; Moral *et al.*, 1998). According to the Lithuania State Standard LST 1591 (LST, 2000), protein content for malting barley should be below 11.5%, and 9.5–11.0% for first class grain. Variation in protein content in barley grain is high. At the Jõgeva Plant Breeding Institute, Estonia, protein content of 57 malting barley varieties varied from 9.9 to 14.4% (Tamm and Tamm, 2002). About two-thirds of malt extractive substances are formed from starch hydrolysis products. The meteorological conditions have a considerable impact on starch synthesis and accumulation per grain, as well as on the grading data (Tamm and Tamm, 2002). Experiments on the malting variety Trumpf revealed that when the weather during the grain maturing period

was sunny, grain was thinner and contained more protein than grain that matured in wet and cloudy weather conditions (Molina-Cano *et al.*, 2001). The chemical composition within the variety for different grain size could vary; however, the variation of those indices is lower than that between the various barley varieties (Elfverson *et al.*, 1999). Consequently, in malting barley breeding it is important to measure yield potential, elements of yield structure, total protein and starch content, as well as grain number per plot and traits such as nitrogen, starch (or carbon calculated from starch data) content, and ratio of Cstarch:N per single grain. Drought tolerance is a primary objective in Mediterranean regions (Forster *et al.*, 2000). However, it is important as a yield and grain quality-limiting factor also in northern parts of medium latitude climatic zones, where mean annual precipitation varies between 620 and 700 mm.

Furthermore, in relation to climate warming, the subject of current research is genotype-dependent spring barley yield and grain quality variation under seasonal dry weather conditions in regions formerly classified as having seasonal wet weather conditions.

The aim of the study was to evaluate spring barley varieties for grain yield and inherent quality under seasonal dry weather conditions and to include extra characters such as grain number per plot, nitrogen, starch (or carbon calculated from starch data) content per single grain, and ratio of Cstarch:N per single grain to be tested for variety choice for warming climate conditions.

Materials and methods

The experiment was conducted with foreign spring barley varieties. Varieties of Lithuanian origin were involved in calculations of yield and grain traits means only. Barley was grown during 2004–2007 in Dotnuva, Lithuania, on an Endocalcari–Endohypogleyic Cambisol (CMg-n-w-can) using the Lithuanian Institute

of Agriculture (LIA) technology designed for breeding. According to the conventional Lithuanian soil assessment method, the soil was of close to neutral, medium in organic matter and plant available phosphorus and potassium. At sowing, 90 kg of N, 60 kg of P_2O_5 and 60 kg of K_2O were broadcast. The plots were harvested by a plot harvester. Combine-harvested grain from each plot was dried and sampled for analyses. Thousand-grain weight (TGW), total grain protein (total nitrogen by Kjeldahl multiplied by 6.25; ICC standard 105/2 (ICC, 1994a)), grading output (fraction on 2.5×20 mm sieve), total starch content (by hydrochloric acid dissolution; ICC 123/1 (ICC, 1994b)) were determined. Extract content was determined according to EBC recommendation, adjusted to hand-technology. Yield data were adjusted to 15% moisture content, grain quality characters were determined in dry grains. Number of grain/ha was calculated from the data of combine-harvested grain yield and TGW. Single grain traits were calculated from consequent parameter percentage in grain and TGW data.

Data were statistically processed using the STAT software package and ANOVA adapted in LIA by Tarakanovas and Raudonius (2003). Mean and standard error of the mean were calculated. A relationship between barley characters was determined and coefficients of linear correlation were presented. The null hypothesis for correlation data among the traits was rejected at the level of significance $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

The varieties were clustered into three entry groups, corresponding to low-, moderate- and high-trait-value varieties. The cluster of moderate-trait-values was formed according to the 2004–2007 mean values \pm the standard error of the mean. The varieties in high- and low-trait-value clusters showed higher and lower trait values than those in the moderate-value cluster. Clusters each contained about one-third of the genotypes for each trait investigated.

Results and discussion

Influence of weather conditions on yield and grain quality variation

The hydrothermal coefficients for April show that there was enough moisture in the soil in all the years (Table 1). In May, a slight drought occurred in 2004 only. Therefore, in all the years the weather conditions were favorable for germination, plant establishment and tillering. The period of stem elongation, booting and beginning of heading (June) was very dry in 2006, with the occurrence of slight drought in 2004 and 2005 and close to optimal weather conditions in 2007. At that stage the drought could reduce the number of florets as well as their development into grain, and it could affect grain yield. At the same time, when the number of grain and grain yield are reduced, the protein content in grain increases (Svobodova and Miša, 2004). The post-anthesis period was sufficiently wet in 2004 and 2007, and dry in 2005 and 2006. Therefore, the diverse weather conditions during the years significantly affected yield and grain quality. The temperature higher than the long-term mean and the lower amount of precipitation are associated with the general phenomenon of global warming. Due to the sunny weather during the post-anthesis period, the highest protein content in grain was identified in 2005 and 2006 (Table 2). Therefore, in those years, the breeder was

able to select the varieties with lower protein content with the aim of combining high productivity and standard malting quality under warming climate conditions.

The grain yield was lowest in 2007, attributed to a high incidence of leaf diseases. The grain yields in 2005 and 2006 were 17–29% less than in 2004, showing that the slight water deficit that occurred in June 2004 did not affect the yield. Above-average yields are achieved only in years when grain filling duration is longer (Schelling *et al.*, 2003). We can conclude that when the HTC during the pre-anthesis period was above 1.0 (moderately dry), the fluctuation of the weather conditions did not significantly affect spring barley grain yield.

The average grain number per hectare ranged from 76.2 to 109.03 million in 2007 and 2004, respectively) and coarse grain yield ranged from 2.7 to 4.87 t/ha in 2005 and 2004, respectively. It is well known that weather conditions have a major effect upon the physiological and biochemical properties of barley grown in the same location. Seasonal weather conditions distinctly affected coarse grain output. The differences in coarse grain yield fluctuated by up to 80% according to season.

In Lithuania, situated in the northwest of Europe, the protein percentage of spring barley grown on fertile soils is the trait most sensitive to weather conditions during grain filling (post-anthesis) period (Leistrumaitė

Table 1. Values of the hydrothermal coefficient (HTC) during 2004–2007 growing periods, Dotnuva, Lithuania.

Year	HTC					Monthly mean during April–September	
	April	May	June	July	August	temperature (°C)	precipitation (mm)
2004	1.3	1.1	1.0	1.6	1.7	14.66	60.26
2005	2.5	1.6	1.1	0.8	1.4	15.64	48.96
2006	2.7	1.3	0.1	0.6	1.8	16.68	54.64
2007	1.8	1.8	1.2	2.2	0.9	16.34	65.58
1924–2007 long-term mean						13.28	58.32

Qualification of weather conditions according to HTC: < 1.0 = drought; 1.0–1.3 = moderately dry; 1.3–1.6 = optimal humidity; > 1.6 = wet.

Table 2. Yield (mean and standard error) of spring barley varieties as affected by seasonal weather conditions. Dotnuva, Lithuania, 2004–2007.

Trait	2004		2005		2006		2007	
	mean (n = 33)	s.e.	mean (n = 34)	s.e.	mean (n = 35)	s.e.	mean (n = 35)	s.e.
Yield t/ha	5.18	0.13	4.44	0.08	4.02	0.07	3.56	0.07
TGW g	47.5	0.50	48.1	0.42	44.3	0.48	46.8	0.63
HLW g/L	676.6	3.60	636.0	3.67	619.8	4.23	626.6	3.42
Grain >2.5 mm %	91.8	1.08	89.4	1.19	67.6	2.00	84.6	1.17
Grain >2.5 mm t/ha	4.87	0.13	3.97	0.09	2.70	0.09	3.03	0.09
Extract %	79.9	0.30	78.5	0.19	76.1	0.29	80.4	0.20
Extract t/ha	4.24	0.10	3.48	0.06	3.06	0.06	2.86	0.06
Protein %	11.91	0.14	13.11	0.13	14.61	0.14	10.52	0.11
Starch %	59.8	0.26	59.8	0.31	58.1	0.30	60.7	0.23
Grain no. 106/ha	109.0	2.56	92.3	1.54	91.3	2.11	76.2	1.46
N µg/grain	904.3	13.63	1010.0	15.17	1036.7	15.19	787.3	12.43
Starch µg/grain	28 408	321.2	28 758	230.4	25 745	258.3	28 429	413.4
Ratio Cstarch:N/grain	14.03	0.20	12.73	0.17	11.09	0.13	16.10	0.21

and Paplauskienė, 2005). In our investigations a significantly higher protein percentage was observed in 2005 and 2006. It is in good agreement with HTC data over the post-anthesis period in those years. In June and July of those years the HTC ranged from 0.1 (in 2006) to 1.1 (in 2005), typical of dry weather conditions. In 2004 and 2007 the protein content in grain was low and it was associated with higher HTC. The components of grain weight and size are related to stressful growing environments (Coventry *et al.*, 2003). In agreement with this, the most stressful conditions were in 2006, when HTC was 0.10–0.60 for the month of the post-anthesis period. As a result, the weather conditions in 2005 and 2006 can be qualified as dry. We can guess that the weather conditions of those years are likely to be very similar to those in the near future due to the effects of global warming. The traits most sensitive to climate conditions were extract percentage, nitrogen content per single grain and ratio of Cstarch:N per single grain. The values of these traits in 2005 and 2006 significantly differed from those in 2004 and 2007. The lowest ratio for

Cstarch:N per single grain was recorded in the dry seasonal weather conditions in 2006, which suggests that in breeding for malting purposes the desirable values of this ratio should be not less than 11.09. The importance of single-kernel quality characteristics in malting barleys was pointed out by Angelino *et al.* (1997).

Correlation among quality traits

The data of grain yield correlated very closely with extract yield, grain number and coarse grain yield (Table 3). There were no correlations between grain yield and protein percentage and nitrogen content per single grain. This indicates that in the spring barley experiments nitrogen supply was sufficient, and nitrogen was not the factor limiting yield. However, for spring barley, the genotype-by-nitrogen interaction was not significant under dryland and irrigated conditions (Emebiri and Moody, 2004). Despite this, the impact of growth-period weather conditions on protein content is essential; the potential of genotypes to accumulate protein in wet and dry climatic conditions should therefore be evaluated.

Table 3. Coefficients of correlation (r) among grain yield and quality traits, Dotnuva, Lithuania, 2004–2007.

Trait	TGW (g)	Grain >2.5 mm (%)	Grain >2.5 mm (t/ha)	Extract (t/ha)	Grain no. (×106/ha)
Yield (t/ha)	0.263	0.335**	0.852**	0.988**	0.926**
TGW (g)		0.610**	0.494**	0.264**	-0.196*
Grain >2.5 mm (%)			0.772**	0.395**	0.100
Grain >2.5 mm (t/ha)				0.878**	0.677**
Extract (t/ha)					0.913**

Trait	Extract (%)	Protein (%)	Starch (%)	N (µg/grain)	Starch (µg/grain)	Ratio Cstarch:N/grain
Yield (t/ha)	0.202	0.017	0.139	0.146	0.290**	-0.052
TGW (g)	0.107	-0.196*	0.047	0.311**	0.916**	0.154
Grain >2.5 mm (%)	0.534**	-0.427**	0.318**	-0.095	0.672**	0.388**
Grain >2.5 mm (t/ha)	0.421**	-0.219*	0.247**	0.042	0.540**	0.171
Extract (t/ha)	0.336**	-0.078	0.219*	0.045	0.323*	0.054
Grain (×106/ha)	0.158	-0.774**	0.668**	0.036	-0.052	-0.118

Trait	Protein (%)	Starch (%)	N (µg/grain)	Starch (µg/grain)	Ratio Cstarch:N/grain
Extract (%)	-0.774**	0.668**	-0.698**	0.364**	0.769**
Protein (%)		-0.611**	0.869**	-0.420**	-0.980**
Starch (%)			-0.575**	0.444**	0.709**
N (µg/grain)				0.047	-0.874**
Starch (µg/grain)					0.423**

* $P \leq 0.05$; ** $P \leq 0.01$.

Grain protein percentage shows a significant negative correlation with malt extract (Qi *et al.*, 2005). Our investigations showed that the ratio of Cstarch:N per single grain was significantly correlated with the coarse grain and extract percentages, and the coefficients of correlation were even higher than those between the coarse grain or extract percentage and starch or protein percentage. The correlation coefficient between extract and protein percentages in 2004 was not significant, but the correlation between extract percentage and N content per single grain in that year was negative and significant at $P \leq 0.01$, and it was significant and high in the other years. Therefore the N content per

single grain and ratio Cstarch:N per single grain can be used as informative characters for evaluating malting barley varieties.

Characteristics of spring barley varieties

Based on the data averaged over four years, the varieties Tocada, Justina and Annabell were high yielding (Table 4). The highest values for coarse grain and extract yields per hectare were obtained for the varieties Tocada, Jersey and Annabell. These varieties were developed under general weather conditions that differ from those in Lithuania.

The coefficients of variation for coarse-grain yield were much higher than those for grain yield. Therefore, for malting barley

Table 4. Variation¹ of yield and grain properties of spring barley varieties. Dotnuva, 2004-2007.

Variety (origin) ²	Yield t/ha	TGW g	HLW g/L	Grain >2.5 mm %	Extract %	Grain >2.5 mm t/ha	Extract t/ha
Annabell (DE)	4.89±0.33 (13.3)	45.4±1.45 (6.4)	643±13.0 (16.8)	80.9±6.95 (17.2)	78.7±0.72 (1.6)	3.83±0.67 (35.0)	3.98±0.36 (16.0)
Barke (DE)	4.49±0.51 (22.5)	48.56±0.99 (4.1)	634±17.2 (12.7)	85.8±3.54 (8.3)	79.3±1.67 (3.7)	3.71±0.54 (28.9)	3.75±0.60 (27.5)
Cruiser (DE) ³	4.30±0.21 (8.6)	44.7±1.09 (4.2)	618±5.0 (1.4)	81.80±7.49 (15.9)	78.6±1.39 (3.1)	3.51±0.41 (0.2)	3.37±0.15 (7.5)
Justina (DE)	5.08±0.27 (10.5)	47.5±1.56 (6.6)	610±17.1 (5.6)	81.7±7.01 (17.2)	77.6±0.99 (2.2)	3.62±0.6 (33.2)	4.04±0.31 (13.4)
Scarlett (DE)	4.57±0.29 (12.9)	44.3±1.64 (7.4)	647±19.7 (6.1)	87.2±6.13 (14.0)	79.9±1.07 (2.3)	3.76±0.55 (29.3)	3.78±0.34 (15.7)
Tocada (DE)	5.09±0.24 (9.5)	47.8±2.3 (9.6)	629±10.8 (3.4)	80.55±4.96 (12.3)	78.7±1.13 (2.5)	3.89±0.45 (23.3)	4.08±0.31 (13.0)
Power ³ (DK)	4.24±0.26 (10.7)	44.1±2.25 (8.9)	633±7.9 (2.2)	67.4±16.15 (41.5)	79.5±0.43 (0.9)	2.81±0.65 (39.9)	3.37±0.20 (10.3)
Sebastian (DK)	4.72±0.33 (14.0)	45.0±1.59 (7.1)	644±9.1 (2.8)	82.9±6.24 (15.1)	79.9±1.20 (2.6)	3.62±0.60 (33.2)	3.94±0.49 (21.4)
Class ³ (FR)	3.96±0.38 (19.7)	46.5±2.02 (7.5)	644±6.1 (1.6)	78.6±10.16 (22.4)	78.5±1.04 (2.3)	3.11±0.57 (31.5)	3.11±0.27 (15.3)
Prestige (FR)	4.25±0.54 (25.5)	49.4±1.14 (4.6)	645±12.6 (3.9)	87.53±5.8 (13.3)	78.8±1.2 (2.7)	3.75±0.69 (36.5)	3.57±0.59 (28.6)
Jersey (NL)	4.61±0.54 (23.5)	47.2±1.22 (5.2)	648±11.8 (3.6)	87.8±4.25 (9.7)	78.7±1.3 (2.9)	3.99±0.73 (36.6)	3.87±0.58 (26.0)
Tolar (NL)	4.58±0.42 (18.5)	47.4±1.29 (5.4)	656±9.7 (13.0)	83.6±9.04 (21.6)	78.3±1.3 (2.9)	3.94±0.69 (34.9)	3.76±0.46 (21.3)
Pongo (SE)	4.46±0.32 (14.6)	42.8±1.63 (7.6)	643±18.7 (12.0)	76.3±9.27 (24.3)	79.0±1.12 (3.8)	3.23±0.82 (51.0)	3.64±0.35 (16.7)
Breamer (UK)	4.17±0.56 (27.0)	46.8±1.28 (5.5)	633±14.4 (4.5)	85.6±6.27 (14.7)	78.7±1.4 (3.1)	3.61±0.66 (36.7)	3.50±0.63 (31.0)

¹ Mean ± standard error of the mean and coefficient of variation (in brackets). ² Countries of origin are DE = Germany, DK = Denmark, FR = France, NL = Netherlands, SE = Sweden, UK = United Kingdom. ³ Mean for the period 2005–2007 only.

breeding, the coarse-grain percentage and yield indices should have priority over grain yield.

The four-year mean, e.g. values averaged for wet and dry years together, showed that there were no significant differences in protein and starch content among the genotypes (Table 5). However, significant differences in nitrogen and starch content were obtained per single grain. The same varieties more often had less protein and a higher ratio of Cstarch:N per single grain. Very high plant population density and a high number of grains per hectare generally result

in lower grain quality. In our study, Annabell and Tocada had more than 100 million grain per hectare.

The next step in our investigations was to evaluate the varieties that have to be used in the breeding programs designed for dryer weather conditions.

The varieties with high trait values

A few varieties (Annabell, Cruiser, Sebastian) were high yielding in diverse weather conditions (Table 6). The varieties Annabell, Tocada, Justina and Cruiser were

Table 5. Variation¹ of grain quality traits of spring barley varieties, Dotnuva, Lithuania, 2004–2007.

Variety	Protein %	Starch %	Grain ×106/ha	N μg/grain	Starch μg/grain	Ratio Cstarch:N per grain
Annabell	12.4±1.04 (16.8)	60.2±0.78 (2.58)	102.0±7.97 (15.6)	903.0±89.94 (19.9)	27342±1022 (7.5)	13.8±1.27 (18.4)
Scarlett	13.0±1.27 (19.6)	60.7±0.39 (1.3)	96.6±8.72 (18.1)	917.8±83.44 (18.2)	27221±1154 (8.5)	13.4±1.32 (19.8)
Pongo	12.0±0.72 (12.0)	59.0±1.12 (3.8)	93.3±12.83 (27.5)	821.4±42.69 (10.4)	25326±1441 (11.4)	13.8±0.91 (13.2)
Tolar	13.0±0.89 (13.7)	58.0±0.80 (2.8)	97.6±8.6 (17.6)	980.22±42.49 (8.7)	27507±1108 (8.1)	13.8±0.91 (13.2)
Prestige	12.4±0.89 (14.4)	59.6±0.85 (2.8)	84.8±9.32 (22.0)	981.5±71.08 (14.5)	29417±971 (6.6)	13.6±1.10 (16.2)
Breamer	12.5±0.80 (12.9)	59.5±0.71 (2.4)	88.16±10.24 (23.2)	933.4±60.11 (12.9)	27837±714 (5.1)	13.4±1.00 (14.9)
Justina	12.4±0.91 (14.6)	58.5±0.88 (3.0)	99.47±8.24 (16.6)	945.9±78.3 (16.6)	27782±1053 (7.6)	13.6±1.16 (17.1)
Barke	12.7±0.99 (15.6)	60.8± (2.5)	88.43±10.86 (24.6)	984.9±78.8 (16.0)	29512±786 (5.3)	13.6±1.16 (17.1)
Sebastian	12.0±0.94 (15.7)	61.0±0.85 (2.8)	96.3±10.5 (21.9)	860.3±71.1 (16.5)	27439±829 (6.0)	13.6±1.16 (17.1)
Tocada	12.3±1.05 (17.2)	59.7±0.42 (1.4)	101.87±10.14 (19.9)	928±52.4 (11.3)	28552±1385 (9.7)	13.9±1.21 (17.4)
Jersey	12.0±0.80 (13.4)	59.1±1.33 (4.5)	94.1±11.9 (25.3)	905.8±71.18 (15.7)	27898±857 (6.2)	14.0±1.24 (17.8)
Class ²	12.6±0.64 (8.8)	59.1±0.38 (1.1)	85.7±9.69 (19.6)	933.1±48.13 (8.9)	27463±1172 (7.4)	13.14±0.77 (10.1)
Cruiser ²	12.8±0.72 (9.8)	59.8±0.74 (2.2)	96.38±5.23 (9.4)	913±42.87 (8.1)	26712±977 (6.3)	13.1±0.87 (11.5)
Power ²	12.5±0.37 (5.1)	60.1±0.93 (2.7)	96.84±8.55 (15.3)	876.61±26.21 (5.2)	26502±1709 (11.2)	13.4±0.49 (6.4)

¹ Mean ± standard error of the mean and coefficient of variation (in brackets). ² Mean for the period 2005–2007

high yielding in both dry years. The varieties Prestige, Breamer, Jersey and Barke had high TGW. Grading data, extract percentage and extract yield are the most important traits for spring barley malting varieties. High >2.5 mm grain yield in wet and dry weather conditions were shown by the varieties Justina, Jersey and Cruiser, and by Jersey, Cruiser and Tocada in dry conditions. According to the extract yield, Annabell,

Cruiser, Justina and Tocada were superior both in dry years and in wet conditions. The highest Cstarch:N ratio was demonstrated by the varieties Sebastian, Justina and Jersey in both seasonal weather conditions. Only Cruiser, Power and Sebastian had a high Cstarch:N ratio in dry conditions in both years. As a result, the inherent properties of the genotypes Annabell, Justina, Tocada, Jersey and Cruiser are of great interest for

Table 6. Performance of spring barley varieties with high trait values¹ in wet and dry seasonal weather conditions. Dotnuva, 2004–2007.

Trait	Wet weather conditions (2004, 2007)	Dry weather conditions (2005, 2006)
Yield (t/ha)	<u>Tolar</u> , Annabell, Barke, Breamer, Cruiser, Jersey, Justina, Prestige, Sebastian, Tocada	<u>Annabell</u> , <u>Cruiser</u> , <u>Justina</u> , <u>Tocada</u> , Jersey, Pasadena, Power, Scarlett, Sebastian
TGW (g)	Tocada, Barke, Breamer, Justina, Prestige, Tolar	Barke, <u>Jersey</u> , <u>Justina</u> , <u>Prestige</u> , Breamer, Class
HLW (g/L)	Class, Jersey, Pasadena, Potter, Prestige, Scarlett, Tolar	<u>Pongo</u> , <u>Potter</u> , <u>Tolar</u> , Annabell, Cellar, Class, Jersey, Power, Scarlett, Sebastian
Grain >2.5 mm (%)	<u>Prestige</u> , <u>Tolar</u> , Annabell, Barke, Breamer, Cruiser, Jersey, Scarlett	<u>Barke</u> , <u>Bremear</u> , <u>Jersey</u> , <u>Prestige</u> , <u>Scarlett</u> , Class, Sebastian
Grain >2.5 mm (t/ha)	<u>Tolar</u> , Annabell, Breamer, Cruiser, Jersey, Justina, Prestige	<u>Cruiser</u> , <u>Jersey</u> , <u>Justina</u> , <u>Tocada</u> , Barke, Class, Scarlett,
Extract (%)	<u>Barke</u> , <u>Scarlett</u> , <u>Sebastian</u> , Breamer, Jersey, Tolar	<u>Pongo</u> , <u>Scarlett</u> , <u>Sebastian</u> , Annabell, Barke, Power, Prestige, Potter, Tolar
Extract (t/ha)	Tocada, <u>Tolar</u> , Annabell, Barke, Breamer, Cruiser, Jersey, Justina, Prestige	<u>Annabell</u> , <u>Cruiser</u> , <u>Justina</u> , <u>Tocada</u> , Jersey, Power, Scarlett, Sebastian, Tolar
Protein (%)	<u>Tolar</u> , Class, Cruiser, Justina, Pongo, Power, Scarlett	<u>Barke</u> , <u>Tolar</u> , Breamer, Scarlett
Starch (%)	<u>Barke</u> , <u>Jersey</u> , <u>Scarlett</u> , Annabell, Breamer, Pasadena, Potter, Prestige, Sebastian, Tocada	<u>Power</u> , <u>Sebastian</u> , Annabell, Barke, Cruiser, Pongo, Potter, Scarlett, Tocada
Grain no. (×106/ha)	<u>Annabell</u> , <u>Tolar</u> , Breamer, Cruiser, Jersey, Pasadena, Pongo, Potter, Power, Scarlett, Sebastian, Tocada	<u>Annabell</u> , <u>Justina</u> , <u>Pongo</u> , <u>Potter</u> , <u>Tocada</u> , Class, Cruiser, Jersey, Pasadena, Power, Scarlett, Sebastian, Tolar
N (µg per grain)	<u>Tolar</u> , Barke, Class, Cruiser, Justina, Prestige, Power, Tocada	<u>Barke</u> , <u>Prestige</u> , Tolar, Annabell, Breamer, Jersey, Scarlett, Tocada
Starch (µg per grain)	<u>Barke</u> , <u>Tocada</u> , Class, Jersey, Justina, Pasadena, Prestige, Tolar	<u>Barke</u> , <u>Prestige</u> , <u>Sebastian</u> , Annabell, Justina, Power, Tocada
Ratio Cstarch:N per grain	<u>Annabell</u> , <u>Jersey</u> , <u>Sebastian</u> , Barke, Justina, Pasadena, Prestige, Scarlett, Tocada	<u>Cruiser</u> , <u>Power</u> , <u>Sebastian</u> , Class, Jersey, Justina, Pongo

¹ Underlined genotypes showed similar results in both years; the other showed high trait values in one year.

Table 7. Performance of spring barley varieties with low trait values¹ in wet and dry seasonal weather conditions. Dotnuva, Lithuania, 2004–2007.

Trait	Wet weather conditions (2004, 2007)	Dry weather conditions (2005, 2006)
Yield (t/ha)	Arve, Cellar, Extract, Henni, Pongo, Scarlett	<u>Antto</u> , <u>Breamer</u> , <u>Prestige</u> , Cellar, Hanka
TGW (g)	<u>Pongo</u> , Annabell, Cellar, Potter, Philadelphia, Scarlett, Sebastian	<u>Potter</u> , <u>Pongo</u> , <u>Cruiser</u> , Power, Scarlett, Sebastian, Tocada
HLW (g/L)	<u>Justina</u> , <u>Pongo</u> , Breamer, Barke, Potter, Extract, Tocada, Cellar	<u>Barke</u> , <u>Justina</u> , <u>Tocada</u> , Breamer, Hanka
Grain >2.5 mm (%)	Barke, Barabas, Gustav, Pasadena, Tocada	<u>Pongo</u> , <u>Power</u> , Barabas, Pasadena, Tocada, Tolar
Grain >2.5 mm (t/ha)	Pasadena, Antto, Extract, Hanka, Pongo, Sebastian	<u>Pongo</u> , <u>Power</u> , Arve, Antto, Cellar, Tolar, Barabas
Extract (%)	Pasadena, Antto, Gustav, Justina, Pongo	Antto, Barabas, Cruiser, Justina, Pasadena
Extract (t/ha)	Cellar, Extract, Gustav, Hanka, Pongo	<u>Antto</u> , <u>Prestige</u> , Breamer, Cellar, Hanka
Protein (%)	<u>Annabell</u> , <u>Sebastian</u> , Jersey, Maurita, Tocada	<u>Power</u> , <u>Pongo</u> , <u>Sebastian</u> , Jersey, Potter, Pasadena, Xanadu
Starch (%)	<u>Justina</u> , <u>Tolar</u> , Pongo, Sebastian, Xanadu	<u>Jersey</u> , Antto, Justina, Prestige, Tolar
Grain no. (×106/ha)	Barke, Class, Cellar, Extract, Hanka	<u>Prestige</u> , Antto, Barke, Cellar, Hanka
N (µg per grain)	<u>Annabell</u> , <u>Sebastian</u> , <u>Pongo</u> , Potter	<u>Pongo</u> , <u>Power</u> , <u>Sebastian</u> , Astoria, Cruiser, Potter
Starch (µg per grain)	Potter, Cellar, Hanka, Pongo, Barabas, Gustav	<u>Cruiser</u> , <u>Pongo</u> , <u>Tolar</u> , Antto, Astoria, Potter
Ratio Cstarch:N per grain	<u>Tolar</u> , Class, Cruiser, Gustav, Hanka, Power	<u>Tolar</u> , Antto, Hanka

¹ Underlined genotypes showed similar results in both years; non-underlined ones showed low-trait-values in one year.

growing barley for malting purposes in warming climate conditions.

Barley varieties with a high grain protein content and high yield suit better for feeding purposes. In this respect, Justina, Tolar and Power exhibited high total grain protein percentage as well as per single grain in

wet weather conditions. In dry and sunny post-anthesis weather conditions, many of the varieties had high protein percentage. However, in both dry experimental years the varieties Tolar and Barke had high protein percentage. The grain yield of these varieties was moderate.

The varieties with low trait values

The low content of protein and high ratio of Cstarch:N per grain are important malting barley characteristics. In both dry years, Pongo, Power, Sebastian, Potter, Pasadena, Jersey, Annabell had the lowest protein content (Table 7). These varieties not only had low N content per single grain but also tended to have a high ratio of Cstarch:N per grain.

The variety Sebastian, which showed low protein percentage in wet and dry weather conditions, could be included in malting barley breeding programs with the aim of reducing protein content.

Conclusions

Subjected to seasonal east European (Lithuanian) weather conditions, the spring barley varieties developed in western and southern regions of Europe exhibited the greatest variation in the following traits: coarse grain and extract yield, grain number per area, and ratio Cstarch:N per single grain. Under warm and dry Lithuanian climate conditions, coarse grain yield and ratio Cstarch:N per grain tended to decrease in the spring barley genotypes developed in western and southern regions of Europe.

The ratio Cstarch:N per single grain and N per single grain are important indicators of spring barley malting quality. Coarse grain and extract percentage correlation with those traits are similar to that between coarse grain or extract percentage and starch or protein percentage. However, the ratio Cstarch:N per single grain and N per single grain better reflected the suitability of spring barley for malting in dry growing conditions.

Foreign varieties with desirable characteristics should be used in breeding for warm and dry climate conditions after estimating yield and grain quality parameters obtained in dry and warm seasonal weather conditions rather than from data averaged over several years.

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Genetic diversity of the qingke (hulless barley) varieties from the Qinghai-Tibet plateau of China detected by SRAP markers

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Abstract

Genetic diversity of 68 accessions of qingke (hulless barley) varieties from the Qinghai-Tibet Plateau of China was analyzed by using sequence-related amplified polymorphism (SRAP) markers. The results showed that 64 primer combinations produced a total of 1056 clear bands with an average of 16.5, of which only one primer pair did not amplify polymorphic bands, while the others (98.4%) produced 311 polymorphic bands (29.8%). Five hundred and eighty-one allelic phenotypes were amplified with an average of 9.22 alleles per primer pair. The mean values of genetic diversity in Sichuan, Gansu, Tibet and Qinghai provinces of China were 0.5207, 0.5091, 0.4859 and 0.3920, respectively, with an average of 0.6226. The genetic differentiation value among different regions ranged from 2.56% to 66.21% with an average of 22.96%. The 68 accessions were classified into six major groups by cluster analysis using UPGMA, which showed a significant relationship with the origin regions of accessions.

Introduction

Hulless barley, locally known as qingke, is very much liked by Chinese Zang-nationality

people, and is also an excellent special germplasm resource in the Qinghai-Tibet plateau of China. Hulless six-row barley has been cultivated in China (Xu, 1982; Xu and Feng, 2001) for a long time. In recent years, qingke breeding and use have received increasing attention. To study the genetic diversity of qingke germplasm resources benefits the protection of qingke resources and the development of new qingke varieties, as well improving the quality of life of Zang-nationality people in China.

Nowadays, more and more DNA markers have been widely used in genetic diversity studies (Petersen *et al.*, 1994; Nevo *et al.*, 1998; Struss and Plieske, 1998; Fernandez *et al.*, 2002; Feng *et al.*, 2006a, b; Pan *et al.*, 2007), germplasm analysis (Powell *et al.*, 1996) and map construction (Graner *et al.*, 1991; Liu *et al.*, 1996) of barley. However, reports about qingke landraces and studies of qingke varieties using molecular markers are rare (Feng *et al.*, 2006b; Pan *et al.*, 2007). Recently, Zhou *et al.* (2007) analyzed the genetic diversity of hordeins in qingke varieties from the plateau regions of Tibet and Sichuan province, using acid-polyacrylamide gel electrophoresis (A-PAGE). It is important to study the genetic diversity of the qingke varieties from the Qinghai-Tibet plateau of

China and efficiently use molecular markers for qingke varietal improvement.

Sequence-related amplified polymorphism (SRAP) markers developed by Li and Quiros (2001) were successfully applied in cultivar identification (Li *et al.*, 2006), map construction (Li and Quiros, 2001; Lin *et al.*, 2003), genetic diversity evaluation (Riaz *et al.*, 2001; Ferriol *et al.*, 2003; Budak *et al.*, 2004; Sun *et al.*, 2006), comparative genomics (Li *et al.*, 2003), and gene location (Wang *et al.*, 2004) of different species. We first reported the use of SRAP markers in the evaluation of genetic diversity of qingke varieties from the plateau regions of Sichuan province (Yang *et al.*, 2008). In this paper, we report the results of a genetic diversity analysis of 68 accessions of qingke varieties from the Qinghai-Tibet plateau regions, including Sichuan (SC), Gansu (GS), Tibet (XZ), Qinghai (QH) and Yunnan (YN) provinces. The objectives of the research were: (1) to evaluate the levels of genetic diversity and differentiation of these accessions; and (2) to further understand the genetic basis of these accessions, and to formulate appropriate strategies for the conservation and utilization of the qingke genetic resources available.

Materials and methods

Plant materials

A total of 68 accessions of qingke varieties, including 25 (SC01 to SC25) from Sichuan province, 6 (GS26 to GS31) from Gansu province, 17 (XZ32 to XZ48) from Tibet, 18 (QH49 to QH66) from Qinghai province and 2 (YN67 and YN68) from Yunnan province, were collected from the Qinghai-Tibet Plateau regions of China (Table 1).

Genomic DNA extraction

The cetyl trimethyl ammonium bromide (CTAB) protocol (Sharp *et al.*, 1988) was used to extract genomic DNA from about 100 mg of fresh young leaf-tissue of each accession. The quality and the concentration

of the DNA were estimated using a Beckman counter DU800 nucleic acid/protein analyzer. The isolated genomic DNA was stored at -20°C for use.

PCR analysis

The SRAP primer sequences used in this study were from Li and Quiros (2001), and are listed in Table 2. Primers were produced by the Dalian Bao Bioengineering company (Dalian, China). The PCR amplification was carried out in a PTC-100TM Thermo Cycler in a 15 µL volume containing 1.5 µL of 10× buffer, 1 µL of Mg²⁺, 0.4 µL of dNTP, 1 µL of forward and reverse primers, and 0.6 U of rTaq DNA polymerase (TaKaRa, China), basically according to the methods described by Li and Quiros (2001), with the following reaction conditions: 95°C × 1 min; 5 cycles of 94°C × 1 min, 35°C × 1 min and 72°C × 1 min; 94°C × 1 min, 50°C × 1 min, 72°C 1 min, 35 cycles; 72°C, 10 min. The products were separated on 6% denatured polyacrylamide gels using 1×TBE buffer. The gel was pre-run at 220 V constant voltage for 20 min before the samples were loaded, using DYY-6C electrophoresis equipment (LiuYi Instrument, Beijing, China). After loading the samples, the gel was run at 450 V constant voltage for 1.5–2.0 hr until the xylene cyanol front reached 2/3 through the gel towards the bottom. DL2000 DNA marker (Dalian TaKaRa Bio Company) was used as a size standard. After electrophoresis, the gel was stained using AgNO₃ solution (Bassam *et al.*, 1991).

Data analysis

Each SRAP primer pair was considered to be one genetic marker. The SRAP profiles were scored for the presence (1) or absence (0) of clear bands for each qingke genotype. The genetic diversity (H) at each primer pair was evaluated with $H = 1 - \sum P_i^2$, where P_i is the frequency of the *i*th allele of the locus (Nei, 1973). The genetic diversity (HT) of the entire sample ($HT = HS + DST$) can be partitioned

Table 1. Cultivar names and origins of the accessions used in this study.

Accession code	Name	Origin (province)	Accession code	Name	Origin (province)
SC01	Kangqing 1	Sichuan	XZ35	Ximala 15	Tibet
SC02	Kangqing 2	Sichuan	XZ36	Ximala 19	Tibet
SC03	Kangqing 6	Sichuan	XZ37	Zangqing 25	Tibet
SC04	Kangqing 7	Sichuan	XZ38	Zangqing 80	Tibet
SC05	Bailiuleng	Sichuan	XZ39	Zangqing 148	Tibet
SC06	Emu 1	Sichuan	XZ40	Zangqing 311	Tibet
SC07	Gangtuoqingke	Sichuan	XZ41	Zangqing 320	Tibet
SC08	Heiqingke	Sichuan	XZ42	Zangqing 690	Tibet
SC09	Dongshenghei	Sichuan	XZ43	Zangqing 3179	Tibet
SC10	Danbaheiqingke	Sichuan	XZ44	Shanqing 7	Tibet
SC11	Qianningluqingke	Sichuan	XZ45	Alidangdiqingke	Tibet
SC12	Minxianqingke	Sichuan	XZ46	Baidichunqingke	Tibet
SC13	98172-2	Sichuan	XZ47	QB23	Tibet
SC14	813	Sichuan	XZ48	Lasagoumang	Tibet
SC15	Songpanzingke	Sichuan	QH49	Beiqing 1	Qinghai
SC16	Qianningqingke	Sichuan	QH50	Beiqing 2	Qinghai
SC17	Baiyuqingke	Sichuan	QH51	Beiqing 3	Qinghai
SC18	33-2-1-1	Sichuan	QH52	Beiqing 4	Qinghai
SC19	Ganziheimawei	Sichuan	QH53	Beiqing 5	Qinghai
SC20	603	Sichuan	QH54	Beiqing 6	Qinghai
SC21	Qipangou	Sichuan	QH55	Beiqing 7	Qinghai
SC22	Cunqingke	Sichuan	QH56	Beiqing 8	Qinghai
SC23	Kangqing 5	Sichuan	QH57	Kunlun 2	Qinghai
SC24	Aqing 4	Sichuan	QH58	Kunlun 3	Qinghai
SC25	Aqing 5	Sichuan	QH59	Kunlun 8	Qinghai
GS26	Ganqing 2	Gansu	QH60	Kunlun 10	Qinghai
GS27	Ganqing 3	Gansu	QH61	Kunlun 12	Qinghai
GS28	Ganqing 4	Gansu	QH62	Dongqing 1	Qinghai
GS29	9516	Gansu	QH63	Dongqing 2	Qinghai
GS30	Dulihuang	Gansu	QH64	Mennong 1	Qinghai
GS31	94-19-1	Gansu	QH65	Moduocha 1	Qinghai
XZ32	Zangqing 1	Tibet	QH66	Nanfan 3	Qinghai
XZ33	Zangqing 21	Tibet	YN67	Diqing 1	Yunnan
XZ34	Ximala 96	Tibet	YN68	Diqing 4	Yunnan

into components, reflecting genetic distance among subgroups (DST) and genetic polymorphism within subgroups (HS) (Nei, 1973), with differentiation among subgroups (GST) being calculated as $GST = 1 - HS/HT$ (Nei, 1973). Pair-wise comparison of genetic diversity between subgroups was carried out according to the Z-test method (Zhang and

Allard, 1986; Zhang *et al.*, 1992).

Genetic similarities (GS) were estimated using the formula (Nei and Li, 1979): $GS_{ij} = 2N_{ij} / (N_i + N_j)$, where N_{ij} is the number of bands shared by accessions i and j , and N_i and N_j are the number of bands from accessions i and j , respectively. The distribution of allelic frequencies among

Table 2. SRAP primer sequences used in this study.

Name of primer	Forward primer (5'→3')	Name of primer	Reverse primer(5'→3')
me5	T GAG TCC AAA CCGG AAG	em12	GA CTG CGT ACG AATT GTC
me6	T GAG TCC AAA CCGG TAA	em13	GA CTG CGT ACG AATT GGT
me7	T GAG TCC AAA CCGG TCC	em14	GA CTG CGT ACG AATT CAG
me8	T GAG TCC AAA CCGG TGC	em15	GA CTG CGT ACG AATT CTG
me9	T TCA GGG TGG CCGG ATG	em16	GA CTG CGT ACG AATT CCG
me10	T GGG GAC AAC CCGG CTT	em17	GA CTG CGT ACG AATT CCA
me11	C TGG CGA ACT CCGG ATG	em18	GA CTG CGT ACG AATT GGT
me12	G GTG AAC GCT CCGG AAG	em19	GA CTG CGT ACG AATT CCG

the subgroups was tested using the Chi-square test (Rong, 1993). Cluster analysis of the similarity matrix was used to reveal associations among accessions using the UPGMA method (unweighted pair group method with arithmetic averages), employing the NTSYS-pc (version 2.10s) program (Rohlf, 1993).

Results

Polymorphism and alleles of PCR products

Figure 1 shows the amplification results of the primer combination “me8/em12” in some of the accessions. Sixty-four pairs of primers, which were formed between one of eight forward primers and one of eight reverse primers, generated a total of 1056 clear fragments, with an average of 16.5. Among these primer pairs, eleven bands which were produced by the primer combination “me9/em14” have no polymorphism (Table 3), while 1045 bands were generated from 63 primer pairs (98.4%) with an average of 16.6 bands per primer pair, except for the primer pair “me9/em14”, of which 311 bands (29.8%) were polymorphic.

The number of polymorphic bands per primer pair ranged from 1 (me7/em15, me11/em13) to 15 (me10/em14) with an average of 4.94. The primer combination “me10/em14” generated 15 polymorphic bands, which was the greatest polymorphism (60.0%), while the lowest primer combinations “me7/em15” and

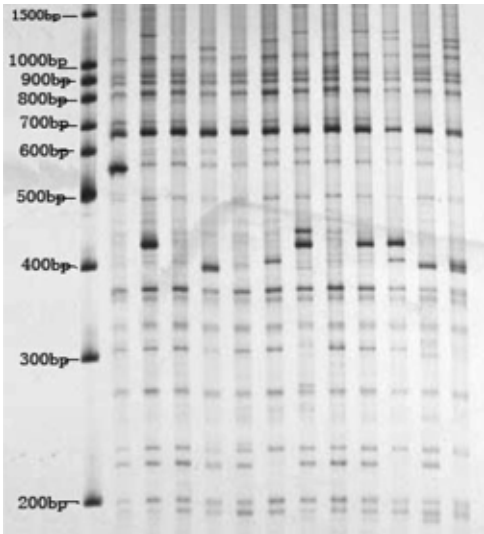


Figure 1. Amplification results of the primer combination “me8/em12” in some of the accessions.

“me11/em13” only generated 1 polymorphic band; the percentage of polymorphic bands was 12.5% and 5.6%, respectively.

A total of 581 alleles were detected in entire samples among 63 primer pairs with an average of 9.22 alleles per primer pair (Table 3). The number of alleles ranged from 2 (me11/em13, me7/em15 and me6/em15) to 26 (me5/em17, me6/em18 and me10/em14) in the entire sample. The average numbers of alleles for SC, GS, XZ and QH provinces were 5.23, 4.40, 4.02 and 2.46, respectively. The number of common alleles between the

Table 3. NPB/TNB¹, genetic diversity/No. of alleles, genetic differentiation (GST) and allelic frequencies distribution (χ^2) from 63 SRAP primer pairs among the developed qingke varieties.

Primer combination	NPB/ TNB	SC	GS	XZ	QH	Entire sample	GST (%)	χ^2
me5/em12	5 / 19	0.5888 / 6	0.6667 / 3	0.8097 / 8	0.6975 / 5	0.8320 / 8	17.97	56.03**
me5/em13	3 / 17	0.5888 / 6	0.6670 / 4	0.8097 / 8	0.6975 / 5	0.8320 / 8	17.97	62.20**
me5/em14	3 / 10	0.4032 / 2	0.5000 / 2	0.0000 / 1	0.5123 / 4	0.3861 / 4	12.49	162.94**
me5/em15	6 / 20	0.8672 / 11	0.4444 / 2	0.6307 / 3	0.5741 / 4	0.8563 / 12	19.48	46.49*
me5/em16	5 / 18	0.5536 / 4	0.0000 / 1	0.4844 / 2	0.6790 / 5	0.6694 / 7	22.37	99.67**
me5/em17	7 / 17	0.8928 / 13	0.2778 / 2	0.7612 / 7	0.8580 / 11	0.9412 / 26	15.69	4.34
me5/em18	5 / 11	0.2784 / 3	0.6111 / 3	0.6574 / 4	0.2901 / 3	0.5840 / 6	29.89	52.76**
me5/em19	6 / 12	0.7008 / 5	0.6111 / 3	0.5536 / 3	0.1975 / 2	0.8067 / 9	35.85	138.52**
me6/em12	5 / 18	0.7968 / 8	0.6111 / 3	0.7266 / 4	0.6975 / 5	0.8558 / 11	14.14	95.91**
me6/em13	7 / 20	0.8736 / 14	0.7778 / 5	0.8097 / 8	0.8395 / 10	0.9206 / 21	8.85	1.67
me6/em14	2 / 10	0.3328 / 3	0.0000 / 1	0.1972 / 2	0.2068 / 2	0.3402 / 3	31.44	40.68**
me6/em15	2 / 8	0.2112 / 2	0.2778 / 2	0.0000 / 1	0.3457 / 2	0.2130 / 2	6.34	19.80**
me6/em16	5 / 16	0.3488 / 5	0.5000 / 3	0.5952 / 4	0.5671 / 4	0.6143 / 7	21.2	81.38**
me6/em17	5 / 20	0.6880 / 6	0.4444 / 2	0.6505 / 6	0.7716 / 6	0.7847 / 9	13.47	59.41**
me6/em18	11 / 16	0.8928 / 15	0.7778 / 5	0.7059 / 6	0.8364 / 8	0.9213 / 26	11.12	10.63
me6/em19	4 / 12	0.1504 / 3	0.7222 / 4	0.1107 / 2	0.4012 / 2	0.3416 / 6	23.72	24.14
me7/em12	5 / 16	0.1472 / 2	0.5000 / 3	0.4983 / 2	0.2307 / 3	0.3508 / 5	11.66	7.54
me7/em13	10 / 21	0.8576 / 10	0.4444 / 5	0.8097 / 7	0.7346 / 8	0.8958 / 21	23.21	10.47
me7/em14	7 / 17	0.6368 / 8	0.5000 / 3	0.8062 / 7	0.7840 / 7	0.8508 / 18	10.20	14.78
me7/em15	1 / 8	0.2112 / 2	0.2778 / 2	0.4567 / 2	0.4444 / 2	0.4444 / 2	17.56	32.97**
me7/em16	4 / 16	0.6560 / 4	0.6111 / 3	0.6851 / 4	0.3796 / 4	0.6823 / 6	47.27	72.86**
me7/em17	9 / 15	0.6976 / 7	0.1944 / 2	0.7474 / 6	0.9352 / 13	0.8306 / 21	48.22	2.89
me7/em18	2 / 10	0.2688 / 2	0.0000 / 1	0.4844 / 2	0.5864 / 3	0.5083 / 4	27.37	95.25**
me7/em19	3 / 16	0.4768 / 4	0.4444 / 2	0.5467 / 3	0.4938 / 2	0.5239 / 4	25.83	36.58**
me8/em12	8 / 22	0.5664 / 7	0.7222 / 4	0.8478 / 8	0.9136 / 14	0.0864 / 20	10.20	6.90
me8/em13	4 / 12	0.7456 / 5	0.4444 / 2	0.4567 / 3	0.4938 / 2	0.5062 / 5	17.56	76.70**
me8/em14	3 / 16	0.2784 / 3	0.5000 / 2	0.5329 / 4	0.3457 / 2	0.6543 / 4	47.27	36.43**
me8/em15	5 / 12	0.0768 / 2	0.0000 / 1	0.4983 / 2	0.0000 / 1	1.0000 / 3	48.22	76.73**
me8/em16	2 / 11	0.3808 / 3	0.0000 / 1	0.6782 / 4	0.5988 / 4	0.4012 / 4	27.37	31.46**
me8/em17	4 / 17	0.7488 / 6	0.2778 / 2	0.6021 / 5	0.6667 / 4	0.3333 / 9	25.83	34.57
me8/em18	6 / 15	0.4064 / 5	0.2778 / 2	0.7128 / 6	0.8025 / 8	0.1975 / 14	31.94	76.31**
me8/em19	3 / 16	0.4576 / 3	0.5000 / 2	0.2076 / 2	0.1975 / 2	0.8025 / 3	21.46	125.13**
me9/em12	3 / 8	0.4352 / 2	0.2778 / 2	0.1107 / 2	0.5494 / 3	0.4506 / 3	18.48	37.89**
me9/em13	4 / 18	0.2176 / 3	0.0000 / 1	0.3945 / 4	0.2778 / 2	0.7222 / 5	17.61	132.27**
me9/em15	4 / 15	0.0000 / 1	0.2778 / 2	0.3045 / 3	0.4012 / 2	0.5988 / 5	26.69	99.79**
me9/em16	4 / 16	0.6560 / 6	0.6111 / 3	0.4844 / 2	0.4753 / 2	0.5247 / 7	24.13	14.90
me9/em17	2 / 17	0.2784 / 3	0.0000 / 1	0.1107 / 2	0.1975 / 2	0.8025 / 3	21.46	46.68**
me9/em18	8 / 19	0.8544 / 10	0.6111 / 3	0.8927 / 12	0.7130 / 5	0.2870 / 21	11.66	86.56
me9/em19	2 / 12	0.2688 / 2	0.6111 / 3	0.6713 / 4	0.5926 / 4	0.4074 / 4	23.21	43.83**
me10/em12	6 / 20	0.5584 / 5	0.5000 / 3	0.5225 / 4	0.5494 / 3	0.7282 / 8	25.65	23.58
me10/em13	4 / 4	0.6080 / 7	0.5000 / 3	0.5467 / 5	0.5494 / 3	0.7241 / 9	21.77	16.05
me10/em14	15 / 25	0.8544 / 11	0.7778 / 5	0.6851 / 8	0.7716 / 4	0.9320 / 26	16.18	7.31
me10/em15	9 / 16	0.6784 / 7	0.5000 / 2	0.5813 / 3	0.7469 / 8	0.7208 / 13	9.02	19.56
me10/em16	5 / 16	0.8032 / 7	0.7778 / 5	0.7820 / 7	0.8086 / 9	0.9008 / 17	11.54	38.16
me10/em17	2 / 12	0.0768 / 2	0.0000 / 1	0.0000 / 1	0.1049 / 2	0.0592 / 3	2.56	111.53**
me10/em18	4 / 17	0.4224 / 3	0.0000 / 1	0.1107 / 2	0.2963 / 4	0.5000 / 7	46.13	47.25**
me10/em19	7 / 18	0.7904 / 9	0.7778 / 5	0.7128 / 6	0.7654 / 7	0.9151 / 22	16.68	102.93**
me11/em12	6 / 16	0.7520 / 5	0.2778 / 2	0.7336 / 4	0.6605 / 4	0.3395 / 10	21.33	99.39**
me11/em13	1 / 18	0.0768 / 2	0.0000 / 1	0.0000 / 1	0.0000 / 1	1.0000 / 2	66.21	106.07**
me11/em14	4 / 18	0.6464 / 5	0.5000 / 3	0.4637 / 4	0.7160 / 6	0.2840 / 9	8.09	43.37**
me11/em15	5 / 24	0.4000 / 4	0.2778 / 2	0.3045 / 3	0.6790 / 6	0.3210 / 8	13.34	4.19
me11/em16	7 / 23	0.7520 / 8	0.6667 / 4	0.6159 / 6	0.8333 / 8	0.1667 / 15	16.38	42.31
me11/em17	5 / 24	0.8416 / 9	0.6111 / 3	0.5121 / 3	0.6111 / 4	0.3889 / 10	15.63	52.30**
me11/em18	4 / 20	0.5344 / 3	0.5000 / 2	0.4567 / 2	0.4259 / 3	0.5741 / 4	13.40	129.71**
me11/em19	3 / 10	0.5504 / 3	0.0000 / 1	0.2907 / 2	0.1049 / 2	0.8951 / 4	24.72	56.57**
me12/em12	7 / 18	0.6080 / 5	0.2778 / 2	0.6298 / 4	0.5679 / 3	0.4321 / 7	9.51	56.52**
me12/em13	3 / 22	0.3648 / 2	0.0000 / 1	0.1107 / 2	0.1049 / 2	0.8951 / 4	26.1	56.52**
me12/em14	3 / 23	0.2176 / 3	0.0000 / 1	0.1107 / 2	0.1049 / 2	0.8951 / 4	27.8	17.19*
me12/em15	5 / 25	0.5056 / 5	0.2778 / 2	0.0000 / 1	0.1975 / 2	0.8025 / 6	20.19	93.09**
me12/em16	5 / 27	0.4768 / 4	0.0000 / 1	0.1107 / 2	0.3765 / 4	0.6235 / 6	41.29	37.51**
me12/em17	5 / 17	0.5888 / 6	0.5000 / 2	0.3875 / 3	0.3457 / 2	0.6543 / 9	42.00	156.03**
me12/em18	2 / 25	0.4352 / 2	0.2778 / 2	0.0000 / 1	0.0000 / 1	1.0000 / 3	56.70	52.97**
me12/em19	10 / 18	0.7712 / 7	0.7222 / 4	0.8927 / 12	0.7963 / 7	0.2037 / 19	7.60	111.7**
Mean	4.9416.59/	0.5207/ 5.23	0.3920/ 2.46	0.4859/ 4.02	0.5091/ 4.40	0.6226/ 9.22	22.96	
SD ²		0.24 / 3.19	0.26 / 1.20	0.27 / 3.24	0.26 / 3.92	0.24 / 14.55	13.11	

¹ NPB is the number of polymorphic bands; TNB is the total number of bands. ² SD = Significant Difference.

** = significantly different at P < 0.01; *** = significantly different at P < 0.001.

two arbitrary subgroups of SC, GS, XZ and QH province also varied with the different SRAP markers (Table 4). The highest average number of common alleles (2.27) was found between XZ and SC provinces, whereas the average number of common alleles between XZ and QH provinces was the lowest (1.27).

Genetic diversity and the distribution of allelic frequencies

The genetic diversity from 66 accessions (two accessions from Yunnan province were not included) of the subgroups SC, GS, XZ and QH was analyzed using 63 pairs of primers. The range of genetic diversity from Subgroup SC, GS, XZ and QH varied from 0 (me9/em15) to 0.8928 (me5/em17, me6/em18) with an average of 0.5207, from 0 (14 primer pairs such as me12/em13, me12/em14 and me12/em16, etc.) to 0.7778 (me6/em13, me6/em18, me10/em14, me10em16 and me10/em19) with an average of 0.3920, from 0 (6 primer pairs such as me12/em15, me12/em18, etc.) to 0.8927 (me12/em19, me9/em18) with an average of 0.4859, from 0 (me8/em15, me11/em13 and me12/em18) to 0.9352 (me7/em17) with an average of 0.5091, respectively, and the genetic diversity range of the entire sample was from 0.0592 (me10/em17) to 1.0000 (me8/em15, me11/em13 and me12/em18) with an average of 0.6226. Generally, the order of genetic diversity mean of the four subgroups was SC > QH > XZ > GS. The Chi-square test (Table 3) indicated that 44 of 63 primer pairs (69.8%) had significant difference, but 30.2% of the primer combinations had no significant difference, in the distribution of allelic frequencies among the four subgroups. The comparisons of genetic diversity (Table 4) indicated that the genetic diversity between two of the four subgroups was significantly different along with different primer pairs. However, on average, significant differences existed between the two arbitrary subgroups of the four subgroups.

Genetic differentiation and cluster analysis

The genetic differentiation (Table 3) among the qingke varieties from the four subgroups ranged from 2.56% (me10/em17) to 66.21% (me11/em13) with different SRAP primer pairs, with an average of 22.96%. This indicated the average genetic differentiation that existed among the accessions from the four provinces.

The dendrogram using UPGMA analysis clustered the 68 accessions into six main groups (Figure 2). Group A comprised the 22 SC collections, except that SC06 clustered into Group F, while SC24 and SC25 clustered into Group B with all 6 GS accessions. All the XZ accessions except for XZ47 were classified into Group C, and all the QH entries and YN67 and YN68 belonged to Group D. The accession “XZ47” was put in Group E by itself. The dendrogram revealed significant relationships with the regions of origin of the accessions.

Discussion

The SRAP markers system was primarily developed for Brassica species, and was then tested in other crops. The previous results showed that SRAP markers have many advantages, such as simplicity, reliability, moderate output ratio and easy sequencing of selected bands (Li and Quiros, 2001). According to Ferriol *et al.* (2003) the information provided by SRAP markers about genetic diversity agreed with the morphological variability and with the evolutionary history of the morphotypes more than that provided by AFLP markers. In barley, the results, which were first reported for the genetic diversity of the qingke varieties from Sichuan province using SRAP markers by us (Yang *et al.*, 2008), also indicated that the SRAP markers had rich polymorphism, and showed a clear correlation between the clusters and the origin of the accessions. In this current study,

63 of 64 SRAP primer combinations (98.4%) produced higher polymorphic bands (29.5%). The number of alleles (581) amplified by 63 primer combinations in 68 accessions was higher than using SSR markers (Feng *et al.*, 2006b; Pan *et al.*, 2007). The dendrogram of SRAP markers given in the present study could classify the geographical origins of the accessions (Figure 2). Our results show that SRAP markers could be efficiently used in studying the genetic variability of barley.

The amount of genetic diversity, which can reflect the extent of genetic variation, depends on the types and the distribution frequencies of alleles. We detected 581 alleles over 63 primer pairs in the entire sample, with an average of 9.22 alleles per primer pair (Table 3). The order of the average for number of alleles based on Table 3 was: SC (5.23 ± 3.19) > QH (4.40 ± 3.92) > XZ (4.02 ± 3.24) > GS (2.46 ± 1.20), and the mean genetic diversity followed the same order, indicating that the qingke varieties from Sichuan province have the widest genetic

basis among the four subgroups, while those from Gansu province have the narrowest. In terms of single subgroups and the entire sample, the genetic diversity values were all low, indicating that it is very necessary to widen the genetic basis of qingke. However, the average number of common alleles was highest (2.27) between the Tibet and Sichuan accessions, while the lowest average number of common alleles (1.27) was found between the Gansu and Qinghai accessions (Table 4), indicating that the Sichuan accessions were largely different from the Tibet accessions, and the Gansu accessions were less different from the Qinghai accessions. The comparisons of genetic diversity all showed significant difference between the two arbitrary subgroups. For most primer combinations (69.8%), the distribution frequencies of alleles showed significant differences (Table 3). Table 3 also shows that a moderate genetic differentiation (22.96%) existed among the four subgroups. The genetic resources from different geographical areas can bring genetic diversity that is required for barley breeding. This study has also illustrated low genetic diversity among the qingke varieties, and suggests that collection and conservation of barley genetic resources are important for the improvement of this crop.

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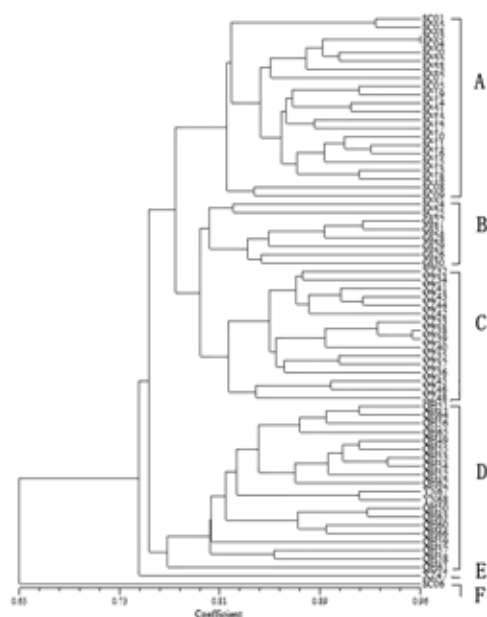


Figure 2. A dendrogram of 68 accessions of hulless barley varieties from the Qinghai-Tibet Plateau of China generated from SRAP markers.

Table 4. The number of common alleles/genetic diversity difference between the two arbitrary subgroups

Primer pair	SC-GS	SC-XZ	SC-QH	GS-XZ	GS-QH	XZ-QH
me5/em12	2 / -0.0779	1 / -0.2209**	2 / -0.1087**	1 / -0.1430**	2 / -0.0309	1 / 0.1122**
me5/em13	3 / -0.0779	6 / -0.2209**	3 / -0.1087**	4 / -0.1430**	3 / -0.0309	5 / 0.1122**
me5/em14	2 / -0.0968	1 / 0.4032**	2 / -0.1091**	1 / 0.5000*	2 / -0.0123	1 / -0.5123**
me5/em15	1 / 0.4228**	2 / 0.2305**	4 / 0.2931**	1 / -0.1922*	0 / -0.1296*	1 / 0.0626*
me5/em16	0 / 0.5536**	2 / 0.0692**	2 / -0.1254**	1 / -0.4844*	1 / -0.6790**	1 / -0.1946**
me5/em17	2 / 0.6150**	2 / 0.1316**	3 / 0.0348**	0 / -0.4835**	1 / -0.5802**	2 / -0.0968**
me5/em18	2 / -0.3327**	2 / -0.3790**	2 / -0.0117	3 / -0.0463	2 / 0.3210**	2 / 0.3673**
me5/em19	1 / 0.0897*	1 / 0.1472**	0 / 0.5033**	1 / 0.0575	0 / 0.4136**	1 / 0.3561**
me6/em12	1 / 0.1857**	3 / 0.0702**	5 / 0.0993**	1 / -0.1155	0 / -0.0864	2 / 0.0291
me6/em13	4 / 0.0958**	4 / 0.0639**	4 / 0.0341**	1 / -0.0319	0 / -0.0617*	3 / -0.0298**
me6/em14	1 / 0.0959**	2 / 0.1356**	2 / 0.1260*	1 / -0.1972*	1 / -0.2068	2 / -0.0096
me6/em15	2 / -0.0666	1 / 0.2112*	2 / -0.1345*	1 / 0.2778	2 / -0.0679	1 / -0.3457**
me6/em16	2 / -0.1512*	4 / -0.2464**	3 / -0.2129**	2 / -0.0952	2 / -0.0617	2 / 0.0334
me6/em17	2 / 0.2436**	4 / 0.0375	4 / -0.0836**	2 / -0.2061**	1 / -0.3272**	4 / -0.1211**
me6/em18	2 / 0.1150**	2 / 0.1869**	3 / 0.0564**	0 / 0.0719*	1 / -0.0586*	0 / -0.1305**
me6/em19	2 / -0.5718**	1 / 0.0397	2 / -0.2508**	1 / 0.6115**	2 / 0.3210**	1 / -0.2905**
me7/em12	1 / -0.3528**	2 / -0.3511**	2 / -0.0565	2 / 0.0017	2 / 0.2963**	2 / 0.2946**
me7/em13	2 / 0.4132**	3 / 0.0479**	0 / 0.1230**	2 / -0.3652**	0 / -0.2901**	0 / 0.0751**
me7/em14	1 / 0.1368*	4 / -0.1694**	3 / -0.1472**	2 / -0.3062**	0 / -0.2840**	1 / 0.0223
me7/em15	2 / -0.0666	2 / -0.2455**	2 / -0.2332**	2 / -0.1790*	2 / -0.1667	2 / 0.0123
me7/em16	2 / 0.0449	4 / -0.0291*	3 / 0.2764**	2 / -0.0740	1 / 0.2315**	3 / 0.3055**
me7/em17	2 / 0.5032**	4 / -0.0498**	3 / -0.2376**	2 / -0.5530**	2 / -0.7407**	2 / -0.1878**
me7/em18	1 / 0.2688**	2 / -0.2156**	1 / -0.3176**	1 / -0.4844*	1 / -0.5864**	1 / -0.102**
me7/em19	2 / 0.0324	3 / -0.0699**	2 / -0.0170	2 / -0.1023	2 / -0.0494	2 / 0.0529*
me8/em12	2 / -0.1558**	6 / -0.2814**	5 / -0.3472**	2 / -0.1255**	2 / -0.1914**	7 / -0.0658**
me8/em13	2 / 0.3012**	3 / 0.2889**	2 / 0.2518**	2 / -0.0123	1 / -0.0494	2 / -0.0371
me8/em14	2 / -0.2216**	2 / -0.2545**	1 / -0.0673	2 / -0.0329	0 / 0.1543*	1 / 0.1872**
me8/em15	1 / 0.0768	1 / -0.4215**	1 / 0.0768	1 / -0.4983**	1 / 0.0000	1 / 0.4983**
me8/em16	1 / 0.3808**	3 / -0.2974**	3 / -0.2180**	1 / -0.6782**	1 / -0.5988**	4 / 0.0794**
me8/em17	1 / 0.4710**	2 / 0.1467**	4 / 0.0821**	1 / -0.3243**	0 / -0.3889**	0 / -0.0646*
me8/em18	2 / 0.1286	3 / -0.3064**	2 / -0.3961**	2 / -0.4350**	2 / -0.5247**	2 / -0.0897**
me8/em19	2 / -0.0424	2 / 0.2500**	2 / 0.2601**	2 / 0.2924*	2 / 0.3025**	2 / 0.0101
me9/em12	2 / 0.1574	2 / 0.3245**	2 / -0.1142**	2 / -0.1671	2 / -0.2716*	2 / -0.4387**
me9/em13	1 / 0.2176**	2 / -0.1769**	1 / -0.0602	1 / -0.3945**	1 / -0.2778**	2 / 0.1167*
me9/em15	1 / -0.2778	1 / -0.3045**	1 / -0.4012**	1 / -0.0267	1 / -0.1235	1 / -0.0967*
me9/em16	3 / 0.0449	2 / 0.1716**	1 / 0.1807**	1 / 0.1267*	1 / 0.1358*	1 / 0.0091
me9/em17	1 / 0.2784**	2 / 0.1677**	2 / 0.0809*	1 / -0.1107	1 / -0.1975	2 / -0.0868
me9/em18	3 / 0.2433**	1 / -0.0383**	3 / 0.1414**	1 / -0.2816**	1 / -0.1019*	3 / 0.1798**
me9/em19	2 / -0.3423**	2 / -0.4025**	2 / -0.3238**	3 / -0.0602	3 / 0.0185	4 / 0.0787**
me10/em12	2 / 0.0584	3 / 0.0359	2 / 0.0090	2 / -0.0225	2 / -0.0494	2 / -0.0269
me10/em13	2 / 0.1080	3 / 0.0613*	2 / 0.0586**	2 / -0.0467	2 / -0.0494	2 / -0.0027
me10/em14	1 / 0.0766**	1 / 0.1693*	0 / 0.0828**	2 / 0.0927*	0 / 0.0062	1 / -0.0865**
me10/em15	2 / 0.1784**	3 / 0.0971**	2 / -0.0685**	2 / -0.0813	1 / -0.2469**	2 / -0.1656**
me10/em16	0 / 0.0254	2 / 0.0212	2 / -0.0054	1 / -0.0042	2 / -0.0309	4 / -0.0266
me10/em17	1 / 0.0768	1 / 0.0768	1 / -0.0281	1 / 0.0000	1 / -0.1049	1 / -0.1049
me10/em18	1 / 0.4224**	1 / 0.3117**	1 / 0.1261**	1 / -0.1107	1 / -0.2963**	1 / -0.1856**
me10/em19	0 / 0.0126	0 / 0.0776**	0 / 0.0250	3 / 0.0650	1 / 0.0123	2 / -0.0526*
me11/em12	1 / 0.4742**	2 / 0.0184	1 / 0.0915**	2 / -0.4558**	0 / -0.3827**	0 / 0.0731**
me11/em13	1 / 0.0768	1 / 0.0768	1 / 0.0768	1 / 0.0000	1 / 0.0000	1 / 0.0000
me11/em14	2 / 0.1464*	3 / 0.1827**	4 / -0.0696**	2 / 0.0363	2 / -0.2160**	3 / -0.2524**
me11/em15	2 / 0.1222	2 / 0.0955*	3 / -0.2790**	2 / -0.0267	2 / -0.4012**	2 / -0.3745**
me11/em16	4 / 0.0853	2 / 0.1361**	4 / -0.0813**	0 / 0.0507	2 / -0.1667**	3 / -0.2174**
me11/em17	3 / 0.2305**	4 / 0.3295**	3 / 0.2305**	2 / 0.0990	2 / 0.0000	2 / -0.099**
me11/em18	2 / 0.0344	2 / 0.0777*	2 / 0.1085**	2 / 0.0433	2 / 0.0741	2 / 0.0308
me11/em19	1 / 0.5504**	2 / 0.2597**	1 / 0.4455**	1 / -0.2907*	1 / -0.1049	1 / 0.1857*
me12/em12	2 / 0.3302*	3 / -0.0218	2 / 0.0401*	2 / -0.3520**	2 / -0.2901*	2 / 0.0619*
me12/em13	1 / 0.3648*	1 / 0.2541**	1 / 0.2599**	1 / -0.1107	1 / -0.1049	1 / 0.0058
me12/em14	1 / 0.2176**	2 / 0.1069*	1 / 0.1127*	1 / -0.1107	1 / -0.1049	1 / 0.0058
me12/em15	2 / 0.2278*	1 / 0.5056**	1 / 0.3081**	1 / 0.2778	1 / 0.0802	1 / -0.1975
me12/em16	1 / 0.4768**	2 / 0.3661**	2 / 0.1003**	1 / -0.1107	1 / -0.3765**	2 / -0.2658**
me12/em17	2 / 0.0888*	2 / 0.2013*	0 / 0.2431**	2 / 0.1125*	0 / 0.1543*	0 / 0.0419
me12/em18	1 / 0.1574	1 / 0.4352*	1 / 0.4352*	1 / 0.2778	1 / 0.2778	1 / 0.0000
me12/em19	3 / 0.0490	3 / -0.1215**	2 / -0.0251*	1 / -0.1705**	2 / -0.0741*	5 / 0.0964**
Mean	1.68 / 0.1287**	2.27 / 0.0348**	2.06 / 0.0116**	1.51 / -0.0939**	1.27 / -0.1171**	1.87 / -0.0232**

* and ** are significant difference at the 0.05 and 0.01 probability levels, respectively.

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Session 2

Molecular breeding

Multi-environmental advanced backcross-QTL analysis in spring barley (*H. vulgare* subsp. *spontaneum*)

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Abstract

For mapping quantitative trait loci (QTLs), the statistical analysis accounting for multi-environmental data as well as multiple QTLs in the model is currently not fully developed. Therefore, in this study a Bayesian multi-locus strategy considering multi-environmental data is derived. Additionally, a Bayesian single-environmental analysis, a single-locus QTL mapping analysis using the Restricted Maximum Likelihood (REML) method and a REML forward selection approach were performed. For that, multi-environmental field data of several agronomic traits of an advanced backcross BC₂DH population were used, where the malting barley cultivar Scarlett was crossed with the wild barley accession ISR42-8 from Israel. In general, similar results were obtained using the REML analyses and the Bayesian multi-locus approaches. The Bayesian QTL mapping approach, accounting for both multiple genes and multi-environmental data, seems to outperform other mapping strategies.

Introduction

Due to genetic erosion, several exotic alleles with positive effects have been lost during

evolution. To identify favorable exotic quantitative trait loci (QTLs), advanced backcross populations were derived by crossing an elite cultivar with an exotic donor. In these populations, the number of negative alleles from the unadapted material should be reduced.

Mapping the QTLs, associations between DNA-markers and phenotypic traits obtained from multi-environmental field trials are examined in a statistical analysis. However, the QTLs are influenced by several genes where the gene effects can differ across environments. This means, in multi-environmental field trials, interactions can occur between QTLs and the environment. In several studies, a composite interval mapping approach was used to analyze multi-environmental data. However, the current QTL mapping strategies available that consider both multiple QTLs and multi-environmental data in the statistical analysis are not fully developed.

In our research we developed a Bayesian multi-locus approach accounting for multi-environmental data. This strategy was compared to a Bayesian single-environmental analysis and to the REML method. The full paper is published in Bauer *et al.*, 2009.

Material and methods

In this study, multi-environmental field data of several agronomic traits of 301 lines of an advanced backcross BC₂DH population were used (von Korff *et al.*, 2006). This population was initiated by crossing the malting barley cultivar Scarlett with the wild barley accession ISR42-8 from Israel. The lines in the BC₂DH population were genotyped with 98 SSR-markers (von Korff *et al.*, 2004). Four different QTL-analyses were computed (Bauer *et al.*, 2009):

1) Mixed model using REML method.

In this analysis, each locus was considered separately by accounting for multi-environmental data. SAS software/Proc mixed (SAS Institute, 2004) was used to calculate the following statistical model:

$$Y_{ijkn} = \mu + M_i + L_j(M_i) + E_k + M_i * E_k + \varepsilon_{m(ijk)} \quad (\text{Eq. 1})$$

where Y_{ijkn} is the observation value m of the i th marker and j th BC₂DH line in the k th environment; and with general mean μ , fixed effect M_i of the i th marker, random effect $L_j(M_i)$ of the j th line nested in the i th marker, random effect E_k of the k th environment, random interaction effect $M_i * E_k$ of the i th marker with the k th environment, residue $\varepsilon_{m(ijk)}$ of Y_{ijkn}

2) Forward selection using a mixed model (REML)

The statistical model used in the forward selection approach was the same as in (Eq. 1). In accordance to Kilpikari and Sillanpää (2003), the QTL analysis was repeated several times until there were no significant marker effects. In each estimation round, the marker with the most significant effect was chosen as a fixed co-factor in the model.

3) Bayesian single-environmental analysis

In this Bayesian approach, multiple genes were taken into account. Then the statistical model was as follows:

$$Y_j = \mu + \sum_{i=1}^n M_{ij} + \varepsilon_j \quad (\text{Eq. 2})$$

where: M_{ij} = effect of the i th marker genotype of the j th line and n = number of markers.

The Bayesian analysis was implemented using Matlab 7 (2007). The Markov Chain Monte Carlo (MCMC) algorithm was run for 50 000 rounds.

4) Bayesian multi-environmental analysis

In a second step, the statistical model (Eq. 2) of Bayesian single-environmental analysis was extended to account for both multiple genes and multi-environmental data:

$$Y_{jk} = \mu + \sum_{i=1}^n M_{ij} + E_k + \sum_{i=1}^n M_{ijk} + \varepsilon_{jk} \quad (\text{Eq. 3})$$

where: M_{ijk} = effect of the i th marker genotype of the j th line in the k th environment.

As this model is more complex, the algorithm was run for 400 000 MCMC rounds.

In both single- and multi-environmental Bayesian analysis, model selection and parameter estimation was based on adaptive shrinkage (Xu, 2003; Hoti and Sillanpää, 2006). For further details see Bauer *et al.* (2009).

Results and discussion

In general, the results of the REML analyses are in accordance with the results of the Bayesian multi-locus approaches (Bauer *et al.*, 2009). Several QTLs were detected in all four analyses. This can be due to the fact that in REML and in Bayesian multi-environmental analysis often QTLs with a significant marker main effect were found, whereas significant marker interaction effects were scarce. In contrast, there are also QTLs that could be detected only with some of the QTL-analyses.

In Bayesian multi-environmental analysis, markers near to a significant QTL on the chromosome were found to have negligible effects. A reason for this observation could be that the power to also detect markers

with smaller effects is increased. In addition, mixing properties of the MCMC-sampler could be improved as there were several coefficients at a single marker in the statistical model.

In analyzing multi-environmental data in a QTL-analysis, genetic correlations among observations of the same line genotype measured in different environments should be considered (Piepho, 2000). As our dataset included no replicated measurements, it was not possible to account for this genetic correlation. However, omitting this correlation in the statistical model could cause spurious QTL effects.

Comparing REML and Bayesian QTL mapping strategies, a Bayesian analysis can be computationally demanding. In our study, on a Pentium IV 2.0 GHz processor, the REML method took about 20 minutes, whereas Bayesian analysis needed about 33 hours for the same trait. So, using a Bayesian QTL method, it is important to program the MCMC-sampler in an efficient way.

In conclusion, applying a Bayesian QTL mapping approach that accounts for multiple genes as well as multi-environmental data seems to be a valuable method.

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Allelic diversity and phenotypic associations at vernalization loci in barley

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Abstract

In order to translate the growing knowledge of the sequence basis for phenotypic variation in cereal vernalization response into outcomes with practical benefits for UK and EU barley breeders, we set out to systematically classify and characterize allelic variation at *VRN-H1* and the *VRN-H2* candidate genes in a set of 429 European cultivated barley varieties. This led to the identification of three new *VRN-H1* alleles, and the development of a diagnostic multiplex PCR assay for allelic state at *VRN-H1*. Full sequencing of the *VRN-H1* allelic series uncovers a complex pattern of mobile element activity and recombination in generating the spectrum of haplotypes observed. We provide evidence that double-stranded break repair by illegitimate recombination plays an important role in the creation of phenotypic diversity by mutation at *VRN-H1*. In addition, using locus-specific markers in conjunction with genome-wide marker data, we demonstrate the ability of association mapping to detect *VRN-H1* and *VRN-H2*, despite their prominent role in determining population sub-structure in the varietal collection. Only four of the ten *VRN-H1* polymorphisms gave significant associations, highlighting the potential for candidate gene evaluation using association mapping, even in highly structured elite variety panels. Finally, comparison of allelic diversity in elite, landrace and wild barley collections shows spring *VRN-H1* alleles are present in wild germplasm, and that the process of domestication has resulted in large

increases in their frequencies. In addition, dramatic reduction of *VRN-H1* haplotype diversity is observed in modern cultivars, indicating that allelic diversity at this locus has yet to be fully exploited by breeders.

Introduction

The timing of flowering in cereal species has major agronomic significance, as alignment of seed development with favorable environmental conditions helps maximize grain yield. Cultivated barley (*Hordeum vulgare* subsp. *vulgare* L.) is predominantly classified as spring or winter growth habit (GH), according to the effects of low temperature on flowering time. Winter varieties require a prolonged period of vernalization (typically 6 weeks below 10°C) to promote subsequent flowering, and are normally planted in the autumn for harvesting the following year. Spring varieties progress to flowering without vernalization treatment, and are typically sown and harvested in the same year in the Northern Hemisphere. The molecular genetics of vernalization requirement in cereal species are relatively well understood (reviewed by Cockram *et al.*, 2007a; Trevaskis *et al.*, 2006). Cloning of the major vernalization requirement loci *VRN-A^m1* and *VRN-A^m2* in the diploid wheat, *Triticum monococcum* L. (Yan *et al.*, 2003, 2004), has led to isolation and characterization of orthologous genes at the collinear barley vernalization response loci, *VRN-H1* and *VRN-H2* (Schmitz *et al.*, 2000; Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003,

2006; Fu *et al.*, 2005; Karsai *et al.*, 2005; von Zitzewitz *et al.*, 2005; Dubcovsky *et al.*, 2005, 2006; Yan *et al.*, 2004, 2005; Szűcs *et al.*, 2006, 2007; Cockram *et al.*, 2007b, c, 2008, 2009). A third barley vernalization response locus, *VRN-H3*, has recently been identified as a homologue of the *Arabidopsis* floral pathway gene FLOWERING LOCUS T (FT) (Yan *et al.*, 2006). Characterization of allelic variation in these genes has led to the discovery that large deletions within *VRN-H1* intron I are associated with spring growth habit, and that the most widespread spring allele at *VRN-H2* co-segregates with a deletion of the entire locus spanning the three candidate genes (*ZCCT-Ha*, *-Hb*, *-Hc*). More recently, it has been shown that *VRN-H1* intron I deletion size confers distinct levels of vernalization sensitivity (Szűcs *et al.*, 2007). This finding implies that the *VRN-H1* locus, which is very often viewed by breeders as having two allelic states ('spring' and 'winter'), could potentially yield an allelic series conferring a continuum of strengths of vernalization requirement.

Knowing the identity of key genes involved in agronomic traits opens up a series of enticing breeding perspectives, ranging from development of diagnostic markers for selection of GH in winter \times spring crosses, to allele mining in diverse germplasm and rational use of allelic variation for fine-tuning flowering time to the target environment. We set out to understand allelic diversity in European barley at *VRN-H1* and *VRN-H2* with three questions in mind:

Which alleles are present in European cultivated barley germplasm, and how are they distributed?

Can we develop diagnostic markers that are predictive of GH? If so, can these markers be used as a test case in refining association genetics methodology?

Does present-day allelic diversity trace through to pools of wider variation in landrace and wild barley gene pools, and are novel alleles present?

Materials and methods

Germplasm, DNA extraction, phenotyping and nomenclature

Samples of 429 spring, winter and facultative barley varieties were obtained from thirteen European Union countries, collected as part of the GEDIFLUX project investigating the impact of modern breeding on crop molecular diversity (Reeves *et al.*, 2004). Protocols for DNA extraction, as well as phenotypic evaluation, collection of passport data, and further details of plant varieties have been previously described (Chiapparino *et al.*, 2006; Cockram *et al.*, 2007b). Details of European barley landraces (195 accessions) and *H. vulgare* subsp. *spontaneum* germplasm (125 accessions) are described by Jones *et al.* (2008). Cereal vernalization locus nomenclature follows that described by Dubcovsky *et al.* (1998). Although formally a candidate gene, due to the volume of experimental evidence we describe the MADS-box transcription factor *HvBM5A* as *VRN-H1*. The three closely linked genes representing the *ZCCT-H* cluster (*ZCCT-Ha*, *-Hb* and *-Hc*) follow the nomenclature established by Dubcovsky *et al.* (2005).

Genotyping and sequencing

Six single nucleotide polymorphisms (SNPs) within *VRN-H1* were selected for genotyping across all barley germplasm: three in the promoter (T-1948/C, A-1881/G, T-1655/C), two in intron VII (T + 14 567/C, G + 14 585/A) and one in the 3' UTR (C + 14 828/G) (subsequently referred to as SNPs 1 to 6). Following the protocols listed in Chiapparino *et al.* (2006), SNPs from varietal material were detected by di-deoxy single-base primer-extension chemistry using an ABI PRISM® SNaPshot® Multiplex Kit (Applied Biosystems), while those in landrace and wild barley accessions were genotyped by direct sequencing of PCR amplicons using primers described by Cockram *et al.* (2007b). Genotyping of the four markers

distributed within the intervening regions are described by Cockram *et al.* (2007b). Assays diagnostic for allelic state at *VRN-H2* were performed as described by Karsai *et al.* (2005). *VRN-H1* and *VRN-H2* genotype data for all varietal material is available on-line at: http://www.niab.com/research_publications. Complete sequencing of *VRN-H1* intron I was conducted from three varieties selected from each *VRN-H1* haplotype. Ultimately, intron I deletion configuration for all lines were determined by PCR/agarose-gel based amplicon analysis, as described by Cockram *et al.* (2007b). All *VRN-H1* polymorphic features are described relative to the translation start site (+1 bp) in the 'Strider' allele (AY750993). *VRN-H1* multiplex PCR reactions were optimized and tested on a panel of 111 modern UK barley varieties, as described by Cockram *et al.* (2009).

Sequence analysis

Sequence data was assembled and inspected for repeat motifs flanking deletion breakpoints as previously described (Cockram *et al.*, 2007b, c). DnaSP (Rozas *et al.*, 2003) was used to identify homologous recombination within the sequenced regions. Prior to input into DnaSP, all InDels ≤ 42 bp were coded. The 9.4 kb region of *VRN-H1* intron I spanning all known major deletions (between the 5' and 3' breakpoints of haplotypes 1B and 4A, respectively) was removed from the analysis.

Results and discussion

VRN-H1 diversity in European cultivated barley

A survey of allelic variation at *VRN-H1* and *VRN-H2* was undertaken in a collection of 429 winter and spring barley cultivars, assembled to represent successful varieties across the main European barley growing areas between the 1940s and 1990s. Initially, partial *VRN-H1* sequencing across a sample of eleven varieties revealed five haplotypes. A

subset of six SNPs able to discriminate these haplotypes was then applied across all 429 varieties. The inclusion of four PCR-based InDel/SSR assays within intron I resulted in screening a total of ten polymorphic features, defining nine distinct *VRN-H1* haplotypes in the complete varietal collection (Cockram *et al.*, 2007b). Twenty-three representative varieties over all observed haplotypes (unrelated by pedigree) were then fully sequenced to establish a robust consensus sequence for each haplotype (although we were only able to sequence across the long TA repeat in the promoter in eight varieties). The three winter varieties with the predominant *VRN-H1* 1A haplotype (found in 97% of winter lines) were identical across all of the 16 kb sequenced region, with no major intron I polymorphisms relative to the winter 'Strider' *VRN-H1* allele. One of the three varieties initially thought to belong to haplotype 5B was found to possess a much smaller intron I deletion, as well as a contrasting phenotype relative to the remaining two lines. This variety ('Express') possessed a 0.4 kb deletion within the solo-LTR found in all winter alleles, as well as a pattern of SNP and additional polymorphisms quite distinct from the 'Igri' 1A haplotype. All other spring varieties were divided among the remaining seven *VRN-H1* haplotypes, almost all of which contain intron I deletions of ≥ 3.9 kb (Figure 1). Spring haplotype 2 was unique in that no large intron I deletion is observed. However, a transposable element (TE) is present within the highly conserved 5' end of intron I. Additionally, the presence of promoter SNPs common to spring haplotypes 4A and 4B indicate homologous recombination within the promoter of a spring. The marker combination used ultimately permitted identification of all major intron I re-arrangements, verified in the complete collection by PCR amplification across breakpoints. These analyses resulted in the discovery of three previously undescribed alleles, and by revealing the existence of

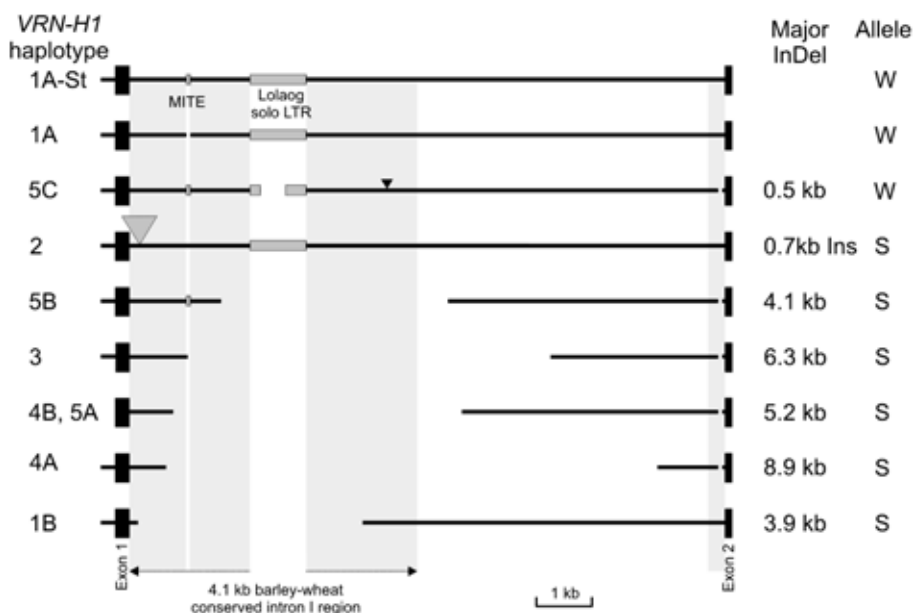


Figure 1. Diagram of intron I configuration in spring (S) and winter (W) *VRN-H1* alleles. The sizes of major intron I deletions (haplotypes 1B, 3, 4A, 4B, 5A, 5B, 5C) and insertions (haplotype 2, grey triangle) are indicated. Regions of intron I nucleotide conservation between barley and *T. monococcum* are shaded in grey. Previous studies (von Zitzewitz *et al.*, 2005) describe a 0.44 kb region immediately downstream of the 5' breakpoint in haplotype 3 as the most likely to harbor the 'vernalization-critical' region. The deletion breakpoint series identified here shows that the minimal 'vernalization-critical' region could be more diffuse, as spring alleles with an insertion upstream (haplotype 2) and downstream (haplotype 5B) of the 0.44 kb region are observed.

rare spring and winter alleles that would previously been mis-diagnosed by existing assays, has paved the way for a predictive diagnostic molecular assay for GH.

Homologous and illegitimate recombination and transposable element activity underlie *VRN-H1* haplotype patterns

Complete sequences of the nine distinct *VRN-H1* alleles described above, ranging in size from 7 to 16 kb, were analyzed as follows: firstly, putative TEs were annotated by searching for inverted and long terminal repeat-type structures using dotplots, einvert (<http://bioweb2.pasteur.fr/>) and homologies to annotated Triticeae repeats in TREP (<http://wheat.pw.usda.gov/ITMI/Repeats/>). A Loloag solo-LTR is

situated within intron I. Two MITEs were identified, both in intron I. The first is found towards the 5' end in haplotypes 2, 5B and 5C. Although this MITE is present in the North American winter variety Strider, it is not found in the three European winter alleles sequenced. The second MITE is located at the 3' end of intron I in haplotypes 1A, 1B and 2, and is responsible for the polymorphic feature previously referred to as the 42 bp InDel (Cockram *et al.*, 2007b). Finally, two additional regions flanked by imperfect inverted repeats were identified: a 317 bp region within the promoter (-1914 to -1598) with high homology to wheat EST whdp15o04 (2e-72), and a 395 bp region in intron II (+12 244 to +12 638) highly

similar to barley TIGR transcript assembly TA49820_4513 (4e-57).

Remaining InDels >50 bp whose sequence did not match known TEs were treated as deletion events, and the border regions analyzed. We found the majority of the large intron I deletions present in spring alleles are flanked by short (3 to 7 bp) nucleotide repeats, characteristic of double stranded break (DSB) repair of DNA by non-homologous recombination (Puchta, 2005). Short sequence repeats are also found flanking promoter and intron I deletions thought to result in spring alleles at orthologues *VRN-1* genes in diploid and hexaploid wheat. Furthermore, the location of all promoter deletions and four of the intron I deletion breakpoints ≤ 16 bp from the borders of TEs suggests insertion/excision events may play a role in the formation of DSBs. The resulting deletions are thought to lead to the mutation of winter alleles into spring alleles by deletion of putative vernalization responsive cis-elements within intron I. We find short sequence repeats also flank deletions that result in loss of photoperiod sensitivity in wheat photoperiod locus *Ppd-D1* (Beales *et al.*, 2007). Although previous studies of truncated transposable elements (TEs) show non-homologous recombination has profound implications for reduction of plant genome size (reviewed by Bennetzen, 2007), the action of this mechanism on sequences other than TEs has not been extensively documented. We report the first examples in which this mechanism is implicated in the creation of naturally occurring adaptive variation in plants (Cockram *et al.*, 2007c).

Homologous recombination within *VRN-H1* was also in evidence. DNASp analysis suggested a minimum of four recombination events where all four gametic types were present, and these could be pinpointed to crossovers within as little as 134 bp. An example of this is illustrated in Figure 2, where a double recombination has occurred in haplotype 4B, resulting in the

‘insertion’ of the 5.2 kb deletion and flanking sequence found in the more frequent 5A haplotype, into a sequence context otherwise characteristic of spring haplotype 4A.

Multi-locus haplotypes diagnostic for growth habit

Genotyping the European varietal collection for the three candidate *VRN-H2* genes allowed prediction of GH based on multi-locus *VRN-H1* and *VRN-H2* haplotypes, according to the model of vernalization control. Only two *ZCCT-H* genotypes were observed: presence (+Z) or absence (-Z) of all three *ZCCT-H* genes. When *VRN-H1* and *VRN-H2* haplotypes were considered together, eight of the nine *VRN-H1* haplotypes divided into +Z and -Z groups, resulting in a total of 17 two-locus haplotypes. A multi-locus haplotype accurately predicted GH in all but one of the 429 varieties. The exception (cv. Xenia), which carried a ‘winter’ 1A+Z haplotype, flowered earlier than winter varieties but later than the latest spring variety assessed. Full sequencing of *VRN-H1* in this variety provided no explanation for the observed phenotype. Sequencing of *ZCCT-H* genes in this variety is in progress. Curiously, all 309 spring varieties investigated contained spring alleles at *VRN-H1*, despite the genetic model predicting that spring alleles at either locus result in spring GH. We suggest this distribution may reflect the relative mutation frequencies, combined with the comparative ease with which dominant alleles (i.e. spring *VRN-H1* alleles) could be selected over recessive ones (spring *VRN-H2* alleles), with the resulting skew in landrace allele frequencies evident in the modern cultivars that succeeded them (Cockram *et al.*, 2007b).

Validation of diagnostic PCR assays for growth habit

Having categorized spring and winter *VRN-H1* haplotypes in European germplasm, we then wished to design a PCR assay able to discriminate between them. Such an assay

VRN-H1 alleles (Figure 3) (Cockram *et al.*, 2009). This assay was tested over a selection of 111 contemporary barley varieties released to the UK Recommended Lists between 2002 and 2006. Unlike the Europe-wide GEDIFLUX barley collection (which relied on gene bank passport data for a description of vernalization requirement), this collection was assessed for GH under unified phenotypic evaluation during the process of varietal registration, following Union for the Protection of New Varieties of Plants (UPOV) procedures (http://www.upov.int/en/publications/tg_rom/tg_index_numerical.html).

Diagram illustrating the structure of the *VRN-H1* haplotype and the deletion analysis. The top part shows a genomic map with Exon I, a Solo LTR, and Exon II, with a 1 kb scale bar. A gray triangle indicates a 'region deleted in spring allelic range'. Below is a haplotype grid for 10 samples (1A, 1B, 2, 3, 4A, 4B, 5A, 5B, 5C). A red arrow points from a specific region in the grid to a detailed view of the 'Major Del' (8.9 kb) and a smaller deletion (5.2 kb) in the 4A and 4B, 5A haplotypes. The 5' and 3' ends of the deletion are indicated.

Figure 2. SNP profile of exemplar varieties selected from each *VRN-H1* haplotype (region shown: -325 to +10 974 bp). Evidence for homologous recombination between haplogroup 4 and haplogroup 5, resulting in haplotype 4B (in which a 5.2 kb intron I deletion and flanking genomic regions is inserted into an otherwise haplotype 4A genomic context). Genomic regions spanning the major intron I deletions present in the *VRN-H1* allelic series (+327 to +9734) have been removed from the analysis. InDels/SSRs ≤ 42 bp are coded. Sequence analysis localizes 5' and 3' recombination points to 105 and 346 bp regions, respectively.



Figure 3. Single-well multiplex *VRN-H1* PCR assay. Representative varieties from *VRN-H1* haplotypes are as follows: 1A ('Pelican'), 1B ('Etu'), 2 ('Dram'), 3 ('Beatrice'), 4A ('Triumph'), 4B ('Maris Cannon'), 5A ('Pohto'), 5B ('Pegasus'), 5C ('Express'). Haplotypes 1A and 5C represent winter alleles; the remaining seven haplotypes are spring. L = 1 kb DNA ladder.

When combined with genotypic scores for the *ZCCT-H* locus, perfect agreement between predicted and recorded GH was achieved for all winter and spring varieties. Interestingly, the only 'alternative' variety (cv. Angela) possessed a winter 5C+Z haplotype, raising the possibility that the rare 5C allele confers a weaker vernalization response than the predominant 1A allele. Further investigation of this hypothesis is under way.

Vernalization requirement as a test case for association genetics

Having empirically reached a near perfect correlation between known vernalization requirement and haplotype, we set out to use this knowledge as a test case for association mapping of a major gene trait. Varietal collections used for association mapping in cereal species invariably possess significant population sub-structure due to factors such as pedigree, geographical origin and phenotype. Initial analysis with 183 genome-wide markers using principle component analysis (PCoA) indicated structure existed, predominantly due to GH and ear-row number. The first two principle components accounted for 23% of

the variation, showing significant structure still remained. Using the program STRUCTURE version 2.2 (Pritchard *et al.*, 2000; Falush *et al.*, 2003), we found the optimum number of sub-populations to be 4. We then contrasted three association analysis approaches: Structured Association (SA), Genomic Control (GC), and a combination of SA+GC. We find that after statistical correction, the genome-wide partitioning effect of allelic status at *VRN-H1* and *VRN-H2* does not result in the high levels of spurious association expected to occur in highly structured samples (Cockram *et al.*, 2008). Furthermore, a combination of SA+GC resolved the *VRN-H1* and *VRN-H2* loci, despite their prominent role in defining population sub-structure (Figure 4). Only a sub-set of the *VRN-H1* markers gave significant associations. These results illustrate the feasibility of association mapping in identifying and prioritizing genetic polymorphisms, even within highly structured samples.

Investigating *VRN-H1* diversity in landraces and wild barley

Towards understanding the effects of domestication on allele variation and frequency, we haplotyped *VRN-H1* in landrace and *H. vulgare* subsp. *spontaneum* germplasm. In wild barley, the mean number of haplotypes per accession was 0.2328. This fell by 18% and 88% in landrace and cultivated European varieties, respectively (Figure 5). A reduction of 78% in haplotype diversity between wild barley and landraces has previously been documented at the *Ppd-H1* locus in barley (Jones *et al.*, 2008). This is comparable to reductions in diversity seen between domesticated maize and its wild progenitor (Tenaillon *et al.*, 2004).

Winter GH is characteristic of the wild ancestor of cultivated barley, and is regarded as the ancestral phenotypic state. Interestingly, we show that spring *VRN-H1* alleles are present in wild germplasm, indicating that pre-existing functional

mutations at *VRN-H1* were utilized during the Neolithic spread of barley domestication. However, evidence that domestication is a continuous process, rather than a single event, is supported by the presence of cultivated *VRN-H1* alleles of apparent recent origin:

the double recombination event thought to have occurred in spring haplotype 4B (Figure 2) is absent from landrace and wild barley germplasm. Similarly, although spring haplotype 1B (which contains a 3.9 kb intron I deletion, and whose sequence is otherwise

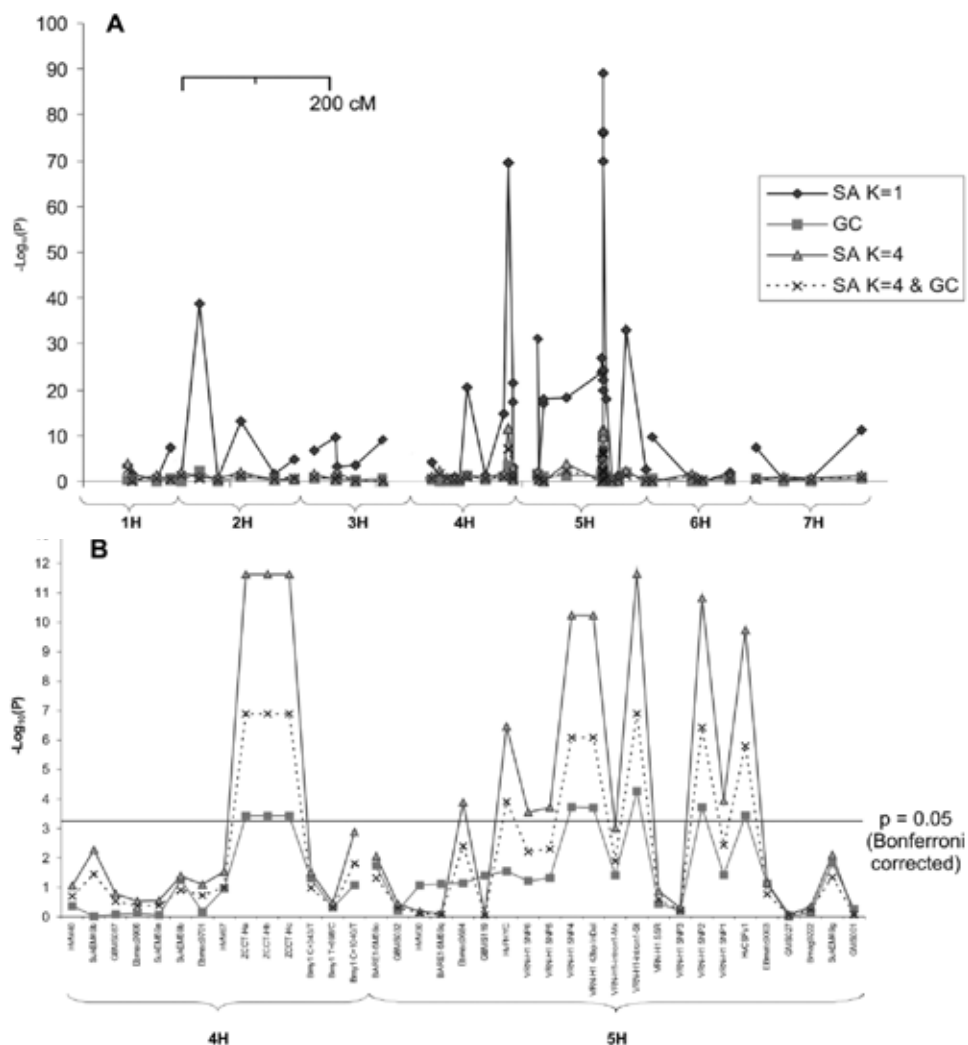


Figure 4. Association mapping of growth habit (GH) in barley. Association of GH with mapped genetic markers using association (SA K = 1), Genomic Control (GC), SA with a population sub-structure = 4 (SA K = 4) and SA with population sub-structure = 4 plus Genomic Control (SA K = 4 & GC). GC was performed using the robust mean of LRT for the unmapped S-SAP markers. (A) All mapped markers. Distance between markers is proportional to scale (cM). (B) Detailed view of mapped markers on chromosomes 4H and 5H (SA K = 1 not shown). Associations between marker and GH are considered significant above $P = 0.05/\text{marker number}$ (Bonferroni correction). Distance between markers is not to scale.

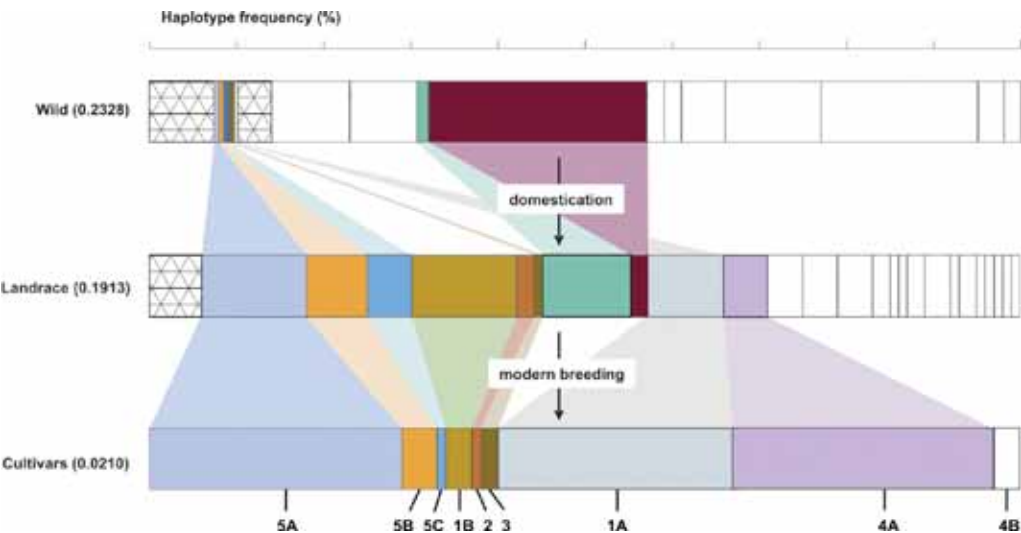


Figure 5. *VRN-H1* haplotype frequencies in *H. vulgare* subsp. *spontaneum* (116 accessions), barley landraces (183 accessions) and elite varieties (429 accessions). The average number of haplotypes per accession is shown in parentheses. Colored haplotypes represent those present in two or more gene pools; white haplotypes are unique to the gene pool in which they are found. Hatched regions present the number of haplotypes with a frequency of 1. *VRN-H1* haplotype designation is indicated for elite cultivars, as described by Cockram *et al.* (2007b).

identical to that of winter haplotype 1A) is relatively common in landraces, it was not observed in *H. vulgare* subsp. *spontaneum*. Of the remaining five cultivated spring intron I re-arrangements, four can clearly be traced back to wild germplasm. In addition, we have identified one novel intron I deletion (possessing flanking short sequence repeats) in wild barley. Further investigation is required in the remaining eleven accessions of undetermined intron I structure.

The predominant cultivated winter allele (haplotype 1A) was observed in only one wild accession, from Afghanistan. A closely related haplotype differing only at SNP2 is well represented in wild germplasm, and is associated with late flowering under long-day photoperiods in the absence of vernalization (JC, HJ, unpublished data). It is possible that gene flow into wild germplasm explains the presence of the 1A haplotype in the Afghan accession. Alternatively, it could indicate the

geographical source of the European winter allele, which co-exists with the closely related wild haplotype in landraces. Further investigation into the genome-wide genetic profile of this accession is needed to help clarify its significance. In contrast, the rare cultivated winter 5C haplotype can be clearly traced back to the wild gene pool via landrace germplasm.

Conclusion

We hope that understanding the allelic range of key genes carried into modern-day varieties during domestication will facilitate the systematic utilization of cultivated and wild germplasm for varietal improvement. This provides the genetic tools to allow pre-breeding activities to deliver phenotypically characterized allelic ranges and combinations to modern breeding programs, for precise tailoring of varieties to agro-environments and end use.

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Additivity of anthesis date QTLs is explained by individual QTLs operative at different periods within the crop growth cycle

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Abstract

We used a doubled-haploid (DH) mapping population derived from the cross BCD47 × Baronesse as a tool to analyze the genetic factors controlling flowering date under Uruguayan conditions. Both parents have similar heading dates but the population shows transgressive segregation. Two QTLs—on chromosomes 2H and 3H—explained most of the phenotypic variation for anthesis date, with Baronesse contributing late alleles in 2H and BCD47 in 3H. We performed QTL analyses on the lengths of three plant developmental periods: plant emergence to tillering (SE-Z20), tillering (Z20-Z30), and end of tillering to anthesis (Z30-Z65). We detected period-specific QTL effects coincident with the main anthesis date QTL. The QTL effects on 2H (in the *EBmac684-Bmac093* interval) were coincident with QTLs for the E-Z20 and Z30-Z65 periods, whereas the QTL effects on 3H (in the *EBmac684-Bmac093* interval) were coincident with QTL effects for the length of the Z20-Z30 period. Each of the two QTLs for the end-point phenotype—anthesis date—was a determinant of flowering at a different developmental stage.

Introduction

Phenology is a key factor in the adaptation of crop plants to environments and management

practices. Heading (anthesis) date is a critical determinant of yield potential in barley (*Hordeum vulgare* subsp. *vulgare*) in Uruguay (because heading date initiates the grain filling period, a trait that is a principal determinant of yield and quality).

Barley is planted in Uruguay in June–July and harvested in November. Winters are mild and temperatures increase rapidly during the spring. Cultivars have little or no vernalization sensitivity (*sensu* Szücs *et al.*, 2007) and varying degrees of photoperiod sensitivity. Anthesis at the end of September to beginning of October leads to grain filling under optimum conditions. Anthesis beyond those dates leads to grain filling under unfavorably high temperatures, with a decrease in yield and grain quality.

Anthesis date in barley is determined by (i) vernalization response (Takahashi and Yasuda, 1970), (ii) photoperiod response (Roberts *et al.*, 1988) and (iii) earliness *per se* (Gallagher *et al.*, 1991). Ellis *et al.* (1989) defined the last-named in terms of temperature-sensitivity genes. Laurie *et al.* (1995) mapped several of the major genes governing these traits. QTL analyses of numerous mapping populations have identified many QTLs that determine heading date, located on all seven chromosomes.

We used QTL analysis to understand the genetic basis of phenology in Uruguay, with the cv. Baronesse × BCD47 mapping

population as a tool (Castro *et al.*, 2007). The two parents of the mapping population have similar anthesis dates but the population shows transgressive segregation. We measured anthesis date in eight field experiments, using different planting dates over three years and two locations, and detected five QTLs, on four chromosomes, for this trait. Two additive QTLs explained most of the phenotypic variation for anthesis and physiological maturity. Non-parental combinations of alleles at these loci account for the phenotypic transgressive segregation. Candidate genes for these QTL effects are *eps2S* (2H) and *denso* (3H). In order to understand the basis for additivity of both QTLs and the population phenology, we pursued more extensive characterization of the different phases of the emergence-anthesis period.

Materials and methods

BCD47 and Baronesse are the parents of the mapping population. BCD47 (Orca/Harrington*2//D-1-72) is a two-row spring habit doubled-haploid (DH) line developed via marker-assisted selection for barley stripe rust (BSR) resistance alleles at Oregon State University. Baronesse (343-6/V34-6//T-427/3/ORIOL/6153-P40) is a two-row spring habit European variety. The mapping population was developed using the *H. bulbosum* method. In this study, a representative sample of 100 lines was used. The whole population was genotyped and a linkage map was constructed using a total of 59 molecular markers, as described in Vales *et al.* (2005).

Two experiments, one in the field and one in the greenhouse, were used to determine the components of the emergence-anthesis period, both performed at the “Dr Mario A Cassinoni” Experimental Station (EEMAC), at Paysandú (58°03' W, 32°55' S). The field experiment was planted on 6 July 2006, using a randomized complete block augmented design with six replications and 20 DH lines

per block plus the parents and six checks (cvs. Clipper, Estanzuela Quebracho, INIA Ceibo, Norteña Daymán, Norteña Carumbé and Perún). Plots were planted using a Wintersteiger plot drill and consisted of six, 4-m-long rows, with 0.15 m between rows, and 0.45 m between plots. Growth stages (Zadoks *et al.*, 1973) recorded were seed emergence (SE), beginning of tillering (Z20), beginning of stem elongation (Z30) and awn emergence. Awn emergence was recorded when awns of 50% of the plants were approximately 1 cm above the flag leaf sheath, and was considered an estimator of anthesis (Z65). We used the length of the different phases of the SE–Z65 period (SE–Z20, Z20–Z30 and Z30–Z65) as dependent variables in our analysis.

The greenhouse experiment was planted on 24 August 2006, using 58 cm × 36 cm plastic containers, with three rows of 10 seeds per container, each row with a different line. The 100 lines plus the parents were included. Seedling emergence and date of emergence of the first tiller were recorded.

All variables were recorded in days and growing degree days (GDD), using a base temperature of 0°C. For the statistical and QTL analysis, GDD data was preferred when available.

The QTL analysis was performed using the composite interval mapping (CIM) procedure (Zeng, 1994) implemented in Windows QTL Cartographer 2.5 (Wang *et al.*, 2005). Up to 15 co-factors for CIM were chosen using a stepwise regression procedure with a significance threshold of 0.05. Walk speed was set to 2 cM and the scan window to 10 cM beyond the markers flanking the interval tested. An LR threshold of 11.5 was used. Epistatic interactions between QTLs were evaluated with the Multiple Interval Mapping (MIM; Kao *et al.*, 1999) tool implemented in Windows QTL Cartographer using Bayesian Information Criteria (BIC-M0). QTLs detected through QTL Cartographer were confirmed using an approach analogous to candidate gene analysis where the genotypes

at the QTL region (determined through the genotype of the markers at that region) are used as independent variables. The treatment design was a $2 \times n$ factorial, where n is the number of genome regions considered, based on QTLs detected by the QTL analysis. Statistical analyses were performed using the GLM procedure of SAS 9.0 (SAS Inst., 2004). QTL and statistical analysis were performed for all traits using days and degree days-1.

Results and discussion

We detected QTL for all the plant developmental periods analyzed, which were coincident with the two major QTLs

previously detected for anthesis (on chromosomes 2H and 3H) (Figure 1 and Table 1). The QTL region on chromosome 2H (located in *EBmac684-Bmac093*) had coincident QTL effects on SE-Z20 and Z30-Z65, while the QTL region on 3H (located in the *Bmag606-Bmag013* interval) had significant QTL effects on Z20-Z30. None of the analyzed periods was affected by more than one QTL region.

In all cases, there was coincidence in the alleles determining shorter periods: BCD47 on 2H, and Baronesse on 3H (Tables 1 and 2). The phenotypic mean values for the DH lines, sorted by their allele genotypes at 2H and 3H (Table 2), reveal the basis of the observed phenotypic variation.

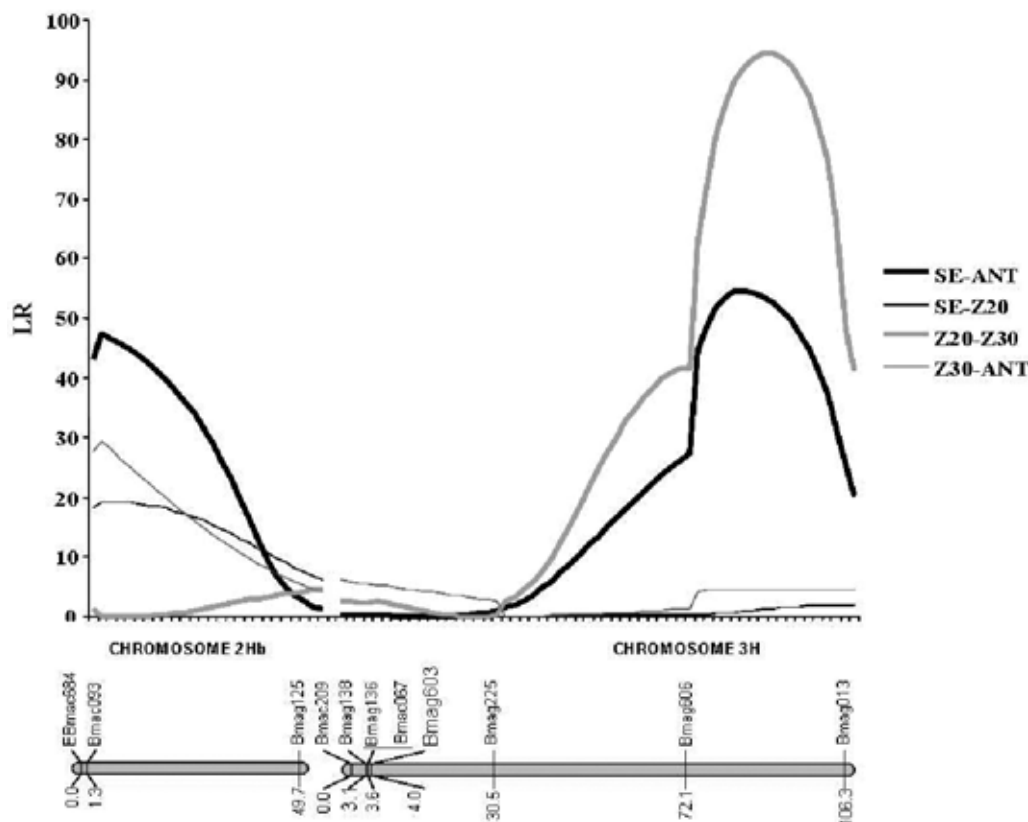


Figure 1. QTL scans for growth stages SE-Z20, Z20-Z30, Z30-Z65 and SE-Z65 (only chromosomes 2Hb and 3H shown) superimposed on the corresponding linkage map of BCD47 \times Baronesse.

Table 1. Summary of QTL effects detected on the duration of growth stages SE–Z20, Z20–Z30, Z30–Z65 and SE–Z65, with chromosome location, marker interval on which the QTL is located and R² explained by each QTL. Empty spaces mean lack of detected QTL effect.

Chromosome	Marker interval	Shorter period allele	Plant developmental period			
			SE–Z20	Z20–Z30	Z30–Z65	SE–Z65
2H	<i>EBmac684–Bmac093</i>	BCD47	12.8		15.7	18.0
3H	<i>Bmag606–Bmag013</i>	Baronesse		53.3		9.8

Table 2. Mean values of SE–Z20, Z20–Z30, Z30–Z65 and SE–Z65 for lines according to their genotype on the QTL regions on 2H and 3H.

Alleles present on the QTL		Average length (in days) of the plant developmental period			
2H	3H	SE–Z20	Z20–Z30	Z30–Z65	SE–Z65
BCD47	Baronesse	14.8	41.5	18.8	72.2
BCD47	BCD47	14.9	46.2	18.5	76.0
Baronesse	Baronesse	15.6	42.0	23.9	77.5
Baronesse	BCD47	15.5	47.0	20.5	79.0

The gene candidate for the QTL on 2H, as mentioned above, was *eps2S*, which is an earliness per se gene (Laurie *et al.*, 1995). The effect of the BCD47 alleles at this QTL indicate that this gene affects the length of the SE–Z20 and Z20–Z30 periods, which roughly means affecting early juvenile and pre-anthesis growth, without affecting the length of the tillering period. The candidate for the QTL on 3H was the *denso* dwarfing gene, with BCD47 contributing the dwarfing alleles. Our results showed that BCD47 alleles at this locus increase the length of the tillering period (Z20–Z30) without affecting the rest of the SE–Z65 period, which agrees with the description of the gene (Laurie *et al.*, 1995).

The causes of the previously detected additive effect on anthesis date of alleles at these large-effect QTLs was intriguing, and our results show that each QTL affected different and specific processes and the additivity was a result of the use of an end point phenotype composed of independent phenotypes, each one affected by specific QTLs.

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Molecular mapping of the short-rachilla-hair gene (*srh*) in barley

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Abstract

The rachilla is an organ found in the inflorescence of grasses. In barley, there are hairs on the rachilla surface and qualitative genetic variation exists in rachilla hair length. Most barley accessions are classified as long rachilla hairs, but there also exists a small proportion of variant with short rachilla hairs. Short rachilla hair is controlled by a recessive gene *srh* located on the long arm of chromosome 5H. It shows a skewed geographical distribution toward the Occidental regions. These characteristics make it an attractive trait to study the phylogeny of barley. The objective of the present study was a rough mapping of *srh* using publicly available markers. A total of 38 markers (one STS, 12 SSR and 25 EST) assigned to the 5HL arm were evaluated for polymorphism between the parents of mapping populations (five long- and three short-rachilla-hair cultivars). Three monomorphic EST markers were converted into polymorphic CAPS markers. An F₂ population of 96 plants derived from a cross between Honen 6 (long rachilla) and Morex (short rachilla) was selected for mapping because of high polymorphism presence. In our genetic map of 5HL, *srh* was mapped between two markers at a distance of 1.6 cM and 0.5 cM on the proximal and distal sides, respectively. These flanking markers will be useful for fine mapping of *srh*.

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world after wheat, maize and rice (FAOSTAT data). It is one of the oldest of the domesticated cereals and had significant roles in the history of agriculture, especially during the development of Neolithic culture (Salamini *et al.*, 2002). Barley is widely grown in many climates of the world, ranging from the Arctic Circle, further north than any other cereal, to the arid climates of Sahara, the high plateaus of Tibet, and the tropical plains of India. It has developed a diversity of easily classifiable hereditary morphological traits during evolution (Poehlman, 1987). These characteristics have made barley a model species for genetic research. Linkage maps were developed by the conventional mapping procedures for each of the seven chromosomes in barley and large number of genes were identified that condition simple morphological characters. Over the last decade, a great number of studies have been directed at research on molecular markers and genetic diversity for conservation and utilization of barley (von Bothmer *et al.*, 2003).

The phylogenetic study of a crop is a multidisciplinary approach combining the use of genetic, archaeological and phytogeographical methods. Barley geneticists are intensively researching

morphological traits that are considered key domestication characters, like brittle and non-brittle rachis, row type and covered vs naked kernels. Takahashi (1955) provided evidence of genetic differentiation between two distinct groups in world barley, an Oriental group and an Occidental group, based on his genetic analyses of rachis brittleness. He placed barleys grown in China, southern Japan and South Korea in the Oriental group, and barleys from the rest of the world in the Occidental group. Komatsuda and Mano (2002) conducted molecular mapping of non-brittle rachis, an important character in studying the evolution of barley. Komatsuda *et al.* (2004) reported that the non-brittle rachis of Occidental barley lines is controlled by *btr1* on chromosome 3H, while non-brittle rachis of Oriental barley lines is controlled by a major gene *btr2* on chromosome 3H and two quantitative trait loci on chromosomes 5HL and 7H. This result suggests multiple mutations of the genes involved in the formation of non-brittle rachis in Oriental lines. Tanno *et al.* (2002) studied the origin of six-row domesticated barley using a DNA marker cMWG699 closely linked to the *vrs1* locus. Kikuchi *et al.*, (2003) developed a fine map around the *nud* locus on the long arm of chromosome 7H using High Efficiency Genome Scanning electrophoresis (HEGS) combined with AFLP. Taketa *et al.* (2004) studied the origin of naked barley by molecular variation of the SCAR marker sKT7 tightly linked to the *nud* locus. It was concluded that naked barley has a monophyletic origin, probably in southwestern Iran, either directly from wild barley or from a hulled domesticated line. The geographical distribution suggested migration routes of naked domesticated barley in central and eastern Asia.

Despite intensive genetic studies of key morphological traits with the help of molecular techniques, little consensus exists regarding the evolution and domestication processes of barley. Many believe, on the

basis of genetic and morphological evidence, that barley was domesticated multiple times (Molina-Cano *et al.*, 1999; Morrell and Clegg, 2007; Saisho and Purugganan, 2007), while others think that the crop was domesticated once only (Badr *et al.*, 2000; Blattner and Mendez, 2001). Therefore, in addition to the key domestication characters, many other morphological traits in barley such as short rachilla hair, smooth awn, leaf pubescence and spike density should be analyzed to accumulate information about evolution and domestication of barley.

The rachilla is the diminutive axis of a spikelet in grasses that bears the florets. The rachilla in barley is markedly reduced to a tiny rod-shaped organ. Genetic variation exists in hairs on the rachilla surface and in rachilla length. Although the adaptive importance of rachilla characters is not known, these are among the best taxonomic characteristics in barley (Takahashi, 1955; Kebebew *et al.*, 2001). Two distinct types of rachilla hairs, long straight and short forked, is a monogenic trait (Figure 1). The long-haired condition is dominant. Short rachilla hair is a simply inherited morphological character that has been used to assess genetic diversity in barley by plant genetic resources scientists and for testing the distinctiveness and uniformity of varieties by barley breeders (Parzies *et al.*, 2000). Short rachilla hair is controlled by a recessive gene *srh* that is located on the long arm of chromosome 5H, about 26.8 cM proximal from the smooth awn gene *raw1* (Kleinhofs *et al.*, 1993; Franckowiak, 1997; Lundqvist *et al.*, 1997; Costa *et al.*, 2001; Kraakman *et al.*, 2006). It was found that whereas all the varieties of the Oriental region and only a part of the Occidental varieties have long rachilla hair, a large number of varieties with short rachilla hair are found in the Occidental region (Takahashi, 1955). This interesting geographical distribution of short rachilla hair makes it a useful character in studying the evolutionary pattern of domesticated barley.

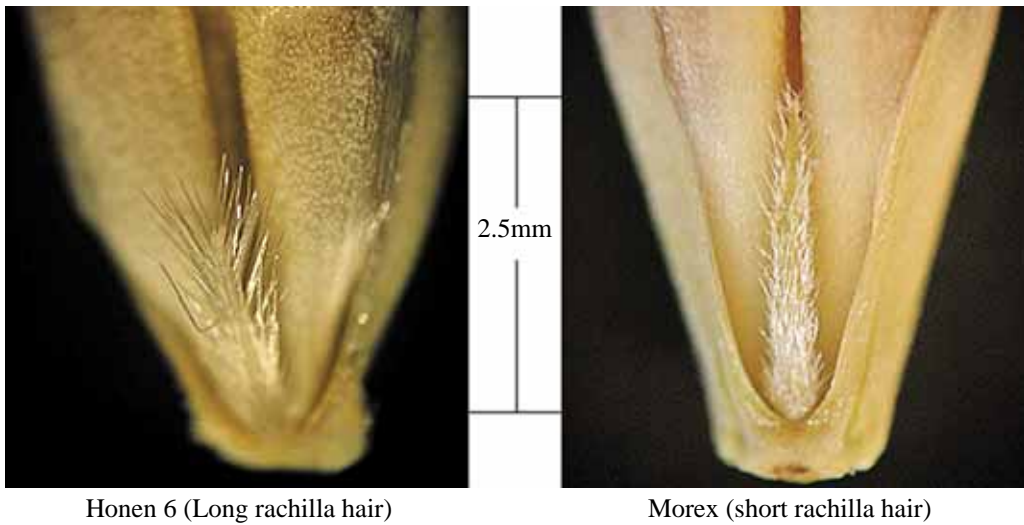


Figure 1. Long rachilla hair in Honen 6 (left) and short rachilla hair in Morex (right).

Molecular markers have been a subject of intensive research. During recent decades, many genetic maps of the barley genome have been developed, based on allelic variation at different loci, using restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), sequence-tagged site (STS), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), and expressed sequence tag (EST) markers (Graner *et al.*, 1991; Kleinhofs *et al.*, 1993; Mano *et al.*, 1999; Ramsay *et al.*, 2000; Costa *et al.*, 2001; Sato *et al.*, 2004; Kraakman *et al.*, 2006). Molecular maps are being continuously enriched by new markers and are being increasingly used in genetic studies. Integration of morphological characters with these maps has facilitated genetic studies of morphological traits. Since a number of marker assays are available now, it provides an opportunity to choose a suitable marker system on the basis of objective, convenience and cost.

The present research was conducted for the genetic analysis of short rachilla hair in barley. Available DNA markers and sequence information was utilized to develop more

tightly linked polymorphic markers in an attempt to provide new insight on the origin and evolution of barley.

Materials and methods

Plant materials and data recording for morphological characters

Five long-rachilla-hair barley varieties—Honen 6 (Japan), Mut 3041 (Europe), Haruna Nijo (Japan), Aizuhadaka 3 (Japan), Kodoku Shirazu (Japan)—and three short-rachilla-hair varieties—Morex (USA), Shogor 3 (Pakistan), Karafuto Zairai (Russia)—were used in the experiment. Seed of these varieties was obtained from the barley germplasm collection of Okayama University, Japan. A segregating F_2 population of 96 plants was developed from the cross combination Morex \times Honen 6 (Figure 1 shows the rachilla hairs of the two parents) and was planted in the field during winter 2005–06. An F_3 progeny test was conducted by planting twenty F_3 plants from each F_2 plant in the field during winter 2006–07 to estimate the genotypes of F_2 plants. Data were recorded in the various varieties, the F_2 population Morex \times Honen

6, and the F_3 population planted for progeny test, for rachilla hair as long or short, and awn barbs as rough or smooth. On the basis of the progeny test, genotypes of plants were determined. Homozygous recessive, homozygous dominant and heterozygous plants were labelled as A, B and H, respectively, for rachilla hair and awn barbs. Segregation analysis of rachilla hair and awn barbs in the F_2 population was performed using Pearson's chi-square test (Tamarin, 2002).

PCR analysis of molecular markers

DNA was extracted from barley varieties and mapping populations using a modified form of an SDS-based method (Csaikl *et al.*, 1998). The eight barley varieties being used in the experiment were evaluated for polymorphism using SSR markers on the long arm of chromosome 5H reported by Ramsay *et al.* (2000). These markers included EBmac0518, EBmac0970, EBmac0684, Bmag0760, HVM30, Bmag0223, Bmag0812, HvLOX, GMS061, EBmac0824, GMS027 and Bmag0222. Genotyping of the Morex \times Honen 6 population of 96 F_2 plants was performed using the SSR primers Bmag0223, Bmag0812 and GMS027.

Parents of the mapping population were evaluated using the STS primers MWG522T7 5' AACCTCTTGTTACAAATGTCATGTG 3' and MWG522T3 5'TGCATGTAGGGCTAGTTACTATAA 3' (Sayed-Tabatabaei *et al.*, 1998) that are derived from the RFLP marker MWG522 that is reported to be linked with a short-rachilla-hair gene (Costa *et al.*, 2001). Direct sequencing of PCR products with primers MWG522T7 and MWG522T3 was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit. TA cloning of PCR products was performed using TOPO TA Cloning® with vector pCR2.1, and three clones of each PCR product were sequenced. Sequence alignment was performed using the software GENETYX Version 7 to check for

polymorphism between the parents.

EST markers reported by Sato *et al.* (2004) on the long arm of chromosome 5H were used to assess polymorphism between Morex and Honen 6. The varieties were evaluated using primers for 25 EST markers reported to be on the long arm of chromosome 5H. The primers for these markers were provided by the Research Institute for Bioresources, Okayama University. Three cleaved amplified polymorphic sequence (CAPS) markers—k06844KU, k06860KU and k06288KU—that showed polymorphism between long-rachilla-hair variety Honen 6 and short-rachilla-hair variety Morex were derived from EST markers and used for genotyping of the mapping population.

Conversion of EST markers to CAPS markers

Direct sequencing of PCR products of Morex and Honen 6 with primers k06844, k03081, k06860, k06288, and k03075 was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit. Polymorphism between sequences of PCR products of Morex and Honen 6 for these primers was examined by sequence alignment using the software GENETYX Version 7.

Polymorphic sites of DNA sequences of Morex and Honen 6 were analyzed for detection by restriction enzymes using NEB Cutter version 2.0 (available at <http://tools.neb.com/NEBcutter2/index.php>), and dCAPS Finder 2.0 (available at <http://helix.wustl.edu/dcaps/dcaps.html>). DNA sequences of PCR products obtained by the three EST primers k06844, k06860 and k06288 showed polymorphic sites that can be detected by restriction enzymes. Three CAPS markers—k06844KU, k06860KU and k06288KU—were developed. The chromosomal locations of these markers were confirmed using wheat-barley whole chromosome addition lines and di-telosomic addition lines.

Construction of the linkage map

Linkage analysis was performed for short rachilla hair (*srh*) and awn barbs (*rawl*) loci with three SSR and three CAPS markers, using the mapping population. Recombination values were calculated by Mapmaker Version 2.0 and a genetic linkage map was constructed based on LOD scores greater than 3.0. Map distances were calculated using the Kosambi function (Lander *et al.*, 1987).

Results

Evaluation of the segregating population

Segregation analysis of rachilla hair and awn barbs in the F_2 population from the cross 'Morex' \times 'Honen 6' was conducted using chi square test (Table 1). The segregation ratio for rachilla hair did not fit the expected Mendelian ratio of 1:2:1, indicating that there is segregation distortion due to an excessive number of heterozygous plants among those with long rachilla hair.

Identification of SSR, STS and EST markers for mapping

PCR analysis of 12 SSR markers reported by Ramsay *et al.* (2000) on the long arm of chromosome 5H using 8 barley cultivars showed a high level of polymorphism between Morex and Honen 6. Three SSR markers were used for genotyping of the mapping population (Figure 2).

STS marker MWG522, developed from an RFLP marker linked with a short-rachilla-hair gene (Costa *et al.*, 2001) was used for PCR analysis of Morex and Honen 6 using primers MWG522T and MWG522G. No length polymorphism was observed. Sequence analysis of PCR products showed that there is no polymorphism that could be detected by restriction enzymes. Therefore, this marker cannot be used to develop a polymorphic CAPS marker for Morex and Honen 6.

Morex and Honen 6 were evaluated for polymorphism using 25 EST markers. Dominant polymorphism was found in the case of k01026 and k04772. Since these markers are linked with short rachilla hair in repulsion phase, these cannot be used for mapping.

Three CAPS markers developed from EST markers (Table 2) were used to study the polymorphism between Morex and Honen 6. These markers were used for the genotyping of the mapping population (Figure 3).

Figure 3. Genotyping of Morex (M) and Honen 6 (H) and the F_2 population of 'Morex' \times 'Honen 6' using CAPS primers (A) k06844KU, (B) k06860KU and (C) k06288KU.

Construction of a molecular map

Three easily distinguishable polymorphic SSR markers and three CAPS markers developed during the experiment were used for linkage analysis of the mapping

Table 1. Chi square analysis of two morphological characters and five molecular markers in the 'Morex' \times 'Honen 6' F_2 population of 96 plants, where tabulated χ^2 at $P = 0.05$ is 5.99 (df = 2).

	Morex genotype (A)	Heterozygous genotype (H)	Honen 6 genotype (B)	χ^2	P
k06844KU	23	57	16	4.40	0.11
k06860KU	26	55	15	4.56	0.10
k06288KU	26	54	16	3.58	0.17
<i>Srh</i>	27	56	13	6.75	0.04
Bmag0223	26	54	16	3.58	0.17
Bmag0812	26	50	20	0.92	0.63
<i>rawl</i>	20	45	21	0.70	0.36
GMS027	24	45	26	0.25	0.88

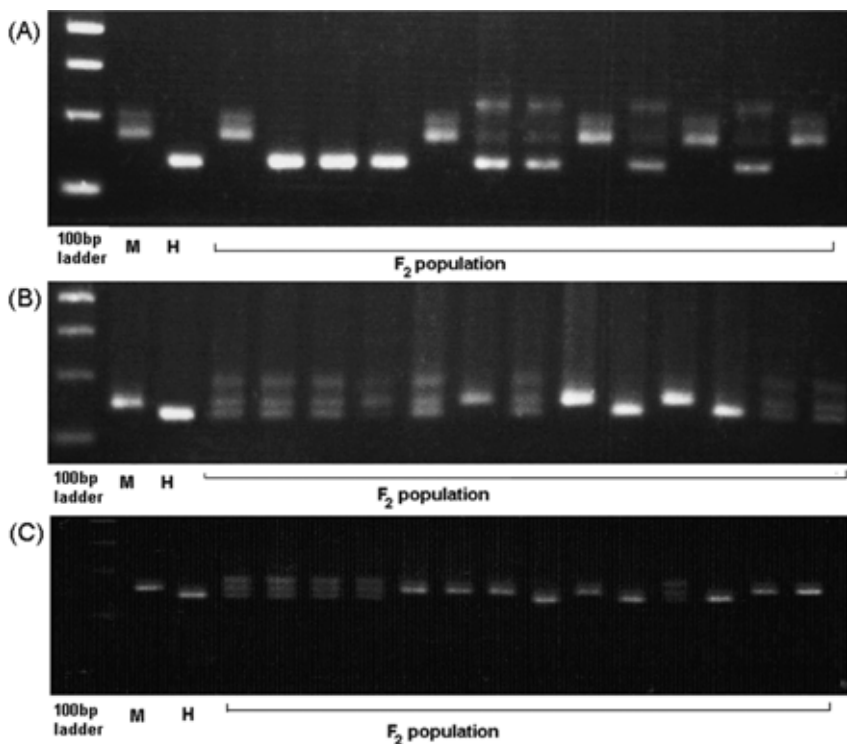


Figure 2. Genotyping of Morex (M), Honen 6 (H) and F₂ population ‘Morex’ × ‘Honen 6’ using SSR markers (A) Bmag0223, (B) Bmag0812 and (C) GMS027.

Table 2. Co-dominant CAPS markers derived from EST markers to study the polymorphism between Morex (M) and Honen 6 (H).

Marker	Primer	PCR product length (bp)	Restriction enzyme	After digestion (bp)
k06844KU	5' CCGGTTCAACGAAATGAATC 3' 5' AAACCATCCATTCAAGCAGG 3'	447 (M) 450 (H)	<i>HinfI</i>	16 + 244 + 21 + 166 (M) 16 + 230 + 17 + 21 + 166 (H)
k06860KU	5' TGTGATCGTCTCCCTCAGTG 3' 5' GGCGTGGTAAGTCTGAATCC 3'	561	<i>EaeI</i>	197 + 364 (M) 561 (H)
k06288KU	5' ATACGCAACAAAGCGGACAC 3' 5' TGGTTGGTCGGTCGTTTACT 3'	262	<i>XspI</i>	262 (M) 133 + 129 (H)

population. Linkage analysis indicated that short rachilla hair (*srh*), smooth awn (*raw1*), three CAPS markers (k06844KU, k06860KU and k06288KU), and two SSR markers (Bmag0223 and Bmag0812) are in one linkage group, while SSR marker GMS027

is in a different linkage group. GMS027 is mapped in the same linkage group with other morphological and molecular markers used in the experiment at LOD score threshold 2.14. CAPS marker k06288KU and SSR marker Bmag0223 were found most tightly linked to

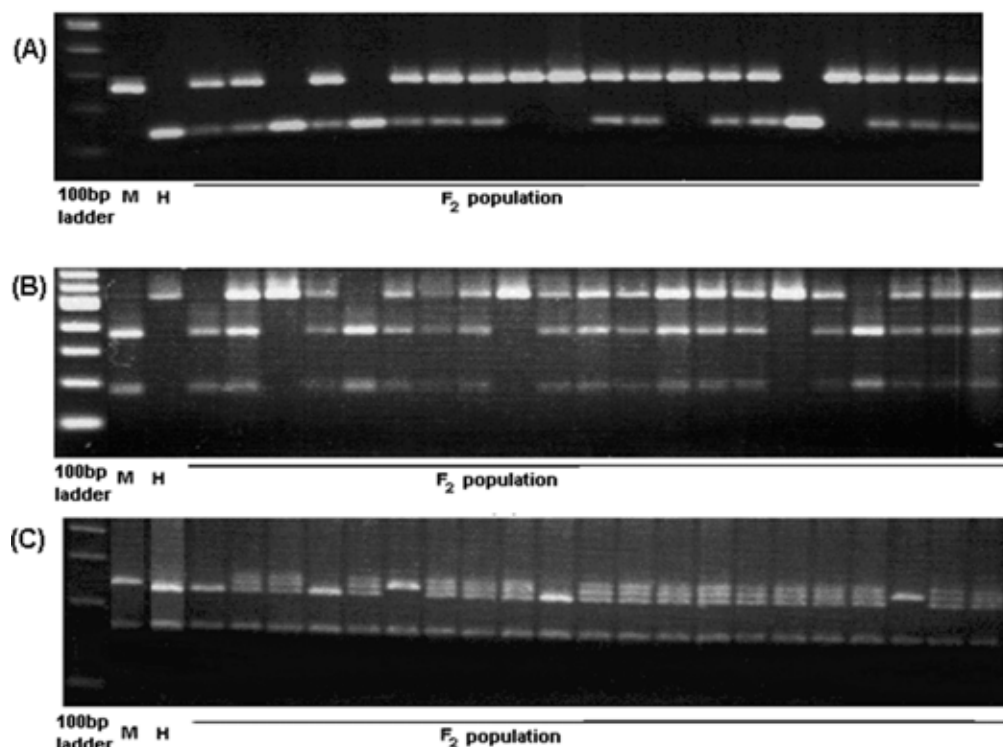


Figure 3. Genotyping of Morex (M) and Honen 6 (H) and the F_2 population of 'Morex' \times 'Honen 6' using CAPS primers (A) k06844KU, (B) k06860KU and (C) k06288KU.

the *srh* locus with three and one recombinants respectively in the mapping population. The linkage map is shown in Figure 4. Three CAPS markers were found proximal to *srh* while three SSR markers and smooth awn locus *raw1* were mapped distal. The *srh* locus was mapped between two flanking markers k06288KU and Bmag0223 1.6 cM proximal and at the 0.5 cM distal side to the *srh*, respectively. Thus, a molecular map of short rachilla hair gene was constructed.

Discussion

Linkage maps including the *srh* gene have been constructed based on morphological markers (Franckowiak, 1997). Although many molecular markers were screened during the experiment, it is difficult to find polymorphic markers near the short-rachilla-

hair locus. None of the previously reported STS and EST markers in this region tested during the experiment showed PCR product length variation among different short-rachilla-hair and long-rachilla-hair varieties. In the case of SSR markers, Bmag0223 is most important as it is reported close to the *srh* locus (Kraakman *et al.*, 2006). Of all barley varieties evaluated during this experiment, only Morex and Honen 6 are those showing variation for rachilla hair length and Bmag0223. These varieties also show polymorphism for SSR markers Bmag0812 and GMS027. An F_2 population obtained from the cross between Morex and Honen 6 was used to study the inheritance of rachilla hair length with SSR markers Bmag0223, Bmag0812 and GMS027 on long arm of chromosome 5H. Similarly the CAPS markers developed during the experiment

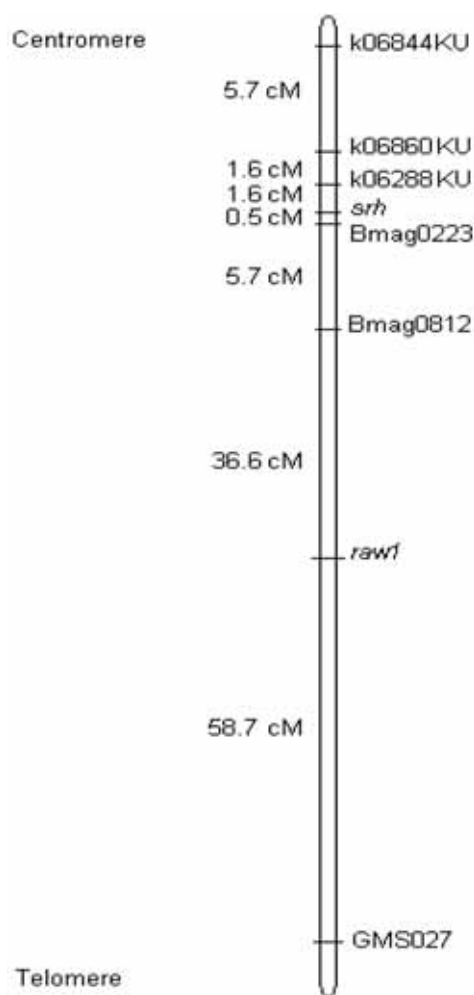


Figure 4. Barley chromosome 5H (7) linkage map constructed during the experiment.

also detected polymorphism after appropriate restriction enzyme digestion between Morex and Honen 6 only. Two flanking markers, k06288KU and Bmag0223, were found at a distance of 1.6 cM and 0.5 cM respectively. Costa *et al.* (2001) reported that Bmag0223 is 0.9 cM distal to the short rachilla-hair gene. Similarly, Kraakman *et al.* (2006) have stated that Bmag0223 is found less than 3.0 cM distal to the short-rachilla-hair gene in different mapping populations. According to the results obtained, k06288KU and

Bmag0223 are probably flanking markers for the short-rachilla-hair gene. The distance between them is 2.1 cM.

The resolution of this molecular map is not sufficient to provide a fine map due to the low density of markers. Therefore, development of a fine map with numerous markers in a large segregating population is required for high-resolution analysis of the *srh* locus (Stein and Graner, 2004). Such markers will be useful for studies on the origin of short rachilla hair in barley. Previous molecular studies for the short rachilla hair were made using RFLP, STS, SSR and AFLP markers (Kleinhofs *et al.*, 1993; Costa *et al.*, 2001; Kraakman *et al.*, 2006). In this study, CAPS markers derived from EST markers are mapped. Since EST markers are conserved among the related species, they may assist identification of syntenic region in the rice genome. Sequence-based markers from syntenic regions of rice can be used for fine mapping and candidate gene identification in barley (Goff *et al.*, 2002).

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Marker-assisted selection in breeding for virus resistance in winter barley

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Abstract

Barley yellow dwarf virus (BYDV) and barley yellow mosaic virus (BaYMV) are serious diseases in many parts of the world, including central and southern Europe. The best prospects for sources of resistance to BYDV appeared to be the advanced lines from Italy (Istituto Sperimentale per la Cerealicoltura, CRI, Fiorenzuola d'Arda) carrying the *Yd2* gene and the cultivars Sibra and Perry, in which resistance is based on other, as yet unidentified, genes. The cultivars Nelly (*rym4*) and Tokyo (*rym5*) were predominantly used to introduce resistance to BaYMV. The resistance genes to BYDV and BaYMV were identified by ASPCR and SSR markers to facilitate the selection of suitable genotypes for further breeding. The resistance level of the selected materials was monitored in field infection tests, which enabled evaluation of gene effects against different genetic backgrounds.

Introduction

The aphid-borne barley yellow dwarf virus (BYDV) and the soil-borne barley yellow mosaic virus (BaYMV) transmitted by the fungus *Polymyxa graminis* can cause serious yield losses. The use of resistant cultivars is generally the most effective means of controlling damage caused by these pathogens. The most widely used sources of resistance to BYDV in barley breeding contain the semi-dominant resistance gene

Yd2 (Rasmusson and Schaller, 1959). The gene was first identified in landraces of Ethiopian origin in 1960, and provides the so far most efficient protection against BYDV (Burnett *et al.*, 1995). Also tolerance to BYDV but not based on the resistance from Ethiopian sources is known. Collins *et al.* (1996) localized the gene *Yd2* on the long arm of chromosome 3H, near the centromere, and a closely linked co-dominant marker *Ylp* was developed for identification of *Yd2* genotypes (Ford *et al.*, 1998).

The genes *rym4* and *rym5*, responsible for the resistance against the complex of mosaic virus, were first detected in the south European landrace Ragusa (*rym4*) and in the Chinese landrace Mokusekko 3 (*rym5*). Using the RFLP technique, *rym4* has been mapped to the distant region of the long arm of chromosome 3H (Graner and Bauer, 1993). *Rym5* was mapped at the same chromosomal region based on its close linkage to the Est1-Est2-Est4 isozyme locus (Konishi *et al.*, 1997).

The objective of this work was to search for sources of resistance to BYDV and BaYMV using molecular markers.

Materials and methods

In total, 150 barley lines Fior from CRI, Fiorenzuola d'Arda, created in a program of gene pyramiding focused on breeding materials with the genes *Yd2* and *rym4*, were tested for presence of the *Yd2* gene, and 28 Russian cultivars were tested for the *rym4*

and *rym5* genes. In addition, the Italian cultivar Doria, created at CRI, Fiorenzuola d'Arda, in the same program, was tested for all three genes: *Yd2*, *rym4* and *rym5*.

Field infection trials to evaluate resistance against BYDV consisted of two blocks of small plots (1 m long) separated by a protective belt (Figure 1). One block represented the infection treatment and the other the uninfected control. At the beginning of the tillering stage infection with the PAV strain of BYDV was carried out by means of *Rhopalosiphum padi* aphids obtained from greenhouse rearing according to the method developed at the Crop Research Institute in Prague (Vacke *et al.*, 1996). Their inoculation suction lasted five to seven days, and the aphids were then killed by an insecticide. Symptomatic reaction to the virus infection was recorded in infected plants during the vegetation period and until the full flowering stage, using the 0–9 scale developed by Schaller and Qualset (1980). Healthy and infected plants are shown in Figure 2.

The DNA for PCR reactions was extracted from first leaves by the CTAB method. Molecular analysis of BYDV resistance was performed using ASPCR (Allele-specific PCR) marker Y1p detecting the *Yd2* gene. The Y1d gene encodes the *Yd2*-linked protein. Genetic analyses showed very close linkage with the gene *Yd2*. The proximity of Y1p and *Yd2* suggested that an assay based upon DNA polymorphisms in *rym4* would provide a valuable predictor of *Yd2* status (Ford *et al.*, 1998).



Figure 1. Fior in the field trial



Figure 2. BYDV infection (infected and healthy plant)

For molecular detection of *rym4* and *rym5*, the microsatellite STS (Sequence Tagged Sites) marker Bmac29 (AC/GT repetition, Graner *et al.*, 1999) was used. Bmac29 can distinguish three PCR products with various lengths. According to the length of these products, it is possible to identify cultivars with the genes *rym4*, *rym5* or susceptible cultivars. Amplification conditions in the UNO II (Schöeller Instruments) thermal cycler were: 3 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C, with final extension of 5 min at 72°C. Then 5 µL of each PCR product was checked by electrophoresis in 2% agarose gel and visualized under UV light after staining with ethidium bromide. After that, 1 µL of each sample was denatured with 0.3 µL of the size standard Tamra-500 (Applied Biosystems) and 4 µL of de-ionized formamid (Sigma) for 7 min at 96°C. Denatured samples were analyzed by capillary electrophoresis on an ABI PRISM 310 (Perkin-Elmer) and the lengths of the PCR products were scored using Genescan and Genotyper software (Applied Biosystems).

Primer pairs and PCR products are listed in Table 1.

Table 1. Primer pairs and the size of PCR products.

Primer pair	PCR product (bp)	Resistance gene
YlpPRCMF, YlpRAS (Ford <i>et al.</i> , 1998)	268	<i>Yd2</i>
Bmac29F-HEX, Bmac29R (Graner <i>et al.</i> , 1999)	144	<i>rym4</i>
	148	<i>rym5</i>
	168	susceptible

Table 2. The analysis of the resistance to BaYMV using the Bmac29 marker

Cultivar	PCR product (bp)	Resistance	Cultivar	PCR product (bp)	Resistance
Allegra	144	<i>rym4</i>	Kondrat – 2	144	<i>rym4</i>
Amarona	144	<i>rym4</i>	Kos	168	Resistant
Anita	168	Resistant	Kozir	169	Resistant
Annette	168	Resistant	Ludmilla	144/168	Heterozygote
Arkona	168	Resistant	Michailo	144/168	Heterozygote
AVC I	168	Resistant	Pavel	168	Resistant
Aviron	144	<i>rym4</i>	Produktiv	144	<i>rym4</i>
Bastion	168	Resistant	Radikal	168	Resistant
Blanca	144	<i>rym4</i>	Sekret	168	Resistant
Cyclon	168	Resistant	Skorohd	144/168	Heterozygote
Dobrynia	144/168	Heterozygote	Stephanie	144	<i>rym4</i>
Eszter	168	Resistant	Strider	168	Resistant
Fakir	168	Resistant	Theda	144	<i>rym4</i>
Fedor	168	Resistant	Theresa	144	<i>rym4</i>
Hutorok	168	Resistant			

Results and discussion

Almost all material from CRI, Fiorenzuola d'Arda, was quite highly resistant in the field trials. 150 lines Fior, from an Italian program focused on breeding material possessing the *Yd2* and *rym4* genes, were tested by the Ylp marker to detect the *Yd2* gene. The analysis revealed 121 resistant lines possessing the *Yd2* gene and 29 susceptible lines without this gene. Molecular analysis of cultivar Doria showed the presence of both *Yd2* and *rym4* genes.

The 28 Russian cultivars were tested using the Bmac29 marker; the results are given in Table 2.

Conclusion

Both markers mentioned above were successfully used for the detection of the barley resistance genes *Yd2* and *rym4* in materials with resistant reaction in field infection tests. Marker-assisted selection is a useful way for searching the sources of resistance that can be used for further breeding.

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Session 3

Barley genomics

TILLING for functional genomics in barley

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Abstract

As part of a larger consortium, funded in the frame of the German plant genome program GABI (GABI-TILL; <https://www.gabi-till.de/>) we have established a TILLING platform for the two-row, malting barley cultivar Barke. In total, 111 540 barley seeds were mutagenized by ethylmethane-sulfonate (EMS) treatment at concentrations between 20 (0.2%) and 60 mM (0.63%), with 24 600 lines advanced to the M_2 generation. M_1 production was streamlined to high-density plant cultivation, keeping barley plants till maturity in 299-well potting plates on flooding tables. M_2 mutants were grown either in the greenhouse or under field conditions, and were regularly observed and screened for visible mutant phenotypes. The mutant population currently comprises 10 492 M_2 plants. Those adjustments affected DNA pool size, enzyme concentration and reaction mixture, as well as cleavage conditions. M_2 -DNA was arranged in 8-fold two-dimensional (2D) pools for high-throughput mutation screening. Ten gene fragments varying in size from 321 to 1496 bp were screened for mutations (October 2007), yielding an average mutation frequency of approximately 1 mutation per 0.5 Mbp. Mutations were confirmed by re-sequencing and functional changes were surveyed by phenotyping the M_3 or M_4 progenies, or both.

In this paper we introduce an EMS-induced mutant population for the barley cultivar Barke, consisting of 10 492 M_2 lines. We have established a TILLING platform and have determined the usefulness of the resource by accomplishing mutation

screenings in a set of 10 fragments of 6 different genes. Based on this work, we conclude that sufficient mutation frequency and density can in principle be obtained in barley by EMS mutagenesis, and this method allows for efficient, large-scale mutation discovery in barley.

Introduction

The economic importance of cereals, like barley, and the demand for improved yield and quality require better understanding of the genetic components that modulate biologically and commercially relevant traits. While the weedy plant *Arabidopsis* has become the preferential model plant system, the spectrum of its traits cannot address all fundamental questions of crop plant development. Unlike *Arabidopsis*, barley is both a crop and a model system for scientific research that is increasingly being used for genetic and molecular investigations into conserved biological processes of cereals.

Chemical mutagenesis is a strong tool in attempts of “forward genetic” gene isolation efforts. A large mutant population is typically developed with the purpose of identifying individual mutants exhibiting an aberration in a single target trait. In the TILLING (Targeting Local Lesions IN Genomes) method, such mutant populations are efficiently employed as a reverse genetics instrument that can be utilized to screen for mutations in any gene of interests for which sequence information has been provided. Plants carrying a mutation in the desired gene are subsequently subjected to phenotypic analysis which eventually (and at low frequency) will yield clear correlation

between observed mutations and phenotypes. Chemical mutagens like ethylmethane-sulfonate (EMS) induce single nucleotide changes at high density, which are randomly distributed in the genome. They induce allelic variation, including mis-sense mutations leading to altered protein function, as well as stop-codon mutations or splice site changes that could result in complete knockout or knockdown of a gene. To obtain clear-cut phenotypes, gene knock-out mutations are often considered as most desirable; however, such mutations could limit the analysis if the effect is lethal or detrimental. Here, mis-sense mutations leading to hypo- or hypermorphic phenotypes could be of great value since they still would allow a useful analysis of functional relevant gene regions (Haughn and Gilchrist, 2006). Besides its relevance in elucidating the genetic basis of biological processes the application of TILLING extends reverse genetics to mutation breeding, since it in principle provides the potential to generate large allelic series of economically interesting genes. Thus, TILLING has become a popular and powerful tool for reverse genetic analyses in plants and other organisms, and populations have been established, among others, for *Arabidopsis* (Greene *et al.*, 2003), hexaploid and tetraploid wheat (Slade *et al.*, 2005), maize (Till *et al.*, 2004), rice (Wu *et al.*, 2005; Suzuki *et al.*, 2007; Till *et al.*, 2007), *Drosophila melanogaster* (Winkler *et al.*, 2005), zebrafish (Wienholds *et al.*, 2003), and the mammals rat and mouse (Smith *et al.*, 2004; Augustin *et al.*, 2005).

Material and methods

Seed material

Seeds of *Hordeum vulgare* L. cultivar Barke (obtained from Saatzzucht Josef Breun GdB.R, D-91074 Herzogenaurach) were used for the development of a barley TILLING library. Barke is a spring-type, two-row, European malting variety.

Creation of barley TILLING library

EMS mutagenesis: Batches of ~1600 seeds were filled into 2000 ml glass flasks and pre-soaked in 500 ml de-ionized H₂O for 4 h at room temperature (20–25°C). Seeds were then incubated in 350 ml dH₂O of varying concentrations (20, 25, 30, 35, 40, 45, 50, 55, 60 mM) of EMS for 16 h at room temperature with gentle shaking (125 rpm) on a tabletop shaker. The seeds were washed as follows: twice with 250 ml of 200 mM sodium thiosulfate for 30 min; and twice with 1 L of dH₂O (for 30 min and then for 1 h). The seeds were transferred to trays covered with Whatman paper and air-dried at 4°C overnight prior to planting.

M₁ cultivation: M₁ plants were cultivated in the greenhouse till maturity in 299-well potting plates on flooding tables. Plants were allowed to self-pollinate, the main spike of each plant was harvested and M₂ seeds were stored until planting. Every M₁ generation was evaluated regarding germination and sterility rates. Germination was measured as percentage of developed seedlings, and sterility was measured as seed set per harvested spike. Those observations were used to determine applicable EMS concentrations.

M₂ cultivation: From each M₁ spike a progeny of 10 individuals was cultivated as an M₂ family. M₂ plants were cultivated as batches, either in the greenhouse (1000 M₂ families) or in the field (3780 M₂ families). After tillering, M₂ families were evaluated regarding viability and one or two individuals of each family were selected for further cultivation. M₂ seedlings were regularly monitored for the presence of chlorophyll defects, and plants were regularly screened for visible mutant phenotypes until maturity. All phenotypes were scored in reference to parent cultivar Barke. M₃ seed from individual M₂ mutants were collected, catalogued, and vacuum-packed and stored at 4°C until use as resource for phenotyping.

Genomic DNA isolation

Genomic DNA was prepared from young leaves after lyophilization of tissue. Twenty to thirty milligram of lyophilized leaf tissue (with the remains of samples stored as backup) were milled in 2 ml tubes and DNA was extracted using the CTAB procedure according to Doyle and Doyle (1987, 1990). DNA was subsequently transferred to 96-well plates and an aliquot was diluted [20 ng/ μ l] for PCR purposes. Diluted DNAs were arranged in 8-fold 2D-pools for mutation screening.

CEL1-based mutation screening

The mutation detection procedure follows a standard CEL1-based hetero-duplex analysis (Colbert *et al.*, 2001) that has been adapted to specific requirements in barley.

Primers, PCR, and sequencing analysis:

Oligonucleotides for PCR were designed either using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) directly or via the program CODDLE (Codons Optimized to Discover Deleterious Lesions; <http://www.proweb.org/coddle/>). Gene-specific primer design was either based on genomic or EST sequence information for the candidate genes. Unlabeled and identical primers labeled at the 5' end with the fluorescent dye IRD700 (forward) or IRD800 (reverse), respectively, were mixed and used in PCR amplification as follows: 3:2 (labeled:unlabeled) ratio for the 100 μ M IRD700-labeled forward primer and 3:1 (labeled:unlabeled) ratio for the 100 μ M IRD800-labeled reverse primer. Primers were designed to fit to melting temperatures between 60°C and 70°C. PCR amplification and heteroduplex formation was carried out according to Colbert *et al.* (2001).

CEL1 nuclease mismatch cleavage assay:

After PCR amplification, samples were digested with 0.06 U of Surveyor CEL1 enzyme (Transgenomics, Omaha, USA) in a 10 \times buffer (10 mM HEPES (pH 7.0), 10 mM

KCl, 10 mM $\text{MgCl}_4 \cdot 7\text{H}_2\text{O}$, 0.002% Triton X-100 and 10 μ g/ml BSA) and incubated for 45 min at 45°C. CEL1 Digestion was stopped immediately by adding 5 μ l of 75 mM EDTA (pH 8.0) followed by freezing probes (-20°C) for 30 min.

Precipitation of 30 μ l samples in 96-well PCR plates is carried out by adding 3M Na-acetate (pH 5.2) and 75 μ l EtOH (99.8%), subsequent shaking for 15–20 min, and centrifugation for 30 min at 4500 rpm. The supernatant was discarded on filter paper and plates were centrifuged face down on a filter paper for 1 min at 500 rpm. Subsequently, probes were washed in 100 μ l EtOH (75%) at room temperature while being centrifuged for 30 min at 4500 rpm. Again, the supernatant was discarded on filter paper and plates were centrifuged face down on a filter paper for 1 min at 500 rpm. Finally, samples were dried for 20 min at room temperature, and re-suspended in 8 μ l formamide loading buffer (33% (w/v) de-ionized formamide, 25 mM Tris (pH 7.5), 25 mM EDTA and ~0.02% (w/v) bromophenol blue) with constant shaking (300 rpm) for 5–10 min, then denatured at 95°C for 5 min and placed on ice. Samples were subsequently electrophoresed in a 6.0% polyacrylamide slab gel (Long Ranger, FMC Corporation, composition according to manufacturer's data) in 1 \times TBE running buffer (89 mM Tris Base, 89 mM Boric Acid, 2 mM $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) at 1500 V, 40 mA and 40 V settings on a Li-COR 4300 DNA Analyser (LI-COR Biosciences, Lincoln, NE). Images were visualized using the GelBuddy software (<http://www.proweb.org/gelbuddy>; Zerr and Henikoff, 2005) and were thus eye-inspected for the presence of cleavage products.

Sequence data processing: For confirmation of putatively mutated loci, amplicons of the respective target gene were generated utilizing the same PCR conditions as established for CEL1 analysis. Only unlabeled primers were used and probes were subjected to the ABI 3730xl DNA Analyzer (Applied

Biosystems) for sequencing. Sequences of identified mutations were processed using Sequencher 4.6 software (Gene Codes, Ann Arbor, MI). Sequences derived from M_2 lines were aligned and compared to the reference sequence that was used to design primers and to Barke wild-type sequences. The PARSESNP (Project Aligned Related Sequences and Evaluate SNPs; <http://www.proweb.org/parsesnp/>) bioinformatics program was used to predict the putative severity of each identified mutation (Taylor and Greene, 2003).

Statistical analysis

Statistical analysis was conducted to investigate possible EMS dosage effects on M_1 germination and sterility, M_2 chlorophyll mutant occurrence, and the respective seed harvest. The STATISTICA 6.0 software (StatSoft, Inc.) was used to conduct analyses of regression and correlation, tests of linear relationships and the one factorial ANOVAs.

Results and discussion

Generation of an EMS-induced mutant population in barley

A mutant population of the malting barley cultivar Barke has been generated by treatment with the mutagen EMS (ethylmethanesulfonate). The cultivar Barke was chosen since it represents an elite central European two-row malting variety and because other barley genomics resources were already established in the same genetic background (~ 150 000 ESTs, DH mapping population). To investigate the optimal EMS concentration for Barke, a range of concentrations between 20 (0.2%) and 60 mM (0.63%) was applied during population construction. To develop a barley TILLING platform, DNA samples and M_3 seeds were collected from 10 492 M_2 individuals. Of these 1949 plants were treated with 20 mM, 2992 plants with 25 mM, 2776 plants with 30 mM, 1933 plants with 35 mM, 469 plants with 40 mM and 373 plants with 45 to 60 mM EMS concentrations.

Although EMS has repeatedly been shown to have a highly mutagenic effect on barley, variation in mutation frequencies were reported for a single mutagen used in different cultivars of a single species. This means that, for a specific mutagen and cultivar relationship, the optimal treatment needs to be determined that will consistently induce the most suitable mutation frequencies (Nilan *et al.*, 1964).

To yield high mutation rates has been a central aim in mutation breeding; at high mutation frequencies, a smaller mutant population size would be required to achieve genome saturation. However, high mutagen dosages also impose practical problems. A high mutation rate does not necessarily result only in a general increase of desirable mutants (Yonezawa and Yamagata, 1975). This may be attributed mainly to considerable biological side effects that are produced during mutagenesis and which affect the general effectiveness of a treatment (Konzak *et al.*, 1964). These effects are mainly caused by the toxicity of EMS metabolites, leading to reduced M_1 seedling germination, growth and survival rates, as well as reduced M_1 spike fertility. In the M_2 generation the random distribution of mutations in the genome results not only in useful but also in undesirable phenotypes, including reduced numbers of spikes per plant and reduced spike fertility, as well as an increased frequency of chlorophyll-deficient seedlings. Consequently, mutation effectiveness can be defined as an optimum between the amounts of physiological injury observed in the M_1 and the mutation frequency and spectrum obtained in the M_2 generation.

In our kill-curve study based on M_1 sub-populations treated with 9 different EMS concentrations, we could observe an unambiguous negative correlation between EMS dosage and germination rate ($P < 0.01$). Barley mutants treated with 20 mM EMS showed on average 8% reduced germination frequency, while this rate increased to 31% if mutants were induced by 40 mM, and

reached a maximum at 60 mM, where on average 60% of the seeds did not grown into plants (Figure 1).

M_1 spike sterility induced by EMS treatment could be scored in three categories: completely sterile (100%), semi-sterile (measured as >50%, 50%, <50%) and no sterility (0%) per examined M_1 spike. The frequencies for the categories complete sterility ($P < 0.001$) and semi-sterility ($P < 0.001$) increased towards the highest EMS concentration, leading to nearly 100% spike sterility observed for treatments with 60 mM EMS (Figure 1). Consequently, the kernel yields of M_1 plants were accordingly variable between the different sub-populations. While from the two lowest EMS concentrations we could harvest M_2 seeds from ~50% of the cultivated M_1 plants, this rate was reduced to 15% for those plants treated with 40 mM and achieved a low point of 3% for the highest EMS level treatment.

As mentioned above, a positive correlation between seedling lethality and M_1 sterility

effects and the EMS concentration used is a conspicuous phenomenon in the M_1 generation. While the germination capacity is supposed to be mainly influenced by the toxicity of EMS metabolites, sterility is rather dependent on a combination of both toxicity and induced mutations. Therefore, germination seems to be a limiting factor for the maximum EMS dosage that can be tolerated by an organism rather than an indicator for the mutation density that can be expected in the next generation (Linnert and Micke, 1997). Based on our observation in the M_1 generation, we found that an EMS dosage greater than 40 mM seems to be problematic for efficient M_2 seed production.

Visible mutations as indicators of variability in mutation populations

Because considerable effort is needed to propagate and advance TILLING populations, it is desirable to have available a phenotypic marker to assess obtained mutation at a very early stage during population development. In former barley mutation experiments, M_2 chlorophyll-deficiency in seedlings was used as a key criterion of genetic effects caused by different mutagens and mutagen concentrations (Sheeba *et al.*, 2003). Chlorophyll-deficient seedling phenotypes are quite suitable for this measurement since they occur most frequently and are easy to score. They comprise three major classes (unicolor, bicolor, convertans) and several sub-classes of phenotypes (Sharma and Bansal, 1972). The occurrence of albina, xantha, viridis (sub-class unicolor), viridoalbina, striata (sub-class bicolor), tigrina and lutescens (sub-class convertans) phenotypes were recorded, and used to survey the variability between M_2 sub-populations produced by 6 different EMS treatments (20, 25, 30, 35, 40 and 50 mM). Chlorophyll mutant rates observed among M_2 seedlings obtained from treatments higher than 50 mM were excluded from statistical considerations,

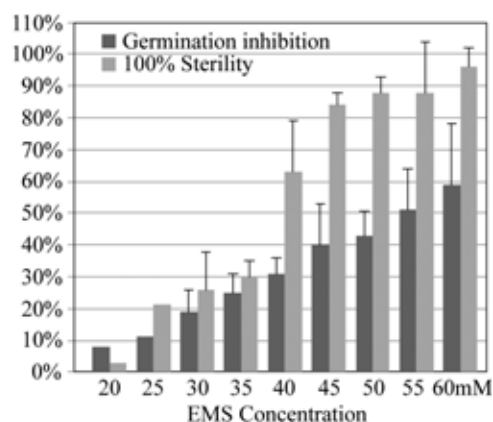


Figure 1. Rates of germination inhibition and full sterility observed among 58 900 M_1 lines obtained from different EMS treatments. All plants were obtained from 7 independent experiments and were cultivated under identical conditions (except for 20 and 25 mM EMS concentrations, which were based on a single M_1 batch). Standard deviations are shown as error bars.

because the general low germination rate of those mutants was insufficient for this purpose. Chlorophyll-deficient mutants were scored among 10 seedlings per cultivated M_2 families. Thereby, the observed relative frequencies were calculated as mutations per germinated 100 M_2 plants according to Gaul (1960). The albina appearance was the most frequently observed in all mutagenized populations. Exclusively for this phenotype, we could demonstrate a significant relationship between EMS treatment and occurrence of chlorophyll-deficient mutants ($P < 0.001$).

The other six phenotypes seem to be distributed randomly. Our interpretation is that there is an evident shift in mutation spectra for chlorophyll deficiencies, especially if M_2 mutants are cultivated under field conditions. Many scientists have reported that it is probable that mutant types other than albina can be classified differently, either by different personal evaluation or according to environmental influences. They have recognized that the environmental conditions under which the seedlings are grown can alter or even induce certain chlorophyll-deficient phenotypes, thus observed frequencies between treatments may not be significant, valid or reproducible (Favret *et al.*, 1964).

During the development of the M_2 TILLING population, 12 682 M_2 individuals, induced with EMS dosages between 20 and 35 mM, were cultivated under field conditions and scored for their phenotype. We surveyed for alterations in spike morphology, heading date (and plant development in general), plant color, plant height, and other variations from the Barke wild-type. In approximately 20% of the cases at least one kind of mutant phenotype could be observed.

Development of a barley TILLING platform

TILLING screens were conducted by using a CEL1-based hetero-duplex analysis for

mutation detection. The optimal mutation detection procedure and pool size for Barke DNA was determined by performing test screenings assaying known single nucleotide polymorphisms (SNPs) in alleles of the gene Hv-eIF4E (Stein *et al.*, 2005) of the barley genotypes Igri and Franka. For pooled DNA, a 1:10 dilution of CEL1 in a 10× buffer solution combined with a 45 min digest at 45°C provides an optimal ratio between background and signal strength. The 8-fold pooling of DNA was found to produce robust and reliable results. To improve the mutation discovery procedure, the population was screened in a two-dimensional format. In each 96-well pool plate the DNA of 384 M_2 individuals were arranged in row and column pools. As every sample is present in a row and a column pool, true mutations will be detected simultaneously in two pools ultimately leading to the mutated individual, which is exclusively present in both pools (Figure 2)

Gene fragments of varying size from 321 to 1496 bp were screened and amplicons were designed to cover either the whole ORFs or selected coding regions. To estimate the frequency of mutations per gene fragment, we divided the total number of mutations identified by the totally screened base pairs. We calculated the density of mutations in the established barley population as an average frequency of all screened fragments. Previous TILLING studies, using LiCOR-based assays for mutation detection, have reported the diminished ability to detect mutations in the upper and lower 100 bp of an amplicon, and consequently considered a respectively reduced size of the actually screened fragments for the estimation of the total number of screened base pairs (Till *et al.*, 2007; Greene *et al.*, 2003). In our screenings we found that the flagged area of ambiguous heteroduplex scoring due to priming and systematic artifacts extended to only an overall of 100 bp. Therefore, the total number of screened base pairs considered was reduced by 100 bp per gene fragment.

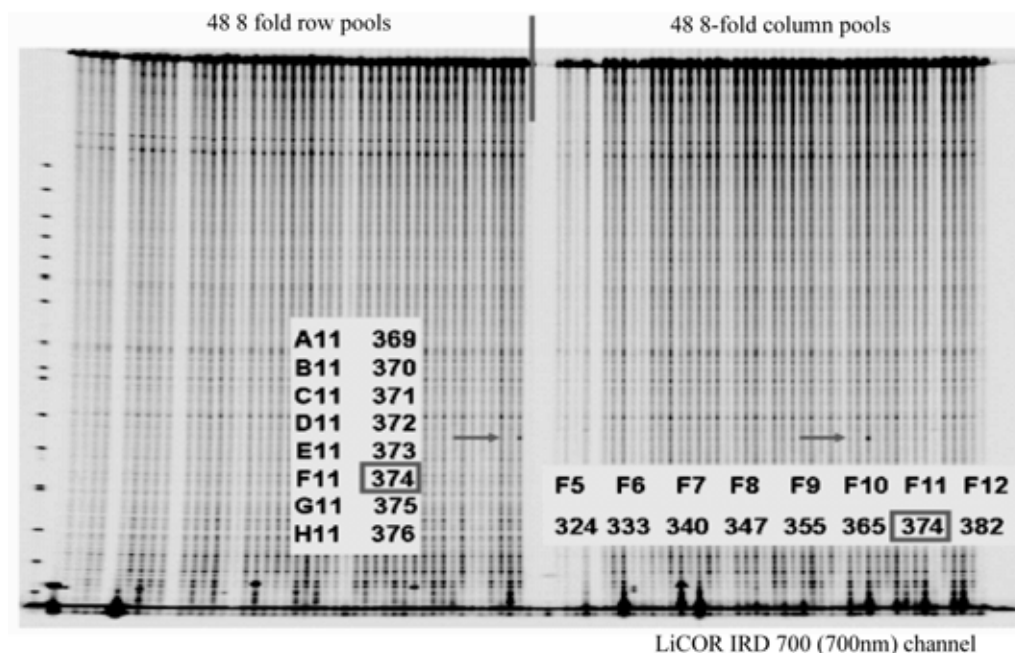


Figure 2. Representative example of a LiCOR gel used to screen 384 M_2 for mutations in the gene *Vrs1*. Each of the 96 lanes of the gel contains 8-fold pooled CEL-1-digested products of 1000 bp amplicon. Red arrows indicate the M_2 mutant 374 detected by its presence in both pools.

The screening of ten gene fragments led to the identification of 81 mutations. This equaled a mutation frequency of one mutation per 0.5 Mbp. The mutation frequency thus corresponded to densities reported earlier for maize (Till *et al.*, 2004) and greater than the rate of 1 mutation per 1 Mbp that was calculated for the barley cv. Optic (Caldwell *et al.*, 2004). Of the 81 identified mutations, 79% (64 of 81) were located in coding regions of the analyzed gene fragments. Among those, 45% (29 of 64) represented mis-sense alleles resulting in a change of amino acid in the protein sequence of the gene, while 5% (3 of 64) of the mutations provided truncation mutations in the reading frame, either by elimination of a splice junction site or by insertion of a premature stop codon.

Conclusions

We have produced a TILLING population of 10 492 M_2 plants as a resource for high-

throughput gene discovery in barley. Pilot screening in 6 target genes has demonstrated a moderate mutation frequency that will allow the identification of series of mutations evenly distributed in the chosen gene regions (Figure 3). The phenotypic evaluations in parts of the M_2 and M_3 generation have revealed the presence of a wide spectrum of morphological mutations implying also a serious potential of the resource for utilization in forward genetic screenings. Collectively, the presented barley mutant population represents an important tool for functional analysis in the crop model system barley.

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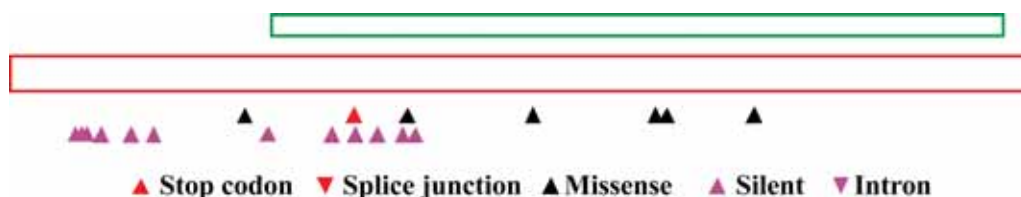


Figure 3. Example gene model showing distribution and type of mutations discovered in a 1300 bp region covering the full length ORF. The figure was designed from PARSESNP (Taylor and Greene, 2003) output files. The red open box indicates the extension of the exon; homology to proteins in the BLOCKS database (Henikoff *et al.*, 2000) is shown as green box above the red box. Purple upright triangles indicate a change that does not affect the amino acid product; black upright triangles indicate a change that induces a mis-sense mutation in the predicted gene product, the red upright triangle indicates a premature stop codon mutation.

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Development of TILLMore: a resource for forward and reverse genetics in barley

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Abstract

TILLING (Targeting Induced Local Lesion IN Genomes) is a reverse-genetics approach combining chemical mutagenesis with a sensitive DNA screening-technique to identify point mutations in target genes. TILLMore is a TILLING resource in barley (cv. Morex) including 4906 M₄ families derived from a sodium azide treatment. Molecular screening for mutation at agronomically important genes, based on the analysis of 8- to 12-fold DNA pools produced from M₂ or M₃ DNA samples, identified an average of about seven alleles per gene and an extrapolated rate of one mutation every 404 kbp. The mutagenized population, although developed for reverse-genetics purposes, is also suitable for forward-genetics analysis. A high frequency of M₃ families (about 33%) showed morphological alterations compared with untreated Morex plants. Furthermore, a preliminary screening for phenotypes at the root level showed altered root morphology for about 7% of the families. Our results indicate the feasibility of using this collection of material to investigate gene function in barley and related crops.

Introduction

Barley (*Hordeum vulgare* L.) is one of the most important cereal crop species and, despite its large genome (more than 5000 Mbp; Bennett and Smith, 1976), is considered a model

species for more complex genomes of the Triticeae tribe. Barley has a long history of genetics and genomics studies, including numerous genetic maps, a large number of genetic stocks and mutant collections (Lundqvist *et al.*, 1996; Caldwell *et al.*, 2004; Varshney *et al.*, 2007) and an extensive assortment of sequence information (Zhang *et al.*, 2004; Isidore *et al.*, 2005). However, translating these data into information on gene function on a large scale remains a challenging task (Varshney and Tuberosa, 2007). Technologies that monitor patterns of differential expression (e.g. micro-arrays) can contribute to categorize genes (Ozturk *et al.*, 2002) but they could not be informative in the context of the comprehensive phenotype of a living organism. At the same time, other technologies, such as gene inactivation by insertional mutagenesis based on transposons or T-DNA, are currently under development in barley (Singh *et al.*, 2006; Zhao *et al.*, 2006). These approaches, although promising, almost invariably suffer from the controversy of growing transgenics in the open field. Consequently, high-throughput methods for the confirmation and validation of gene function by target gene inactivation are currently recognized priorities.

One of the most utilized approaches in biological research is mutational analysis where chemical or physical mutagenesis is used for achieving a large-scale random gene inactivation in a population of plants.

However, although forward-genetics based on the phenotypic identification of individual mutations has been the most widely used tool to understand the genetic control of plant development, there are limitations to this approach, such as the considerable efforts needed to identify rare mutations (Stemple, 2004). These limitations can be overcome by high-throughput reverse genetics tools that allow us to identify mutations in genes that are known only by their sequence.

In this context, TILLING (Targeting Induced Local Lesion IN Genomes) is a reverse-genetics technique that enables the recovery of individuals carrying allelic variants at candidate genes (McCallum *et al.*, 2000). TILLING can be applied to either chemically mutagenized populations or collections of genotypes (e.g. cultivars, collection of ecotypes, landraces and wild accessions; this latter approach is also known as EcoTILLING (Comai *et al.*, 2004). TILLING can be applied essentially in any species, without significant differences in protocol. Since its first introduction, TILLING has been successfully used as a functional genomics discovery platform both in plants (McCallum *et al.*, 2000; Perry *et al.*, 2003; Till *et al.*, 2004, 2007; Slade *et al.*, 2005; Suzuki *et al.*, 2007) and animal species (Wienholds *et al.*, 2003; Winkler *et al.*, 2005; Gilchrist *et al.*, 2006). Among the Triticeae, TILLING platforms have been generated in barley by EMS-chemical mutagenesis on cultivar (cv.) Optic (European two-row malting variety; Caldwell *et al.*, 2004) and in wheat by EMS treatment of both the hexaploid cv. Express and the tetraploid cv. Kronos (Slade *et al.*, 2005). The wheat TILLING platform has been successfully screened for mutations in functional genes for starch production, representing a good example on how TILLING can directly enable the recovery of agronomically useful alleles (Slade *et al.*, 2005; Slade and Knauf, 2005).

This study describes the assembly of a sodium azide mutagenized barley population

in a cv. Morex background suitable for a TILLING approach and the identification of artificially induced allelic variants at target genes.

Material and methods

Assembly of the mutagenized population

Seed of cv. Morex was used for the mutagenesis experiment. Three batches of about 20 000 seeds were treated with different concentrations of sodium azide (1, 5 and 10 mM concentration) following the protocol described in Nilan *et al.* (1973) and modified according to Hodgdon *et al.* (1979). The mutagenized populations were grown as M_1 plants and allowed to self-pollinate. Early indications of the mutagenic effect of the three NaN_3 concentrations were obtained by measuring seed germination, coleoptile length, frequency of foliar chimeras and spike fertility in the M_1 generation. Untreated seed was always included as control.

One M_2 seed per M_1 plant was collected from the 10 mM treatment and planted to produce the M_2 population and the 4906 $M_{3/4}$ families. Figure 1 provides an overview of the procedures adopted to assemble the TILLMore resource.

Forward-genetics screening

The entire TILLMore M_3 population was grown in the field and scored for visible phenotypes in reference to untreated Morex. Phenotypic information was collected as to habit, heading data, leaf appearance, presence of necrotic spots, plant color, plant height, plant morphology, spike appearance and tillering.

To detect root morphology alterations, a preliminary analysis on about 1000 M_3 families was carried out using a paper-roll approach (Woll *et al.*, 2005). About 12 seeds per family per paper roll and replicated were grown under controlled conditions

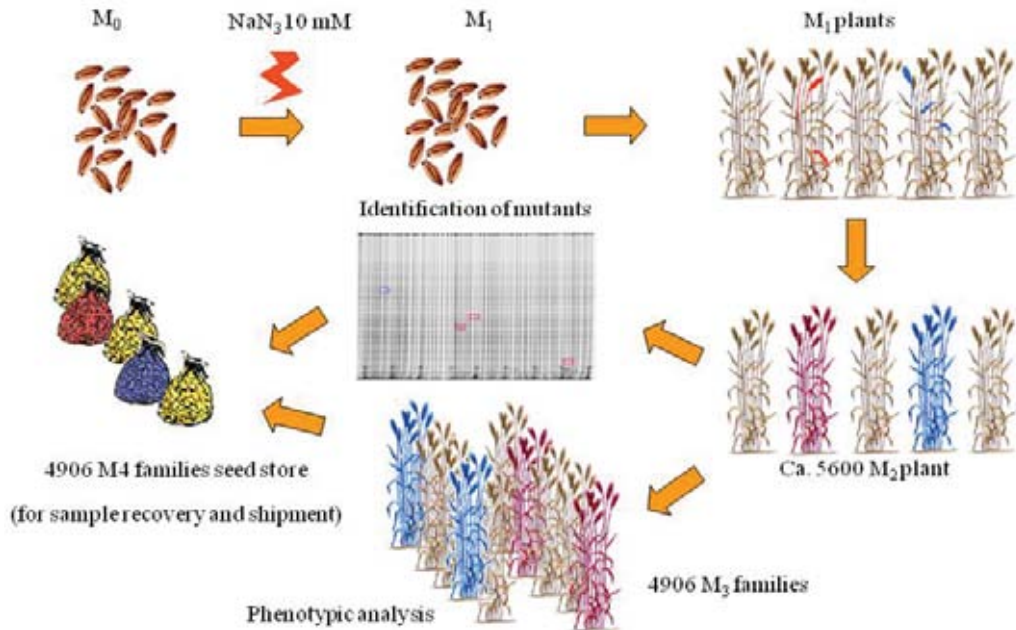


Figure 1. Workflow followed to produce TILLMore. Seeds of cv. Morex were mutagenized and immediately planted in the field. Plants were allowed to self-pollinate. One seed per M_1 plant was collected and grown in the following year to produce the M_2 population, which provided leaf samples for DNA extraction for molecular analysis and M_3 seeds. In the third year, M_3 families were grown for seed increase and traits evaluation for forward genetics purposes.

(16/8 h photoperiod and 24/22°C day/night, respectively). Seminal roots observations and measurements in reference to untreated Morex were performed on eight-day-old seedlings.

Genomic DNA preparation

DNA isolation was performed from leaf tissue samples collected from plants at the tillering stage from leaf tissue. DNA isolation was performed following a standard CTAB protocol (Saghai-Marouf *et al.*, 1984). Aliquots of genomic DNA were separated on 0.8% agarose gel to check for DNA quality. Genomic DNA was diluted to the concentration of 30 ng/ μL and used for serial dilution stocks of 10 and 2.5 ng/ μL . Two different DNA stocks were prepared: one referred to 3148 samples collected from M_2 plants, while the second DNA stock

referred to the whole TILLMore population of 4906 M_3 families. The first DNA stock was pooled four-fold and then combined to obtain eight-fold pools. The second DNA stock was pooled according to a two-dimensional gridding strategy combining all samples both in 8- and in 12-fold pools.

Mutation screening

The primer design, based on the Morex genomic sequence, was carried out using CODDLE (<http://www.proweb.org/coddle>; Taylor and Greene, 2003) and Primer3 (<http://frodo.wi.mit.edu/>; Rozen and Skaletsky, 2000). PCR amplification was conducted as described in Colbert *et al.* (2001). Amplified samples were digested with *CelI* nuclease according to the manufacturer's directions for the Surveyor® Mutation Detection Kit for agarose gel (Transgenomics Inc., Omaha,

NE, USA) and to the protocol described in Colbert *et al.* (2001). Electrophoresis was performed either on a LI-COR4200 gel analyzer (LI-COR Inc, USA) according to the protocol described in Colbert *et al.* (2001) or on ABI3730 capillary DNA Sequencer (ABI, USA) according to Wienholds *et al.* (2003), with minor modifications. Images were analyzed visually for the presence of cleavage products using Adobe Photoshop software (Adobe Systems Inc.). Presumptive mutants identified were subsequently confirmed by sequencing genomic DNA using the ABI BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences of mutant alleles were analyzed with SIFT (Ng and Henikoff, 2001) and PARSESNP (Taylor and Greene, 2003) programs in order to rank mutations based on probability of affecting protein function.

Results and discussion

Production of the TILLMore resource

TILLMore, a barley TILLING resource in the genetic background of the cv. Morex, was obtained by means of a sodium azide (NaN_3) treatment. The mutagenic agent sodium azide was chosen because it has been shown to be particularly effective in barley (Nilan *et al.*, 1973). Although its exact mechanism of action is not completely understood, NaN_3 is known to generate mainly single point mutations (Kleinhofs *et al.*, 1978) which can be easily identified by TILLING. M_1 plants derived from the seed treated with different NaN_3 concentrations were scored for seed germination, reduction of coleoptile length, frequency of leaf chimeras and fertility of M_1 spikes. Based on these results, we concluded that the 10 mM treatment would have guaranteed the highest mutation density. Therefore, only the M_1 plants derived from the 10 mM treatment were considered to produce the TILLMore resource.

To prevent redundancy of mutations, we harvested a single M_2 seed for each M_1 plant. The M_2 population was subsequently grown in the field to allow for leaf-DNA sampling and seed increase. A further generation was carried out to increase the amount of stock DNA and seed for each family. For 4906 families (the current dimension of the TILLMore resource) it was possible to prepare DNA suitable for molecular screening and obtain at least 100 M_4 seeds for stock purposes.

TILLMore reverse-genetics screening

We screened TILLMore with assays designed around six target genes involved in different aspects of the barley development and metabolism. The molecular screening of the first set of three genes was carried out on 3148 samples using a *Cell*-based heteroduplex assay coupled with gel-electrophoretic detection on LI-COR analyzer screened for mutations in 8-fold sample pools. For molecular screening of the second group of three genes we used an ABI3730 sequencer system on the entire population of 4906 samples screened for mutations in 8- and 12-fold sample pools.

For each TILLED gene, the molecular screening yielded an allelic series of mutants with an average of about seven alleles per gene, corresponding to a mutation density of one mutation every 404 kbp. This parameter was estimated by dividing the number of base pairs screened by the total number of identified mutations. Reflecting that mutations placed in the terminal 80 bp of both ends of the amplicon can escape identification due to PCR priming and electrophoresis artefacts, a correction on the effective screening window was applied by subtracting 160 bp from the length of each amplicon (Greene *et al.*, 2003).

A total of 41 point mutations were identified at the target genes. In nine cases the mutations were in non-coding regions. Of the 32 point mutations identified in coding

regions, some (about 28%) are predicted to be silent because they affect the third base of a codon, which does not change the amino acid encoded by that codon, while 72% of them are mis-sense alleles resulting in a change in one of the amino acids in the protein encoded by the gene (Table 1). No nonsense (truncation) mutation was identified for the genes tested. Of the 41 lesions, only one was a C/G-to-A/T transversion, while 40 were G/C-to-A/T transitions. Although a base transition mechanism has been postulated to explain the mutagenic effect of sodium azide (Kleinhofs *et al.*, 1978), the exact type of NaN_3 -derived mutants has not been exhaustively described in terms of nucleotide-change typology. To our best knowledge, to date only one study investigating the nucleotide changes induced by NaN_3 in barley has been published (Olsen *et al.*, 1993). These authors proposed that NaN_3 causes mutations of transition type. Although limited to a few genes, our results combined with those reported by Olsen *et al.* (1993) reinforce the hypothesis that barley NaN_3 -induced sequence alterations are mainly transitions. Due to these considerations and to the finding that all but one of our mutations are G/C-to-A/T transitions, we feel confident to rule out the possibility that they are naturally occurring polymorphisms due to seed contamination of the original Morex seed stock used for this study.

In order to predict the impact of mutations on protein function we utilize bio-informatic methods like SIFT and PARSESNP (Ng and Henikoff, 2001; Taylor and Greene, 2003). It

is expected that values of SIFT or PSSM (for PARSESNP) scores above specific thresholds indicate mis-sense mutations that are more likely to have a deleterious effect on protein function. In our case, three mutations showed significant PSSM score values, while the application of the SIFT algorithm predicted a possible deleterious effect for only one mutation.

Forward-genetics screening

The main purpose of our barley-mutagenized population was the implementation of a TILLING resource facility to be used for reverse genetics. However, the same population can also be used for forward-genetics studies.

For this purpose, the 4906 M_3 families were grown in the field and visible phenotypes were scored during the growing season with reference to untreated Morex plants. A visible variant phenotype was recorded for 32.7% of the M_3 families (1605/4906), either fixed or segregating within the family (Figure 2). Changes in plant color, including families showing segregation for albino seedlings, was the phenotype most frequently observed (27% of mutated families; 12% of total families).

Furthermore, a preliminary morphological analysis of 1000 M_4 families by means of a paper-roll approach allowed the detection of putative root mutants showing, for instance, a reduction in root length or severe modifications in root appearance and growth. About 7% of the families tested showed a clearly altered root phenotype (Figure 3). In order to confirm the observed phenotype, the paper-roll screening will be repeated for the putative mutants identified during this preliminary screening.

An on-line database with further information on phenotypes observed in the forward-genetics screening is publicly accessible at www.distagenomics.unibo.it/TILLMore/ and seed is available upon request.

Table 1. TILLMore mutation screening.

Gene	PCR size (bp)	Mis-sense	Silent	Non coding	Total
1	525	3	—	2	5
2	990	7	3	—	10
3	908	3	—	1	4
4	940	3	2	4	9
5	670	4	3	2	9
6	996	3	1	—	4
Total	5029	23	9	9	41

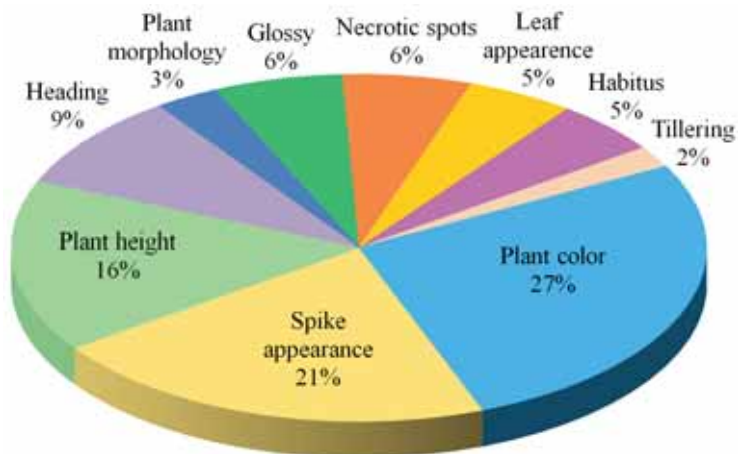


Figure 2. Proportions of different types of altered phenotypes in reference to cv. Morex recorded among M_3 families grown in field conditions.

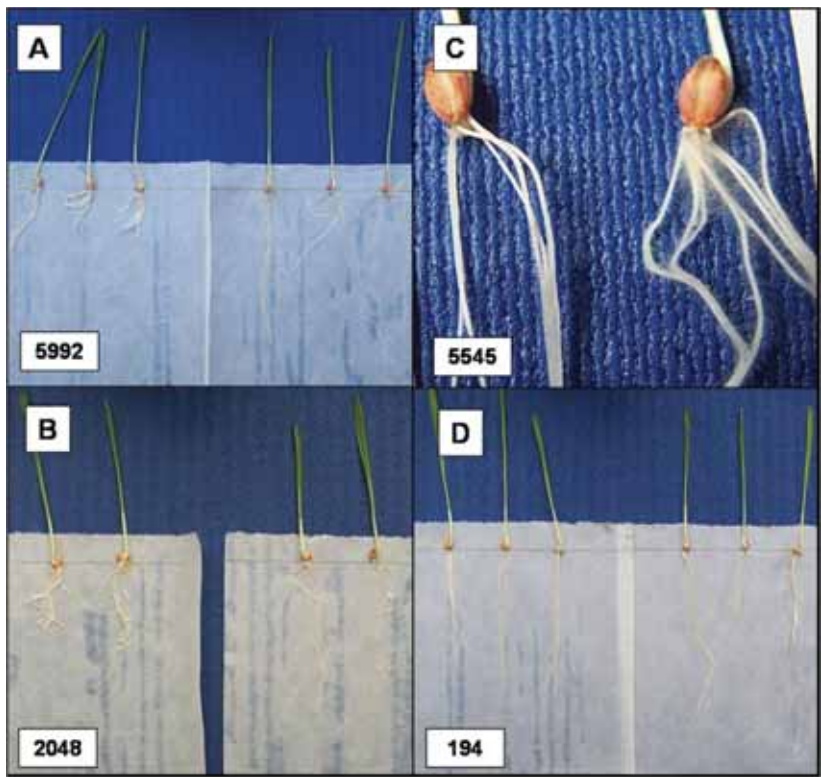


Figure 3. Examples of phenotypes observed within TILLMore for the root morphology categories. TILLMore accession numbers are given for each image. A. Short and thick root. B. Short and curly root. C. Hairless root. D. Highly-geotropic root. Each image includes the untreated genotype (cv. Morex) on the right.

Conclusions

Our report describes TILLMore, a new TILLING resource in barley based on a mutagenized population derived from cv. Morex. The use of NaN_3 provided an efficient alternative to more commonly used mutagenic agents to obtain a high mutation density suitable for TILLING. Additionally, the high frequency of visible phenotypes will make this population useful for forward-genetics screening. The TILLMore resource is available as a reverse-genetics platform to the research community on a cost-recovery basis and/or through collaborations (for details, see www.distagenomics.unibo.it/TILLMore/). TILLMore will efficiently complement existing functional genomic resources within the cereal research community and help move closer to a more tangible impact of TILLING on breeding programs.

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Candidate gene and reverse genetics approaches for the analysis of development in barley

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Abstract

Research in our group focuses on the genetic and molecular dissection of barley development as a mean to identify useful genes for the manipulation of plant architecture. Although progress has been made in rice and maize, the molecular bases of development are largely unknown in Triticeae species. Traits under analysis include tillering, spike morphology, and development of leaves and floral bracts. In order to identify and characterize genes involved in these processes, we have been exploiting a wide collection of developmental mutants, combining candidate gene (CG) and reverse genetics approaches. CGs for inflorescence branching and foliar development were identified based on synteny between the barley and rice genomes. CGs for meristem function and floret development were identified through molecular approaches, using as a starting point the *Hooded* (*K*) mutant. Support for specific CGs was provided by phenotypic similarities between rice and barley mutants. Validation of barley CGs is based on a range of complementarity between forward and reverse genetics approaches.

Introduction

Crop plants with desirable architectures are able to produce higher grain yields, and

understanding the molecular mechanisms that underlie plant architecture will facilitate breeding of more productive crop varieties.

Plant development depends on the activity of meristems, pluripotent cell populations that give rise to all vegetative and reproductive organs according to the characteristic architecture of the species. The architecture of the aerial part of a plant is elaborated by the shoot apical meristem (SAM) that repetitively generates lateral organs on its flanks. Studies on *Arabidopsis*, rice and maize have provided insight into the molecular mechanisms regulating meristem function and plant development, attributing a crucial role to transcription factor (TF) networks.

Class I Knotted1-like homeobox (KNOX I) genes play pivotal roles not only in SAM formation and maintenance, but also in morphogenetic processes throughout development (reviewed by Hake *et al.*, 2004). In *Arabidopsis* and maize, the negative modulation of knox genes in leaf primordials is mediated by genes belonging to the MYB class, *rough sheath2* (*rs2*) and *asymmetric leaves* (*AS1*; Byrne *et al.*, 2000), respectively. Recessive mutations in those genes are the cause of non-appropriate foliar development and of the formation of ectopic meristems. Upon the transition from vegetative to reproductive growth, the SAM is converted into an inflorescence meristem:

depending on the inflorescence architecture of the species, this will produce a series of intermediate meristems and finally flowers. In maize, the *ramosa1* (*ra1*), *ramosa2* (*ra2*) and *ramosa3* (*ra3*) genes define a pathway that plays a fundamental role in inflorescence architecture (McSteen, 2006). The maize *Branched silkless1* (*bd1*) and rice *fizzy panicle* (*fzp*) genes encode ERF (ethylene-responsive element binding factor) transcription factors controlling the transition from spikelet to floral meristem identity (Chuck *et al.*, 2002; Komatsu *et al.*, 2003).

Barley (*Hordeum vulgare* L.) is along with wheat a crop of primary importance in the world, and due to its diploid genome is considered a good model system for the dissection of morpho-physiological traits in the Triticeae. Although progress in this field has been made in rice and maize, the molecular bases of spike development are largely unknown. In order to identify and characterize genes involved in barley inflorescence architecture we took advantage of existing collections of developmental mutants. Forty mutant loci were positioned on a linkage map of the barley genome (Castiglioni *et al.*, 1998; Pozzi *et al.*, 2000; Roig *et al.*, 2004) opening the opportunity for positional isolation of the corresponding genes.

Results and discussion

Syntenic-based approach

Map positions provide an ideal starting point for isolation of target genes based on information deriving from interspecies synteny. A synteny-based approach, comparing the genomes of barley and rice, was adopted for the identification of the candidate gene (CG) for the developmental mutants previously mapped (Rossini *et al.*, 2006). Such approaches are now facilitated by availability of dense genetic maps integrating molecular markers and gene sequences, as well as information derived from genomic

approaches (e.g. over 462 000 ESTs in barley). The analysis of synteny with the completely sequenced rice genome facilitates the association of target loci with candidate genes of barley. The approach has made it possible to identify rice orthologous regions for 23 barley loci. In a subsequent step, the regions were scanned for CGs following the annotation of rice genomic sequences.

This strategy led to the isolation of the barley orthologue of *FZP*, considered to be a good candidate for *branched1* (*brc1*), a mutation producing branched spikes. Allelic comparisons and co-segregation analysis on the progeny of a wild-type \times *brc1* cross support the correspondence between *HvFZP* and the mutant locus responsible for alteration in inflorescence architecture.

RT-PCR analysis showed that *HvFZP* transcripts are absent from leaves and expressed in young developing inflorescences. Transcript levels are comparable in wt and *brc1* mutant plants.

Allelism tests between *brc1* and six other inflorescence branching mutants obtained from the Scottish Crop Research Institute (SCRI) are underway. In parallel, a reverse genetics approach was adopted for the identification of mutant alleles of *HvFZP*: three M₄ TILLING lines carrying amino acid substitutions in *HvFZP* were identified from the TILLMore resource developed for the barley cultivar Morex by the group of Roberto Tuberosa at the University of Bologna.

Molecular approach

In barley, the dominant hooded phenotype is conditioned by the duplication of 305 bp enhancer element in intron IV (Muller *et al.*, 1995) that causes ectopic expression at the lemma-awn transition zone of *Bkn3*, the orthologue of the maize *Knotted1* gene. This lesion leads to the formation of an ectopic meristem and subsequently to the development of an extra flower in place of the awn present in wild-type spikelets (Muller *et al.*, 1995). Enhancement of *KNOX*

gene transcription due to the activity of intron-located regulatory elements has been reported in both dicots (Golz *et al.*, 2002) and monocots (Greene *et al.*, 1994; Muller *et al.*, 1995).

In order to gain insight into intron-mediated *KNOX* gene regulation, a one-hybrid screen, based on the 305 bp element as the DNA target, allowed the isolation of a set of protein candidates for binding the element, collectively indicated as KIBPs (K Intron Binding Proteins). The first of these proteins, BBR, was extensively characterized and demonstrated to bind the 305 bp element *in vitro* and *in vivo*; moreover, the over-expression of BBR in transgenic tobacco plants is associated with morphological alterations in leaf and flower development (Santi *et al.*, 2003).

Three additional KIBPs identified from the one-hybrid screen were confirmed to bind the 305 bp element *in vitro*. They localize to the nucleus of plant cells and share high sequence similarity with previously characterized transcription factors known to play roles in the ethylene and gibberellin pathways. Analysis of KIBP gene expression following hormone treatments supports their potential involvement in mediating the control of *Bkn3* activity by ethylene.

Comparison of map positions within the barley genome led us to exclude the correspondence of KIBP genes and developmental mutant loci previously characterized and mapped in our group.

In order to analyze their developmental role, KIBP genes were introduced in rice plants by *Agrobacterium*-mediated transformation. Phenotypic characterization of transgenic lines is in agreement with an involvement of KIBP genes in controlling development, possibly through cross-talk between *KNOX* and hormone pathways.

In addition, the TILLMore resource was screened in a search for mutations in CGs for *Bkn3* regulation, yielding 3 and 6 lines carrying mutations in BBR and *Brs2*, respectively.

Conclusions

This CG integrated approach is proving effective in the identification and characterization of regulatory genes. It will contribute to understanding gene functions and to dissect plant developmental pathways in different plant species.

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Patterns of structure and LD in Mediterranean barley for adaptation to drought

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Abstract

A collection of 192 barley accessions providing a comprehensive coverage of the past and present of the crop in the Mediterranean basin were assembled. Population structure and genome-wide linkage disequilibrium (LD) were investigated using 52 nuclear microsatellite and 1131 DArT[®] markers. Both clustering and coordinate analyses clearly subdivided the sample into five distinct groups clustering around key ancestors and regions of origin of the germplasm. A large variation in LD values and extent was observed, ranging from closely linked markers completely at equilibrium to unlinked marker pairs 50 cM away from each other and still showing significant LD. Genome coverage and LD decay are sufficient for whole genome association mapping, and the population formed is a valuable resource that can be used in basic and applied research in barley.

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world, with a long history of breeding and research. Domestication from its wild relative *Hordeum vulgare* subsp. *spontaneum* (C. Koch) in the Fertile Crescent occurred around 10 000 years ago. It is cultivated in a wide range of environments and the variability of the conditions therein and consequent differences in breeding objectives have played major roles in producing the diversity found in world collections. Within defined regions, plant breeders focus their efforts on local elite gene pools, which reflect the accumulated effects of crossing, selection and recombination. By contrast, it is now widely recognized that the wild progenitors, wild relatives and landraces of crops have accumulated, during their long existence, a rich reservoir of genes for adaptation to a range of abiotic stresses, including drought, that they experience in the

harsh natural environment (for a review of barley landraces, see Grando *et al.*, 2001). In spite of the recent advances in biotechnology, wild progenitors and landraces are still, and will remain for the next few decades, the most immediate acceptable source of useful genes for conventional breeding programs, provided they still exist.

Drought is the most prevalent abiotic stress limiting barley production in Mediterranean areas (Ceccarelli and Grando, 1996). It affects plant processes over time scales ranging from a few minutes to weeks or months, with physiological consequences from minor stomatal adjustments to significant yield loss (for a review, see Belhassen, 1996). Alleviation of the consequences of drought will increase food security, diminishing poverty and marginalization. A key factor for sustainable agricultural production is, therefore, our ability to understand and utilize the mechanisms of germplasm adaptation to dryland conditions found in genetic resources such as local landraces.

Over the last few years, candidate gene studies and high-throughput genotyping platforms have promoted association mapping strategies as a viable alternative for Quantitative Trait Locus (QTL) mapping. These strategies differ from meiotic mapping approaches with bi-parental crosses fundamentally in three key ways. First, the genotyping of a representative collection of material automatically provides an analysis of the diversity within the collection and thus identifies key introgressions. Second, the extensive recombination history present in many breeding gene pools allows whole genome variation to be connected with phenotypic variation, providing more meaningful QTL identification. Third, QTLs can be detected in different genetic backgrounds, including those which are representative of the present cultivated germplasm, providing the right tools for marker assisted selection in breeding programs.

The study of non-random association of alleles at different loci, i.e. Linkage Disequilibrium (LD), plays a central role in

association mapping because it will determine the number and density of markers needed for whole-genome mapping, and the possibility of fine mapping. In barley, comparison of gene-based studies (Caldwell *et al.*, 2006; Morrell *et al.*, 2005; Stracke *et al.*, 2006) and genome-wide studies (Kraakman *et al.*, 2004; Malysheva-Otto *et al.*, 2006; Rostoks *et al.*, 2006) provide insight about the strong influence of selection and recent population history on the observed structure of LD. LD is rapidly eroded in genes not subjected to strong directional selection exerted in plant breeding in ancestral *Hordeum spontaneum* populations (Caldwell *et al.*, 2006; Morrell *et al.*, 2005). In contrast, LD extends over large linkage distances (sometimes greater than 50 cM) for a few loci in related elite germplasm (Rostoks *et al.*, 2006; Kraakman *et al.*, 2004).

We therefore assembled a collection of 192 barley genotypes embracing most of the diversity present in the cultivated gene pool in the Mediterranean region to provide a meaningful survey of the diversity of adaptation to drought. This collection comprised landraces and historically important varieties as well as modern varieties, together with representative genotypes from central and northwest Europe, and we have termed it Diverse Barley Germplasm (DBG). Genetic variation at the DNA sequence level was screened initially with Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP) molecular markers. This was later augmented by screening the collection with Diversity Array Technology (DArT®) molecular markers. DArT®, described by Wenzl *et al.* (2004), provides cost-effective, repeatable, high-throughput, multi-locus dominant bi-allelic markers for medium-density genome scans at low cost.

By applying these molecular marker technologies to the DBG, we can: (1) estimate genetic diversity within different germplasm groupings; (2) provide whole marker genome coverage of the sample,

which can be used in association-mapping studies; (3) understand and describe patterns of population structure between and within the groupings detected; and (4) investigate patterns of LD for further characterization of germplasm clusters, in the light of their ancestral history. Ultimately, the goal is to provide the barley research community with a genetically well characterized population for mapping adaptation to the drought-prone areas throughout the Mediterranean.

Materials and methods

Genotyping

The collection of 192 accessions of *H. vulgare*, selected to represent the past and present germplasm grown in various regions around the Mediterranean basin (Table 1), were multiplied for harvest 2003 by ICARDA. From a sub-sample of this seed, a single plant was grown in the glasshouse at SCRI under standard growing conditions.

DNA was then extracted from seedling leaf material of each genotype using the Mixer Mill MM300 (Retsch) in combination with DNeasy plant extraction kits (Qiagen) according to manufacturer’s instructions.

Genotyping was conducted in two phases. In phase 1, 53 molecular markers, comprising 31 derived from expressed sequenced tags, 30 of which were SSRs and one was an SNP, and 22 SSRs derived from genomic DNA were used. These were distributed reasonably evenly amongst the seven barley chromosomes (Figure 1). In phase 2, further DNA aliquots, containing between 0.5 and 1 µg of total DNA from each accession were sent to Triticarte™ (<http://www.triticarte.com.au/>) for whole-genome profiling with DArT®, with each polymorphic probe being scored as a binary variable.

Data analysis

For each of the markers in the set used in phase 1, diversity statistics, including total

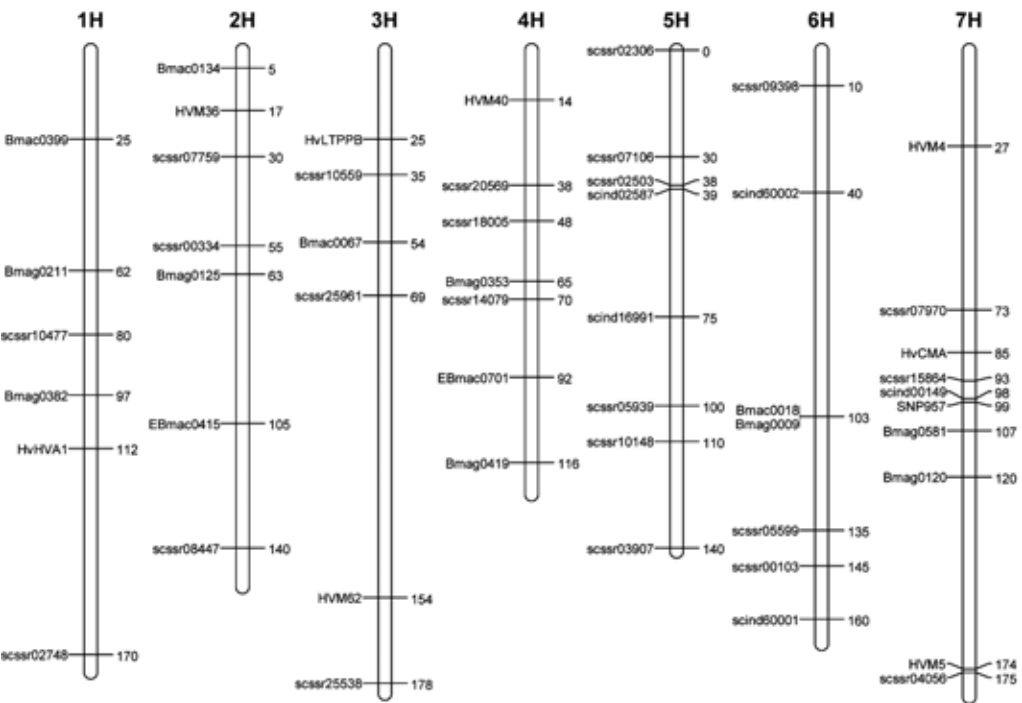


Figure 1. Location of molecular markers used in phase 1 genotyping of the DBG.

Table 1. Genotypes and geographical origin of germplasm sampled and genotyped.

	<i>A priori</i> groupings	Cultivated region	Genotype
Landraces	East Mediterranean	Jordan	Entry191, entry192, entry193, entry194, Shoubak, Al Mansoura, Mafrag, Jordan1, Kafar Rakeb, Jordan2, Al Rama, That Rass, Al Azraq Al Janoubi, Modeibeia, Bir Al Dabaghat, Al Hisha, Twal Al Janoubi, Al Gdieda village
	North Mediterranean	Dalmatia	Ragusa
		France	Hatif de Grignon
		Italy	Agello, Amiterno, Locale Aquila, Locale Leccese, Locale Siciliano, Orazio
		Spain	Albacete, Almunia, Berta, Macotera (SA), Pane, Pamula de Artes (L), Precoz de Cadreira (NA), 206-33, 206-62, 206-94, 206-44, 206-24, 206-78, 206-99, 206-16
	Other	Czech	Hanna, Kneifel, Vollkorn
		Denmark	Kenia, Binder
		Germany	Tschermaks 2row, Heils Franken, Haisa
		Sweden	Gull
		UK	Pioneer
	South Mediterranean	Algeria	Saida, Tichedrett, Algerian Landrace
		Morocco	Sountate 1/1, Sountate 1/31, Sountate 1/64, Sountate 1/61, Sountate 1/167, Taghighachte 8/1, Taghighachte 8/2, Taghighachte 8/9, Taghighachte 8/33, Taghighachte 8/49, Motzli 9/4, Motzli 9/28, Motzli 9/39, Motzli 9/46, Motzli 9/55, Taounate 10/2, Taounate 10/8, Taounate 10/9
Modern cultivars	Turkey	Turkey	Amb-192, Amb-196, Amb-307, Amb-19, Amb-199, Amb-191, Amb-135, Amb-134, Amb-133
	East Mediterranean	Jordan	ASCAD 176, Rum
	North Mediterranean	France	Manitou, Siberia, Hispanic, Reinette, Tremois
		Italy	Aliseo, Amillis, Dasio, Federal, Gothic, Grecale, Grosso, Kelibia, Mattina, Nure, Naturel, Solen, Sonora, Tea, Tidone, Ultra, Vertige
	Other	Spain	Candela, Dobla, Kika, Orria, Zaida
		Germany	Alexis, Igri, Scarlett, Triumph, Barke, Otis, Magda, Regina
		Holland	Atem, Intro, Aramir, Apex
		UK	Chariot, Fanfare, Optic, Graphic, Kym, Nevada, Tipper
		USA	Steptoe
	South Mediterranean	Algeria	Alanda-01, Manel
		Morocco	Arig8, Aglou, Amalou, Merzaga-07, Massine, Rabat-01, Oussama
	Turkey	Turkey	Efes-98, Anadolu-98, Aydanhanim, Sahin-91, Karatay, Tarm-92, Orza-96, Bulbul-89, Yesevi-93
Old cultivars	East Mediterranean	Jordan	Entry190
	North Mediterranean	France	Plaisant, Ager, Alpha, Astrix, Aurore, Barberousse, Beka, Logra, Monlon, Baraka, Elan
		Italy	Arda, Onice, Vetulio
	Other	Germany	Vogelsanger Gold, Dea, Volla, Union
		Netherlands	Vada, Emir, Zephyr, Hassan
		Sweden	Balder, Ingrid, Pallas, Formula
		UK	Proctor, Georgie, Maris Otter
	South Mediterranean	Algeria	Martin, Harmal, Assala-04, ER/Apm
	Turkey	Turkey	Tokak, Yesilkoy, Hamidiye, Cumhutiye, Efes-3, Obruk, Zafer-160, Anadolu-86

number of alleles and mean number of alleles per locus, were calculated using the Microsatellite Toolkit for Excel (Park, 2001). Polymorphism information content (PIC) was calculated in Microsoft Excel using the following formula: $PIC = 1 - \sum P_i^2$ where P_i is the frequency of the i th SSR allele (Smith *et al.*, 2000).

Based on data from phase 1 genotyping, population structure was examined using three different methodologies. Firstly, phylogenetic analysis was conducted by using an allele sharing distance matrix (Bowcock *et al.*, 1994), computed using the Microsatellite Toolkit (Park, 2001), as input for the neighbor-joining (NJ) tree building clustering algorithm implemented in the PHYLIP package (Felsenstein, 1997). The resulting dendrogram was rooted using Jordan landrace 'entry194' as the out-group, based on its proximity to wild barley (*Hordeum spontaneum*) genotypes from the Fertile Crescent (data not shown). Secondly, principal coordinates analysis, PCA, based on simple matching of fragment product sizes was performed with GenStat 9 (Payne *et al.*, 2006). Thirdly, Bayesian clustering, using simple matching, was applied to identify clusters of genetically similar individuals using STRUCTURE software version 2.1 with admixture (Pritchard *et al.*, 2000; Pritchard and Donnelly, 2001).

Linkage disequilibrium analysis

An interim consensus map of barley DArT markers (A. Kilian, Triticarte, pers. comm.) was used to study Linkage Disequilibrium (LD) amongst the 192 accessions. LD between mapped DArT loci was calculated by the squared allele frequency correlation coefficient, R^2 , (Weir, 1979), implemented in Tassel, version 1.9.4 (Buckler *et al.*, 2006). In this way, P-values from all pairs of DArT® bi-allelic markers within the same chromosome after removal of loci with rare alleles ($f < 0.10$) were estimated. The extent and distribution of LD along each of barley's

seven chromosomes was visualized by plotting R^2 values against the genetic distance in centi-Morgans

Results

Genetic diversity

One SNP and one EST-SSR marker proved monomorphic across all 192 accessions and were therefore excluded from further analyses. The remaining 51 markers used in phase 1 genotyping were able to distinguish all accessions. Over the whole sample of 192 accessions, 476 alleles were detected with an average of 8.98 alleles per locus and an overall mean diversity value of 0.522, ranging from 0 to 0.909. Comparisons between landraces and cultivars (modern and old pooled together) showed a general decrease in diversity from mean values of 7.79 to 5.57 alleles per locus for 84 and 108 accessions for landraces and cultivars, respectively. Presence of alleles specific to landraces, old cultivars and modern cultivars was investigated with 124, 11 and 40 rare alleles (<5% frequency) respectively. Two frequent alleles (>5% frequency) from SSRs HVM62 and Bmag0353 were specific to landraces.

Visual inspection of the accessions growing in trials derived from the same seed lots used to produce leaf samples for DNA extraction identified five aberrant accessions, namely cultivars Steptoe, Tipper, Regina, Hanna and Pioneer. These differences were confirmed by analysis of their phase 1 marker genotypes. Two other accessions, cv. Zephyr and a Jordanian landrace, were duplicated amongst the 192 entries and each duplicate pair produced identical genetic fingerprints. The five aberrant accessions and one accession from each of the duplicate pairs were therefore excluded from further genotyping and analyses. DNA from cv. Alexis failed to produce consistent hybridization signals in DArT® analysis and this accession was also excluded from further analysis, leaving 184 accessions that produced unique DArT

genotypes. DArT analysis produced 1131 bi-allelic markers with corresponding PIC values ranging from 0.126 to 0.499. For a bi-allelic marker, the minimum and maximum PIC values are 0 and 0.5, respectively. In our study, most DArT[®] markers had PIC values over 0.450, with an average of 0.407. Mean DArT[®] diversity values for landraces, old cultivars and modern cultivars were 0.390, 0.386 and 0.386, respectively. From the interim consensus map, 811 of the 1131 DArT[®] markers had a known map location. Only 31 markers (23 of known location) had minimum allele frequencies (MAF) lower than 0.1. DArT[®] markers provided an average genome coverage 116 markers per chromosome with a minimum of 52 markers for 4H and a maximum of 164 for chromosome 3H. The more sparse coverage of chromosome 4H can also be seen in the published DArT consensus map (Wenzl *et al.*, 2006) and, in our case, led to four gaps of 20 cM or more.

Population stratification and admixture

STRUCTURE vs.2.1. Bayesian cluster analysis (Pritchard *et al.*, 2000), using the SNP and SSR data, produced population estimates for each individual genotype. STRUCTURE was run several times independently with the number of independent groups, k , ranging from 1 to 10 in each to verify that the estimates were consistent across runs, after which we concluded that k should take a value of either 5 or 6. The composition of the clusters at $k=5$ also made biological sense so we have utilized this grouping for the remainder of the study. The five groups produced from STRUCTURE comprised: (i) 61 southwest Mediterranean six-row accessions; (ii) 21 Turkish accessions; (iii) 16 east Mediterranean accessions; (iv) 42 north Mediterranean six-row winter accessions; and (v) 52 north Mediterranean two-row spring accessions. The groups are relatively diverse in growth habit and spike morphology with

an observable gradient from “pure” two-row spring accessions to “pure” six-row winter accessions, but admixture within each grouping can be observed (Figure 1). For instance, varieties that share ancestors from both groups can be picked up from the estimated membership probabilities produced by STRUCTURE (Figure 2). Admixture can also be observed between southwest Mediterranean and north Mediterranean six-row varieties (Figure 2). All Turkish accessions are two-row springs, except for six-row Sahin91. The southwest Mediterranean group is diverse in origin. Most accessions within this group are six-row spring types. Within the southwest Mediterranean group, there is a single cluster of 8 accessions that share a unique chloroplast haplotype (data not shown) containing the genotype Macotera (SA), and the collection of Spanish landraces labeled ‘206’ have a winter growth habit. Three old Italian varieties, Orazio, Locale Leccese and Agello, are clustered with southwest Mediterranean varieties but, according to their pedigree, they originated from a selection of a heterogeneous African landrace. In this study, the group of Turkish accessions shows some similarity to north Mediterranean two-row spring accessions, but is clearly a distinct subgroup.

Linkage Disequilibrium

Comparing LD between all pairs of mapped DArT markers, the general trend is a rapid decay with genetic distance (Figure 3). The proportion of markers with $R^2 > 0.4$ decreased from 60 to 10% within the first 2 cM, although some unlinked markers showed high levels of LD. Whilst mean LD across the whole population sample level decayed below 0.2 within 3 cM, high levels of LD extended across the entire genome in the East Mediterranean and Turkish germplasm groups (Figure 4). For these two groups, the small sample size and high levels of similarity between accessions were the main reasons for high levels of LD.

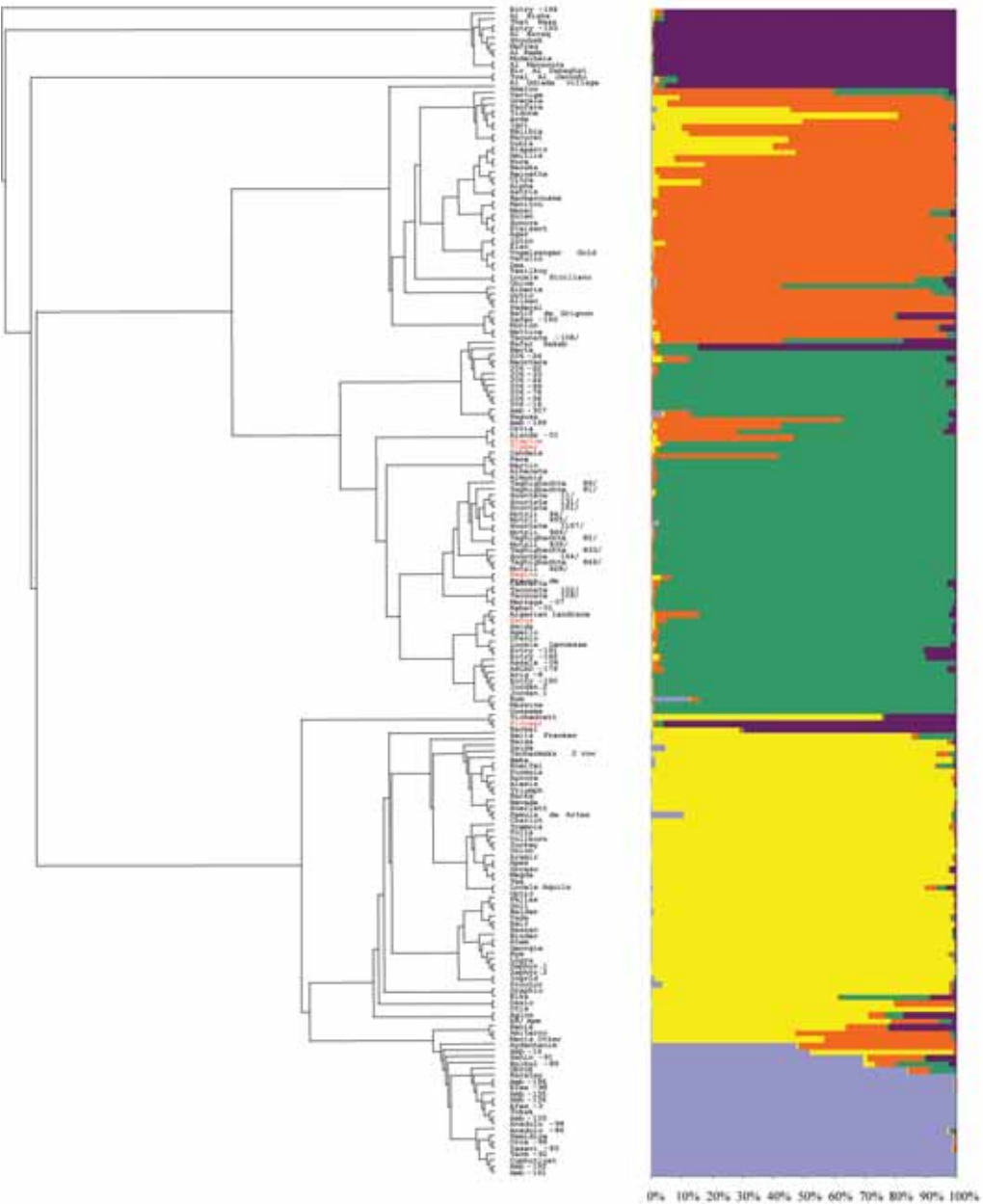


Figure 2. Dendrogram (neighbor joining) and inferred population structure based on 192 individuals and 50 markers using STRUCTURE (Pritchard *et al.*, 2000a). Each individual is represented by a line partitioned in k colored segments that represent the individual's estimated membership fractions in K cluster. Names in red refer to aberrant genotypes due to selection of contaminants at some stage in the selection and multiplication process.

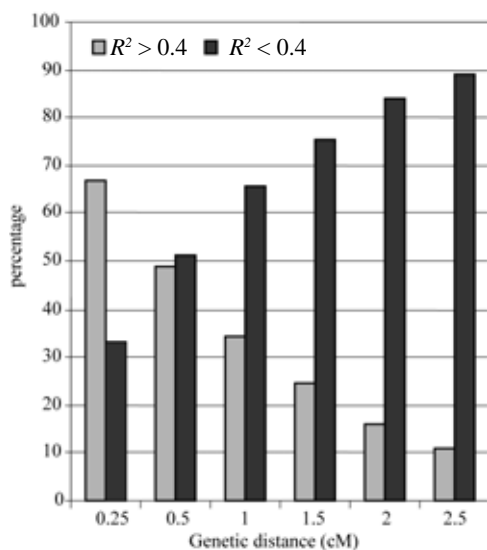


Figure 3. Linkage Disequilibrium (R^2) as a function of genetic distance for the first 2.5 cM.

Comparison of LD values (Figure 4) and diversity values between the STRUCTURE groupings (Figure 5) offer the opportunity to further explore the population structure of the sample. North Mediterranean six-row winter and southwest Mediterranean groups could be stratified. In fact, the North Mediterranean

six-row winter group, where high levels of admixture are observed, is not completely homogeneous in row number, and the group encompassing southwest Mediterranean accessions is heterogeneous in growth habit, with some accessions being winter and the rest spring.

Discussion

This study is among the first to deliberately develop a specific population representing the available germplasm of the Mediterranean basin to provide a platform for association mapping in barley. The level of polymorphism observed using bi-allelic markers exceeds those of previous studies, with 1130 DArT® markers (788 mapped with MAF >0.1) providing an average genome coverage of 116 markers per chromosome (i.e. <2 cM average distance between markers), with 319 markers of unknown map position. Previous studies in barley have reported average densities of 33.7 markers per chromosome (Kraakman *et al.*, 2004), and more recently Rostoks *et al.* (2006) reported an average of 93.7 markers per chromosome. Considering that significant LD extends up to 2 cM in our sample, this constitutes reasonable genome

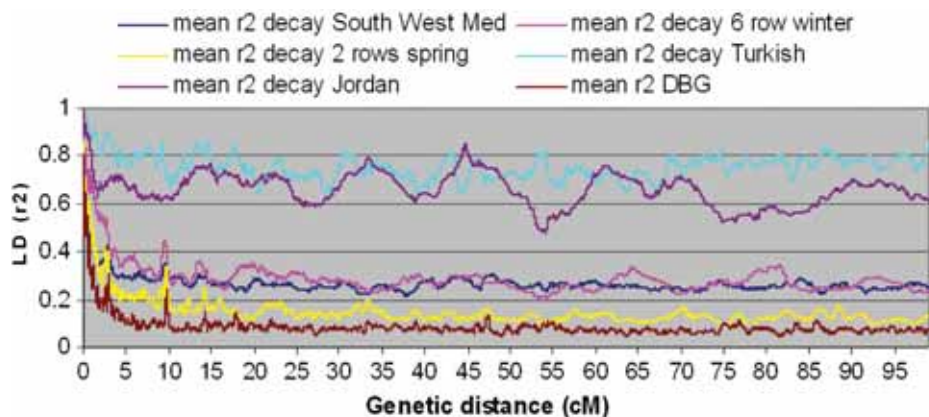


Figure 4. Plots of R^2 measurements as a function of genetic distance (in centimorgans) between informative ($f > 0.1$) polymorphic DArT® loci sharing chromosome in five different germplasm pools.

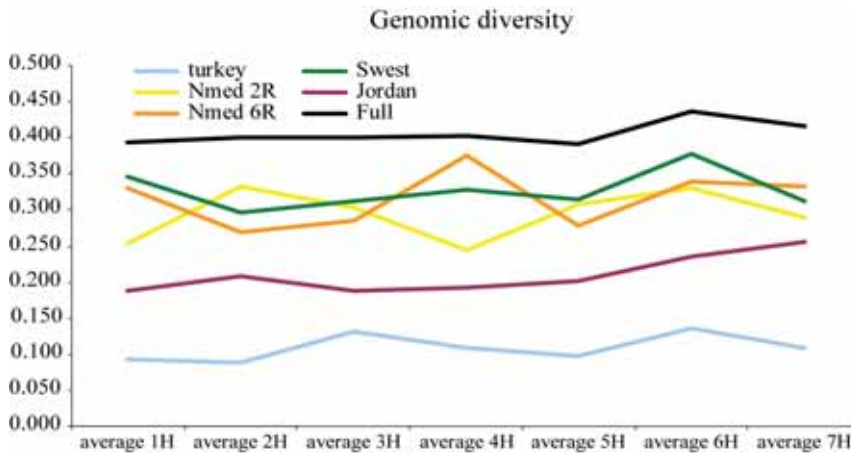


Figure 5. Genomic diversity: average PIC values of 811 mapped DArT® loci in barley germplasm subsets.

marker coverage for attempting a whole genome association scan in barley.

Our mapping population is genetically and physiologically diverse, sampling alternative alleles at several major developmental loci, and consequently has a strong population structure based on an individual's pedigree or origin, or both, which reflects different environmental and breeding selection practices shaping barley diversity. The strong population stratification in Mediterranean cultivated barley, reported also by Malysheva-Otto *et al.* (2006), underlines the extensive use of a few key ancestors well adapted to specific target regions. The old Swedish landrace Gull features strongly in the pedigrees of north Mediterranean two-row spring accessions, as do older accessions developed from it, such as Heils Franken, Binder and Kenia. North Mediterranean six-row winter accessions are often derived from Groninger and Mammut (not present in our sample). Moreover, two-row winter barleys have Carstens two-row or Tschermaks two-row in their pedigrees, which were derived from winter six-row crosses to spring two-row (Fischbeck, 2002). Turkish accessions that were sampled cluster strongly together because most were developed from Tokak, and Kilian *et al.* (2006), analyzing haplotype

structure at 7 barley genes, also reported a strong cluster of the two-row spring Turkish cultivars. The strong Turkish identity has also been observed in wheat accessions (Kim and Ward, 2000) and may reflect singularity of Turkish breeding program strategy and selection for cold hardiness, which would have been sufficient to give distinctness from other two-row barleys (T. Akar, pers. comm.).

Previous studies have highlighted contrasting patterns of LD between genes *a priori* closely linked to a polymorphism but subjected to distinct selection pressures, and recombination histories in the population under study (Caldwell *et al.*, 2006). Our results suggest that in barley, whilst LD might decay rapidly in some regions of the genome, selfing and strong selection pressures generally result in persistence of LD for at least 2 cM. Presumably, regions of interest have already been under selection pressure and will be in higher LD, thus facilitating association studies. For instance, disparate and contrasting LD values can be observed but may just reflect different recent history of selection and drift in different genomic regions.

Despite mean basal LD values of the whole sample level being low (Figure 5) the distribution of genetic diversity is not

random and is highly structured within groups. The lower basal R^2 values of north Mediterranean two-row springs in respect to north Mediterranean six-row winter and southwest Mediterranean six-row accessions may reflect the existence of unaccounted population sub-structure within North Mediterranean six-row winter and southwest Mediterranean six-row groups as a source of significant LD. We have already observed that both groups are not completely homogeneous in terms of growth habit and may have two genetic sources for the six-row spike phenotype, as reported by Casas *et al.* (2005) among six-row Spanish landraces.

In highly structured samples like the one used in the current study, accounting for population structure in association mapping studies is essential to minimize the discovery of false-positive associations. The persistence of LD that was detected suggests that whilst association mapping approaches in such barley populations may not be suitable for fine mapping of traits, they are much more likely to locate QTLs of relevance than classical linkage mapping, as the germplasm screened can be considered as representative of the actual cultivated germplasm.

In conclusion, association studies provide methodologies to improve the characterization of germplasm collections, as their genetic characterization with large numbers of markers can be used to identify genomic locations of the genetic determinants of traits of interest. This work provides a detailed description of an association mapping population that represents the past and present of cultivated barley diversity around the Mediterranean rim. Furthermore, it encompasses a large proportion of the genetic variation underlying the different mechanisms and strategies for adaptation to drought-prone environments around the Mediterranean basin.

We have demonstrated that the association mapping population is genetically and physiologically diverse,

and strongly structured due to pedigree relationships providing contrasting levels of population stratification. Current genome coverage, together with observed LD decay values, should not represent a problem for attempting a whole-genome scan, and makes the population an ideal QTL mapping resource for Mediterranean environmental conditions, provided that population structure is effectively and appropriately controlled within the association analysis.

Acknowledgements

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Evolution of barley *vrs1*

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Abstract

Archeological evidence has revealed that barley (*Hordeum vulgare* L.) is one of the oldest crops used by ancient farmers. One of the remarkable changes during barley domestications was the evolution of six-row barley. The six-row spike 1 (*vrs1*), the most important locus in duration of the domestication of this trait, is located on barley chromosome 2H, which is syntenous with rice (*Oryza sativa*) chromosomes 4 and 7. Elucidation of the origin of six-row barley has been a long-term goal of barley scientists. We isolated the *vrs1* gene by means of positional cloning. Expression of *Vrs1* was strictly localized in the lateral-spikelet primordia of immature spikes. *Vrs1* encodes a transcription factor that includes a homeodomain with a closely linked leucine zipper motif (HD-ZIP). Loss of function of *Vrs1* results in complete conversion of the rudimentary lateral spikelets in two-row barley into fully developed fertile spikelets in the six-row type. Sequence analysis of the *vrs1* gene among a worldwide collection of six-row cultivars revealed three independent origins for six-row barley. Two of them (alleles *vrs1.a2* and *vrs1.a3*) were derived from their immediate ancestor two-row cultivated barley (alleles *Vrs1.b2* and *Vrs1.b3*) by single nucleotide mutation. The origin of *vrs1.a1*, the most widespread allele and probably the first allele of six-row barley, was not found among two-row cultivars tested in this study. This study revealed that six-row barley (*vrs1*) originated repeatedly, at different times and in different regions, through independent single-nucleotide mutations of *Vrs1* from wild barley.

Background

During the process of cereal domestication, humans have selected within wild species toward the general direction of increased yield (Harlan *et al.*, 1973). One of the most conspicuous selections for increased seeds was the appearance of a six-row spike during barley domestication. Six-row barley produces three times as many seeds per spike as two-row barley and is a change of dramatic agronomic importance. Spike architecture of *Hordeum* species is unique among the Triticeae and simply symmetrical, where each rachis node has one central and two lateral spikelets. All three spikelets are fully fertile and able to develop into grains in six-row barley, but the two lateral spikelets are reduced in size and sterile in two-row barley (von Bothmer and Jacobsen, 1985; von Bothmer *et al.*, 1995). Various theories have been proposed for the evolutionary pathway of six-row cultivars. Åberg (1938) assumed that six-row cultivated barley derived from six-row form with brittle rachis known as *H. agriocriton* found in the early 1930s in western China. Another theory, which is nowadays favored by barley scientists, assumed a single evolutionary line from subsp. *spontaneum* to subsp. *vulgare*.

An excavation at Ali Kosh in the foothills of Kurdistan detected twisted kernels, an indication of six-row barley, at 7800 yBP, and a few kernels of non-brittle, two-row barley at 9000 yBP, indicating that two-row barley was cultivated earlier than six-row barley (Helbaek, 1959, 1969). This is a sign that the six-row character in barley was derived from two-row barley during domestication. Six-row spontaneous mutants do not have

a survival advantage in the wild and they are eliminated naturally and rapidly from wild barley populations (Zohary, 1963; von Bothmer *et al.*, 1995).

The two- and six-row spikes of barley are determined by a single gene locus, *Vrs1* (dominant allele exists in two-row barley) or *vrs1* (recessive allele exists in six-row barley) (Ubisch, 1916; Lundqvist *et al.*, 1997). Although *Intermedium spike-c.h* (*Int-c.h*), which occurs in six-row barley, is involved in enlarging the size of lateral spikelets, the presence of the recessive gene *vrs1* is by itself sufficient to modify two-row barley into six-row barley (Lundqvist *et al.*, 1997). There are at least three additional genes that have been identified on different barley chromosomes through artificially induced mutations; however, none of these genes has yet been found in known six-row cultivars, probably due to the phenotypic disadvantages, such as reduced size and/or fertility of the lateral spikelets on the upper and lower portions of the spikes that occur in those mutants (Lundqvist *et al.*, 1997; Pourkheirandish and Komatsuda, 2007). These observations indicate that *Vrs1* has been the primary target of mutation during the appearance of six-row barley. It has been assumed that six-row barley developed from domesticated two-row barley by means of spontaneous mutation (Zohary and Hopf, 2000; Harlan, 1968), but discovery of the origin of six-row barley has been a long-term goal of barley scientists.

Scope

Based on the concept of diffuse centres of differentiation (Harlan, 1968), barley evolution has been a continuous and gradual process and not a single event. Among the main key traits of barley domestication, the six-row spike gene, *vrs1*, has a significantly high value in this aspect. Because wild and cultivated barley are interfertile (von Bothmer *et al.*, 1995; Linde-Laursen *et al.*, 1997), reciprocal gene introgression between

them and between cultivars are frequent (Abdel-Ghani *et al.*, 2004). Therefore, using random markers in the barley genome does not appear to represent the two- and six-row types of barley, and evolutionary studies based on DNA markers that are inherited independently from the domestication genes might provide different insights into barley specialization (Kilian *et al.*, 2006; Badr *et al.*, 2000). In this study, we focus exclusively on the origin of six-row barley, using the *vrs1* gene itself. In other words, we concentrate on the *vrs1* gene rather than the whole barley genome to elucidate the origin and evolution of six-row barley, even though our data might have some implications for evolution of the species.

Isolation of the six-row gene, *vrs1*

To isolate the *vrs1* gene, we used a map-based approach. We mapped the *vrs1* gene to a 0.90 cM interval between cMWG699 and MWG865 in previous publications (Komatsuda and Tanno, 2004; He *et al.*, 2004). The two *vrs1* flanking markers cMWG699 and MWG503 share homology with rice genes Os04g45490 and Os04g46300, which lie in a 980 kbp contig of nine rice bacterial artificial chromosome (BAC) clones on rice chromosome 4. Twenty of the informative barley ESTs were successfully mapped to an interval between cMWG699 and MWG503 including *vrs1*. The linear order of these markers in barley and rice was identical. When high-resolution mapping was used, BC12348 (an EST contig consisting of AJ468022 and CB881790) placed 0.053 cM distal to *vrs1*. The mapping narrowed the location of *vrs1* to a 0.06 cM interval between e40m36-1110S and BC12348 using segregating progeny equivalent to 13 093 gametes (Komatsuda *et al.*, 2007; Pourkheirandish *et al.*, 2007). The candidate genomic region between two flanking markers was completely covered with six BAC clones by chromosome walking strategy. Sequencing of six independent BAC

clones revealed a contig containing *vrs1* composed of 518 343 bp. The contig spanned eight recombination events, which were not evenly distributed in this region. The central 220 kbp segment between e34m13-260S and BJ242706, and harboring *vrs1* showed no recombination (Pourkheirandish *et al.*, 2007).

The *vrs1* contig of the 518 343 bp combined sequence (accession number EF067844) revealed over 82% of nested transposable elements (Komatsuda *et al.*, 2007). Seven “islands” of a few kbp in size can be classified as a non-repetitive sequence and considered as candidate location of *vrs1* (Pourkheirandish *et al.*, 2007). Sequences showing homology to known genes were identified in islands 3 (*HvHox1*), 6 (*YHvEP1*) and 7 (*HvEP2*), whereas the other islands showed no sequence homology to known genes. *YHvEP1* appears to be degenerated and interrupted by several insertion events involving transposable elements. *HvEP2* is an expressed gene with unknown function, which was recombined with *vrs1* genetically. Thus *HvHox1*, in island 3, is the only gene that lies between the flanking markers. *HvHox1* encodes a 222 amino acid polypeptide, including a homeodomain of three helices and a leucine zipper motif in its centre (Figure 1). Orthologue of *HvHox1* was *Oshox14* (OS07g39320), which was located on rice chromosome 7. The barley and rice amino acid sequences are largely identical, containing a small number of conserved amino acid changes (Pourkheirandish *et al.*, 2007). The rice-barley *in silico* map around the *vrs1* gene was perfect without any exception; however, a rice orthologue of *Vrs1* was missing in this co-linear rice interval. This co-linearity was disrupted by some chromosomal re-arrangement in barley or rice,

which happened during the evolution process after separation of rice and barley.

Identification of the *Vrs1* gene

To investigate the biological function of *HvHox1*, *Vrs1* mutant lines were used. Among 91 reported mutant lines (Lundqvist *et al.*, 1997), we analyzed 57 mutants, which were derived from five different two-row cultivars. These mutants were categorized into two main groups of *Intermedium spike-d* (*Int-d*) and *hexastichon* (*hex-v*) based on the development of lateral spikelets (Figure 2). In *Int-d* mutants, lateral spikelets are sterile or partially fertile, with variable awn length, which appear intermediate to those of two-row and six-row barley (Gustafsson and Lundqvist, 1980), whereas in *hex-v* the lateral spikelets are fertile and fully developed, with long-awns (Gustafsson and Lundqvist, 1980), and phenotypically very similar to normal six-row barley (Figure 2). Based on a previous study (Lundqvist and Lundqvist, 1988), these mutants are allelic to the *vrs1* gene. Thus morphological changes in the mutants can be considered as changes in the *Vrs1* gene. Lesions in *Vrs1* DNA sequence

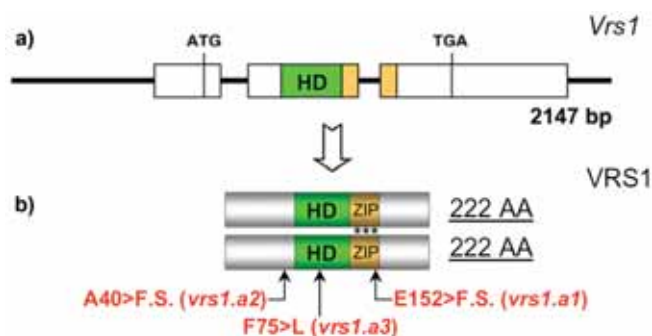


Figure 1. *HvHox1* from two-row cultivar encoding a protein with conserved DNA-binding homeodomain (HD) and the dimer-forming leucine zipper region (ZIP). (A) Genomic structure of 2147 bp including *HvHox1* with exon/intron junctions. (B) Primary structure of *HvHox1* subunit and homodimer formation through ZIP.



Figure 2. One spikelet at a rachis node. Normal two-row barley used as a parent of mutant lines (*Vrs1*), *Intermedium spike-d* (*Int-d*) and *hexastichon* (*hex-v*).

of 49 mutant lines were correlated with their morphological changes. These changes consisted of 22 cases with a single amino acid substitution, 12 mutant lines with truncation of the protein due to the introduction of a new stop codon, 3 lines with a single nucleotide substitution in the conserved splicing signals which cause alternate splicing (change of splicing was confirmed by sequencing their transcripts), 5 lines had a frameshift mutation caused by a deletion, and 7 revealed complete deletion of the *Vrs1* region (based on flanking marker analysis). All lines with a complete

deletion of *Vrs1* (>182 kbp) as a result of irradiation, always shows *hex-v*-type six-row spikes under a range of growing conditions. Since the 7 deletion mutants showed no developmental lesions, *Vrs1* appears to be dispensable in barley. Thus, it is plausible that *Vrs1* evolved through duplication of a vital “master” gene, which may still present in its ancestral location on chromosome 2H. We found 8 mutants without any DNA changes throughout the coding region. Expression analysis revealed that 5 of the 8 mutants have reduced or no expression of *Vrs1*, which may be caused by some mutational change in transcription machinery. The remaining 3 mutants without any changes in coding sequence and which showed almost the same level of expression as in the two-row cultivars were subjected to an allelism test. These lines (*hex-v.46*, *hex-v.47* and *hex-v.48*) were derived from cv. Bonus by neutron bombardment, ethyl methanesulfonate exposure and x-ray treatment, respectively. The allelism test revealed that these three mutants were not allelic to *vrs1*, as confirmed by test crosses with *hex-v.23* and *hex-v.49*. Mutant analyses clearly confirm the identity of *HvHox1* candidate gene with our target six-row spike gene, *Vrs1*.

Expression analysis of HD-ZIP I homeobox gene

Our study revealed that *Vrs1* encodes a member of the HD-ZIP class of transcription factors. Transcription of homeodomain leucine zipper (HD-ZIP) I gene (*Vrs1*) was specifically detected in shoot apices or immature inflorescences by RT-PCR. A deletion mutant (*hex-v.3*) showed no gene expression, in accordance with the hypothesis that HD-ZIP I lies at the *vrs1* locus. It is interesting that both two-row and six-row barleys show almost equal levels of transcription, indicating that transcriptional machinery is intact in the six-row cultivars analyzed. Apices or immature inflorescences in the early stage (1–16 mm long) showed a

stronger transcription of HD-ZIP I than those in the middle stage (20–50 mm), supporting a prediction that expression of *Vrs1/vrs1* is related to differential development between central and lateral spikelets in two-row barley (Komatsuda *et al.*, 2007). *In situ* hybridization using a 315-bp RT-PCR fragment including only the 3'-UTR of *Vrs1* revealed that this gene was expressed only in the lateral spikelet primordia. *Vrs1* was not detectable before differentiation of central and lateral spikelet (double-ridge stage), but its expression was clearly detectable at the triple-mound stage and the glume primordium stage (after differentiation of central and lateral spikelets). During these *Vrs1*-expressing stages (when the immature spikelets are 1.5 to 2.0 mm long), the

primordium of the central spikelet became larger than the primordia of the lateral spikelets (Komatsuda *et al.*, 2007).

HD-ZIP proteins are unique to the plant kingdom and are classified into four classes, I to IV (Meijer *et al.*, 2000). Barley *Vrs1* belongs to class HD-ZIP I based on the phylogenetic tree of HD-ZIP homeodomain proteins in plants (Figure 3). Members of classes I and II form homodimers with members of the same class and function as transcriptional activators and repressors (Sessa *et al.*, 1993; Meijer *et al.*, 2000). Some members of classes III and IV are involved in the development of the apical meristem, vascular development, and establishing cell fates in the epiderm (Henriksson *et al.*, 2005). The biological function of class

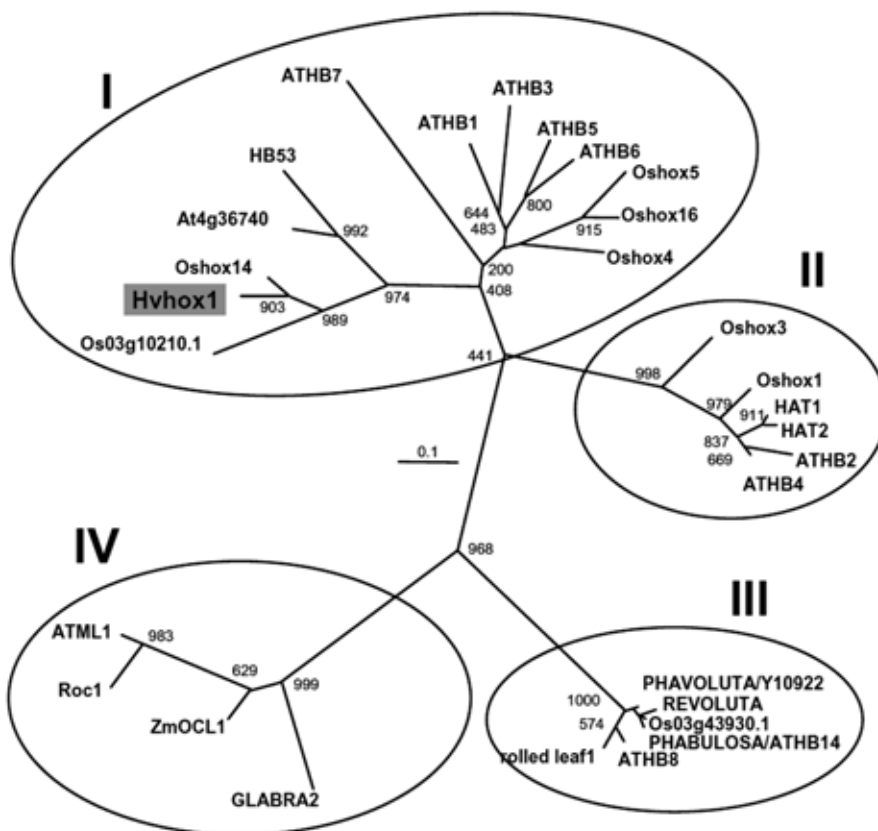


Figure 3. Neighbor-joining phylogenetic tree of HD-ZIP homeodomain proteins in plants including *Vrs1/Hvbox1*.

I and II proteins has not yet been clarified. Our study shows a conspicuous association between a HD-ZIP I gene and a plant developmental process. The spatial and temporal specificity of *Vrs1* gene expression suggests that VRS1 is a transcription factor involved in the development (suppression) of lateral spikelets in two-row barley. Thus, our study is the first to show that a class I protein is associated with a plant developmental process. Most domestication genes have been revealed to encode transcription factors (Doebley, 2006), and our result agrees with these observations.

Rudimentary spikelets or flowers of wild species have been recovered to their maximum capacity to increase seed number through the process of wheat and barley domestication (Zohary and Hopf, 2000; Harlan *et al.*, 1973). Deterioration of *Vrs1* is sufficient to convert the undeveloped lateral spikelets of two-row barley into the fully developed lateral spikelets of six-row barley. The loss of function of the *Vrs1* gene implies that six-row barleys are genetically recessive and that two-row barleys are controlled by the wild-type allele, which exists naturally in wild barley. The dominant nature of *Vrs1* and DNA-binding ability of HD-ZIP I proteins suggest that *Vrs1* may suppress gene(s) involved in the development of lateral spikelets. Sub-cellular localization of *Vrs1* proteins will be useful to test the hypothesis.

Six-row spike evolution

Row-type is a key characteristic in analyzing the origin of cultivated barley. There is a long history of discussion between scientist as to whether the progenitor of modern cultivated barley was six-row (Åberg, 1940), two-row (Harlan, 1968; Bakhteyev, 1964), or both (Freisleben, 1940; Takahashi, 1955). The two-row progenitor hypothesis was favored by barley scientists because it was supported by archaeological specimens, which show the existence of domesticated two-row remains earlier than six-row barley, and by

the dominance nature of the two-row spike gene versus six-row (von Bothmer *et al.*, 1995; Zohary, 1963; Helbaek, 1959; Harlan, 1968).

To figure out the six-row barley origin, we analyzed the sequence of the *HvHox1/Vrs1* from 15 two-row and 16 six-row cultivars, one variety (*deficiens*), and three wild barley lines selected from distant geographical areas. In this study we find two alleles in two-row barley (*Vrs1.b2* in 1 cultivar and *Vrs1.b3* in 14 cultivars) and three alleles of six-row barley (*vrs1.a1* in 10 cultivars, *vrs1.a2* in 5 cultivars, and *vrs1.a3* in 1 cultivar) using haplotype analysis (Figure 4). In each allele, sequences were identical between cultivars, except that the *vrs1.a1* shows three sub-haplotypes. For *vrs1.a2* and *vrs1.a3*, we could deduce an immediate ancestor, *Vrs1.b2* and *Vrs1.b3*, respectively, as a result of a single point mutation. Phylogenetic analysis supported the hypothesis that the six-row alleles were derived from two-row alleles, rather than vice versa, because the wild barley lines (OUH602, OUH630 and OUH743) were outgroups (Komatsuda *et al.*,

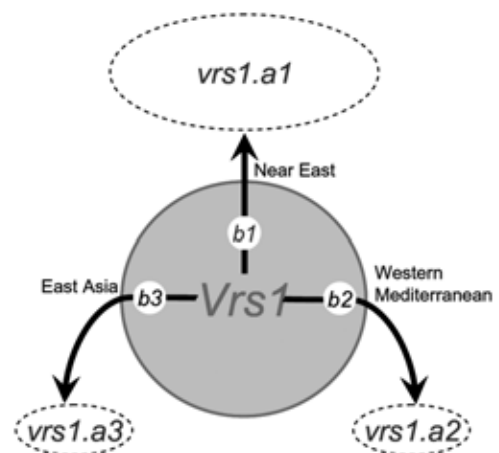


Figure 4. Scheme of the origin of six-row barley (*vrs1.a1*, *vrs1.a2*, and *vrs1.a3*) from the two-row ancestor (*Vrs1*). *b1*, *b2* and *b3* indicate alleles *Vrs1.b1*, *Vrs1.b2* and *Vrs1.b3*, respectively.

2007). The *vrs1.a2* allele has been created through an insertion of a single nucleotide (A/T) in the second exon, which results in a frame shift from Alanin (A) at position 40 (Figure 1B). The *vrs1.a3* allele has a substitution of one nucleotide which caused non-synonymous amino acid change of phenylalanine (F) at position 75 to leucine (L) (Figure 1B). This amino acid is highly conserved in *HvHox1* homeodomains of plants, animals and yeasts, based on BLASTP alignment analysis (Marchler-Bauer *et al.*, 2005). Three *hex-v* mutants (*hex-v.12*, *hex-v.13*, and *hex-v.14*) also caused by the replacement of F by another amino acid at position 75 confirm the importance of this amino acid in the DNA-binding domain of barley *Vrs1* gene. The *vrs1.a3* allele was detected in a six-row cultivar (Natsudaikon Mugi) from eastern Asia. The most common allele, *vrs1.a1*, which was detected in six-row barley distributed around the world (10 cultivars), has a deletion of one nucleotide (G/C) in the third exon, which results in a frame shift from Glutamin (E) at position 152 (Figure 1B). The progenitor of the *vrs1.a1* allele remains to be identified. In this allele we have found three sub-haplotypes, which were differentiated from each other by point mutations in 5'-non-coding region. Two- and six-row barley cultivars analyzed in this experiment revealed equal levels of transcription of *Vrs1*, thus point mutations described here in these *vrs1* alleles seem to be the only reason for the functional changes observed in all three natural six-row alleles (Komatsuda *et al.*, 2007).

Sequence analysis of the *vrs1* gene among a worldwide collection of six-row cultivars revealed three independent origins for six-row barley (Figure 4). This result agrees with the hypothesis of Tanno *et al.*, (2002) that six-row barley originated more than once during barley domestication. Even though we did not find the progenitor of the *vrs1.a1* six-row allele among 15 two-row barley cultivars (hypothetically *Vrs1.b1*, Figure 4), three sub-

haplotypes were found in this allele. Based on the assumption of the minimum number of changes, this allele must have originated from a hypothetical *Vrs1.b1* allele through a single unknown two-row ancestor. On account of *vrs.a1* predominance in worldwide six-row barley and higher haplotype diversity among the six-row members of this allele, we hypothesize that the *vrs1.a1* allele may represent the most ancient six-row allele (dating to 7000 to 6000 yBP), which was widely present in Neolithic agriculture in the Near East. The result of this study is straightforward because we used the DNA sequence encoding the *Vrs1* gene itself. These three alleles (*vrs1.a1*, *vrs1.a2* and *vrs1.a3*) were created independently because they cannot be deduced by intragenic recombination through hybridization of six-row barleys. Since these alleles are fairly diverse, simple mutation is not enough to generate new alleles from each other. Therefore, six-row barley origination must have taken place repeatedly from two-row barley (cultivated or wild) by means of a loss-of-function of an HD-ZIP I-class homeobox gene. Six-row barley evolved at different times and in different regions (Figure 4), probably as a result of conscious selection by interested farmers who greatly appreciated the improved seed yield.

Implications for the evolution of barley

Barley originated from the Fertile Crescent, but its precise origin remains unknown. The DNA sequence of *Vrs1* may trace the origin and migration of two-row barley. Based on the archeological evidence, two- and six-row barleys were cultivated in Greece between 8000 and 6000 yBP, in the Balkans and central Europe between 5000 and 2000 yBP, and in southern Europe and north Africa between 7000 to 4000 yBP (Zohary and Hopf, 2000). After two-row barley disappears from ancient Mesopotamian and Egyptian

archaeological evidence, it does not reappear in the archaeological sites of these regions till 1100 yBP (Helbaek, 1959). There is no evidence of two-row barley cultivation in central and northern Europe until 1000 yBP, and it has been assumed that two-row barley was introduced into Europe at 900 to 800 yBP by Crusaders from the Near East (Fischbeck, 2002). In this study, 14 two-row cultivars (*Vrs1.b3*) show an identical DNA sequence. This suggests that *Vrs1.b3* originated recently and that the allele has spread out through the world and become dominant, which may partly support Fischbeck's hypothesis (2002). This explanation agrees with the fact that only one *vrs1.a3* six-row cultivar (among 16 six-row cultivars) has been detected so far. This hypothesis does not necessarily disagree with the hypothesis of Helbaek that two-row barley was cultivated earlier than six-row barley (Helbaek, 1959), because the DNA sequences of archaeological specimens remain unknown. Moreover, the type of lateral spikelet in the ancient domesticated two-row cultivars (*deficiens*, *Vrs1.t*, *distichon*, *Vrs1.b*) is not clear.

The inflorescence domestication in the Poaceae is a continuous story of reduction from a more original "panicle" of spikelets (as seen in rice and oats) to a "spike" of spikelets (Vegetti and Anton, 1995), resulting in three sessile spikelets per node in barley and a single sessile spikelet per node in wheat and rye. In wild *Hordeum* species, the three spikelets and their slender awns form a light arrow-head dispersal unit that both facilitate seed dispersal by animals and aids seed burial (von Bothmer *et al.*, 1995). In two-row barley, strict temporal and spatial regulation of *Vrs1* expression leads to reduction and sterility of the lateral spikelets. We speculate similar impacts of *Vrs1* orthologs on the inflorescence architecture of other species in the Poaceae could lead to complete repression of lateral spikelet formation at inflorescence nodes, as is found in wheat and rye. Study of *Vrs1* orthologs in Poaceae

would be an exciting topic for elucidating the inflorescence evolution of this family.

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Session 4

Abiotic stresses

Integrated aspects from breeding to genomics of the abiotic stress response: the case of drought

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Abstract

Plant adaptation to environments with sub-optimal growing conditions is a multi-level concept. At the crop level, adaptation to stress has been interpreted as the ability of the plants to achieve high yield in stress-prone environments. At the physiological level, given the number of physiological traits contributing to stress tolerance, it is expected that there is no single response pattern highly correlated with yield under stress conditions; instead, different 'resistant plants' can achieve similar levels of tolerance by using different mechanisms. Furthermore, different developmental stages show different sensitivity to stress, the reproductive stage probably being the most critical. It is therefore expected that different tolerance strategies operate at various developmental stages. At cellular level, adaptation to stress is linked to the variation in gene expression and to the identification of genes and gene functions involved in the response to stress that provide the molecular bases of the stress resistance. Key genes in the stress response pathways might explain the genetic variability for stress tolerance and might confer stress tolerance when introduced into transgenic plants. Understanding the role of the different mechanisms involved in tolerance strategies found in different cultivars is a crucial task for the understanding of the abiotic stress tolerance and for knowledge-based breeding of plants for the future.

Introduction

The evolution of crops since their domestication has been driven by the selection of desired traits recognized at the phenotypic level. Nevertheless, direct selection for grain yield under stressed conditions has been hampered by low heritability, polygenic control, epistasis, significant genotype-by-environment (G×E) interaction and quantitative trait loci (QTLs)-by-environment (QTL×E) interaction. The complexity of stress-tolerance mechanisms explains the slow progress in yield improvement in stress-prone environments. In recent years, crop physiology and genomics have led to new insights in stress tolerance, providing breeders with new knowledge and tools for plant improvement (Cattivelli *et al.*, 2008). This article aims to highlight future perspectives in plant breeding that could result from the integration of the recent advances in physiology and genomics, with specific attention to drought stress.

Yield improvement in drought-prone environments

In mild to moderate drought conditions characterized by a wheat or barley grain yield between 2 and 5 t/ha, selection for high yield potential has frequently led to some yield improvements under drought conditions (Araus *et al.*, 2002). In these cases

the breeders selected plants characterized by high yield potential and high yield stability, with the latter being attributed to a minimal G×E interaction. This implies that traits maximizing productivity normally expressed in the absence of stress can still sustain a significant yield improvement under mild to moderate drought (Slafer *et al.*, 2005; Tambussi *et al.*, 2005). The rationale of this breeding strategy is also supported by several retrospective studies where the yield of large sets of cultivars was evaluated in parallel fields under different water regimes, thus enabling a direct comparison of the performance of the same cultivars in drought and non-drought (usually irrigated) conditions. When 89 barley genotypes representing a sample of the germplasm grown in Europe were evaluated in southern Italy under both rainfed and irrigated conditions, eight genotypes with high yield potential and minimal G×E interaction were identified. They ranked among the best in both rainfed and irrigated treatments and, although considerably reduced in the absence of supplementary irrigation, their yield was superior under all conditions tested (Rizza *et al.*, 2004). Old varieties were characterized by low yield in rainfed conditions and by a minimal ability to improve yield when water became available. In contrast, modern cultivars showed a higher yield in rainfed conditions and strong yield increases in response to irrigation. A highly significant correlation ($r = 0.73^{***}$) between yield in rainfed and irrigated conditions was found (Rizza *et al.*, 2004), suggesting that, in a typical Mediterranean environment, selection based on the absolute performance of the genotypes across environments is more successful than selecting for the minimum yield decrease under stress with respect to favorable conditions.

Many reports suggest that, during the last century, yield in water-limited environments was mainly determined by the inherent yield potential. This can be explained by considering that the main targets of selection

(high harvest index in wheat and barley, resistance to pests and diseases, nitrogen use efficiency) are equally beneficial under dry and wet conditions and, often, the best performances for these traits were overriding the differences in drought adaptability. Nevertheless, examples where selection for stress resistance was effective in improving yield in limited environments are also known (Morgan, 2000).

Further progress will depend on the introduction into high yielding genotypes of traits able to improve drought tolerance without detrimental effects on yield potential, thus reducing the gap between yield potential and yield in drought-prone environments. This goal can be achieved via the identification of drought-tolerance-related traits and the subsequent manipulation of the corresponding genes using Marker Assisted Selection (MAS) or gene transformation, or both.

Physiological bases for yield under drought

The physiologically relevant integrators of drought effects are the water content and the water potential of plant tissues. They in turn depend on the relative fluxes of water through the plant within the soil-plant-atmosphere continuum. Thus, apart from the resistances and water storage capacities of the plant, it is the gradient of water vapour pressure from leaf to air, and the soil water content and potential that impose conditions of drought on the plant. Once a drop in water potential develops, responses of a wide range of physiological processes are induced. Some of these responses are directly triggered by the changing water status of the tissues, while others are brought about by plant hormones that are signalling changes in water status. Physiological traits relevant for the responses to water deficits or that are modified by water deficits span a wide range of vital processes (Table 1). As a consequence, it can be expected that there is no single response

Table 1. Physiological traits relevant for response to drought conditions.

Plant traits	Effects relevant for yield	Modulation under stress
Stomatal conductance / Leaf temperature	More / less rapid water consumption. Leaf temperature reflects the evaporation and hence is a function of stomatal conductance	Stomatal resistance increases under stress
Photosynthetic capacity	Modulation of concentration of Calvin cycle enzymes and elements of the light reactions	Reduction under stress
Timing of phenological phases	Early / late flowering. Maturity and growth duration, synchrony of silk emergence and anthesis, reduced grain number	Wheat and barley advanced flowering, rice delayed, maize asynchrony
Partitioning and stem reserve utilization	Lower / higher remobilization of reserves from stems for grain filling, effecting kernel weight	Compensation of reduced current leaf photosynthesis by increased remobilization
Single plant leaf area	Plant size and related productivity	Reduced under stress (wilting, senescence, abscission)
Rooting depth	Higher / lower tapping of soil water resources	Reduced total mass but increased root/shoot ratio, growth into wet soil layers, regrowth on stress release
Cuticular resistance and surface roughness	Higher or lower water loss, modification of boundary layer and reflectance	
Photosynthetic pathway	C ₃ / C ₄ / CAM, higher WUE and greater heat tolerance of C ₄ and CAM	
Osmotic adjustment	Accumulation of solutes: ions, sugars, poly-sugars, amino acids, glycinebetaine	Slow response to water potential
Antioxidative defence	Protection against active oxygen species	Acclimation of defence systems
Accumulation of stress-related proteins	Involved in the protection of cellular structure and protein activities	Accumulated under stress

pattern that is highly correlated with yield under all drought environments.

Osmotic adjustment (OA) is a key mechanism enabling plants under drought to maintain water absorption and cell turgor pressure, thus contributing to sustained higher photosynthetic rate and expansion growth. A general analysis of OA under several drought stress conditions has shown that osmoregulation can be an effective selection criterion for drought tolerance, and that it has a role in reducing drought-dependent yield loss, especially when water deficit occurs during the reproductive growth stage (Morgan, 1983; Moinuddin *et al.*, 2005). A comparative analysis of many studies dedicated to OA has suggested that OA cannot be considered

equally useful in all crops or drought conditions, or both, but that a general positive association between yield and OA can be found under severe water stress where yields tend to be low (Serraj and Sinclair, 2002).

A relevant example of a successful breeding program for dry environments, based on a physiological trait, was reported by Rebetzke *et al.* (2002). They used carbon isotope discrimination (Δ) as a surrogate for water use efficiency to select wheat lines with high water use efficiency in drought-prone environments. During photosynthesis, plants discriminate against the heavy isotope of carbon (^{13}C) and, as a result, in several C₃ species, Δ is positively correlated with the ratio of internal leaf CO₂ concentration

to ambient CO₂ concentration (C_i/C_a) and negatively associated with transpiration efficiency. Thus, a high C_i/C_a leads to a higher Δ and lower transpiration efficiency (Farquhar and Richards, 1984).

Molecular markers to dissect drought-tolerance-related traits

Molecular markers can be used to explore germplasm through segregation and association mapping to identify useful alleles in both cultivated varieties and wild relatives. Although association mapping is intrinsically more powerful than 'classical' genetic linkage mapping because it scrutinizes the results of thousands of generations of recombination and selection, most of the data available on drought tolerance are based on segregation mapping and QTL analysis. Many efforts have been dedicated to understanding the genetic basis of physiological traits conferring advantages in dry environments, while less attention has been given to the understanding high yield stability in dry and wet environments.

Drought tolerance is a typical quantitative trait; however, single genes, such as those controlling flowering time, plant height, ear type and osmotic adjustment, may have important roles in adaptation to drought-prone environments (Forster *et al.*, 2004). During the last ten years, the application of QTL analysis has provided unprecedented opportunities to identify chromosome regions controlling variations in almost all the physiological, morphological and developmental changes observed during plant growth in water-limiting conditions. Particular attention has been paid to: (i) genetic variation of the osmotic adjustment (Teulat *et al.*, 1998); (ii) genetic bases of phenological traits (Verma *et al.*, 2004); (iii) the ability of the roots to exploit deep soil moisture to meet evapotranspirational demand (Johnson *et al.*, 2000; Nguyen *et*

al., 2004); (iv) the limitation of water-use by reduction of leaf area and shortening of growth period (earliness); (v) isotope discrimination (Juenger *et al.*, 2005); and (vi) the limitation of non-stomatal water loss from leaves, e.g. through the cuticle (Lafitte and Courtois, 2002).

Comparative analysis of QTL results clearly shows that chromosomal regions determining variation in agronomic and physiological drought-related traits cover a large proportion of the whole genome. For many crop plants, information on drought-related QTL findings have been collected in open source databases, such as GRAMENE (<http://www.gramene.org/>) or GRAINGENES (<http://wheat.pw.usda.gov/GG2/>).

The association between variation in drought-related quantitative traits and, ultimately, the effects of these traits on yield in drought and favorable environments is the main goal for present and future research. Looking for the coincidence of loci for specific traits and loci for yield under drought stress and in stress-free environments, it is possible to test more precisely whether a specific trait is of significance in improving drought tolerance and yield potential. These results may suggest that selection for drought tolerance could become more efficient thanks to the availability of handle markers tightly linked to loci for stress-related traits. Marker diagnostics of individual QTLs represents an important surrogate for physiological trait measurements, and may ultimately improve breeding efficiency through MAS.

One of the possible disadvantages of molecular markers is that the genetic linkage between a specific random DNA marker and a target locus allele, established by QTL studies, can be broken by genetic recombination, although a QTL or individual gene can be tagged by two flanking markers to reduce the recombination risk. Furthermore, an accurate QTL mapping usually resulting in a small QTL interval is also a pre-requisite to improve MAS-

QTL efficiency. These intrinsic difficulties, together with the polygenic nature of drought tolerance and the interaction with the environment, makes MAS for drought-tolerant QTLs extremely difficult (Francia *et al.*, 2005) due to the number of genes involved and the interactions among them (epistasis). The fact that numerous genes are involved in the expression of polygenic traits means that the individual genes generally have small effects on the plant phenotype. This implies that several regions (i.e. QTLs) must be manipulated at the same time in order to obtain a significant impact, and that the effect of individual regions is not readily identifiable. Replicated field tests are needed in order to accurately characterize the effects of QTLs and to evaluate their stability across environments. Although significant QTL effects can be detected across a range of environments, the evaluation of the QTL×E interactions remains a major constraint on the efficiency of MAS (Beavis and Keim, 1996).

The contribution of genomics-assisted breeding to the development of drought-resistant cultivars has so far been marginal and only a few significant examples of MAS for traits associated with drought tolerance have been reported. No examples have been published in barley so far, while in rice, MAS was used to transfer several QTLs for deep roots from the japonica upland cultivar Azucena, adapted to rainfed conditions, to the lowland indica variety IR64. MAS selected lines showed a greater root mass and higher yield in drought-stressed trials (Courtois *et al.*, 2003). In sorghum, molecular markers were exploited to develop near-isogenic lines each containing one of four stay-green QTLs previously identified (Harris *et al.*, 2007). Favourable alleles in each of the four loci contributed to the lower rate of leaf senescence under post-anthesis water deficit.

An important step towards the application of molecular markers in breeding for drought tolerance is the cloning of DNA sequences underlying QTLs. To date most plant QTLs

have been cloned by the positional cloning approach, although alternative strategies based on candidate genes and linkage disequilibrium may represent an interesting shortcut to QTL cloning (Salvi and Tuberosa, 2005). A candidate gene for drought tolerance usually refers to a sequence for which the expression profile or protein function can be associated with the stress response or adaptation process, and the position on the genome co-maps with a QTL conferring drought tolerance. Candidate genes can be selected from literature data by mapping of known stress-responsive genes (Tondelli *et al.*, 2006), or using bio-informatic analysis of all genes present in QTL-underlined genomic regions. A perfect example of candidate gene associated to a QTL for stress tolerance is represented by the coincidence between one QTL for frost tolerance and the CBF gene family in barley (Francia *et al.*, 2004) and in wheat (Vágújfalvi *et al.*, 2003, 2005).

So far, no QTL for drought tolerance has been cloned in crop species, although a recent study in *Arabidopsis* has led to the cloning of the ERECTA gene, a sequence beyond a QTL for transpiration efficiency (Masle *et al.*, 2005). In plants with large genomes, the generation of molecular-linkage maps based on candidate genes (molecular-function maps) is one way to identify the genetic determinants of QTLs, i.e. functional markers, in spite of the time-consuming fine mapping. This candidate gene strategy shows promise to bridge the gap between quantitative genetic and molecular genetic approaches to study complex traits and, for example, has been applied to find genes potentially involved in barley and rice drought tolerance (Nguyen *et al.*, 2004; Diab *et al.*, 2004; Tondelli *et al.*, 2006). The identification of the QTL corresponding genes will also provide the best markers for MAS, those designed to directly tag the different alleles of the drought-related genes.

In most QTL studies the work has not been extended beyond their detection for a

given trait under drought. The development of consensus QTL maps generated from a number of crosses is an important step towards the identification of regions commonly associated with drought tolerance. A major challenge remaining is to confirm that QTLs discovered in a given mapping population will improve drought tolerance when introduced into high-yielding elite genotypes. This is particularly difficult when the traits are governed by “context-dependent” gene effects (i.e. interaction with other genes or the environment, or both). In these cases, the value of the QTL alleles can differ depending on the genetic structure of the current germplasm set in the breeding program. Under these conditions, the value of a given QTL allele can change during selection due to changes in the background effects at any given time in the breeding process. As a consequence, when the background effects are important, the stacking of desirable alleles by MAS becomes inadequate because the initial target combination of alleles may no longer be the best target, or even a relevant target, for increasing trait performance in subsequent breeding cycles. The “Mapping As-You-Go” strategy (Podlich *et al.*, 2004) involves repeated re-estimation and validation of the QTL effects throughout the breeding process to ensure that they remain relevant throughout. This method results in substantial increases in MAS efficiency compared with standard approaches based on the evaluation of the QTL effects only at the beginning of the breeding program, particularly when epistasis or G×E interactions play a significant role.

Genes and metabolites conferring drought tolerance

New chances to further improve yield or yield stability under limiting conditions come from the last ten years of progress in the identification of the genetic determinants of

the physiological responses related to stress tolerance. Adaptation of plants to drought and to the consequent cellular dehydration induces an active plant molecular response. This response significantly improves the tolerance to negative constraints and it is to a great extent under transcriptional control. Many stress-related genes have been isolated and characterized in the last two decades in a variety of crop species (Cattivelli *et al.*, 2002; Marè *et al.*, 2004; Mazzucotelli *et al.*, 2006; De Leonardis *et al.*, 2007). However, the complexity of the whole molecular response to abiotic stress in crop plants has only recently been revealed by large transcriptome analyses (see Svensson *et al.*, 2006, for cold response; and Talamè *et al.*, 2007, for drought response in barley). Molecular analysis in *Arabidopsis* has sketched the complex network constituting cell communication during drought response. From model plants, genetic information is being moved to crops exploiting genome synteny, taking advantages of conserved molecular pathways, including those controlling stress tolerance. Following this approach the regulatory components of the drought response are being sought and identified in crop plants (Shen *et al.*, 2003; Kizis and Pages, 2002; Marè *et al.*, 2004).

Transgenic plants have been developed either to up-regulate the general stress response or to reproduce specific metabolic or physiological processes previously shown to be related to drought tolerance by classical physiological studies. Transcription factors as well as components of the signal transduction pathways that coordinate expression of downstream regulons are thought to be optimal targets for engineering of complex traits such as stress tolerance. Successful examples are transgenic crops engineered with genes encoding the DREBs/CBFs transcription factors (in rice: Dubouzet *et al.*, 2003, and Ito *et al.*, 2006; and in wheat: Pellegrineschi *et al.*, 2004). The transgenic plants showed increased stress tolerance as well as the over-induction of downstream stress-related genes and/or

higher levels of soluble sugars and proline. A recent report has shown that rice plants over-expressing the SNAC1 (Stress-Responsive NAC1) transcription factor showed improved drought tolerance and yield potential under field conditions. The leaves of SNAC1-over-expressing plants lost water more slowly, showing an increased stomatal closure and ABA sensitivity (Hu *et al.*, 2006). Ectopic expression of a stress-induced rice gene encoding a calcium-dependent protein kinase (OsCDPK7) also resulted in enhanced levels of stress-responsive genes that contribute to improved salt and drought tolerance (Saijo *et al.*, 2000).

Over-expression of transcription factors may also activate additional non-stress-related genes that adversely affect the normal agronomic characteristics of a crop, producing deleterious effects on the phenotype and thus yield (Wang *et al.*, 2003). Common detrimental effects due to constitutive expression of regulative factors are growth retardation, and reduced seed numbers in transgenic plants under normal conditions. Alternative strategies based on stress-inducible promoters cause minimal negative effects under normal growth conditions and enhance stress tolerance, although it remains to be established if the threshold stress under which the promoter is active corresponds well to stress levels in target environments (Wang *et al.*, 2005).

Metabolic engineering for increasing osmolyte contents was successful in several plants subjected to stress (Wang *et al.*, 2003), although the real advantages of such a strategy are always a subject of debate (Serraj and Sinclair, 2002). Given that the target compounds did not achieve levels sufficient to sustain a role in osmotic adjustment, chaperone-like activity and scavenging of reactive oxygen species were proposed as alternative functions in plant protection during stress exposure. The first example of metabolic engineering for drought tolerance was the overproduction of

proline in transgenic plants (Zhu *et al.*, 1998), resulting in an enhanced biomass under stress conditions. Garg *et al.* (2002) developed drought-tolerant transgenic rice lines showing tissue- or stress-inducible accumulation of trehalose, which accounted for higher soluble carbohydrate levels, a higher capacity for photosynthesis and a concomitant decrease in photo-oxidative damage, and more favorable mineral balance under both stress and non-stress conditions, without negative effects. A significant improvement in wheat tolerance to water deficit was also achieved by Abebe *et al.* (2003), through the ectopic expression of the mannitol-1-phosphate dehydrogenase (mtlD) gene that caused a small increase in the level of mannitol.

Since an earlier-than-normal stomatal closure in a crop is considered a positive trait to improve water use efficiency in drought environments, developing transgenic plants with a drought-avoidance phenotype represents a possible strategy for crop improvement. A phenotype with decreased conductance and higher water use efficiency was obtained in tobacco plants over-expressing a maize NADP-malic enzyme, the primary decarboxylating enzyme in C_4 photosynthesis (Laporte *et al.*, 2002). The implication of abscisic acid hormone as a molecular signal in drought-activated pathways and in the control of stomatal closure makes ABA synthesis and response a possible target for improving drought tolerance. When a farnesyl-transferase acting as a negative-regulator of ABA sensing was down regulated in a drought-inducible manner in *Brassica napus*, the transgenic plants showed enhanced ABA sensitivity, as well as a significant reduction in stomatal conductance and transpiration under drought-stress conditions. Furthermore, transgenic plants were more resistant to water-deficit-induced seed abortion during flowering (Wang *et al.*, 2005).

A more robust root system enables plants to take up greater amounts of water during

water deficit stress, resulting in a more favorable plant water status and less injury. Although this consideration has been obvious for many years, only recent studies have found the way to increase root size through single gene transformation. The gene coding for the vacuolar H⁺-pyrophosphatase (H⁺-PPase) – *AVP1* – plays an important role in root development through the facilitation of auxin fluxes. Over-expression of *AVP1* in *Arabidopsis* and tomato resulted in more pyrophosphate-driven cation transport into root vacuolar fractions, an increased root biomass, and an enhanced recovery of plants from an episode of soil water deficit stress (Gaxiola *et al.*, 2001; Park *et al.*, 2005).

Integration of different approaches for stress improvement

When phenotypic selection was the only tool available to improve yield under drought, the improvements in crop yield observed were probably due to an increase in yield potential through the unconscious pyramiding of yield-related traits or loci. Research in the last three decades has opened up three main approaches: (i) plant physiology studies have provided new tools to understand the complex network of drought-related traits, and several drought-related traits useful to improve selection efficiency have been proposed (see Table 1); (ii) molecular genetics has led to the discovery of a large number of loci affecting yield under drought or the expression of drought-tolerance-related traits; and (iii) molecular biology has provided genes that are either useful as candidate sequences to dissect QTLs or for transgenic approaches. The integration of molecular genetics with physiology is leading to the identification of the most relevant loci controlling drought tolerance and drought-related traits. Routine cloning of the genes underlying the QTLs is still a long way off, but it will, ultimately, provide simple markers for effective MAS.

Nevertheless, MAS for drought tolerance will not be an easy task because dozens of QTLs for drought-related traits have been identified. Selecting which QTLs or traits follow with MAS is now crucial. The improvement of drought tolerance should not be achieved with a parallel limitation of yield potential. Hence, drought-tolerance traits should be tested in both stressed and non-stressed environments before being introduced into a MAS breeding program. QTLs for drought-related traits coincident with QTLs for yield potential should be considered as priority targets for MAS.

The success of any selection process relies on the availability of superior alleles for the target trait. Most QTLs for drought tolerance, rather than being chosen for their overall agronomic value, have been identified in segregating populations derived from parental lines chosen to maximize the differences in the target traits. Typically, a segregating population from a cross between modern and old varieties allows the identification of many QTLs; nevertheless, a majority of the positive QTL alleles might derive from the modern parental line and therefore are already present in the best performing cultivars. A chance to find new useful alleles is represented by the exploitation of wild germplasm. During the domestication process, wild plants carrying promising traits were cultivated, leading to locally adapted landraces. These lost many undesirable alleles and useful alleles became enriched in the cultivated gene pool (Tanksley and McCouch, 1997). Many studies have demonstrated the value of alleles originating from non-cultivar germplasm (Tanksley and McCouch, 1997), showing that centuries of selective breeding have thrown away useful alleles in addition to many useless ones. The effect of a given QTL or locus can also be influenced by the background of the genotypes used in the breeding program. Epistatic interactions among QTLs, for example, might hamper the development of an efficient MAS program. Notably, when

four genomic segments carrying QTLs for root length were transferred from the rice cultivar Azucena into Kalinga III, only one of them significantly increased root length in the new genetic background (Steele *et al.*, 2006).

Transgenic breeding will also have a role in the future and the possibility of cloning stress-related QTLs will enable the simultaneous engineering of multiple genes governing quantitative traits. However, the scarcity of field trials for drought-tolerant transgenic plants does not allow for final conclusions. New transgenic plants where the gene is introduced into elite genotypes have to be tested under optimal as well as drought conditions to evaluate the impact of the transgenes on yield potential and stress tolerance.

A significant example showing how the integration of stress physiology and genomics can lead to an integrated view of plant breeding is represented by the studies on transpiration efficiency. During drought stress, plants generally coordinate photosynthesis and transpiration, although significant genetic variation in transpiration efficiency has been identified both between and within species (Rebetzke *et al.*, 2001). After the demonstration that Δ of plant matter is a reliable and sensitive marker negatively related to variation in transpiration efficiency, several selection programs based on Δ were carried out (Rebetzke *et al.*, 2002; Juenger *et al.*, 2005) and new wheat cultivars with improved water use efficiency have already been released. The isolation of a gene, ERECTA, that regulates transpiration efficiency in *Arabidopsis* (Masle *et al.*, 2005), and the transcriptional analysis of wheat genotypes with contrasting transpiration efficiency (Xue *et al.*, 2006) are providing the molecular bases for the isotopic discrimination parameter. Hence, in the near future, an integrated approach for transpiration efficiency could involve the use of the physiological test (analysis

of Δ), the markers for QTLs controlling Δ , and the ERECTA gene, as well as of other genes, either to search for allelic variations in the germplasm or as a tool for plant transformation. Every day, it becomes more evident that successful breeding for stable high yield under drought conditions will only be possible when a true integration of traditional breeding with physiology and genomics is achieved. Thus, to face drought stress and to achieve sufficient grain yield in the future requires a multidisciplinary approach based on plant genomics, physiology and modeling.

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Investigating root architecture in barley and responses to salinity and high boron

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Abstract

Barley root architecture determines adaptation to different environments and may be modified in response to subsoil constraints. Subsoil constraints influencing root architecture in southern Australia include salinity and boron. Root architecture was investigated using hydroponics, sand culture, and field soil coring. Advanced breeding lines and mapping population parents were examined in hydroponics and a probe set of individuals in sand culture with the aim of investigating root architecture *per se* and Na \times B interactions. An RT-PCR method was developed to quantify barley root tissue, and calibrated with conventional root measurements to develop a more cost-effective approach to examine root morphology in field trials. Soil coring experiments to quantifying root biomass and DNA were conducted in 2005 and 2006 using mapping population parents with different root morphology. The implications of this research is a better understanding of inherent root architecture, root responses to salinity and boron, and the development of a new methodology to investigate root architecture in the field. Ultimately this will lead to the development of varieties with a robust root structure and improved adaptation to environments with these subsoil constraints.

Introduction

Hostile subsoils are prevalent throughout the cropping zone in southern Australia and include physical, biotic, and chemical constraints that inhibit healthy root growth and consequently water use and nutrient capture (Sadras *et al.*, 2002). Improving root growth through tolerance to subsoil constraints should therefore translate into yield improvement. Previous studies have found that subsoil chemical toxicities reduce water use or that correcting deficiencies improves root growth. Sodium (Na) and boron (B) toxicity occurs on a large range of soils, particularly the neutral to alkaline soils of southern Australia (Adcock *et al.*, 2007).

The traits identified as contributing to B toxicity tolerance in barley are low B uptake, foliar tissue tolerance, and maintained root growth under B toxicity (Jefferies *et al.*, 1999). Detailed genetic studies and long-term breeding efforts on B toxicity tolerance has failed to deliver any agronomic improvement in Australia (Jefferies *et al.*, 1999; Eglinton *et al.*, 2003; Sutton *et al.*, 2007), although deleterious linkage drag cannot be unequivocally excluded. Extensive studies have been undertaken on a range of aspects of salinity tolerance in barley; however, an advantage in agronomic performance is yet to be demonstrated. The combined influence of

Na and B on productivity in neutral-alkaline soils has not been systematically examined.

Combining Na and B tolerance may be the answer to improved productivity through maximized water use, assuming improved root growth translates to a yield advantage. However, without appropriate tools to investigate root architecture in the field, it can only be assumed that breeding for tolerances to chemical, physical and biotic constraints, and maximum root growth, will maximize resource capture. Clearly there are different root ideotypes for different environments, and ignoring the subsoil constraints, the overriding factor would be rainfall amount and distribution during the growing season. In a stored moisture environment (i.e. northern Australia and many continental environments), deep roots are needed to extract soil water at depth, but for the variable moisture environments of southern Australia and other Mediterranean environments, it is only beneficial to have a deep root system to capture residual stored moisture if physiochemical constraints can be tolerated.

A multidisciplinary approach is needed to address the complex issues of Na and B tolerance in relation to productivity and root architecture. In this paper we examine Na and B tolerance using a combination of controlled environment and field trials. A novel approach to measure root architecture in field trials is also examined.

Sodium uptake experiments using hydroponics

Hydroponic screening was conducted using the method of Genc *et al.* (2007) to investigate the effects of different levels of Na, Ca and pH on Na uptake. A probe set of genotypes was selected that showed putative or reported Na tolerance by different mechanisms. Ten days after sowing, NaCl was added in 25 μM increments with supplemental CaCl_2

twice daily until the required concentrations were obtained. Plants were exposed to the treatments for 10 days, after which the third fully expanded leaf was harvested, and examined for Na and K content by flame photometry. All factors had an effect on the rankings for Na and K uptake, and on the NA/K ratio. The combination of factors associated with the best ranking of probe genotypes without inducing osmotic stress or other ionic imbalances, and best reflecting field subsoil environments, was the treatment 150 mM NaCl + 7.5 mM CaCl_2 + pH 7.5.

As illustrated in Figure 1, Mundah had consistently low tissue Na across treatments. This is important if the uptake mechanism is to be applicable to a range of subsoil environments in the field. Mundah had consistently lower leaf Na than Keel across treatments. These genotypes are parents of an Australian mapping population (Long *et al.*, 2003). Mundah becomes more differentiated from the other genotypes with increasing Na concentration with the exception of JE001*02D/20 at 200 mM Na. This is a line from the ABQTL population Barque-73/CPI71284-48 (Hearnden *et al.*, 2007) previously characterized with Na exclusion from CPI71284-48 (Shavrukov, pers. comm.). At 150 mM Na (7.5 mM CaCl_2 , pH 7.5), JE001*02D/20 did not maintain low leaf Na concentration relative to Barque-73 since they both had low Na accumulation. Skiff was used as the positive control as it accumulates high Na and exhibits tissue tolerance (Munns and James, 2003).

The treatment 150 mM NaCl + 7.5 mM CaCl_2 + pH 7.5 shows that Mundah has the lowest Na accumulation (Figure 2). Most lines accumulated 150 mM of Na, with Keel and WI3788 having the highest Na accumulation. The Na/K ratio indicates discrimination between Na and K. The lower the ratio the lower the proportion of Na to K, although it must be cautioned that the Na/K ratio does not explicitly relate to a 1:1 discrimination between these ions. For example, Mundah

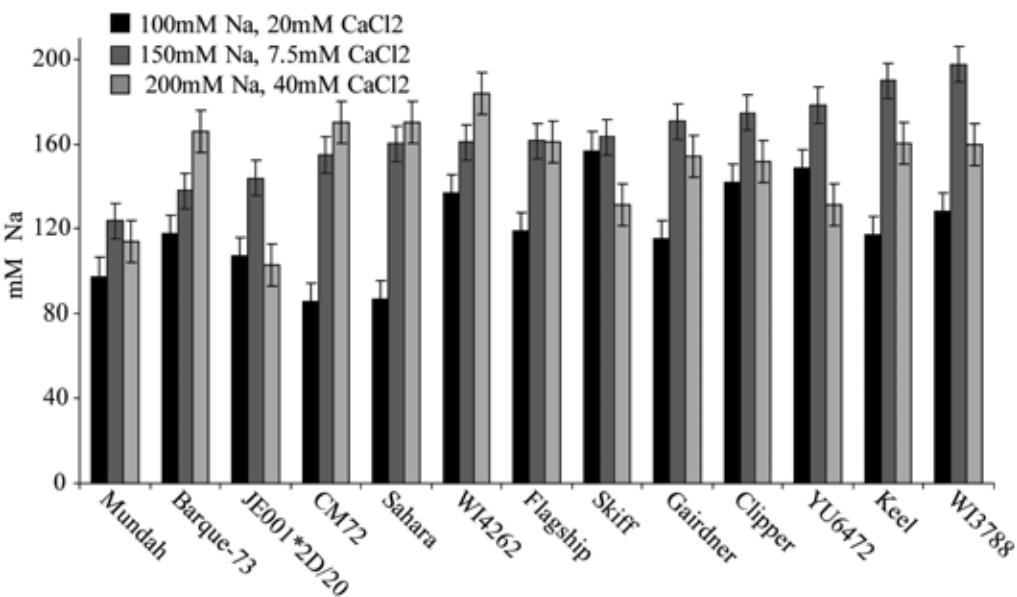


Figure 1. Na concentration (mM) of 13 barley genotypes grown in hydroponics at pH 7.5 and different sodium and calcium (Ca) treatments: (a) 100 mM NaCl + 20 mM CaCl₂; (b) 150 mM NaCl + 7.5 mM CaCl₂; (c) 200 mM NaCl + 40 mM CaCl₂. Results are in ascending order of leaf-Na concentration in the 150 mM NaCl + 7.5 mM CaCl₂ treatment. Differences between genotypes at LSD (0.05) = (a) 17.6; (b) 16.8; (c) 19.8.

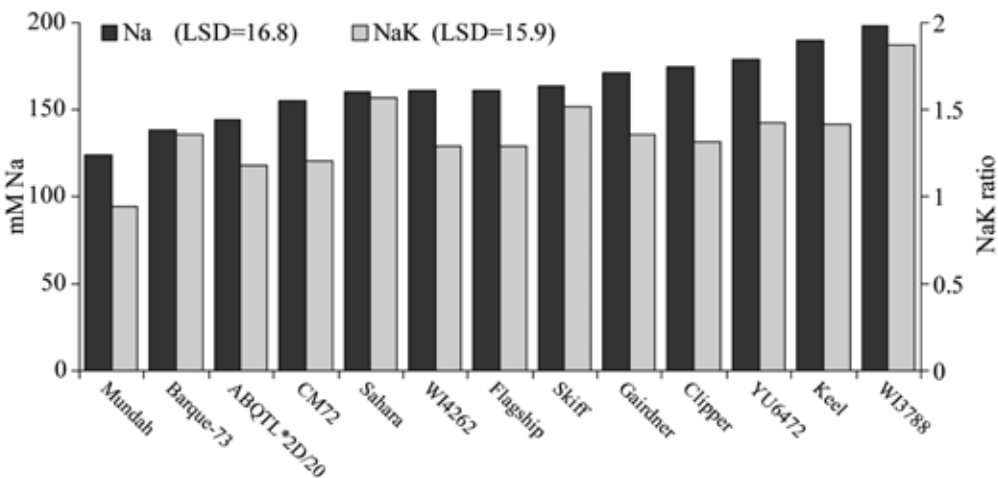


Figure 2. Na uptake (mM) and Na/K ratio of 13 barley genotypes grown in hydroponics at 150 mM NaCl + 7.5 mM CaCl₂, pH 7.5. Results are in ascending order by leaf Na concentration.

and Keel do not significantly differ for K accumulation (136 and 138 mM respectively), yet Mundah has lower Na uptake resulting in the ratio difference. The only varieties that accumulated Na at the expense of K were Sahara, WI3788 and Skiff. The results show that Mundah has lower Na uptake at the same K concentration as Keel.

B and Na uptake using soil culture

The seedling tolerance of Mundah and Keel to high B and Na was assessed by growing plants in Glenthorne soil (Paull *et al.*, 1988) with high levels of B and Na (60 mg/kg extractable B, Electrical Conductivity [ECe] ~20 dS/m). Five plots with five plants per plot were sown for Mundah and Keel and grown for 35 days. Leaf necrosis due to B was scored, and each plot harvested and analyzed for elemental composition by inductively coupled plasma spectrometry (ICP) (Zarcinas *et al.*, 1987). As shown in Table 1, analysis revealed no difference in B uptake between genotypes, although they differed significantly for B leaf symptom expression. Keel exhibited reduced leaf symptom expression indicating tissue tolerance to high boron. There was a significant difference between Na concentration, with Mundah accumulating less Na. The difference in K concentration was just not significant, but Mundah accumulated significantly less Ca.

The Na/K ratio was lower for Mundah due to accumulating less Na at the same level of K, consistent with the findings from the hydroponics experiments. Together these experiments show that Keel exhibits tissue tolerance to B and Mundah accumulates less Na. There was no inter-correlation between B leaf expression, B uptake, and Na uptake, indicating independent mechanisms.

Field trials to investigate Na tolerance

Yield under salinity, Na and K uptake were investigated using field trials on high pH (7.5-8.5) clay loam saline soils at Whitwarta and Georgetown in South Australia. Trials were sown in an area with uniform Electrical Conductivity of a saturated soil Extract, used as an indicator of salinity, in the 0–30 cm and 30–60 cm soil layers. The ECe (sampled mid-growing season) at Whitwarta and Georgetown respectively for the 0–30 and 30–60 cm depths was 3.6/8.5 and 1.9/7.2, and exchangeable Na levels of 1205/2024 and 655/2300 mg/kg. This indicates mild salinity at 0–30 cm and high salinity at 30–60 cm depth. Soils with >4 dS/m are considered saline (Sumner, 1993), and an ECe of 9 dS/m causes a 25% yield reduction in barley (Royo *et al.*, 2000).

A germplasm set of 180 barley lines, including the probe set plus Australian and international genotypes with putative Na

Table 1. Differences between Mundah and Keel for boron leaf symptoms (Mundah is more sensitive), and elements in whole seedlings as measured by ICP (mg/kg) from plants grown in a box containing Glenthorne soil with toxic levels of B and Na.

Measured traits	Mundah	Keel	P value
Boron leaf symptom expression	6.3	3.3	<0.001
Boron (mg/kg)	538	567	0.79 ns
Sodium (mg/kg)	15 240	21 400	<0.001
Potassium (mg/kg)	67 500	62 000	0.08 ns
Na/K ratio	0.22	0.35	<0.001
Calcium (mg/kg)	7 017	7 850	0.02

ns = not significant.

tolerance, were planted as a randomized block design with three replications. Trials were sown on 4 June and 31 May for Whitwarta and Georgetown, respectively, at a seeding rate of 145 plants/m² in 3.2 × 1.2 m plots. The fully expanded penultimate leaf was sampled from 15 random plants in each plot at growth stage Z45 (Zadoks *et al.*, 1974) and analyzed for Na and K content by flame photometry. At Whitwarta, each plot was assessed for B leaf symptom expression, and the site was characterized by very low rainfall, with moisture stress from spring. Georgetown experienced sufficient

moisture during the growing season. Yield, Na and Na/K were analyzed using restricted maximum likelihood (REML) to adjust for spatial trends. The results of a selected few genotypes are presented.

Mundah was the top yielding variety in both saline environments (Table 2), with Fleet, WI3788 and Keel not significantly different at Georgetown. The top yielding malting variety was Flagship, not significantly different from Keel at both sites. Sodium levels at both sites were discriminative, and were highest at Georgetown. The variety Chevron had lowest Na uptake and Na/K ratio at both sites, so

Table 2. Yield (t/ha), Na uptake (μmol/g dry weight leaf tissue) and Na/K discrimination (Na/K) of the penultimate leaf, and B leaf symptom score of barley lines (9 = severe symptoms) grown in saline sites (Whitwarta and Georgetown). Those in bold are not significantly different from the best lines: Chevron for Na (within 2 significance intervals at Whitwarta) and Na/K (within 2 significance intervals at Georgetown), and Mundah for yield.

WHITWARTA					GEORGETOWN			
Barley Line	Yield	Na	Na/K	B	Barley Line	Yield	Na	Na/K
Mundah	1.85	103	0.13	7	Mundah	2.63	162	0.22
Keel	1.64	102	0.13	4	Fleet	2.56	208	0.33
Flagship	1.62	165	0.17	8	WI3788	2.51	187	0.26
CM72	1.60	133	0.20	2	Keel	2.49	195	0.25
Sloop	1.55	147	0.18	6	Flagship	2.43	232	0.35
Chevron	1.51	83	0.10	7	Capstan	2.42	136	0.22
Schooner	1.41	180	0.20	5	Buloke	2.39	230	0.35
WI3788	1.40	114	0.14	5	WI3416-1572	2.30	200	0.30
Gairdner	1.36	142	0.17	5	Sloop	2.29	169	0.25
Rihane-03	1.24	143	0.10	4	Rihane-03	2.26	180	0.23
Fleet	1.24	190	0.20	4	WI4262	2.23	243	0.39
Buloke	1.17	182	0.20	6	Skiff	2.22	216	0.33
Skiff	1.13	160	0.16	3	Gairdner	2.11	181	0.29
Capstan	1.11	143	0.17	6	CM72	2.03	173	0.27
YU6472	1.09	166	0.19	5	Barque-73	2.02	172	0.25
Barque-73	0.94	145	0.17	5	Maritime	1.89	176	0.27
WI4262	0.94	159	0.17	1	Schooner	1.73	194	0.25
WI3416-1572	0.91	169	0.19	7	Chevron	1.73	141	0.20
Franklin	0.85	224	0.28	4	Taixing 9425	1.65	142	0.23
Maritime	0.83	161	0.19	4	Franklin	1.42	168	0.25
Taixing 9425	0.72	114	0.15	9	YU6472	1.39	156	0.19
Yuyaoxiangtiixerleng	0.09	105	0.14	3	Yuyaoxiangtiixerleng	0.87	148	0.24
LSD = (2*SED)	0.12	12.4	0.05		LSD = (2*SED)	0.17	12.4	0.02

varieties are compared to Chevron. For leaf Na content and Na/K ratio, the varieties not significantly different from Chevron at Whitwarta (within 2 significance intervals for Na) and Georgetown (within 2 significance intervals for Na/K) were Mundah, Taixing 9425, and Yuyaoxiangtiixerleng. Taixing 9425 and Yuyaoxiangtiixerleng are Chinese varieties with low Na uptake reported by Chen *et al.* (2007). Rihane-03 had a low Na/K ratio at both sites, but not low Na content. Keel and WI3788 had low leaf Na content and Na/K at Whitwarta. Capstan had low leaf Na content and Na/K ratio, and YU6472 low Na/K ratio at Georgetown. Mundah had high yield combined with low leaf Na content and Na/K ratio at both sites. Keel had high yield combined with low leaf Na content and Na/K ratio only at Whitwarta, an interesting observation since Keel was characterized with high Na uptake in controlled experiments. Flagship maintained yield across these saline environments despite having high Na uptake, an interesting parallel to its excellent yield performance on B toxic soils despite exhibiting high levels of B toxicity symptoms. There was no correlation between leaf Na content, Na/K ratio and yield, owing to the diversity of the germplasm evaluated, so only casual relationships can be drawn.

Investigating root architecture

Controlled environment experiments conducted using long PVC pots and basal nutrients added to a sandy loam showed that Mundah and Keel differ for maximum rooting depth but not root length density (a measure of root volume) after 31 days growth (Long, 2003). Figure 3 shows the differences in maximum rooting depth and that Keel has a shallow root system, with a plateau at 25 cm deep by 24 days, while Mundah's root depth is still increasing at 31 days. A shallow root system would be advantageous to avoid subsoil constraints

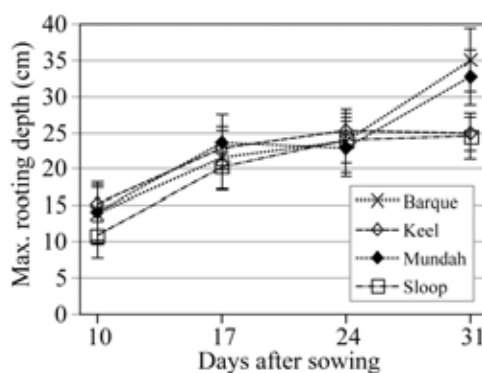


Figure 3. Maximum rooting depth of four barley varieties after 31 days growth in sand culture.

at depth, while a deeper root system would allow water extraction at depth. However, root architecture is influenced by the presence of subsoil constraints. For example Clipper has inherently longer roots than Sahara, but under high B, morphological changes lead to increased root elongation and longer roots in Sahara (Choi *et al.*, 2007).

Controlled environment experiments are used to characterize root growth due to inherent error associated with field phenotyping techniques. However field studies more appropriately reflect the dynamics of root growth in response to the subsoil environment and the relationship between root morphology and productivity. Root architecture was investigated in the field by determining root biomass and DNA from collected soil cores. A quantitative TaqMan® assay was developed to quantify barley root DNA in live root cells in soil samples. The assay does not detect closely related species and can detect less than 1 mg dry roots per 500 g soil. To validate the application of this methodology in the field, strip plots of Mundah, Keel, Clipper and Sahara were grown in alkaline soils at Roseworthy (clay loam) and Callington (sandy loam) in South Australia during 2006. Soil cores were collected from positions within and between plant rows to a depth of 80 cm and sectioned into 10 cm intervals to allow investigation

of maximum root depth and distribution. Soil cores were collected as pairs, one to measure root DNA content and one for root biomass, with samples bulked at each 10 cm depth, making pairs of 500 g composite soil bulks for each genotype \times position \times depth. Composite bulks were used to account for within-plot spatial variation in root DNA and biomass.

The DNA and biomass data was log transformed to examine differences due to genotype, position and depth. At both sites, root DNA content and biomass decreased in amount with depth. The overall effect of depth was significant at both sites, with root DNA and biomass declining with depth and significantly higher in the 0–10 cm depth at Roseworthy, and the 0–10 cm, 10–20 cm and 20–30 cm depths at Callington. Root DNA and biomass had a significant positive correlation at these depths, and the relationship in the 0–30 cm layers at Roseworthy and Callington is shown in Figure 4. Root DNA within rows was higher than that between plant rows at Roseworthy but not significant at Callington. Position had

no significant effect on root biomass. Due to composite bulking, genotypic effects can only be inferred from the overall plot effect. There were differences between genotypes for root DNA content, with Mundah and Keel lower than Clipper and Sahara at Roseworthy, and Sahara lower than Mundah, Keel or Clipper at Callington. The relationship with depth and position for root DNA and biomass of Mundah and Keel at Roseworthy is shown in Figure 5. Depths below 40 cm did not give meaningful results and are not shown. The results at Roseworthy when measured within the row are comparative with soil culture, with Keel having more roots in the 0–10 cm depth, and Mundah higher in the 10–30 cm depth. Between rows, Keel had higher biomass than Mundah but lower root DNA. This may reflect finer roots for Mundah between rows that are lost during root washing. Clearly more work is needed to utilize the DNA methodology to investigate roots below 40 cm and between plant rows.

The DNA root screening methodology was implemented at the saline sites of Georgetown and Whitwarta (see above for

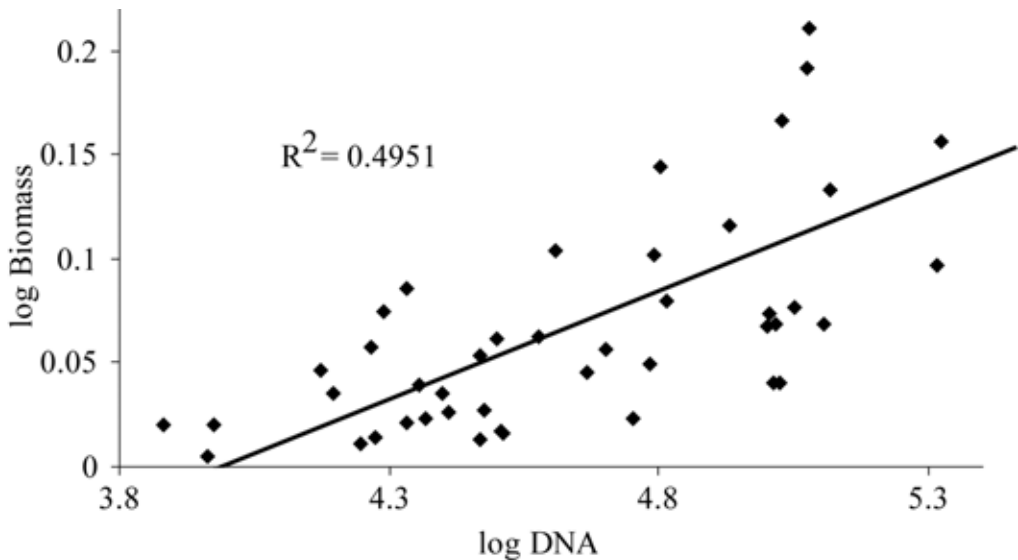


Figure 4. Relationship between DNA content and biomass from the 0–30 cm layers from Callington and Roseworthy, South Australia.

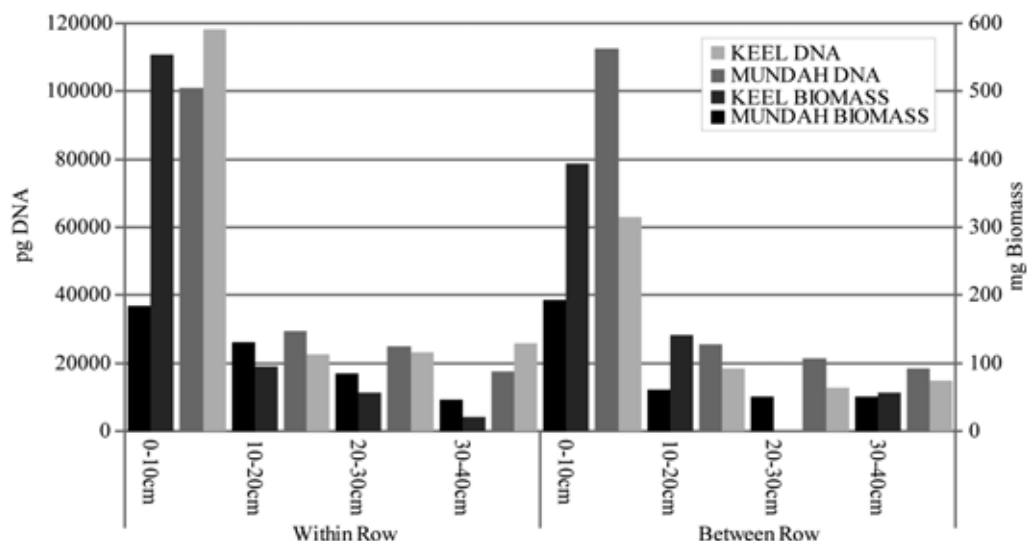


Figure 5. DNA (pg) and root biomass (mg) within a row and between rows at 10 cm depths to 40 cm for Mundah and Keel grown at Roseworthy, South Australia, in 2006.

site details) for Mundah and Keel, with roots sampled to a depth of 45 cm within plant rows. Three intervals were chosen to represent the cultivated topsoil layer (0–15 cm) and subsequent subsoil layers (15–30 cm and 30–45 cm). Sampling below 45 cm between plant rows was not warranted based on the previous results. Four replications of each genotype were grown, and at growth stage Z50 (Zadoks *et al.*, 1974), four soil samples were collected for each replication from within plant rows and bulked at each depth interval. Soil barley root DNA content was measured and log transformed to analyze the data, as shown in Figure 6.

The amount of root DNA decreased with depth, and the rankings between genotypes changed with depth. Keel had higher root DNA in the 0–15 cm depth, and Mundah was higher at the 30–45 cm intervals at Georgetown. Mundah had higher root DNA at 30–45 cm and 45–60 cm at Whitwarta. This corresponds to Roseworthy in 2006, with higher root DNA in the topsoil layer (0–15 cm) for Keel and higher in the subsoil layers for Mundah. If subsoil salinity affects root architecture, it is postulated that the low Na uptake of Mundah would translate to less inhibition of root growth in the field and more root DNA at depth beyond its genetic potential

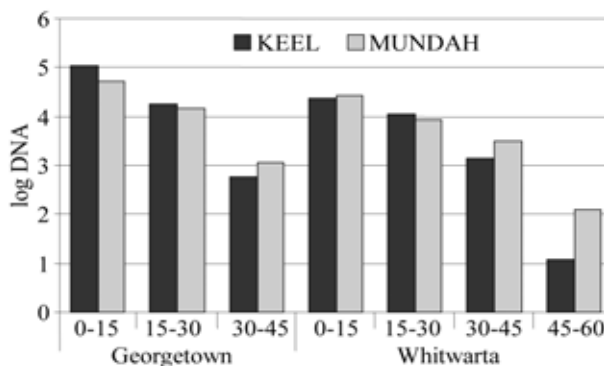


Figure 6. Barley DNA in soil samples collected within rows from Mundah and Keel plots at Georgetown and Whitwarta in 2007 at growth stage Z50 (Zadoks *et al.*, 1974).

for deeper roots. This novel DNA assay has potential to provide a better understanding of root growth and morphology in the field. However more validation is needed to test its robustness for in-field root phenotyping, especially of root architecture below 45 cm.

Conclusion

The implications of this research are a better understanding of inherent root architecture, root responses to salinity and boron, and the development of a new methodology to investigate root architecture in the field. Mundah and Keel are shown to differ for B and Na tolerance, root architecture, and traits contributing to high productivity. This research has developed the methodologies to investigate differences between these genotypes, and a mapping population has been developed (Long *et al.*, 2003) to investigate the genetics of B, Na, and root architecture interrelationships. The Mundah × Keel population is ideal to investigate the ‘root ideotype’ for southern Australia and Mediterranean environments generally. This root ideotype would have root volume in the cultivated topsoil (0–15 cm) to capture within-growing-season rainfall and nutritional resources added to the farming system, and root volume in the subsoil to exploit stored nutrients and water in favorable subsoil environments or hostile environments if physiochemical constraints can be tolerated. This ‘root ideotype’ involving maximization of soil water use through balancing subsoil constraint tolerance with avoidance is part of the bigger picture of ‘drought tolerance’ or better-termed general adaptation.

An understanding of adaptive traits from this germplasm will lead to relationships with yield and physical grain quality. However, even with combined Na and B tolerance there is no guarantee there will be a yield advantage since other physiochemical constraints may become inhibitors to root growth. Root growth responds dynamically

to soil physiochemical constraints, rainfall amount and distribution during the growing season, and exploits regions of the soil where conditions are favorable. This makes a multidisciplinary approach necessary to investigate these complex issues to ultimately develop varieties with a robust root structure, improved adaptation to Mediterranean environments with subsoil constraints, and improved productivity.

Acknowledgements

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Vernalization-independent regulation pathway of the *VRN-H1* vernalization response locus

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Abstract

The effects of various environmental factors on flowering and on the activities and interactions of the photoperiod sensitivity (PPD) and vernalization response (VRN) loci were examined applying systematic phenotypic characterization in controlled growth chamber tests and functional QTL analyses based on gene-specific primers. The experimental materials were Dicktoo (D; facultative), Morex (M; spring), and Kompolti korai (KK; winter), together with the D × M and D × KK mapping populations. Morex carries the spring allele of *VRN-H1*: a 5.2 kb deletion in intron 1. The functional winter alleles of Dicktoo and Kompolti korai are identical in the coding regions and in the vernalization-critical region of intron 1. Promoters are identical except for SSR polymorphisms. Small modifications in the controlled environment conditions led to dramatic changes in the flowering-time phenotype. A genetic dissection of these changes via QTL analysis revealed novel effects and interactions of barley VRN and PPD genes. We hypothesize that the phenotypic reactions given to low light intensity, to sub-optimal temperature and to the synchronous application of photo- and thermo-cycles are connected with the mechanism and action of the circadian rhythm, which, in turn, alter the activity and role of *PPD-H1*, *VRN-H2* and *VRN-H1* in a manner distinct from that attributable to vernalization and photoperiod duration.

Introduction

In cereals, the major genetic determinants of flowering are the VRN (vernalization response) and the PPD (photoperiod sensitivity) genes (Cockram *et al.*, 2007; Trevaskis *et al.*, 2007). Two major genes for vernalization response, *VRN-H1* and *VRN-H2*, have been identified in barley (Danyluk *et al.*, 2003; Yan *et al.*, 2003; von Zitzewitz *et al.*, 2005). The candidate gene for *VRN-H1* is the MADS-box gene *HvBM5A*, which is the barley orthologue of the wheat *AP1* (*VRN1*) floral meristem identity gene. In genotypes with spring growth habit the *VRN-H1* gene product can be detected early in plant development, resulting in early flowering, while in genotypes with winter growth habit the AP1 protein does not appear until the vernalization requirement has been met (Danyluk *et al.*, 2003; von Zitzewitz *et al.*, 2005). A region in the first intron of *HvBM5A* is thought to include the binding site for the repressor encoded by *VRN-H2*, and accordingly was named as the vernalization critical region (Fu *et al.*, 2005; von Zitzewitz *et al.*, 2005). Deletion of this region leads to the dominant allele for spring growth habit. One or more of the three physically linked *ZCCT-H* genes on chromosome 4H, which are present in winter genotypes and deleted from facultative and spring genotypes, are candidate genes for *VRN-H2* (Yan *et al.*, 2004; von Zitzewitz *et al.*, 2005). Based on the flowering regulation model, vernalization saturation represses the activity of the

dominant *VRN2* allele, allowing recessive alleles to be expressed at *VRN1*. This two-gene epistatic model has been demonstrated to be responsible for the vernalization requirement and thus for the growth habit in cereals (Yan *et al.*, 2004; Dubcovsky *et al.*, 2006; Kóti *et al.*, 2006; Szűcs *et al.*, 2007). Gene expression, QTL and segregating population studies proved that low temperature is the primary environmental factor that controls the activity of the *VRN* loci (Yan *et al.*, 2003; Danyluk *et al.*, 2003; Yan *et al.*, 2004; Yan *et al.*, 2006). There is increasing evidence, however, that secondary and interaction effects may also regulate these loci (von Zitzewitz *et al.*, 2005; Dubcovsky *et al.*, 2006; Karsai *et al.*, 2006; Szűcs *et al.*, 2006; Trevaskis *et al.*, 2006). Thus *VRN-H1* has been shown to respond to photoperiod as well; in facultative barley there was no transcription of the gene under short photoperiod (Danyluk *et al.*, 2003; von Zitzewitz *et al.*, 2005). In carrying out comparative mapping and QTL analyses in two barley populations, we identified the *VRN-H1* gene as a significant source of variance, even though in both cases it was not expected, based on the recent genetic regulation model of flowering in cereals (Yan *et al.*, 2006; Cockram *et al.*, 2007; Trevaskis *et al.*, 2007). The Dicktoo (facultative) × Morex (spring) population segregates for the presence or absence of the *VRN-H1* vernalization critical region but it lacks the *VRN-H2* gene, which codes for its repressor (von Zitzewitz *et al.*, 2005). In spite of this, the winter *VRN-H1* allele of Dicktoo resulted in later first node appearance and flowering in the range of 12- and 18-hour photoperiods and showed a significant interaction with the allele type of the *PPD-H1* gene (Karsai *et al.*, 1997). The other population of Dicktoo (facultative) × Kompolti korai (winter) was segregating for the presence of the *VRN-H2* gene, but carried the same vernalization-critical regions of the *VRN-H1* gene (Karsai *et al.*, 2005). An allelic variation at the promoter

of *VRN-H1*, not known to affect phenotype, allowed us to monitor allelic segregation at this locus. Again, *VRN-H1* proved to be significant component of flowering in field-grown experiments, and under long photoperiod, showing strong interaction with the allele composition in the *VRN-H2* locus (Karsai *et al.*, 2005). In this population, too, it was the Dicktoo type of *VRN-H1*, which resulted in later plant development.

In order to examine the role of the *VRN-H1* locus in determining plant development, we combined the functional mapping of the major genes (*VRN-H1*, *VRN-H2* and *PPD-H1*) of flowering, and the functional QTL analyses with systematic phenotypic characterizations under various sets of environmental cues in controlled climatic chamber tests. This investigation was facilitated by the availability of (i) allele-specific primers for these genes; (ii) very well-characterized barley accessions representative of the three growth habit types; and (iii) two well-characterized doubled-haploid (DH) mapping populations.

Materials and methods

Plant materials

The Morex (M; spring), Dicktoo (D; facultative) and Kompolti korai (KK; winter) cultivars and the two DH mapping populations derived from the cross of D × M (DM) and D × KK (DK) used for these experiments have been well-characterized at the genotypic and phenotypic levels (Pan *et al.*, 1994; Karsai *et al.*, 2005, 2006, 2007, 2008; von Zitzewitz *et al.*, 2005; Szűcs *et al.*, 2006).

Phenotypic characterizations

Controlled environment experiments were carried out in the Phytotron facilities of the Agricultural Research Institute of HAS, Martonvásár, Hungary, using CONVIRON PGV type growth chambers (CONVIRON Ltd., Winnipeg, Canada). The technical parameters of the growth chambers, including

light sources and control systems for temperature and light intensity, are detailed in Karsai *et al.* (2004). The individual effects of the following environmental factors were examined: two levels of light intensity at two photoperiod regimes; two levels of ambient temperature; and the effect of daily fluctuating vs. constant temperatures. The combinations of various environmental cues used for testing the developmental patterns of each population and for carrying out functional QTL analyses are listed in Table 1.

Genotypic characterizations

The DM linkage map consists of 165 loci of various types (e.g. AFLP, RFLP, SSR, STS and ASGTs (allele-specific gene tags)) with a total recombination length of 1040 cM and an average marker spacing of 6.3 cM (Pan *et al.*, 1994; Szűcs *et al.*, 2006). The DK linkage map consists of 236 loci of various types, with a total recombination length of 1107 cM and an average marker distance of 4.5 cM (Karsai *et al.*, 2005; Szűcs *et al.*, 2006; Karsai *et al.*, 2007). The *VRN-H1*,

VRN-H2 and *PPD-H1* loci were mapped, when possible with allele-specific primers in the DM and DK populations (von Zitzewitz *et al.*, 2005; Karsai *et al.*, 2005; Turner *et al.*, 2005). Linkage maps were constructed using JoinMap 4.0 (van Ooijen, 2006). QTL analyses were performed using composite interval mapping (CIM) Model 6, with forward regression and backward elimination as implemented in WinQTL Cartographer v. 2.5 (Wang *et al.*, 2007). Threshold levels were set using 500 permutations.

Results

Effect of *VRN-H1* on plant development in the absence of *VRN-H2*

In the Dicktoo × Morex population there is functional segregation in the *PPD-H1* locus, in addition to the *VRN-H1* functional polymorphism. These two loci were the major determinants of flowering in the various temperature treatments applied under long photoperiod (Table 2). Under

Table 1. Lists of environmental factors studied in the two barley mapping populations

Photoperiod (hours)	Temperature treatment (°C)	Light intensity $\mu\text{mol m}^{-2} \text{s}^{-1}$	DM	DK
12	18 constant	340		+
12	18 constant	170		+
16	18 constant	340		+
16	18 constant	220	+	+
16	18 constant	170		+
16	18/16 thermo-cycle	220	+	+
16	10 constant	220	+	

Table 2. Effects of the *VRN-H1* vernalization response and *PPD-H1* photoperiod sensitivity genes on flowering time in the Dicktoo × Morex barley mapping populations under various temperature treatments.

Temperature treatment	<i>VRN-H1</i>			<i>PPD-H1</i>		
	LOD	R^2	Add. eff.	LOD	R^2	Add. eff.
18 °C Constant	22.7	30.3	9	31.2	55.0	-12
18 °C Thermo-cycle	22.3	41.6	24	12.0	17.2	-13
10 °C Constant	8.7	17.1	7	18.6	47.6	-12

all conditions, the Dicktoo-type winter allele in the *VRN-H1* locus and the Morex-type insensitive allele in the *PPD-H1* locus significantly delayed plant development. Of the two genes, *VRN-H1* determined a greater proportion of the phenotypic variance at 18°C thermo-cycle and *PPD-H1* at 18°C constant and at 10°C constant temperature treatments. Compared with the 18°C constant temperature, the application of thermo-cycle increased the phenotypic effects of *VRN-H1*, while significantly decreased that of *PPD-H1* to a ratio of one-third. The low constant temperature of 10°C did not influence the effect of *PPD-H1* but significantly decreased the effect of the *VRN-H1* locus.

The combined effects of *VRN-H1* and *PPD-H1* explained most of the phenotypic variation in the experiments (two-locus R^2 values were 83.5%, 74.0%, and 83.1% in the 18°C constant, 18°C thermo-cycle, and 10°C constant treatments, respectively). As a result, the mean flowering times of lines with the parental allele combinations at the two loci were statistically the same as the respective parents under the two constant temperature treatments (18°C and 10°C) (Table 3). At 18°C thermo-cycle, however, the DD (*VRN-H1* / *PPD-H1*) lines headed significantly earlier than Dicktoo (84 vs. 128 days, respectively), while the MM lines were significantly later than Morex (66 vs. 54 days, respectively).

The non-parental allele combinations were responsible for the significant phenotypic transgressive segregation, which were apparent at each temperature treatment.

Lines with MD alleles at *VRN-H1* / *PPD-H1* headed significantly earlier, while lines with DM alleles at *VRN-H1* / *PPD-H1* headed significantly later than the parents and parental allele combinations. In addition, the reactions of the non-parental combinations to a sub-optimal temperature or thermo-cycle were significantly different from those of the parents or parental allele combinations. The MD (*VRN-H1* / *PPD-H1*) was the only subclass with a relatively uniform reaction to all the treatments, including the thermo-cycle, but its flowering was delayed to a great extent by the sub-optimal temperature. Conversely, the sub-optimal temperature had the least delaying effect on the flowering of the DM (*VRN-H1* / *PPD-H1*) subclass, but the largest scattering was observed in this subclass when the thermo-cycle was applied (Figure 1).

Effect of *VRN-H1* on plant development in the presence of *VRN-H2*

In the Dicktoo × Kompolti korai population there is only functional polymorphism in the *VRN-H2* gene, as all the lines carry the sensitive allele in the *PPD-H1* locus based on the SNP22 haplotype (Turner *et al.*, 2005).

Table 3. Flowering time values of the four *VRN-H1* / *PPD-H1* allele classes and the two parents at the various temperature treatments.

<i>VRN-H1/PPD-H1</i> allele combination	Flowering time at			% change from 18°C constant temperature to	
	18°C constant temperature	18°C thermo-cycle	10°C constant temperature	18°C thermo-cycle	10°C constant
Dicktoo	36	128	61	356	169
DD / DD	38	84	60	221	159
DD / MM	75	121	89	161	119
MM / DD	30	44	53	148	176
MM / MM	45	66	71	145	157
Morex	41	54	70	132	171

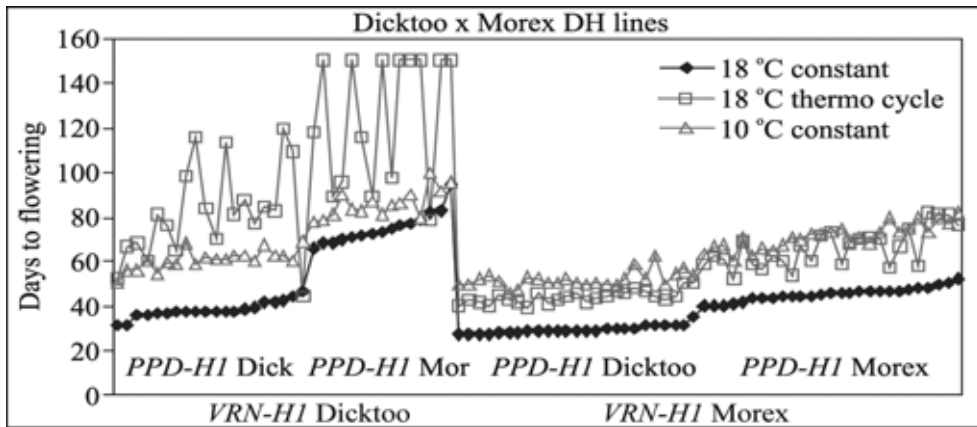


Figure 1. Effect of the sub-optimal temperature and the synchronous photo- and thermo-cycles on the major developmental genes in the Dicktoo (facultative) \times Morex (spring) barley population.

There was allelic variation at region of *VRN-H1* not known to affect phenotype and this variation allowed us to monitor allelic segregation at this locus.

When the role of the two *VRN* loci in flowering time was examined, it was found that photoperiod, light intensity, and the application of thermo-cycle influenced their activity (Table 3). When active, the presence of the *VRN-H2* gene and the Dicktoo-type winter allele at *VRN-H1* delayed plant development, irrespective of the environmental conditions.

Under a long photoperiod (16 h), *VRN-H2* explained the largest portion of the phenotypic variance, irrespective of the light intensity. The *VRN-H1* gene alone was only a significant though minor source of variance under high light intensity. The two genes together contributed more than 90% of the variance at both light intensities (R^2 high = 96.9%; R^2 low = 91.9%). Light intensity had the strongest effect on the *VRN-H* genes under the 12-hour photoperiod regime, which represents the borderline between long and short photoperiod regimes. While the effect of *VRN-H2* was highly significant under high light intensity, the activity of this gene could not be detected when low light intensity was applied. The effect of *VRN-H1*,

in contrast, was tripled at low light intensity. Thus under a 12-hour photoperiod, more than 50% of the phenotypic variance in the flowering time was explained by *VRN-H2* under high light intensity and by *VRN-H1* under low light intensity. The bi-locus effect was highly significant at both light intensities (R^2 high = 78.8%; R^2 low = 52.8%).

In comparing the effects of constant temperature and daily thermo-cycle, *VRN-H2* had a very large effect on flowering time at 18°C constant temperature, accounting for 64% of the phenotypic variance (Table 4). The *VRN-H1* locus had a significant effect under this condition, but it explained a very low portion of the phenotypic variance. The application of both light and thermo-cycles resulted in a shift in the significance of the effects of these two loci: at 16T *VRN-H1* explained close to 60% of the phenotypic variance and *VRN-H2* only 16%.

As the bi-locus effects of the two *VRN-H* genes contributed the highest proportion of the phenotypic variance under 16- and 12-hour photoperiod regimes, the flowering characteristics of the four possible allele combinations were compared (Figure 2). Under a long photoperiod, the light intensity did not influence the type or degree of interaction between the allele phases of the

Table 4. Effects of the *VRN-H1* and *VRN-H2* vernalization response genes on flowering time in the Dicktoo × Kompolti korai barley mapping populations under various environmental conditions.

Treatments	<i>VRN-H1</i>			<i>VRN-H2</i>		
	LOD	R^2	Add. eff.	LOD	R^2	Add. eff.
Photoperiod and light intensity treatments						
16 hours @ 340 $\mu\text{mol m}^{-2} \text{s}^{-1}$	5.1	2.0	3	32.8	89.6	-16
16 hours @ 170 $\mu\text{mol m}^{-2} \text{s}^{-1}$		ns		32.3	70.3	-14
12 hours @ 340 $\mu\text{mol m}^{-2} \text{s}^{-1}$	5.8	16.6	8	12.8	48.2	-14
12 hours @ 170 $\mu\text{mol m}^{-2} \text{s}^{-1}$	13.5	51.6	10		ns	
Temperature treatments						
18°C Constant	3.7	3.2	3	34.5	63.8	-12
18°C Thermo-cycle	24.6	49.5	12	11.0	15.5	-7

ns = not significant.

two *VRN-H* genes. The Kompolti allele in *VRN-H2* (presence of the gene) resulted in later flowering irrespective of the light intensity level applied, and this effect was not modified by the allele composition of the *VRN-H1* gene. The lack of the *VRN-H2* gene caused earlier flowering and made the effect of the allele composition of *VRN-H1* significant under both light intensities. Under a 12-hour photoperiod, however, the light intensity exerted a strong modifying effect on the interaction between the two *VRN-H* genes. At high light intensity the interaction between *VRN-H2* and *VRN-H1* was similar to that observed for the 16-hour photoperiod, except that the importance of the *VRN-H1* allele composition increased. At low light intensity level, the quantitative effect of the *VRN-H2* gene in repressing flowering diminished significantly. The presence or absence of the *VRN-H2* gene only influenced flowering when the Kompolti Korai allele was present in the *VRN-H1* gene. In the case of the Dicktoo *VRN-H1* allele, the *VRN-H2* gene had no apparent effect on flowering. When the effects of the two light intensities on flowering were compared under the 12-hour photoperiod regime, it became apparent that low light intensity only resulted in earlier flowering when the *VRN-H2* gene was

present. In this case, however, its effect was mostly independent of the allele composition of *VRN-H1*.

Similar environment dependent interaction between the *VRN-H2* and *VRN-H1* genes was apparent in the comparisons of constant vs. daily fluctuating temperatures. *VRN-H2* and *VRN-H1* jointly accounted for most of the phenotypic variation, irrespective of growth condition: the two-locus R^2 values were 0.83 for 18°C constant temperature and 0.69 for 18/16°C thermo-cycle. The average flowering times of lines with parental allele combinations at these two loci were statistically the same as respective parent under all the three conditions, with one exception. At 18°C constant temperature the average flowering of the DD lines was again significantly earlier than that of Dicktoo (86 vs. 109 days).

As shown in Figure 3, there is a pattern of growth condition-dependent epistasis between these two loci. Two features are noteworthy. First, the K allele at *VRN-H1* always resulted in significantly earlier flowering than the D allele, regardless of growth condition or allele phase of *VRN-H2*. Second, the winter allele (K) at *VRN-H2* delayed flowering, with one exception: under 18/16°C thermo-cycle, the D allele at

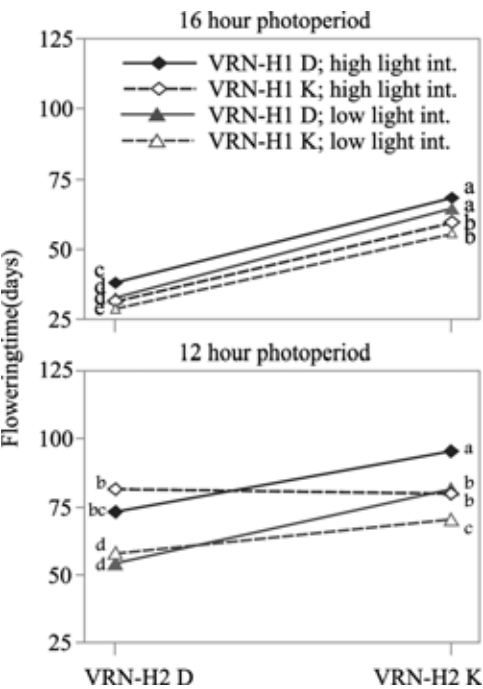


Figure 2. Effects of photoperiod (12, 16 h) and light intensity (high: $340 \mu\text{mol m}^{-2} \text{s}^{-1}$, low: $170 \mu\text{mol m}^{-2} \text{s}^{-1}$) on the association between the allele phases of the *VRN-H2* / *VRN-H1* genes in the Dicktoo (D) × Kompolti korai (K) barley population, measured in terms of flowering time. Within each photoperiod, data points labelled with the same letter were not significantly different from each other at the $P = 0.05$ level.)

VRN-H1 locus resulted in extremely delayed flowering irrespective of the allele phase at *VRN-H2*.

Discussion

Gene expression, QTL and segregating population studies proved that the presence or absence of the vernalization-critical region in the intron 1 of the *VRN-H1* gene basically determines the growth habit (von Zitzewitz *et al.*, 2005; Fu *et al.*, 2005; Karsai *et al.*, 2005; Kóti *et al.*, 2006; Szűcs *et al.*, 2007). The spring allele (deletion of the vernalization-

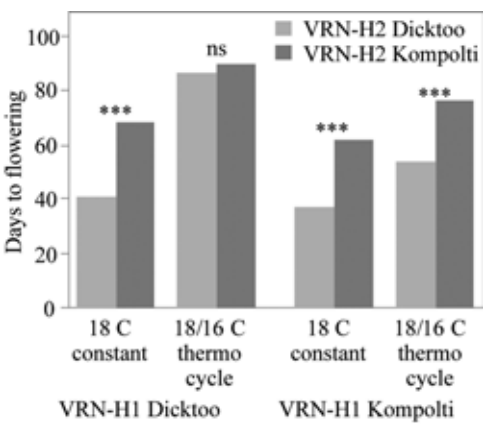


Figure 3. Effects of daily fluctuating environmental factors on the association between the allele phases of *VRN-H2* and *VRN-H1* genes in the Dicktoo (facultative) × Kompolti korai (winter) barley mapping population.

critical region) shows complete dominance over the winter allele (Yan *et al.*, 2004; Dubcovsky *et al.*, 2006; Kóti *et al.*, 2006; Szűcs *et al.*, 2007). Our results emphasize that, in addition to determining the growth habit, the *VRN-H1* gene also quantitatively influences the flowering time, and that the *VRN-H1* gene is also subject to regulation by environmental stimuli other than low-temperature vernalization. The site(s) of this additional regulation is partly different from that of the vernalization regulation site (Karsai *et al.*, 2005; Kóti *et al.*, 2006; Szűcs *et al.*, 2007). Photoperiod, low light intensity, the ambient temperature and the various combinations of daily fluctuating factors all produced modifying effects on the *VRN-H1* gene in an allele-specific way (von Zitzewitz *et al.*, 2005; Karsai, 2008). The dominant spring allele showed greater sensitivity to sub-optimal temperature, while the synchronous photo- and thermo-cycles had the strongest effect on the recessive winter allele. In addition, significant differences were identified between the reaction types of two recessive winter alleles from the facultative

Dicktoo and from the winter Kompolti korai, which were completely the same in the vernalization-critical region (von Zitzewitz *et al.*, 2005). This may be due to as yet uncharacterized functional polymorphisms in other regions of the 17 kb gene. The Dicktoo-type *VRN-H1* allele was more sensitive to the application of synchronous photo- and thermo-cycle than the Kompolti-type allele. In addition, low light intensity differentially influenced the activating effect of the two parental recessive alleles on flowering under an intermediate photoperiod regime.

The various environmental factors influenced not only the activity of the *VRN-H1* gene, but also its specific interactions with the allele types of the *PPD-H1* and *VRN-H2*. The non-parental allele combinations in the *PPD-H1* and *VRN-H1* were responsible for the significant phenotypic transgressive segregation resulting in the early and late flowering genotypes (Pan *et al.*, 1994; Karsai *et al.*, 1997). These combinations also showed specific reactions to the various environmental cues. The environmental-dependent allele interactions were the most characteristic under the sub-optimal temperature, and under the synchronous application of the photo- and thermo-cycle. There was also a pattern of growth condition-dependent epistasis between the *VRN-H2* and *VRN-H1* loci, which became evident under two growth conditions: applying low light intensity under an intermediate photoperiod regime, and the synchronous application of photo- and thermo-cycles.

In summary, the systematic phenotypic characterizations combined with functional QTL analyses proved to be efficient in identifying environmental-factor-dependent gene functions and allele interactions. Thus this approach produces valuable additional information for gene expression studies. The genetic dissection of the phenotypic changes via QTL analysis revealed novel effects and interactions of the barley *VRN* and *PPD* genes.

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Physiological mechanism and quantitative trait loci associated with waterlogging tolerance in barley

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Abstract

Waterlogging (WL) is a major environmental constraint severely limiting crop production worldwide. Breeding programs need to incorporate exotic germplasm and develop WL-tolerant barley varieties. A series of morphological, anatomical and physiological mechanisms contributing to WL tolerance were studied in detail. The results showed that the key features targeted by breeding should include both morphological (production of more adventitious roots and formation of larger aerenchyma area in adventitious roots), and physiological (high capability for O₂ uptake and K⁺ acquisition in plant roots) traits, as well as the plant's ability to withstand soil-borne phytotoxins. Foliar application of nutrients can be used in practice to alleviate the adverse effects of WL. To identify molecular markers associated with WL tolerance, two barley doubled-haploid populations were used to analyze QTLs of WL-tolerance-related traits. Seven QTLs for barley WL were detected and validated under different stress durations, between both different experiments and different populations. Some of these QTLs affected multiple WL-tolerance-related traits, for example, QTL Qwt4-1 contributed not only to reducing barley leaf chlorosis, but also to increasing plant biomass under WL

stress, whereas other QTLs controlled both leaf chlorosis and plant survival.

Introduction

Water logging (WL) is one of the major restrictions for barley production in high-rainfall areas. It causes chlorophyll, protein and RNA degradation, and a decrease in concentration of nutrients such as nitrogen, phosphorus, metal ions and minerals in barley shoots can occur rapidly after the onset of flooding and precede leaf chlorosis (Drew and Sisworo, 1977; Wang *et al.*, 1996). As a consequence, shoot and root growth, dry matter accumulation and final yield are all reduced (Huang *et al.*, 1994a, b; Malik *et al.*, 2002).

Barley cultivars differ in their WL tolerance. Barley collections from China, Japan and Korea contained many tolerant cultivars, while those from North Africa, Ethiopia and southwest Asia showed few tolerant cultivars (Takeda and Fukuyama, 1986). In our studies, some Chinese cultivars showed significantly better tolerance than Australian cultivars (Pang *et al.*, 2004, 2006, 2007a, b; Zhou *et al.*, 2007). Thus it is possible to breed for tolerance. However, WL tolerance is likely to be a complex trait affected by several mechanisms and complicated by confounding factors such

as temperature, plant development stage, nutrient status, soil type and sub-topography of the subsoil. It is critical to study the morphological, anatomical and physiological mechanisms contributing to WL tolerance to identify some useful selection criteria and molecular markers associated with WL tolerance. This paper presents a summary of physiological mechanisms associated with WL tolerance, and the identification of QTLs controlling WL tolerance in barley.

Physiological mechanisms for WL tolerance

Growth and physiological responses of barley genotypes to WL

Barley plant growth was adversely affected by WL. As WL stress developed, chlorophyll content, net CO₂ assimilation and maximal photochemical efficiency of PSII (Fv/Fm) decreased significantly. Among these characters, chlorophyll fluorescence of dark-adapted samples (Fv/Fm values) was found to be the most efficient screening parameter for future use in large-scale programs selecting for WL tolerance. Some Chinese cultivars, such as TX9425, were found to have much better tolerance to WL, which showed less reduction in plant growth, chlorophyll content, chlorophyll fluorescence and photosynthetic parameters. Naso Nijo (a Japanese cultivar) and Franklin (an Australian cultivar) were found to be the most susceptible to WL.

The formation of aerenchyma as an indicator of WL tolerance

Two barley cultivars with contrasting WL tolerance, TX9425 and Naso Nijo, were selected to study the changes in the morphological and anatomical characteristics under WL stress. Most seminal roots of both cultivars died under WL conditions, while adventitious roots were produced at the

shoot base. More adventitious roots were found in the WL-tolerant cultivar than in the susceptible one. In adventitious roots, cortical cell breakdown and the formation of air channels (aerenchyma) were observed from 0.5 cm behind the root tip and persisted along the entire adventitious root axis in both genotypes after 3 weeks of WL. No aerenchyma was found in the roots of drained plants. The percentage of aerenchyma to root cross-section area in TX9425 along the entire adventitious root was obviously larger than that in Naso Nijo. At about 2 cm from the root-shoot junction, the proportions of aerenchyma were 23.9% and 7.1% for TX9425 and Naso Nijo, respectively. This aerenchyma facilitated O₂ transportation from the aboveground parts into the submerged roots, increasing the plant's tolerance to WL.

Micro-electrode ion and O₂ flux measurements reveal differential sensitivity of barley root tissues to hypoxia

To understand the effects of WL on nutrient acquisition and potential involvement of plasma membrane ion transporters in WL tolerance in barley, the non-invasive microelectrode MIFE system was used to concurrently measure net O₂ and ion fluxes from the root surface. Oxygen deprivation caused the decline of O₂ uptake and had an immediate and substantial effect on root ion flux patterns. These effects differed between WL-sensitive and WL-tolerant cultivars. The O₂ uptake in the WL-tolerant cultivar TX9425 remained much higher than in the WL-sensitive cultivar Naso Nijo in the root mature zone under hypoxia stress. However, there was no significant difference in the root elongation zone between these two cultivars. In the mature zone, hypoxic treatment caused a very sharp decline in K⁺ uptake in Naso Nijo, but did not reduce K⁺ influx in the WL-tolerant TX9425 cultivar. In the elongation zone, onset of hypoxia enhanced K⁺ uptake from roots of both cultivars. Hypoxia

also caused qualitatively different effects on the activity of plasma membrane ion transporters in mature and elongation zones. Pharmacological experiments suggested that hypoxia-induced K^+ flux responses are likely to be mediated by both KIR and NSCC channels in the elongation zone. In the mature zone, KOR channels are the key contributors.

Ability to tolerate secondary metabolites associated with WL soil conditions is a useful trait for WL breeding in barley

WL stress is traditionally associated with O_2 depletion. Accordingly, breeding programs routinely target the plant's ability to tolerate O_2 deprivation or increase O_2 supply to roots, or both. However, significant accumulations of toxic substances from the microbial reduction processes have been widely reported in waterlogged soil. To understand the importance of these accumulations, the effects of several secondary metabolites (phenolic acids, monocarboxylic acids and Mn^{2+}) on nutrient (K^+ , H^+ and Ca^{2+}) acquisition of barley roots were investigated. In the WL-sensitive variety Naso Nijo, all three lower monocarboxylic acids (formic, acetic and propionic acids) and three phenolic acids (benzoic, 2-hydroxybenzoic, 4-hydroxybenzoic acids) caused a substantial shift towards steady K^+ efflux accompanied by immediate net influx of H^+ . Detrimental effects of secondary metabolites on K^+ homeostasis in root cells was absent in the WL-tolerant TX9425 variety. Root treatment with Mn^{2+} caused only temporary K^+ loss, which returned to the initial level 10 min after treatment. Plant roots showed different responses of ion fluxes and membrane potential to these chemicals in the long term (24 h), as 24-hour treatment with all chemicals significantly reduced the K^+ uptake, and the adverse effects of phenolic acids were smaller than those that arose from treatment with monocarboxylic acids and Mn^{2+} . Treatment with monocarboxylic

acids for 24 h reversed H^+ from net efflux to net influx. None of the three phenolic acids caused significant effects on H^+ flux compared with the control. Phenolic acids caused significant net Ca^{2+} efflux from roots pre-treated for 24 h (Pang *et al.*, 2007a).

Foliar spray of nutrient solution can significantly alleviate adverse effects of WL

In order to alleviate the adverse effects of WL, the possibility of using foliar nutrient sprays was investigated. Foliar application of full strength Hoagland solution significantly improved plant growth, reduced leaf chlorosis and increased chlorophyll content, photochemical efficiency of PSII, net CO_2 assimilation, and production of adventitious roots. N and K content also increased, not only in shoots but also in roots, suggesting the translocation of nutrient occurred from the shoot to root. This may be partly the reason for the greater production of adventitious roots in sprayed plants. Another contributing factor to the alleviation of the adverse effects of WL may be significantly higher amounts of auxin, accumulated in the shoot base of waterlogged plants (increased by 18.4%) after foliar nutrient application (Pang *et al.*, 2007b).

Identification of QTLs associated with WL tolerance

To identify QTLs associated with WL tolerance, leaf chlorosis under WL stress was chosen as the main indicator for WL tolerance. This trait was measured three times for each population across the two experimental years (2004 and 2005). Leaf chlorosis was measured as follows: the proportion of yellowing or chlorosis on each leaf was scored, and then the length of each leaf was measured to weigh the overall average proportion of chlorosis in each plant. The control plants of both populations in both years had no leaf chlorosis. The second

trait measured was plant biomass reduction after three weeks of WL treatments. The third measured trait was plant survival after eight weeks of WL.

QTLs identified in the population of Franklin × TX9425

Three QTLs (tfy1.1-1, tfy1.1-2 and tfy1.1-4) controlling leaf chlorosis after two-weeks of WL stress (2004) were identified. For all these QTLs, the Franklin alleles increased leaf chlorosis while the TX9425 alleles decreased it. One QTL (tfy1.2-1) was identified for leaf chlorosis after a four-week WL treatment. This is likely to be the same QTL as tfy1.1-2 as it was mapped to the same position and the Franklin allele also increased leaf chlorosis. Two QTLs (tfy2.1-1 and tfy2.1-2) were found for leaf chlorosis in the experiment carried out in 2005. QTL tfy2.1-1 is likely to be the same as tfy1.1-2 and tfy1.2-1 and again the Franklin alleles increased leaf chlorosis.

One QTL (tfmas) was identified for plant biomass reduction after three-weeks of WL stress. This QTL was mapped to chromosome 4H and explained 16.3% of the genetic variation for this trait. The Franklin allele leads to a greater reduction of plant biomass following WL.

Two QTLs (tfsur-1 and tfsur-2) were found for plant survival rate after eight weeks continuous WL stress. Both of these were located on chromosome 2H and explained 19% and 13.2% of the genetic variation for this trait, respectively. These QTLs were located onto different regions of chromosome 2H compared with the QTLs for leaf chlorosis. For the detected QTLs, the Franklin allele increased the survival rate of the plant at tfsur-1 locus, whereas TX9425 allele increased plant survival at the locus of tfsur-2.

QTLs identified in the population of Franklin × Yerong

Three QTLs (yfy1.1-1, yfy1.1-2 and yfy1.1-3) controlling leaf chlorosis after two-

weeks of WL stress (2004) were found on chromosome 3H, 2H and 5H. The Franklin alleles increased leaf chlorosis at two QTLs (yfy1.1-1 and yfy1.1-2), whereas at the yfy1.1-3 locus the Yerong allele increased leaf chlorosis. Three QTLs (yfy2.1-1, yfy2.1-2 and yfy2.1-3) were found for leaf chlorosis after two weeks of WL in the experiment carried out in 2005. These QTLs were located on chromosomes 7H, 3H and 4H. The Franklin alleles increased leaf chlorosis in all three cases. QTL yfy2.1-2 may be the same as yfy1.1-1, as both were located at a similar position on chromosome 3H. Three QTLs (yfy2.2-1, yfy2.2-2 and yfy2.2-3) were found for leaf chlorosis after four weeks of WL stress in the 2005 experiment, these QTLs were located on chromosomes 3H, 1H and 4H. The Franklin allele increased leaf chlorosis at yf2.2-1 and yf2.2-3 loci, whereas the Yerong allele did so at the yf2.2-2 locus. QTL yfy2.2-1 is likely to be the same as yfy2.1-2 as it is in an identical position on chromosome 3H. The same applies to QTL yfy2.1-1 and yfy2.2-3 on chromosome 4H.

One QTL (yfmas) was identified for the reduction of plant biomass following WL in this population. This QTL explained 8.2% of the genetic variation for this trait and was mapped on chromosome 4H to almost the same position as QTL yfy 2.2-3 and yfy2.1-1 and is probably due to pleiotropy.

Two QTLs (yfsur-1 and yfsur-2) were identified for plant survival rate after 8 weeks of continuous WL stress. They were located on chromosomes 2H and 5H. The Yerong allele increased plant survival rate at the yfsur-1 locus while the Franklin allele increased plant survival rate at the yfsur-2 locus. Yfsur-1 was located near yfy1.1-2, while yfsur-2 was located near yfy1.1-3.

Comparison of identified QTLs between populations.

In order to compare the QTLs identified in different populations, a consensus map was constructed using four barley DH

populations, including the two populations used in this study. Comparison of the QTLs identified between the two populations (Figure 1) showed that many of the QTLs identified in Franklin \times TX9425 mapped to similar chromosomal regions compared with those identified in Franklin \times Yerong (such as QTLs identified on chromosomes 1H, 3H, and 7H), or were mapped to a very close location (QTLs identified on chromosomes 2H and 4H) with distances of less than 10 cM between QTL maxima (Figure 1).

Overall, this study suggests that substantial genetic potential exists to improve WL tolerance in barley. Key features targeted by breeding should include morphological (production of more adventitious roots and

formation of larger aerenchyma area in adventitious roots) and physiological (high ability of O_2 uptake and K^+ acquisition in plant roots) traits, as well as the plant's ability to withstand soil-borne phytotoxins. Foliar application of nutrient can be used in practice to alleviate the adverse effects of WL. At least seven QTLs for barley WL were detected and validated under different stress durations, between different experiments and different populations. Some of these QTLs affected multiple WL-tolerance-related traits; for example, QTL Qwt4-1 contributed not only to reducing barley leaf chlorosis, but also to increasing plant biomass under WL stress, whereas other QTLs controlled both leaf chlorosis and plant survival.

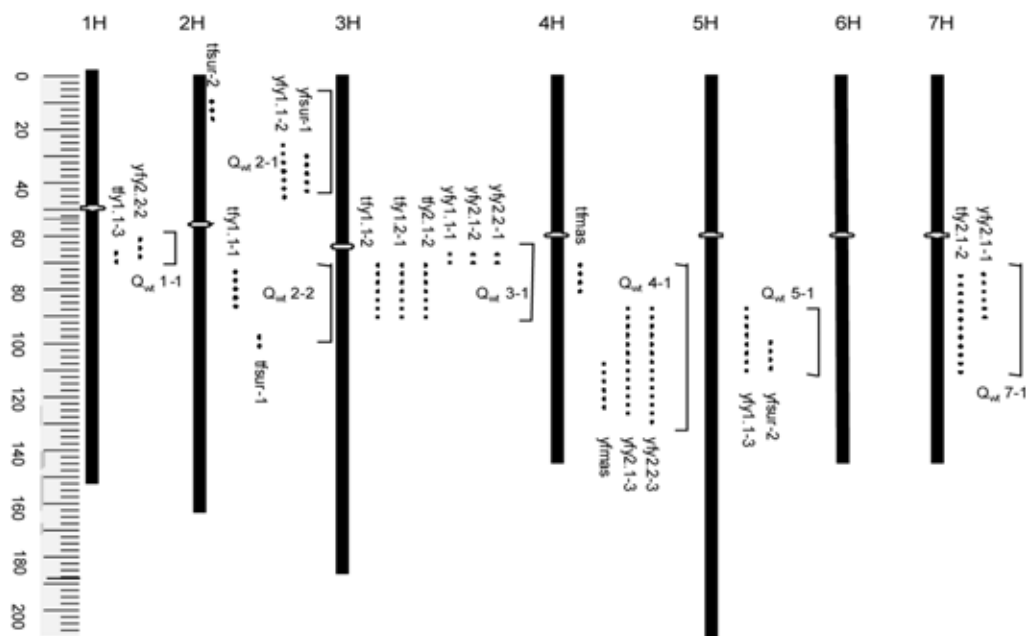


Figure 1. Comparison of quantitative trait loci (QTLs) identified for WL tolerance in two different barley DH populations: tf = Franklin \times TX9425; yf = Franklin \times Yerong. A general name (such as Qwt11-) was given to each chromosome region that was associated with different WL-tolerance-related traits, the first number is the chromosome number, the second number is the serial number of regions identified on that chromosome

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Barley adaptation to Mediterranean conditions: lessons learned from the Spanish landraces

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Abstract

Barley evolved as a crop under a Mediterranean climate. Therefore, it should be equipped with the genetic components necessary to cope with the hardships and constraints of Mediterranean environments. The study of old landraces, well adapted to their environments, should improve our knowledge of the basis of adaptation, and could deliver some useful lessons for barley breeders.

The Spanish Barley Core Collection (SBCC) is a representative sample of inbred lines derived from landraces. This collection is composed mainly of two large populations of individuals. Most likely, these populations correspond to the entry of two different sets of barley ancestors into the Iberian Peninsula, followed by only partial admixture. Their distribution across the range of local climates indicates differential adaptation of these populations to prevailing environmental factors. The study of the main vernalization and photoperiod response genes in these

materials provides some clues to explain, at least partially, differential adaptation patterns found in the landraces.

The conclusions being drawn from the study of the SBCC reveal its usefulness to contribute valuable traits, and are relevant for the management of this germplasm in breeding programs.

Introduction

During the 20th century, barley producers in Europe chose to substitute their landraces or primitive cultivars with newly bred cultivars. These new materials were readily adopted because they were specifically improved for the challenges of modern agriculture and consumer demands. Old landraces were gradually abandoned, and are currently kept mostly in germplasm banks. Therefore, although barley is a highly diverse species, much of the genetic variation existing in primitive landraces was abandoned in Europe following the advent of modern plant breeding (Fischbeck, 2003).

Nevertheless, some landraces and old cultivars persisted in some areas in Europe usually considered marginal from an agronomic point of view. The instability of modern varieties in these zones was the main reason for the maintenance of the more stable landraces. Old cultivar Albacete, a straight selection out of a landrace, continues to be quite popular in Spain. In the semi-arid region of Aragón, it occupied 69% of the area devoted to 6-row barley in 2002, and still close to 40% in 2004 (DGA, 2004, latest year available). The greater stability of local varieties of cereals, especially under unfavorable environmental conditions, is a recurrent motif in plant breeding literature (for instance, Ceccarelli and Grando, 1991; Ceccarelli *et al.*, 1991; van Oosterom *et al.*, 1993; Voltas *et al.*, 1999). Spanish barleys have long been known as a distinct plant genetic resource. The genetic singularity of the Spanish barleys was described by Tolbert *et al.* (1979), Moralejo *et al.* (1994), Lasa *et al.* (2001), and especially by Yahiaoui *et al.* (2008). This genetic distinctiveness may be related to the presence of adaptation traits specific to Mediterranean conditions, with potential application in breeding.

The Spanish barley landraces have not been widely used in modern breeding. Some of the reasons for this oversight are an insufficient effort on phenotyping, the clear presence of undesirable agronomic traits, together with a lack of knowledge on their genetic control. In Spain, the National Centre for Phytogenetic Resources holds a collection of over 2000 accessions of cultivated barley. Most are native landraces, collected in the first half of the 20th century (Lasa *et al.*, 2001). Given their history of selection under Mediterranean conditions, probably for a long period (as barley cultivation in Spain dates back to prehistoric times), they may harbour adaptive genes and alleles that may have escaped mainstream breeding. The evaluation of this wealth of genetic resources for their use in cultivar development is a sensible

pre-breeding goal. The Spanish Barley Core Collection (SBCC) was assembled as a tool to study the large local genetic diversity of this crop (Igartua *et al.*, 1998). It comprises a set of inbred lines derived from Spanish landraces, representative of the accessions stored at the Phytogenetic Resources Centre (CRF-INIA), in Madrid.

This collection was the result of a collective effort between INIA, CSIC, IRTA-UdL and ITACyL, and this report is a brief summary of the most relevant findings that might have a future impact in barley breeding.

SBCC was assembled as 159 inbred lines derived from landraces (148 6-row; 11 2-row), mostly collected in the first half of the 20th century, prior to introduction of modern cultivars, representing all barley growing regions of the country in a proportional manner. Also, 16 cultivars widely grown in Spain for long periods during the 20th century were included in the SBCC (Igartua *et al.*, 1998).

This collection has been characterized for over 30 agronomic, quality and morphological traits. Field trials were carried out at several Spanish locations over three seasons (a total of ten trials), to provide a thorough characterization of the agronomic characteristics of these accessions. Ten modern cultivars were also included in these field trials, as checks.

The agronomic evaluation revealed the causes that led farmers to abandon, in most cases, the cultivation of landraces. Most of the entries were too tall (Figure 1) and prone to lodging.

Though barley dryland agriculture in Spain does not make use of large amounts of agrochemicals, possibly the amount of fertilizers used is enough to provoke excessive vegetative growth in landraces when water is not too limiting, and thus lodging may occur quite often. Other negative characteristics of a majority of Spanish accessions were poor quality traits (small grain and high protein content), and lateness.

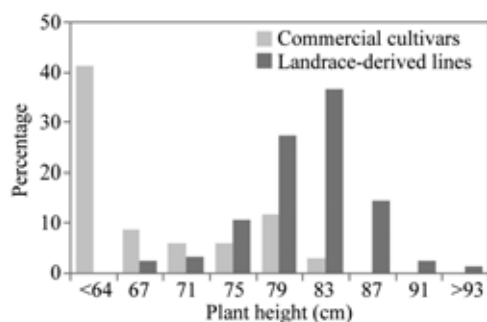


Figure 1. Distribution of plant height averages (in cm up to the base of the spike) for landrace-derived lines of the SBCC and a set of commercial cultivars, evaluated in field trials across Spain for three seasons.

On the positive side, there seemed to be ample genetic variability for all traits measured, and some traits revealed better characteristics than expected in landrace-derived material. The most striking result was the similar grain yield production of landrace-derived lines, old cultivars (the 16 included in the SBCC) and new cultivars (the 10 checks), in low-productivity trials (Figure 2). Field trials were split into two subsets, corresponding to high and low productivity, with the grain yield threshold arbitrarily established at 2500 kg/ha. These two subsets comprised five trials each, which did not follow geographical patterns.

At high productivity level, the effect of plant breeding is evident, as there is a gradual increase of mean and maximum grain yield from landrace material to old cultivars and then again to modern cultivars. In low productivity trials, however, there was no gain at all, and even the maximum yield was achieved by landrace-derived lines. Several of these lines out-yielded even the best check cultivars across low productivity sites. This observation confirms the findings of long running research at ICARDA by Ceccarelli and Grando and co-workers (Ceccarelli and Grando, 1991; Ceccarelli *et al.*, 1991, 1992; Ceccarelli, 1996), who found that local

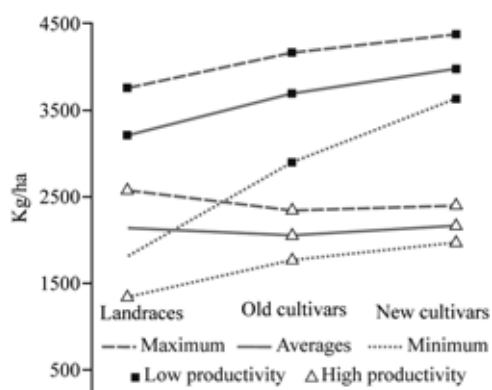


Figure 2. Grain yield averages, maximum and minimum, at 10 field trials, divided into high- and low-production environments, for three sets of genotypes: landrace-derived inbred lines of Spanish origin, old cultivars widely grown in Spain in the past, and new cultivars.

landraces from the ICARDA mandate region frequently out-yielded modern cultivars in low productivity environments.

The genetic diversity of Spanish barleys was compared with the diversity present in sets of mostly European cultivars (Table 1). These sets were assembled to include cultivars that contributed most in the pedigree of current cultivars. These results are reported in detail in Yahiaoui *et al.* (2008). It turned out that the Spanish barleys hold a large amount of diversity, when compared with other collections of similar size. They also present a high proportion of unique alleles, not present in the sets of European cultivars. Some of these unique alleles were present in a large proportion of the Spanish barleys, suggesting either a common ancient origin of these barleys, or a selective advantage of some loci which resulted in fixation of particular alleles. Both hypotheses are intriguing and deserve further investigation, as they may affect the management of this germplasm in breeding programs.

The homogeneity of the SBCC was investigated by means of a cluster analysis

Table 1. Summary of diversity analysis of the SBCC landrace-derived lines, compared with two sets of reference cultivars of mostly European origin.

Results for 64 SSRs	SBCC Spanish 6-row	Reference 6-row	SBCC Spanish 2-row	Reference 2-row
Number of accessions	152	33	11	29
Total number of alleles	591	383	249	272
Number of unique alleles	184	21	11	12
Average HT	0.62	0.58	0.54	0.53

Table 2. Means of several agronomic traits for the populations of Spanish landrace-derived lines (I-IV) and three groups of cultivars, evaluated at 10 field trials in Spain over three years.

Groups (n)	Heading date (days)	Plant height (cm)	Powdery mildew (score)	Test weighth (kg/hl)	Grain yield low prod. (q/ha)	Grain yield high prod. (q/ha)
I (17)	122.9	a 85.4	a 4.6	b 65.5	d 18.86	b 28.8
II (9)	118.5	b 73.7	c 4.5	b 69.3	b 18.90	b 30.4
III (48)	118.8	b 81.4	b 5.9	a 62.7	f 21.63	a 31.4
IV (82)	116.9	c 80.8	b 4.2	b 63.6	e 22.08	a 33.3
Old 2-row cultivars (8)	112.5	e 63.0	d 2.9	c 72.1	a 18.97	b 36.5
Old 6-row cultivars (8)	114.2	d 74.1	c 3.9	b 67.0	c 21.59	a 36.6
New cultivars (10)	114.7	d 59.8	e 2.5	c 69.3	b 21.84	a 40.0

Means followed by different letters are significantly different at $P \leq 0.05$.

using the “STRUCTURE” software (Falush *et al.*, 2003). After this analysis, four populations were clearly evident (Yahiaoui *et al.*, 2008): 6-row cultivars of European origin, together with a few Spanish landrace-derived lines; 2-row cultivars of European origin, together with most 2-row Spanish landrace-derived inbred lines; and 2 large populations made up exclusively of Spanish landrace-derived 6-row lines. This result was unexpected, as there was no prior report on the existence of different groups of Spanish 6-row barleys, and triggered many new questions. Did these populations have the same origin and diverged locally? Or did they come into the Iberian Peninsula at different times, and there was not enough time for total admixture? Do they present different agronomic and adaptive characteristics?

The agronomic evaluation already reported in Figure 2 showed significant differences

among the Spanish landrace-derived lines, differences that were accounted for to some extent by their ascription to the four populations found in the “STRUCTURE” analysis (Table 2). The differences in agronomic traits were also evident between the landrace-derived lines and the groups of cultivars. To illustrate this point, just a few traits have been summarized in Table 2. The two main populations of Spanish materials (III and IV) presented differences for traits that may bear adaptive relevance, such as heading date or powdery mildew resistance score. Population IV was the most productive among the landrace-derived material in high productivity trials, but well below levels of old and new commercial cultivars. Under low productivity levels, however, both most abundant populations presented grain yield means that differed from the cultivar groups. One conclusion of these evaluations is that

Table 3. Means and standard deviations of geographical and climatic factors for the landrace-derived inbred lines of the SBCC of the four populations, deduced from the population structure analyses.

Population (no. of lines)	Altitude (m)			Latitude (degrees N)			Temperature (°C)		
	Mean	SD		Mean	SD		Mean	SD	
I (18)	689	a	337	40.8	a	1.7	14.6	b	2.5
II (9)	817	a	411	41.6	a	0.7	13.7	b	2.3
III (50)	757	a	219	40.6	a	1.1	14.9	b	2.0
IV (82)	490	b	329	38.9	b	2.8	16.8	a	2.3

Means followed by different letters are significantly different at $P \leq 0.05$.

the four genetically distinct populations found in Spanish barleys differ also in agronomic terms.

Where did these populations come from? We did some statistics on passport and climatic data of the places of collection (Yahiaoui *et al.*, 2008). There were few differences between the places of collection for the accessions belonging to populations I, II and III (Table 3), but population IV presented clearly distinct values from the other three regarding altitude, latitude (lower for both), and mean temperature (higher). Therefore, it seems that the two main populations of landrace barleys in Spain were grown in quite different environments, with population IV coming from southern and coastal regions, with warmer temperatures, and populations I to III coming from more northern, inland, higher elevation regions with colder temperatures.

The STS marker MWG699 is closely linked (0.1 cM) to the *vrs1* locus, and it has been proposed as a marker of domestication in barley (Komatsuda *et al.*, 1999; Tanno *et al.*, 2002). The *vrs1* locus controls the formation, development and fertility of lateral spikelets of barley. The fertility of the lateral spikelets is one of the diagnostic genetic traits for the domestication process of barley. We tested this marker in the SBCC and found that its haplotypes were not evenly distributed over the collection. We found the two haplotypes (A and D) formerly found in 6-row barleys, but with a large proportion of

the rarer haplotype D. Also the geographical distribution of haplotypes A and D showed differences (Casas *et al.*, 2005), which apparently matched the geographical distribution of populations III and IV. A two-way table crossing MWG699 with population assignment revealed that the D haplotype was predominant in population III, whereas population IV had both haplotypes in large frequencies (Table 4).

A hypothesis that may explain this would be the arrival of different barley germplasm into the Iberian Peninsula, each one carrying different MWG699 haplotypes, which have gone through a process of partial admixture over the years, without erasing the boundaries between the populations. Why has this admixture not been complete?

Table 4. Frequencies of haplotypes for marker MWG699 across the populations of Spanish landrace-derived lines, established through cluster analysis of genetic similarities using the “STRUCTURE” software suite.

Population	MWG699 haplotype			Total
	A	D	K	
I	7	11	–	18
II	–	1	8	9
III	9	40	1	50
IV	47	35	–	82
Total	63	87	9	159

One of the critical phenomena shaping the life cycle of small-grain cereals is the reaction to low temperatures. In agronomy, cultivars are classified as winter if they have a vernalization requirement to reach flowering without delay, or spring if they lack this requirement. In barley, this trait is apparently controlled by three loci, *VRN-H1*, *VRN-H2* and *VRN-H3* (for a review of these issues, see Cockram *et al.*, 2007a, and Distelfeld *et al.*, 2009). The polymorphism at the first intron of *VRN-H1* has been identified as responsible for phenotypic variation in vernalization requirement (von Zitzewitz *et al.*, 2005).

According to the classification of Cockram *et al.* (2007b), most of the Spanish barleys fall in the categories 5B+Z and 5C+Z (Casas *et al.*, 2006). Though Cockram *et al.* (2007b) classify the former as spring and the latter as winter, we hypothesize that polymorphism at *VRN-H1* causes a gradation of vernalization requirements, and that this is one of the main tools of small-grain cereals to adapt to areas of different winter temperatures. For 5B+Z and 5C+Z, we believe that both require some vernalization, and that their distribution matches the harshness of winters in Spain (Figure 3). Cold winters regions (*Triticum* and *Avena*) present mostly landraces with haplotype 5C (4850 bp), whereas regions with Citrus or Tropical winters present a majority of accessions with haplotype 5B (1200 bp).

The SBCC (and a number of cultivars as checks) were tested under controlled conditions in the greenhouse, to find the responses to photoperiod and cold temperatures. A great majority of landrace-derived lines showed some vernalization effect when comparing the duration of cycle until flowering (or its surrogate measure, the number of leaves) with or without vernalization (under long days). When these results were split by *VRN-H1* type, the result was clear-cut (Figure 4): the lines with spring type *VRN-H1* (150 or 1900 bp) presented almost no response to vernalization. Lines

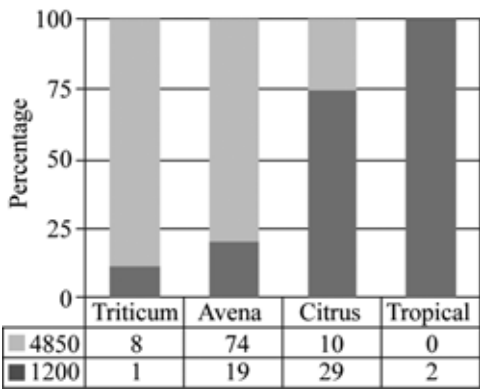


Figure 3. Percentage of Spanish landrace-derived lines described by their polymorphism in HvBM5A (size of the 1st intron of *VRN-H1*, 4850 bp or 1200 bp), broken-down by winter type of their collection sites. *Triticum* winter is the coldest one, Tropical the warmest. The 4850 bp fragment corresponds to haplotype 5C, and the 1200 bp fragment to 5B of those reported by Cockram *et al.* (2007b), respectively (see text).

with haplotype 5B (1200 bp) presented an average of around 2.19 leaves of vernalization effect, whereas lines with haplotype 5C (4850 bp) presented an effect close to 5 leaves (4.65). The distributions for the three types represented had little overlap.

To summarize, the populations of Spanish barleys discovered with a set of SSR seemed driven mainly by the presence of two distinct populations of barley, carrying different events at MWG699. Also, there is polymorphism at *VRN-H1* that affects the vernalization requirement of these landrace-derived lines. And the geographical distribution of this polymorphism matches the harshness of the winters of the collection sites. Finally, as shown in Table 5, MWG699 and *HvBM5A* (marker for *VRN-H1*) presented a rather high linkage disequilibrium ($R^2 = 0.24$). This linkage disequilibrium (LD) was larger than the LD found for all possible pairs of markers in a set of 64 SSR. There seems

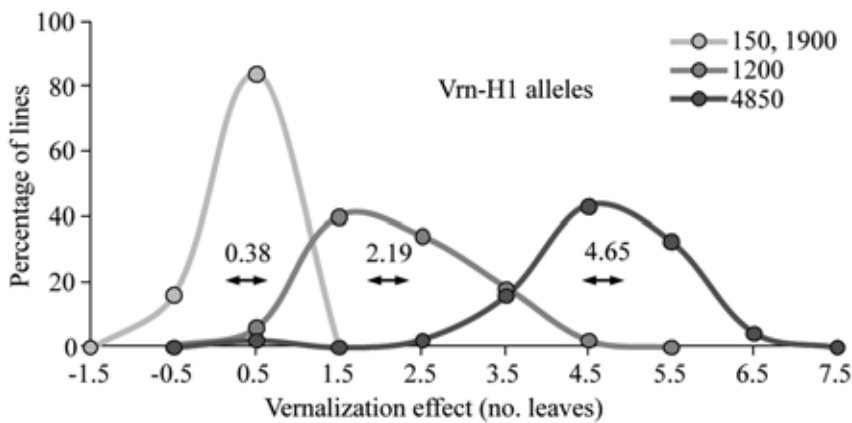


Figure 4. Vernalization effect, expressed as the difference in number of leaves produced in the main stem, between a treatment without vernalization and long days, and a treatment with vernalization and long days. The three curves represent the distribution of lines with different *VRN-H1* alleles.

to be a relationship between germplasm origin, vernalization requirement and barley adaptation in the Iberian Peninsula.

What about the other major genes that control vernalization and daylength responses? *PpdH1* and *PpdH2* are almost fixed in Spanish landraces, but *VRN-H3* is highly polymorphic. This gene has spurred a lot of interest recently. In barley, expression of its candidate gene *HvFT1* is induced by long-day conditions and promotes flowering (Hemming *et al.*, 2008). *HvFT1* alleles have

polymorphisms in the first intron. Yan *et al.* (2006) identified two SNPs that correlated with phenotypic responses, and suggested that these regions may play an important role in the regulation of the gene. The polymorphism at this gene is described as spring or winter, according to the prevalence of each SNP haplotype in these type of cultivars (Yan *et al.*, 2006). When we analyzed the distribution of *VRN-H3* polymorphism in the SBCC, the result was quite striking. The geographical distribution of this polymorphism was

Table 5. Distribution of absolute frequencies for markers MWG699 (closely linked to *vrs1*) and *HvBM5A* (perfect marker for *VRN-H1*), across climate types of Spain.

Climate	MWG699	A		D		K	
	<i>HvBM5A</i>	1200	4850	1200	4850	1200	4850
Mediterranean Tropical		1		1			
Mediterranean Maritime		11	1	1	2		
Mediterranean Subtropical		20	5	1	11		
Mediterranean Continental		3	4	2	20		
Mediterranean Temperate		5	4	2	21		
Mediterranean Fresh Temperate			7	1	16	1	1
Temperate Warm					1		
Patagonian Humid		2			1		
Total		42	21	8	72	1	1

the most extreme of all markers studied (a complete set of DArT markers, data not shown) according to a self-correlation analysis (Figure 5). This distribution was certainly latitudinal, and suggests a role of *VRN-H3* in adaptation through response to daylength. As most of these lines present the responsive allele at *PpdH1*, we can speculate that it provides a further mechanism to fine tune the response of barley to daylength.

Finally, we have explored the possibility of performing association mapping with the SBCC. The LD was lower than that usually reported for commercial cultivars, as expected, but it was still noticeable at distances between 2 and 3 cM (Figure 6). This fact suggests that association mapping is feasible, provided genetic structure is controlled to avoid an excess of false-positive associations. Actually, the correspondence between preliminary association mapping and results published in the literature is quite good for flowering time loci, and for disease resistance (Igartua *et al.*, 2006). In a work recently submitted for publication (Silvar *et al.*, 2010), we found a large powdery mildew resistance QTL in chromosome 7H in a mapping population derived from a resistant

accession from the SBCC. An association study with just 64 SSR found the strongest, and significant, association of any marker with powdery mildew resistance, for a marker in the same region (Igartua *et al.*, 2006). Thus, it seems possible to identify true associations in the SBCC, with good marker coverage. Current marker systems such as Illumina oligo pool assay (Rostoks *et al.*, 2006) and DArTs (Wenzl *et al.*, 2004) provide enough coverage to be optimistic about the potential of finding relevant new genes or alleles for barley breeding in the SBCC.

Conclusions

The main conclusions of this study, listed in chronological order as they were being gleaned from the experiments summarized in this report, are the following:

1. Potential to improve agronomic traits still present in Spanish landraces.
2. Spanish landraces hold greater genetic diversity than European reference cultivars.
3. Not all Spanish landraces are the same, neither genetically nor agronomically.
4. The genetic diversity distribution follows geographical patterns.

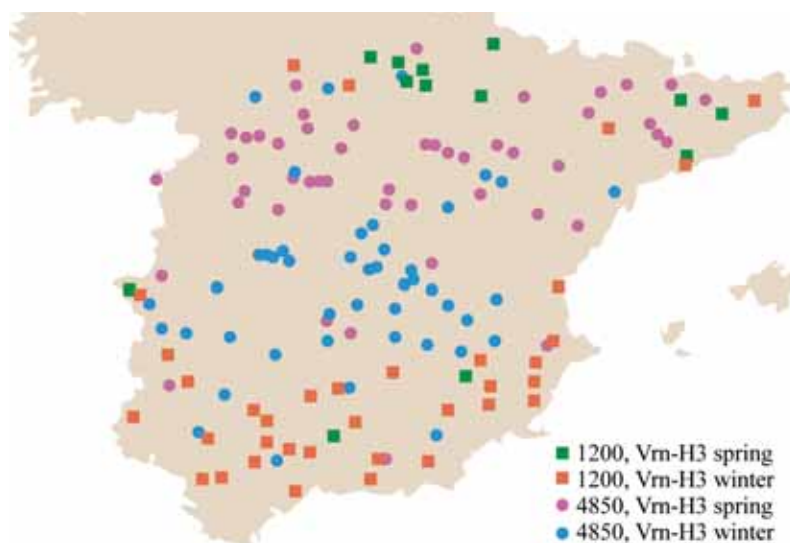


Figure 5. Distribution of *VRN-H3* and *VRN-H1* haplotypes over Spain.

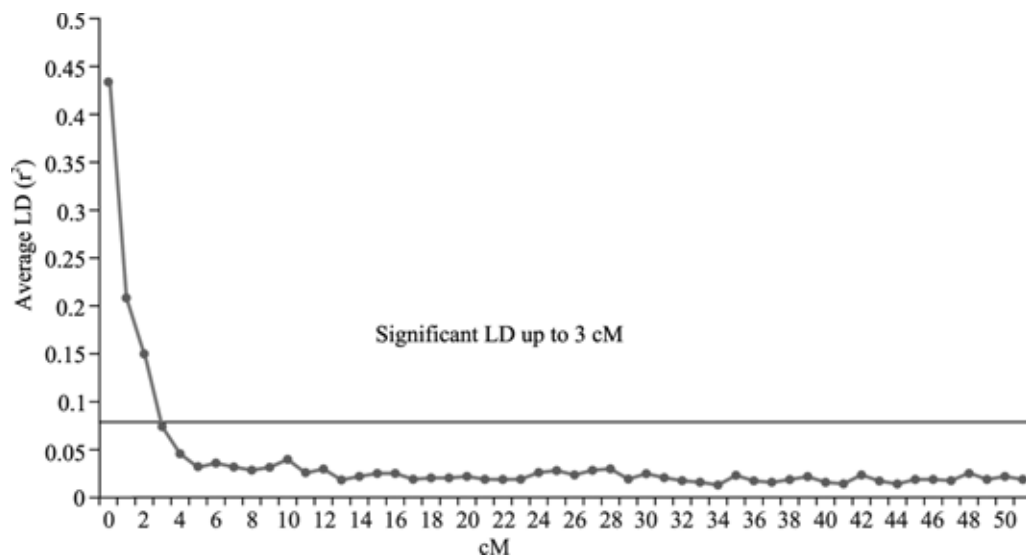


Figure 6. Average linkage disequilibrium between markers in the same chromosome in the SBCC. Calculations were made in 1 cM intervals, for a set of over 700 DArT markers.

5. Current populations probably derive from founder populations coming into the Peninsula at different times, still only partially admixed.
6. Functional diversity at *VRN-H1* may be responsible for adaptation to winter temperatures.
7. Selection for adaptation to winter temperatures may have shaped genetic diversity of Spanish barleys.
8. The SBCC is a suitable laboratory for the study of crop adaptation.
9. Association mapping in the SBCC is feasible, given levels of LD found and current marker availability for barley.

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Introgression of boron tolerance genes from Sahara 3771 into two-row barley: impact on grain yield and malt quality

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Abstract

Toxic levels of boron (B) can seriously diminish grain yield in cereal crops by affecting root growth, and thus restricting water extraction from the subsoil. Amelioration of high B concentrations in soils is expensive and not always feasible, so breeding for boron tolerance is the most viable alternative. This article reports the marker-assisted (MAS) transfer of favorable alleles from an unadapted 6-row barley (*Hordeum vulgare* L.) variety, Sahara 3771, into 2-row lines adapted to southern Australia. During the backcrossing process, the SSR marker EBmac679 located on chromosome 4H was used to control the target region in foreground selection, but no background selection was applied. Gene introgression was confirmed with 40 BC₆F₁-derived doubled-haploid lines segregating for the SSR marker EBmac679. We used a combination of molecular and conventional assays to unequivocally classify the 40 BC₆F₁-derived DH lines as B tolerant or sensitive, and then compared their means in a balanced ANOVA for grain yield measured over two years and four locations. Results showed very modest and inconsistent improvements in grain yield of lines carrying B tolerance genes in boron toxic environments. Based on DArT polymorphisms at 700 loci, the BC₆F₁-derived lines were found to have retained approximately 3.8% of the Sahara 3771 genome, but there was no correlation between donor genome content and grain

yield at any of the sites or years, either on a whole-genome basis or at the chromosomal level. We concluded that less-than-expected improvements in grain yield might not be due to linkage drag. Our results also showed no significant negative impact of gene introgression on the existing malting quality profile. This is important because it suggests that boron tolerance from Sahara 3771 has been incorporated in two-row barley germplasm without any penalty on quality.

Introduction

High levels of boron (B) occurring on the alkaline soils can seriously diminish yields by affecting root growth, and thus restricting water extraction from the subsoil. Amelioration of the high soil concentrations is expensive and not always feasible, so breeding programs targeting subsoil constraints in these regions currently include B tolerance as a desirable trait.

In barley (*Hordeum vulgare* L.), breeding for tolerance to B toxicity is greatly aided by the work of Jefferies *et al.* (1999) in the identification of specific tolerance traits, a range of methods that can be used to screen breeding populations, and associated molecular markers. The source of desirable alleles conferring B tolerance is Sahara 3771, an unadapted six-row landrace variety from North Africa. It is known to exhibit high levels of tolerance to B toxicity by extrusion of B from the root (Hayes and Reid, 2004), resulting in lower concentrations in roots and shoots.

Gene introgression from exotic germplasm can result in undesirable progeny, either due to linkage drag or pleiotropic effects associated with the introgressed novel alleles. Marker-assisted (MAS) backcrossing is an ideal approach, as it can greatly facilitate the transfer of targeted chromosomal segments while circumventing many of the difficulties inherent in using conventional breeding techniques. We used MAS backcrossing to transfer B tolerance from Sahara 3771 into an adapted breeding line (VB9104+), and in the process developed a population of backcross (BC_6F_1)-derived doubled-haploid (DH) lines differing in the presence or absence of a QTL associated with boron tolerance. Our objectives in the present study were to confirm the QTL effect using this independent population and to characterize possible pleiotropic effects on grain yield and malt quality.

Materials and methods

Parent materials and crossing scheme

VB9743 was developed from the backcross (Sahara 3771 \times WI2723) \times Chebec, by selecting progeny with a high level of B tolerance and cereal cyst nematode (CCN) resistance. VB9743 was crossed to an adapted line, VB9104 (Europa \times IBON#7.148) and the F_1 backcrossed twice to VB9104, to develop VB9104+, an adapted germplasm with B tolerance and CCN resistance. VB9104+ was crossed with doubled-haploid (DH) lines derived from simple crosses involving Chebec \times Harrington and Franklin \times Morex, a strategy designed to introgress favorable malting quality alleles from Canadian (Harrington), North American (Morex) and Australian (Franklin) sources.

F_1 progenies from the inter-cross were selected using markers for the presence of all desired marker alleles for the malting quality traits, B tolerance and CCN resistance traits, and used as donor parents in three

further backcrosses to VB9104+. During the backcrossing process, we used the SSR marker EBmac679 (Karakousis *et al.*, 2003) to control transfer of B tolerance QTL on chromosome 4H, but no background selection was applied. Given that the final cross in the backcrossing program involved VB9104 rather than VB9104+, the subsequent derived population was segregating for the SSR marker EBmac679 linked to the B tolerance QTL on chromosome 4H derived from Sahara 3771. From the 19 resulting final backcross F_1 plants, 758 DH lines were produced. The number of DHs created from each of the selected final backcross F_1 plants varied from 2 to 109.

Selection using markers reduced the population of 758 to 97, of which seeds of 81 were successfully multiplied to provide seed for field trials. All 81 lines possessed favorable alleles at malting quality QTLs, but it was not possible to select for CCN resistance using marker alleles due to a lack of polymorphism with the marker panel used between VB9104+ (CCN resistant) and VB9104 (CCN susceptible). A CCN resistance bioassay was subsequently used to identify a subset of 40 DH lines from the population of 81, which possessed CCN resistance and were progressed to field assessment.

Boron tolerance assessment

We conducted two separate bio-assays for B tolerance to confirm effects of transferred genes in the new genetic background. First, we used the filter paper assay described by Chantachume *et al.* (1995) to measure root growth of seedlings grown on filter papers soaked in boron-toxic versus control solutions. Since high levels of tolerance to B toxicity in Sahara 3771 is due to its ability to accumulate less B in the shoot (Hayes and Reid, 2004), we sought further confirmation of gene transfer by comparing shoot B concentration in a soil-based assay. Two genotypes (ABBD03-015*4 and ABBD03-

015*20), selected for the presence or absence of Sahara 3771 alleles at the EBmac679 locus on 4H, but possessing maximum similarity at all other background genetic loci (facilitated by graphical phenotyping), were tested in a replicated pot experiment with varying levels of applied B. The different levels were created by adding boric acid at different rates to a soil mixture consisting of an equal mix of river sand and a standard soil mixture containing basal dressings. Boric acid was added at the rates of 0, 5, 10, 20 and 40 mg B/kg soil mixture, and thoroughly mixed in a concrete mixer. The treatments are designated B0, B5, B10, B20 and B40 respectively.

Seeds of 3 barley varieties (Sahara 3771, VB9104 and VB9104+) and the 2 selected lines (ABBD03-015*4 and ABBD03-015*20) were surface-sterilized in 0.5% sodium hypochlorite and germinated on damp filter paper in Petri dishes. After 2 days, three germinated seedlings were transferred to pots containing soils with different levels of boron. The experiment was designed as a 5×5 factorial, and arranged in a randomized complete block layout with three replications. The soil in each pot was maintained at 80% field capacity by watering to weight with reverse-osmosis water. Plants were harvested at ground level after five weeks, oven-dried and weighed. Dried shoots were ground, and digested with nitric acid and hydrogen peroxide in a closed vessel using the HotBlock digestion system (Zarcinas *et al.*, 1987). Sample solutions were analyzed using ICPAES Method - 3VR by Radial CIROS Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES) (Hayes and Reid, 2004).

Field experiments

Field experiments were conducted in 2004 and 2005 at four locations in southern Australia with naturally occurring high concentrations of boron in the subsoil. Genotype entries comprised the 40 BC₆F₁-derived DH lines along with 18–24 checks, which included

the two recurrent parents (VB9104 and VB9104+). The experiments were located at Horsham and Birchip in Victoria, and Roseworthy and Pinery in South Australia, in a randomized complete block design, with two replications. In all trials, plots measured 6 m in length at sowing, with six rows of plants spaced 15 cm apart, at a seeding rate of 40 g per plot (≈ 50 kg/ha). The trials were maintained free of weeds, fully fertilized, and mechanically harvested at maturity. Data were recorded on grain yield per plot.

For malting quality assessment, grain samples from the trials at Horsham (Victoria) and Roseworthy (South Australia) were passed through a 2.2 mm screen before micro-malting. The malting schedules were as described in Emebiri *et al.* (2004).

Statistical analysis

The method of restricted maximum likelihood (REML), developed by Patterson and Thompson (1971) and implemented in the Gen Stat v10 (Lawes Agricultural Trust) statistical package, was used for data analyses. Each experiment was first subjected to spatial analysis to account for field heterogeneity, by using spatial information of the plot layouts (rows and columns) and modeling trends using the separable autoregressive (AR) process (AR1 \times AR1) proposed by Cullis and Gleeson (1991). The analyses were carried out with all terms (replicates and genotypes) considered fixed. The adjusted means obtained from the individual spatial analyses were then combined to form input data for analysis of genotype \times environment interaction, and to calculate BLUPs (best linear unbiased predictors) of genotypes.

Results

Confirmed transfer of genes for B tolerance

There was no difference in the root growth of lines possessing alternate alleles at the EBmac679 locus in the control (water)

treatment; in the boron-toxic treatment, however, a highly significant difference ($P < 0.001$) was observed in root length (Figure 1A), such that individuals carrying the Sahara 3771-derived allele at EBmac679 were longer by an average of 13.6 mm. Relative root length, considered by Jefferies *et al.* (1999) as the appropriate indicator of B tolerance, also showed a highly significant difference ($P < 0.001$) between lines possessing the EBmac679 marker allele derived from Sahara 3771 versus those with the alternate allele (Figure 1B).

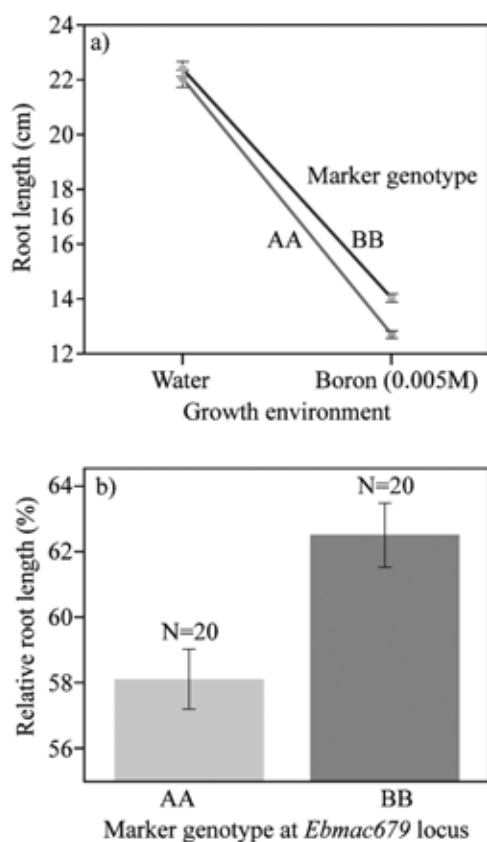


Figure 1. Root length and relative root length of BC_6F_1 -derived doubled-haploid lines contrasting for alleles at EBmac679. Relative root length was calculated as the root length in toxic boron concentration expressed as a percentage of the root length in water (control).

Shoot B concentration

Pot assay confirmed the successful transfer of B tolerance from Sahara 3771 to VB9104+ (Figure 2A), and ultimately to their BC_6F_1 -derived progeny (Figure 2B), as demonstrated by the differences in shoot B concentration with increasing concentration of soil B. Boron accumulation in Sahara 3771 and the BC-derived tolerant line, VB9104+, were on average, fairly similar across the range of applied B in the soil, but significantly ($P = 0.014$) lower than the sensitive line, VB9104 (Figure 2A). Amongst the BC_6F_1 -derived progeny, differences in B tolerance were clearly evident from as low as 5 mg B/kg soil concentration, and became highly significant ($P < 0.001$) as soil B concentration was increased to 40 mg/kg (Figure 2B).

At 10 mg/kg B soil applied treatment, tissue concentrations in the line carrying VB9104 allele at EBmac679 locus (ABBD03-015*4) exceeded 500 mg/kg whilst concentrations in the sister line (ABBD03-015*20) possessing Sahara 3771 allele at the QTL locus were generally below 350 mg/kg (Figure 3). Although there is a wide range quoted for critical tissue B concentrations in many crops and trees (Gupta, 1993), these statistically significant contrasts confirmed the successful transfer of a major gene for B tolerance at the QTL region on chromosome 4H.

Impact of introgressed boron QTL on grain yield and malt quality

We used a combination of molecular and conventional assays to unequivocally classify the 40 BC_6F_1 -derived DH lines as B-tolerant or B-sensitive, and then compared their means in a balanced ANOVA for grain yield measured over two years and four locations. Although differences were not statistically significant, mean values showed patterns consistent with a trait of adaptive advantage at some locations, but not others (Figure 3). For instance, lines possessing B tolerance had consistently higher grain yield at Roseworthy

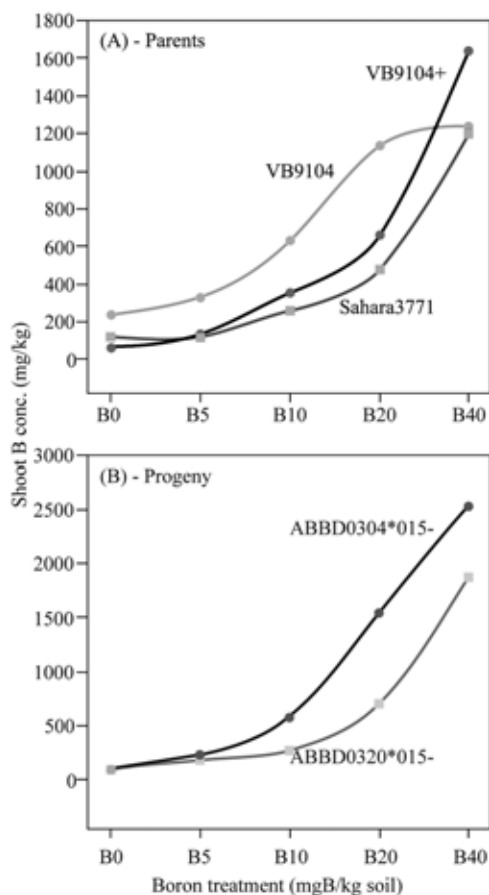


Figure 2. Shoot boron concentrations of donor (Sahara 3771), recurrent parents (VB9104, VB9104+), and BC_6F_1 -derived doubled-haploid lines contrasting for alleles at EBmac679. Shoot B concentrations were measured on seedlings grown under different levels of applied B, in a soil-based assay, as described in Materials and methods.

(3.3% across years), but were consistently inferior at Horsham (-7.0%).

Based on separate and combined analyses across sites, there was no evidence of a significant impact on malt extract, which is arguably the most important quality parameter. Differences in other quality parameters were also rarely significant, although a significantly ($P < 0.01$) negative impact on α -amylase activity was verified at Horsham (not shown).

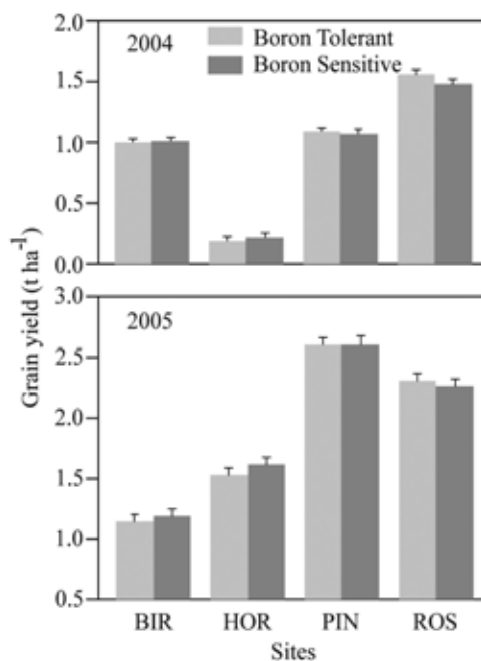


Figure 3. The average grain yield of BC_6F_1 -derived doubled-haploid lines classified as B-tolerant or B-sensitive, based on cluster analysis with a combination of molecular and phenotypic data. Trial sites in southern Australia were Birchip (BIR), Horsham (HOR), Pinery (PIN) and Roseworthy (ROS).

Discussion

Our results demonstrate successful transfer of genes for boron tolerance into recipient lines using linked, but very modest and inconsistent improvements in grain yield of lines carrying B-tolerance genes in boron toxic environments. The extensive backcrossing strategy used in this study was aimed at recapturing the agronomic attributes of the recurrent parent, VB904, a line widely recognized for its excellent grain plumpness (Paynter *et al.*, 2004), good foliar disease resistance and wide adaptation, particularly in the low rainfall environments. Yield assessment of the entire BC_6F_1 -derived lines indicated that none had recaptured the yield performance of the original line VB9104

(Moody, pers. comm.). These results have led to suggestions that linkage drag from Sahara 3771 of deleterious alleles might be masking any yield improvement (Eglinton *et al.*, 2004; Jefferies *et al.*, 2001; Sutton *et al.*, 2007). Based on DArT (Wenzl *et al.*, 2004) polymorphisms at 700 loci, the BC₆F₁-derived lines used in this study were found to have retained approximately 3.8% of the Sahara 3771 genome. With the exception of chromosome 4H, there was no difference in donor genome content (DGC) of lines possessing the EBmac679 marker allele derived from Sahara 3771 versus those with an alternate allele at this locus. Furthermore, there was no correlation between DGC and grain yield at any of the sites or years, on either a whole-genome basis or at the chromosomal level. We concluded that the less-than-expected improvements in grain yield in boron toxic environments might not be due to linkage drag.

Although B toxicity is reported to cause up to 17% yield loss in barley (Cartwright *et al.*, 1984), yield benefits from gene introgression are hard to establish. Earlier studies to introgress B tolerance from Sahara 3771 found that lines carrying the introgression can potentially be lower-yielding than the recipient cultivars (Eglinton *et al.*, 2004; Jefferies *et al.*, 2001). Alleviating subsoil constraint to increase potential rooting depth can have a positive, negative or no effect on grain yield, depending on season and soil type (Wong and Asseng, 2007), as there are many interacting factors, such as sodicity and salinity (Nuttall *et al.*, 2005). Genetically improving tolerance to B toxicity can, in fact, depress grain yield in some years or sites by promoting early crop growth and increased water demand not matched by water supply later in the season, a common problem in Mediterranean-type environments. Although yield benefits were modest, our results also showed no significant negative impact of gene introgression on existing malting quality profile (not shown). This is important

because it suggests that boron tolerance from Sahara 3771 has been incorporated in two-row barley germplasm without any penalty on quality.

Acknowledgement

Financial support from ABB Ltd through the Molecular Plant Breeding Cooperative Research Centre is gratefully acknowledged.

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Introgression and validation of reproductive frost tolerance

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Abstract

Frost damage regularly causes significant financial losses to Australian cereal production by affecting crops at the most sensitive reproductive stages of development. Certain genotypes have been identified as having significantly higher reproductive frost tolerance and subsequently populations were developed to genetically map the trait. The level of tolerance identified provided significant and commercially relevant reductions in reproductive frost damage under field conditions. Two genomic regions associated with frost tolerance were introgressed into a southern Australian-adapted genetic background using an aggressive MAS breeding strategy. Individuals with a high proportion of the recurrent parent and small introgression segments were selected to fast track through the breeding program. These lines were tested at multiple sites across southern Australia in 2006, with grain yields equal to or higher than current commercial checks. Results obtained from controlled environment experiments and 2006 field trials suggest the progeny have retained a level of reproductive frost tolerance comparable to the donor and significantly better than the recurrent parent. Results from trials to validate frost tolerance will be presented and discussed in the context of agronomic performance and potential to commercialize the first frost-tolerant barley varieties in Australia.

Introduction

Past frost tolerance research in the northern hemisphere has focused on the vegetative stages of development in winter-grown cereal varieties. However, in Australia the significant economic impact of frosts for cereal farmers occurs during reproductive stages of development in spring-grown varieties. Significant frost events at flowering or early grain filling also occur periodically in Canada, and major economic losses were incurred during 2007 in Argentina. Frost events that occur close to flowering can cause floret sterility, significantly reducing yield. Subsequent frosts during grain filling can result in downgrading of grain quality from increased screenings and scalloping of the grain.

Radiation frosts are meteorological events that are the main cause of frost damage to cereal crops in Australia. They occur in late winter and in spring when flowering cereal crops are most susceptible. A sequence of specific weather conditions is conducive to a significant event of this type occurring. Following a cold front, large high-pressure systems can move in causing wind disturbance to drop and cloud cover to dissipate. This generates conditions that allow most of the heat that was absorbed into the ground during the day to easily radiate into clear night skies. As a result, temperature falls rapidly until the sun, wind or cloud cover returns. Damaging radiation frosts occur at canopy temperatures

ranging from 0°C (minimal floret sterility) to -6°C (high to complete floret sterility), although typical events generally range between -2°C and -4.5°C (floret sterility varies between genotypes).

Between 2000 and 2003, Reinheimer *et al.* (2003, 2004) screened a diverse array of barley varieties, landraces and breeding lines in a dedicated field screening nursery to identify genotypic variation for reproductive frost tolerance (RFT). Marked differences were identified, with two Japanese varieties (Haruna Nijo and Amagi Nijo) exhibiting particularly low rates of frost induced sterility (FIS) compared with commercial Australian varieties. Haruna Nijo and Amagi Nijo had FIS at rates of 4.5% and 5.4%, respectively, compared with Schooner at 79.1%, Arapiles at 27.2% and Galleon at 40.3%. The Japanese varieties are not immune to reproductive frost damage; however, the level of RFT exhibited at typical -4°C potentially represents a significant economic advantage to barley farmers in frost-prone regions.

Both Haruna Nijo and Amagi Nijo were varieties used to develop two of three Australian barley mapping populations (Arapiles × Franklin, Amagi Nijo × WI2585, Haruna Nijo × Galleon) with alternate parents that were shown to contrast significantly for FIS. Using these three mapping populations, Reinheimer *et al.* (2003, 2004) identified two genetic locations controlling the frost-tolerance trait. Quantitative trait loci (QTL) on chromosome 2HL were identified for FIS in the two Japanese parent populations at the same genomic location. QTL on chromosome 5HL were identified for FIS and frost-induced grain damage in all three of the populations studied.

The germplasm identified as carrying the RFT QTLs are of Japanese origin and poorly adapted to Australian conditions. An aggressive breeding strategy was developed to fast track the development of a population for potential commercial relevance with highly adapted background germplasm and

the donor level of frost tolerance. In this paper we will outline the breeding strategy used and review the progress of these breeding lines in dedicated field trials to gauge the potential future release of well adapted, frost tolerant barley for susceptible production regions of southern Australia.

Materials and methods

Parent selection

The University of Adelaide Barley Program-developed line WI3806 was selected as the recurrent parent due to its broad adaptation and, more specifically, its high relative yields in the environments in southern Australia that are frequently exposed to frost. WI3806 was derived from a cross between Mundah and Keel, which was then top-crossed with Barque. These three parents have been leading feed barley varieties grown in southern Australia and were able to produce progeny of high yield stability and physical grain quality.

The donor of RFT was sourced from the Galleon × Haruna Nijo mapping population. Selection of this donor individual considered field-based frost tolerance, Haruna Nijo segments at the 2H and 5H loci and the largest proportion of Galleon genetic background as the donor parent. This population had been phenotyped in the Loxton field-based frost screening nursery, so detailed RFT scores had been collected on progeny of this cross. Progeny of the Galleon × Haruna Nijo population had been comprehensively genotyped, which enabled the selection of an individual with the 2 identified RFT loci and a high proportion of the better adapted parent, Galleon, in the genomic background. With the phenotypic and genotypic information combined, the line GH-129 was selected as the donor parent. A single backcross to the adapted recurrent parent was made as the donor GH-129 already possessed approximately 60% of the genome from the Australian adapted parent Galleon.

Marker Assisted Selection

Linked SSR markers were used to select BC₁F₁ individuals heterozygous at the chromosome 2H and 5H loci for use as doubled-haploid (DH) donors. A population of 300 DHs was generated and screened with the same SSR markers to select individuals carrying Haruna Nijo alleles at both loci. Selected lines were subjected to whole-genome analysis to identify individuals carrying small introgression segments and a high proportion of recurrent parent genetic background, for summer seed multiplication. From the surviving DH individuals after the final MAS for the two RFT loci, 34 individuals were selected for genotyping with Diversity Array Technology (DArT) markers, with 686 DArT markers screened over the 34 DH individuals plus 4 parental lines: Galleon, Haruna Nijo, GH-129 and WI3806. Only a limited number (2) of lines carrying WI3806 alleles at the 2H and 5H loci were retained. The 34 DH lines were progressed through seed multiplication for subsequent evaluation.

Adaptation field trials

Yield trials to assess genetic validation in terms of yield (kg/ha) were undertaken in the 2007 season. Several regions (5) in the target production environment were selected to reflect the frost-prone Mallee environments of South Australia and Victoria. The sites are Geranium, Lameroo, Pinnaroo, Peebinga and Cooke Plains, which collectively span a high proportion of the southern Australian Mallee cereal farming region.

A randomized complete block design with 2 replications was used at each site. Planting rates of 145 plants/m² were calculated using thousand grain weight and germination information. Plots of 16 m² were harvested and yields were measured then converted to kg/ha. Due to seed limitations, not all entries were tested in all trials. Benchmark check lines Schooner (malt), SloopSA (malt) and Keel (feed) were included as controls.

Results

Development of the WI3806*2 × GH-129 population

Using the isolated-microspore method (Hoekstra *et al.*, 1992), 307 regenerated DH plants were produced from the BC₁F₁s. The F₁s used for donors were selected via MAS to be heterozygous for the 2 RFT loci. After screening the regenerated DH for the 2 RFT loci using MAS, 66 positive individuals were identified as carrying the target loci. Of the 66 individuals that were positive for the 2 RFT loci, 37 were confirmed as DH (i.e. producing seed) and 29 as haploid (i.e. sterile, not producing seed). The 37 surviving individuals were seed increased over summer and notes were taken on growth habit. Lines of winter growth habit were dug up, placed in pots, and grown in a growth room to meet vernalization requirement. Three of the 37 lines were lost during this process. Of the remaining lines, 19 displayed spring growth habit and 15 displayed winter growth habit. The introgression strategy is outlined in Figure 1.

Whole genome analysis

The donor parent, GH-129, had 44.6% of the RFT parent in its genetic background carrying whole chromosomes of 2H and 5H from Haruna Nijo. The progeny from the WI3806*2 × GH-129 population had, on average, 16.3% of the donor parent in the genetic background, with a range from 8.9% to 25.9% that was normally distributed around the mean (Figure 2). The expected percentage of donor genome retained if no selection were applied would have been 11.15%. As MAS was applied to retain the 2 RFT loci, an increase in donor genome retention was observed.

DArT marker genotypes were produced for each of the 34 lines and 4 parents (Figure 3). The high resolution marker data was quality controlled prior to producing graphical genotypes. As DArT markers can only identify two alleles, a level of interpretation needed to be undertaken before assigning alleles to any

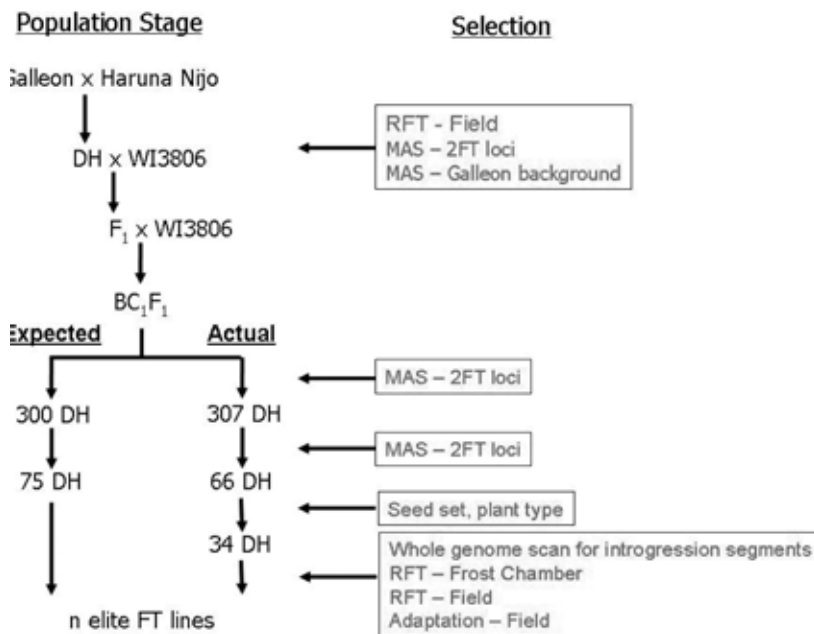


Figure 1. Breeding strategy employed to rapidly introgress RFT loci into adapted germplasm, achieved in only six years following the initial identification of genetic variation for this complex trait.

of the 3 parents, i.e. WI3806, Galleon and Haruna Nijo. As Galleon and WI3806 were closely related, polymorphism between these two lines was low (36%) when compared to the polymorphism between WI3806 and Haruna Nijo (56%). To simplify the visual characterization of the introgression segments from the poorly adapted but frost-tolerant donor line Haruna Nijo, alleles were either assigned WI3806 × Galleon or Haruna Nijo. Although the progeny were initially selected with SSRs that were flanking the 2H and 5H RFT loci, after high resolution DArT analysis it was found that several lines were missing part or all of the critical RFT loci: S-97 missing 2H; S-116, S-130 and S-236 missing 5H. Note that two lines missing both RFT loci (S-191 and S-305) were deliberately retained.

Adaptation field trials

The five sites selected for field trials (Geranium, Lameroo, Pinnaroo, Peebinga

and Cooke Plains) included 30, 20, 13, 6, and 7 RFT introgression lines, respectively.

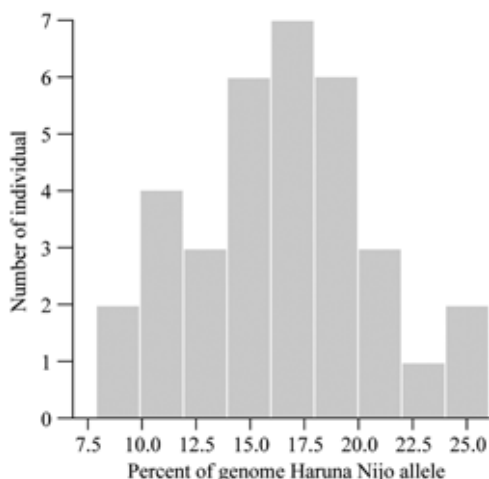


Figure 2. Histogram of the proportion of donor genome retained in lines derived from the WI3806*2 × GH-129 population.

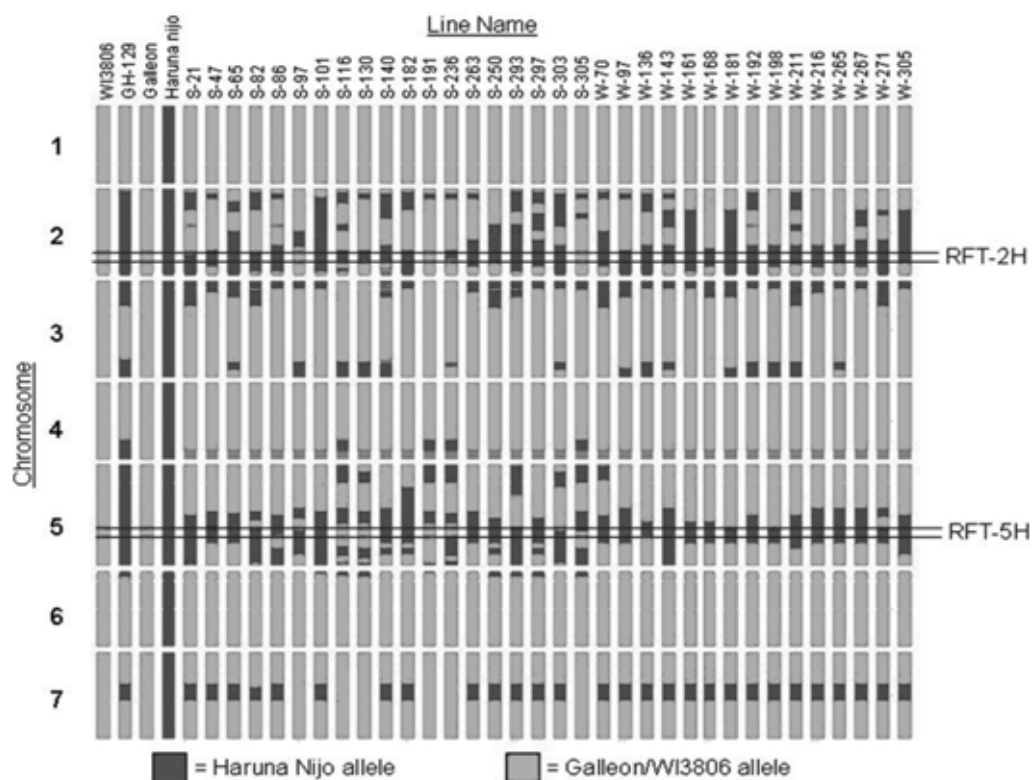


Figure 3. Graphical genotype of parents and lines from the WI3806*2 \times GH-129 population. Red bars indicate either Galleon or WI3806 genotype. Blue bars indicate Haruna Nijo genotype. Grey bars indicate missing data. Black lines indicate the location of the 2H and 5H RFT loci.

Benchmark check lines cv. Schooner (malt), cv. SloopSA (malt) and cv. Keel (feed) were included and each has several years of yield results from this region. It must be noted that the 2007 season suffered a drought, which may have significantly affected yield results. Multiple frost events occurred at all of the sites, with various lowest temperatures and dates (Table 1).

Peebinga experienced a lot more frost ($<0^{\circ}\text{C}$) events (24) than the other 4 sites (8 or 9). Several frost events of minimum temperatures below -2°C occurred at Peebinga (11) and Geranium (4), whereas Lameroo, Cooke Plains and Pinnaroo each had 2 events. FIS scores were taken for all the sites by taking ten random spikelet samples from each variety prior to harvest.

Growth development stage scores (Zadoks growth development stages; Zadoks *et al.*, 1974) were taken on 12 September 2007 for the multiple sites (data not shown). A large range of maturities was observed within the population. Growth development stages ranged from flag leaf sheath extending (Z41) to kernel late milk development (Z77). FIS results could not then be interpreted due to the differential effect of plant maturities coupled with the timing and severity of multiple frost events.

Yield data from the five trials was analyzed to predict genotype yield means with spatial adjustment (Gen Stat version 6.1). Strong heritability and small standard error (SE) values from Geranium, Lameroo and Peebinga of 0.52 (SE 75.7), 0.71 (SE

Table 1. 2007 frost (below 0°C) occurrences for the five Mallee field trials. Temperatures were taken at canopy height and are the lowest minimum temperature for the morning of the noted date. Results highlighted in color: green are temperature events between 0 and -0.9°C; yellow are temperature events between -1 and -1.9°C; orange are temperature events between -2 and -2.9°C; and red are temperature events below -3°C.

Event	Date	Geranium	Lameroo	Cooke Plains	Pinnaroo	Peebinga
1	18/08/2007					-0.3
2	19/08/2007					-1.6
3	20/08/2007				-1.7	-4
4	21/08/2007					-3.1
5	23/08/2007					-0.9
6	24/08/2007					-2.7
7	25/08/2007					-0.6
8	29/08/2007		-1.6		-0.1	-2.1
9	30/08/2007					-0.2
10	1/09/2007	-1.2	-2.2		-2.8	-4.6
11	2/09/2007	-2.7	-1.7	-1.5	-1.5	-4
12	3/09/2007	-0.6				
13	4/09/2007	-3.4	-0.6	-2	-0.6	-2.5
14	5/09/2007		-1.1	-0.5	-0.6	-4
15	15/09/2007	-1.9	-3.3	-0.7	-2.9	-4.7
16	18/09/2007					-1.9
17	21/09/2007	-1.4	-0.8		-0.7	-2.8
18	24/09/2007		-0.6			-0.9
19	25/09/2007			-0.2		-1.3
20	4/10/2007					-1.6
21	5/10/2007					-0.8
22	8/10/2007	-2.9	-1.9	-2.3		
23	9/10/2007	-2.4		-1.4		-1.1
24	14/10/2007					-0.8
25	17/10/2007	-1.1		-1.3	-0.8	-2.9
26	18/10/2007					-0.2
	Total < 0°C	9	9	8	9	24

65.9) and 0.64 (SE 69.29), respectively, was observed. Geranium and Lameroo were the only sites considered in detail due to the large number of lines tested and large heritability values. Geranium and Lameroo yield results had a significant correlation coefficient of 0.61 ($P = 0.01$)

FIS scores at Lameroo ranged between 3 and 21% and were found to be not statistically significant ($P = 0.23$). FIS scores at Geranium ranged between 2 and 58% and were statistically significant ($P = 0.04$). The Lameroo site was then considered in further detail. The Lameroo yield results were found to be

consistent with regional yield predictions from National Variety Trials (NVT) for the control lines (Table 2).

The predicted yields (kg/ha) for Keel, Schooner and SloopSA in this region are 1799, 1579 and 1578, respectively. Adjusted yield results from 2007 Lameroo trials were very similar, at 1714, 1586 and 1660 kg/ha. Twelve out of 20 of the WI3806*2 × GH-129 population retained grain yield at least matching the current dominant malting varieties in the region, SloopSA and Schooner. Three lines outperformed the current dominant feed variety Keel.

Table 2. Yield data from Lamerloo 2007 field trial. Data is sorted in descending order of yield (kg/ha) at Lamerloo. Standard error (SE) is in kg/ha. National Variety Trials (NVT) yield predictions are based on 22 site×year combinations of trials in the target production region. Values of %Schooner are a percentage of yield when compared to Schooner.

Variety Name	Lamerloo 2007			NVT Yield Prediction	
	Yield (kg/ha)	S.E.	%Schooner	Yield (kg/ha)	%Schooner
BX03S;198DMW-198	2008	65.9	127		
BX03S;198DMS-097	1877	65.9	119		
BX03S;198DMS-191	1826	65.9	116		
Keel	1799	65.9	114	1714	108
BX03S;198DMW-143	1769	65.9	112		
BX03S;198DMS-303	1766	65.9	112		
BX03S;198DMW-265	1765	65.9	112		
BX03S;198DMW-305	1700	65.9	108		
BX03S;198DMW-216	1685	65.9	107		
BX03S;198DMS-130	1684	65.9	107		
BX03S;198DMW-271	1671	65.9	106		
BX03S;198DMW-097	1623	65.9	103		
BX03S;198DMS-182	1585	65.9	100		
Schooner	1579	65.9	100	1586	100
SloopSA	1578	65.9	100	1660	105
BX03S;198DMS-263	1536	65.9	97		
BX03S;198DMS-297	1515	65.9	96		
BX03S;198DMW-267	1501	65.9	95		
BX03S;198DMS-305	1428	65.9	90		
BX03S;198DMW-136	1404	65.9	89		
BX03S;198DMS-250	1364	65.9	86		
BX03S;198DMS-293	1251	65.9	79		
BX03S;198DMS-047	1229	65.9	78		

Discussion

The fast-track breeding strategy employed was able to produce fixed lines incorporating the donor loci within 2 years from the first cross. The use of MAS within this breeding strategy enabled a larger population size to be maintained, as donor F_1 s for DH production were all carrying the 2 RFT loci in a heterozygous state. This integrated approach has enabled progress from the identification

of tolerance, genetic mapping, a crossing and selection to potential variety release within only 9 years. This is significantly faster than the time taken for equivalent research and breeding steps taken to deploy cereal cyst nematode resistance and thermostable forms of β -amylase.

Germplasm derived from this breeding strategy can be used for the validation of RFT in a genetic background that is commercially relevant to barley production environments

that experience frequent frost events. The introgression of these 2 RFT loci via MAS and the identification of introgression segment size via DArT markers have provided a foundation to further identify and validate high yielding, well adapted RFT lines for increasing production in the frost-prone areas of southern Australia.

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Effect of post-anthesis heat stress on grain yield of barley, durum and bread wheat genotypes

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Abstract

In order to study the effects of heat stress after anthesis on grain yield and yield components of two barley (six- and two-row) and six wheat genotypes, two field experiments were conducted with optimum and delayed sowing dates. With delayed sowing, plants experienced heat stress in the post-anthesis growth stages. The stress tolerance index (STI) and stress susceptibility index (SSI) for grain yield and 1000-grain weight indicated that Jonoob (six-row barley) and Stork (durum wheat) had the highest and the lowest STI for grain yield, respectively. Barley genotypes had a higher tolerance to post-anthesis heat stress than wheat genotypes. Average grain yield reduction in barley and wheat genotypes exposed to heat stress after anthesis was 17% and 24%, respectively. Higher SSI for 1000-grain weight in late-maturity genotypes was related to delay in anthesis and coincidence of grain growth period with heat stress. In early maturity genotypes with early anthesis, the grain growth period took place before heat stress.

Introduction

Barley and wheat are traditionally grown as cool-season crops, but with the increased availability of more widely adapted semi-dwarf cultivars, barley and wheat production has expanded into warmer regions of countries where production had previously been restricted to higher altitudes or cooler

latitudes (Badrruuddin *et al.*, 1999). Heat and drought are the most important stresses that limit crop production (Entez and Flower, 1990; Rawson, 1988). Optimal crop growth requires a non-limiting supply of resources (water, nutrients and radiation) and, as temperature rises, the demand for growth resources increases due to higher rates of metabolism, development and evapotranspiration (Rawson, 1988). In Mediterranean conditions, such as Khozestan in the western part of Iran, heat stress after anthesis is the major factor limiting grain yield in winter-sown barley and wheat. The optimum temperature range to achieve maximum kernel weight is 15–18°C; higher temperatures (up to 30°C) reduce the duration of grain filling and this reduction is not compensated by the increase in rate of assimilate accumulation (Wardlaw *et al.*, 1980, 1989; Stone *et al.*, 1995). Heat stress during the growth stages following anthesis mainly affect assimilate availability, translocation of photosynthates to the grain, and starch synthesis and deposition in the developing grain. The net result is a lower grain yield due to a lower 1000-grain weight (TGW) (Gibson and Paulsen, 1999; Rao *et al.*, 2002; Modhej and Behdarvandi, 2006). Some studies indicated that barley genotypes had higher tolerance to post-anthesis heat stress (Modhej *et al.*, 2003, 2005). Modhej *et al.* (2005) showed that the higher heat stress tolerance of barley after anthesis was due to its shorter crop growth duration compared to

wheat. As in this study, the barley genotypes used were early, so the grain growth period occurred before the heat stress.

The objective of this investigation was to examine the effects of high temperature stress during the grain filling period on the grain yield of barley and wheat genotypes, and also to identify heat stress after anthesis on tolerant and sensitive genotypes using the stress tolerance index (STI) recommended by Fernandez (1992), and the stress susceptibility index (SSI) recommended by Fischer and Maurer (1978).

Materials and methods

The trial was conducted at Ahvaz, in the southwest of Iran, in the 2005–06 growing season. Ahvaz is located at 20 m above sea level (32°20' N, 40°20' E). Wheat was sown on optimum (22 November) and delayed (20 January) sowing dates. Wheat and barley genotypes were sown on optimum (22 November) and delayed (22 December) sowing dates. Plants with delayed sowing date experienced heat stress in the growth stages after anthesis. Two short-season barley cultivars and six wheat cultivars with different growth durations were used (Table 1). Treatments of each individual experiment (sowing date) were arranged as

Table 1. Characteristics of the genotypes examined.

Crop	Genotype	Growth Duration
Barley	Jonoob (six-row)	Short-season
	Sarasary (two-row)	Short-season
Bread wheat	Fong	Short-season
	Chamran	Middle-season
	Star	Long-season
Durum wheat	Showa/malt	Middle-season
	Stork	Short-season
	Green	Long-season

a randomized complete block design with three replications. The soil was clay loam in texture, alkaline in reaction, pH 8.0 with low organic carbon (less than 1%), moderate phosphorus (7.2 ppm) and high potassium (220 ppm) status. The experimental site had a hot climate with mild winters and dry, hot summers.

Mean temperatures during the grain growth period were 22°C and 28°C, in optimum and delayed sowings, respectively (Figure 1).

Normal cultural and management practices (fertilizers, irrigation and pest control) for wheat plants were used. Plot size was 1.2 m by 3.0 m, and based on research recommendations and differing tillering capacities of the barley and bread and durum wheat genotypes, seeds were drilled in 18 cm rows at about 350, 400 and 500 seeds/m² for barley, bread and durum genotypes, respectively. There were 8 rows in each plot. Yield and yield components were estimated by harvesting after physiological maturity, excluding border rows and at least 0.5 m

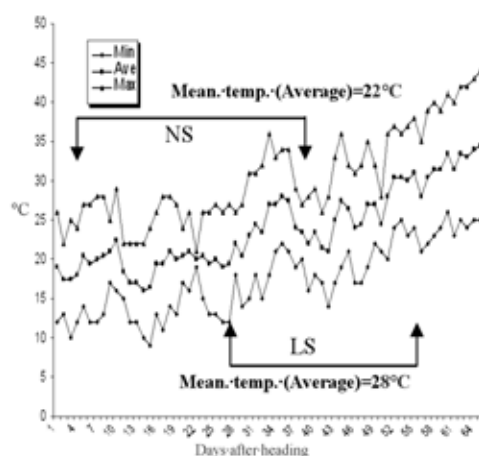


Figure 1. Maximum daily air temperature (°C) during heading and grain growth. NS = normal sowing date and LS = late sowing date. Arrows indicate the beginning and end of the grain filling period for each sowing date.

from either end of the rows. The harvested area was 1.2 m² and TGW was estimated on a sample of 200 grains.

Grain yield and individual TGW in heat stress after anthesis were investigated using the stress susceptibility index (SSI) recommended by Fischer and Maurer (1978) and stress tolerance index (STI) recommended by Fernandez (1992). SSI was calculated as below:

$$SSI = \frac{1 - \frac{Y_s}{Y_n}}{1 - \frac{Y_s}{Y_p}}$$

where Y_{st} , Y_{pi} , Y_s and Y_p are the grain yield or TGW of each cultivar in stress condition, the grain yield or TGW of each cultivar in optimum condition and the mean grain yield in all the genotypes in stress and optimum conditions, respectively. STI was calculated using the formula:

$$STI = \frac{Y_s \cdot Y_n}{Y_p^2}$$

where Y_{st} , Y_{pi} and Y_{2p} are the grain yield or TGW of each genotype in stress condition, in optimum condition and the mean square of grain yield of all the genotypes in optimum condition respectively. Statistical analysis was carried out using the SPSS statistical program.

Results and discussion

Optimum sowing date (optimum conditions)

Results indicated that the differences between genotypes for grain yield in the optimum sowing date group were not significant (Table 2). However, Jonoob (six-row barley) and Fong (bread wheat) tended to have the highest and the lowest grain yields in optimum conditions, respectively, but the difference was not significant (Table 3). Mean grain yields in barley, bread wheat and durum wheat genotypes under optimum conditions were 515, 473 and 479 g/m², respectively (Table 3). Statistical differences for spike per unit area, spikelet per spike and TGW were significant at the 1% probability level (Table 2). Jonoob and Fong had the highest and the lowest number of spikes/m². Jonoob and Chamran (bread wheat) did not differ significantly for number of spikes/m². Among the wheat genotypes, Chamran had the highest number of spikes per unit area. Green (durum wheat) had the highest and Jonoob and Chamran genotypes had the lowest TGW for optimum sowing date (Table 3). Modhej *et al.* (2008) indicated that there was negative correlation between grain number per spike and TGW in durum and bread wheat genotypes. Sattore and Slafer (2000) reported that yield components were correlated with each other and changes in one

Table 2. ANOVA for grain yield (GY), number of spikes/m² (S), 1000-grain weight (TGW), spikelets per spike (s/S) and number of grains per spike (G/S) for optimum sowing date.

S.O.V	DF	Mean of square				
		GY	S	TGW	s/S	G/S
R	2	372999	357	2.50	0.13	6.5
G	7	261531 ns	14421**	61.23**	2.02 ns	67.2 ns
Error	14	377016	1110	2.25	0.01	18
CV (%)		1.6	6.5	3.3	5.8	13.5

S.O.V. = sources of variation; DF = degrees of freedom; ** = significant at 1% probability level; ns = not significant; GY = grain yield (g/m²); S/m² = spikes per m²; TGW = 1000-grain weight (g); s/S = spikelets per spike G/S = grain per spike.

Table 3. Grain yield (GY), 1000-grain weight (TGW), stress susceptibility index (SSI) and stress tolerance index (STI) for barley and wheat genotypes under optimum and late sowing dates

Genotype	STI Gw	SSI Gw	TGW (g/m ²)	(TGW(OP (g/m ²)	STI GY	SSI GY	(GY(S (g/m ²)	(GY(OP (g/m ²)
Barley								
Jonoob	0.63c	0.78c	31ab	41e	1.06	0.63de	461a	535a
Sarasary 6	0.71bc	0.80c	33a	44cde	0.76	0.72d	387a	495a
Mean	0.67	0.79	32	42	0.91	0.67	424	515
Durum wheat.								
Showa	0.84ab	1.03bc	34a	50e	0.78b	1.34b	358a	504a
Green	0.88a	1.25ab	33a	54a	0.71c	1.23b	434a	473a
Stork	0.59c	1.4a	26c	46c	0.66d	1.00c	346a	442a
Mean	0.77	1.22	31	50	0.71	1.19	379	473
Bread wheat								
Star	0.60c	0.91bc	30b	42de	0.70c	1.50a	332a	492a
Fong	0.73bc	0.80c	33a	45cd	0.71c	0.53e	383a	432a
Chamran	0.61c	0.86bc	30b	41e	0.90a	0.97c	405a	513a
Mean	0.64	0.85	31	42	0.77	1.00	373	479

Means in each column followed by similar letter(s) are not significantly different at 5% probability level using Duncan's Multiple Range Test. S = Stress; OP = Optimum; SSI = Stress susceptibility index; STI = Stress tolerance index

character may lead to changes in the other. The mean TGW in durum wheat was higher than in bread wheat and barley (Table 3).

Late sowing date (post-anthesis heat stress conditions)

Results indicated that the differences between genotypes for grain yield in late sowing date were not significant (Table 4).

TGW showed significant difference between genotypes in post-anthesis heat stress conditions (Table 4). Cvs. 6 Sarasary (two-row early maturing barley), Fong and Showa (durum wheat) had the highest TGWs for the late sowing date (Table 3). Modhej *et al.* (2008) reported that TGW had a strong relation with grain yield under post-anthesis heat stress conditions. They also reported that, for an optimum sowing date, the highest correlation was between grain yield and grain number per unit area.

Comparison of the two environments

The combined ANOVA indicated that the effect of the treatment (sowing date) on spike per unit area (Figure 2), grain number per spike and fertile floret per spikelet characteristic was not significant. The treatment effect was significant for GY and TGW at $P = 1\%$. In the late sowing, an average increase of 6°C in the average temperatures reduced TGW and GY by approximately 34% and 30% compared with the optimum sowing date, respectively. GY reduction was due to significant reduction in TGW (Table 3).

Stork (durum wheat) and Jonoob had the highest and the lowest SSI for TGW (Table 3). SSI for individual TGW was highly correlated ($P < 0.01$) with final individual TGW reduction in heat stress ($r = 0.99^{**}$) rather than with TGW in optimum condition (data not shown).

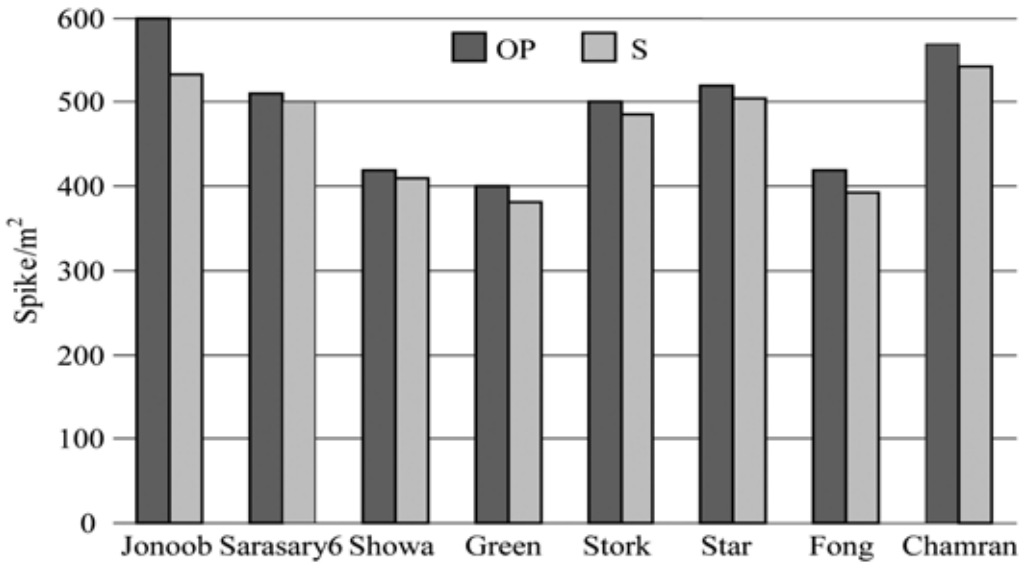


Figure 2. Mean spike number per m² for optimum(OP) and late sowing (S) dates.

Table 4. ANOVA for grain yield (GY), number of spikes/m² (S), 1000-grain weight (TGW) and number of grains per spike (GS) for the late sowing date.

S.O.V	DF	GY	S	Mean of square			GS
				TGW	s/S		
R	2	616479	2686.6	23.0	6.4		5.8
G	7	815120**	11331.4**	28.3**	212.2**		71.3ns
Error	14	292511	735.2	5.5	4.9		13.0
CV (%)		13	5.6	7.5	12.2		13.8

** = significant at the 1% probability level; ns = not significant; S.O.V. = sources of variation; DF = degrees of freedom; GY = grain yield; TGW = 1000-grain weight; s/S = spikelets per spike; GS = grains per spike.

Cvs. Green and Fong had the highest and the lowest STIs for TGW (Table 3). STI for TGW was highly correlated ($P < 0.01$) with final individual TGW in optimum condition ($r = 0.99^{**}$) (data not shown). Modhej and Behdarvandi (2006) reported that genotypes with high TGW in optimum and post-anthesis heat stress conditions had higher STI than other genotypes.

The STI and SSI values for grain yield indicated that Jonoob (six-row early maturing barley) and Stork (durum wheat cultivar) had the highest and the lowest STI for grain yield,

respectively (Table 3). Star (late maturing genotype) had the highest and Fong (early maturing genotype) had the lowest SSI for grain yield, respectively (Table 3). There was no significant difference between Fong and Jonoob for SSI for grain yield (Table 3). However, Star had the greatest grain yield reduction in stress condition (Table 3). The problem with using SSI as a measure of adaptation to the stress is that there are cases where SSI is positively correlated with TGW reduction in those genotypes whose yield was affected little by the stress but also had

a very low yield potential (Modhej, 2006). This means that genotypes with low SSI may also have low stress resistance yield and would not be useful to farmers (Modhej *et al.*, 2005).

Generally, among all the entries, barley genotypes had higher tolerance to post-anthesis heat stress compared with wheat genotypes. The average reduction in grain yield in barley, durum wheat and bread wheat genotypes in post-anthesis heat stress were 17%, 20% and 23%, respectively. Higher SSI for TGW in late-maturity genotypes was related to delay in anthesis and overlapping of grain growth period with heat stress. In early maturing genotypes with early anthesis, the grain growth period occurred before heat stress developed.

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Comparative effects of cadmium on some metabolic changes during the germination of barley grains

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Abstract

Heavy metals, especially cadmium (Cd), are major environmental pollutants. When present in high concentration in soil, they have potential effects on growth metabolism of plants at different stages of development, and more especially at germination, being a crucial stage for future plant growth. In this perspective, we attempted to evaluate the effects of increasing levels of Cd (0, 5, 10, 25 and 50 μM applied for 12, 24, 48, 72 and 96 h) on germinating barley grains on the growth (expressed in mg dry matter) and the proteolytic activity (expressed in units per g fresh weight of the radicles of two six-row varieties, Manel and Rihane). Higher growth and proteolytic activity levels were found in the control variety Manel compared with Rihane. Under Cd stress, radicle growth is inhibited (compared with the control) in the two varieties when Cd concentration exceeds 10 μM , but more in Rihane than in Manel, whatever the duration of treatment. The proteolytic activity level in radicles of the variety Rihane appeared to be higher when compared with Manel, with a significant increase observed from Cd treatment of 48 h in the two barley varieties.

Introduction

Among all toxicants, cadmium (Cd) is considered one of the major pollutants in the current environment. The widespread use of Cd in a large number of products and industrial

process, such as mining, metal-working industries, mineral fertilizer and other sources (Hsu and Kao, 2003), has resulted in discharge and dumping of this element in both aquatic and soil environments, leading to severe environmental contamination with cadmium.

Although cadmium is highly toxic to plants, it can be taken up easily by roots and interrupt a wide range of biological processes, including enzyme activity (Chugh and Sawhney, 1996), photosynthesis and respiration (Mihoub *et al.*, 2005). The inhibitory effect of cadmium on seed germination, which represents an initial and crucial phase of plant development, has been reported in several vegetable species (Mrozek, 1980; Mihoub *et al.*, 2005; Ahsan *et al.*, 2007a) but not in barley. Moreover, plant response to Cd toxicity, more especially the interaction of this heavy metal with biochemical processes in germinating barley seeds, has not been well documented by previous investigations.

The aim of this paper is to evaluate the effects of increasing levels of Cd (0, 5, 10, 25 and 50 μM) applied for 12, 24, 48, 72 and 96 h on germinating grain on the growth and the proteolytic activities in radicles of two barley varieties, Manel and Rihane.

Material and methods

Mature barley (*Hordeum vulgare* L.) seeds were disinfected by calcium hypochlorite (2.5%) for 20 min, followed by a thorough

washing in distilled water. Seed germination was tested on moist filter paper. In each Petri dish (14 cm diameter), thirty seeds were randomly placed on the filter paper, and 5 ml of the cadmium solution with a wide range of concentrations (0, 5, 10, 25 and 50 μM) were added. Controls were maintained by moistening the filter paper with 5 ml pure distilled water. The seeds were germinated for 12, 24, 48, 72 and 96 hr in the dark at 25°C and the fresh weight was recorded each sample taking. Proteolytic (or protease) activity was measured by the method of Weckenmann and Martin (1984), using azocasein as substrate. Absorbance of the azo dye released was measured at 340 nm and one unit of activity was defined as the activity producing an increase of 0.01 units of absorbance during 1 hr incubation.

Results and discussion

Effects of cadmium on radicle growth as a function of dose

The relative effect of Cd on radicle growth of germinating grains was analyzed by determining the dry weight production in the two barley varieties Manel and Rihane. The control, Manel, had a growth level about 26% higher than Rihane at 72 hr of germination (Figure 1).

In the Cd-treated germinating barley grain (Figure 2), radicle dry weight (RDW) decreased (compared to the control) in the two varieties exposed to increasing Cd concentrations of 10, 25 and 50 μM during at least 48 hr of germination. In addition, growth reduction was concentration-dependant and more marked in the variety Rihane (Figure 2). Therefore, RDW production in the varieties Manel and Rihane treated with the highest level of Cd during 48 hr of germination declined by about 24% and 32%, respectively. The lowest dose of 5 μM Cd, in contrast, enhanced slightly radicle growth (Figure 2). This is in agreement with data in the literature showing a stimulatory

effect on plant growth and cell proliferation of low doses of Cd (Aina *et al.*, 2007). The stimulation of cell proliferation by low doses of Cd could be connected to the capacity of Cd to functionally substitute Zn^{2+} , which

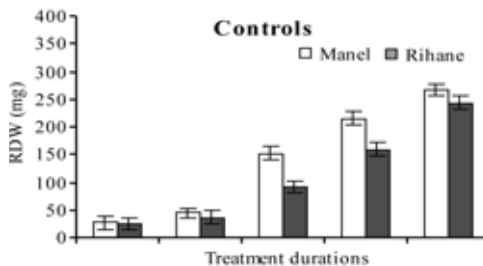


Figure 1. Radicle dry weight (RDW) production of the barley varieties Manel and Rihane germinated without Cd (controls) during 12, 24, 48, 72 and 96 hr. Data are the means of five individual repetitions \pm SE at 0.05 significance level.

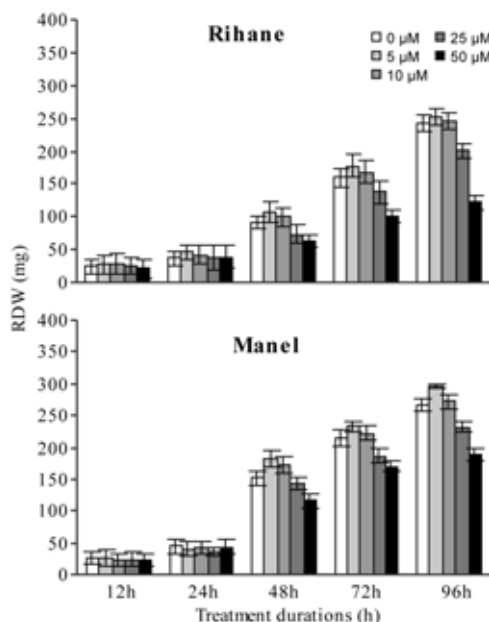


Figure 2. Effects of increasing Cd doses of 0, 5, 10, 25 and 50 μM applied during 12, 24, 48, 72 and 96 hr of germination on RDW production of the barley varieties Manel and Rihane. Data are the means of five individual repetitions \pm SE at 0.05 significance level.

allows the binding of multiple transcription factors to the regulatory regions of genes and is also a co-factor of important key enzymes involved in replication and translation (Aina *et al.*, 2007).

Effects of cadmium on radicle growth in the course of time

Results presented in Figure 3 show that the RDW production of germinating barley grain Manel and Rihane exposed to increasing external Cd levels during short-term treatment of 12 hr was not significantly affected. In contrast, in germinating barley grain exposed until 96 hr, a significant decrease was registered in the RDW production of the two barley varieties Manel and Rihane by about 30% and 50%, respectively. Radicle growth declined, especially in the long-term treatment, probably because of the tendency of Cd to accumulate in higher amounts in the roots (Chugh and Sawhney, 1996; Ahsan *et*

al., 2007b). The result suggests that seedlings emerging in cadmium-contaminated soils are likely to have a stunted and poorly developed root system (Gianazza *et al.*, 2007). This would restrict the capacity of the seedling to absorb nutrients and moisture from soil, which might hamper their further growth.

Effects of proteolytic activity as a function of doses

Rihane showed a higher proteolytic activity level in the control than Manel (Figure 4) at all treatment durations, especially at 12 hr of germination, in which the proteolytic activity increased by about 57%.

The effect of increasing Cd concentration in the germination medium on proteolytic activity in radicles of germinating barley exposed to Cd treatment during 12, 24, 48, 72 and 96 hr is shown in Figure 5. The highest dose of Cd stimulated proteolytic activity, expressed in units per g fresh weight (FW) compared with the control, with an increase of about 2 to 2.5 times in Manel and 2 to 3 times in Rihane.

Effects of cadmium on proteolytic activity in the course of time

As illustrated by Figure 6, proteolytic activities gradually increased with increasing external Cd level. Concomitantly to the

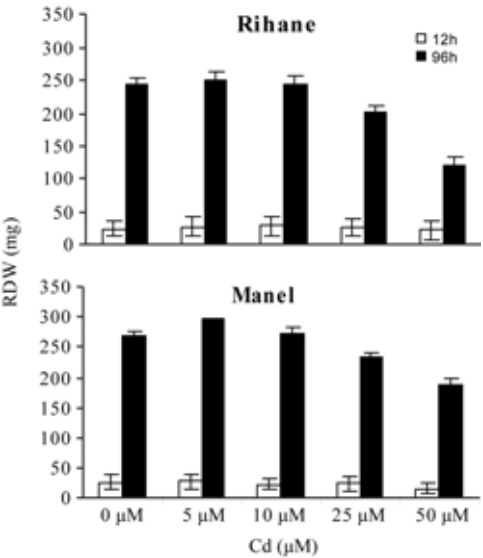


Figure 3. Effects on RDW production of Cd treatments (0, 5, 10, 25 and 50 μM) applied for the two extreme durations of 12 and 96 hr of germination in Manel and Rihane. Data are the means of five individual repetitions ± SE at 0.05 significance level.

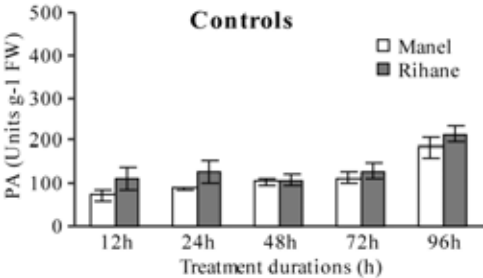


Figure 4. Changes of proteolytic activity (PA) in radicles of barley grains Manel and Rihane germinated without Cd (controls) during 12, 24, 48, 72 and 96 hr. Data are the means of five individual repetitions ± SE at 0.05 significance level.

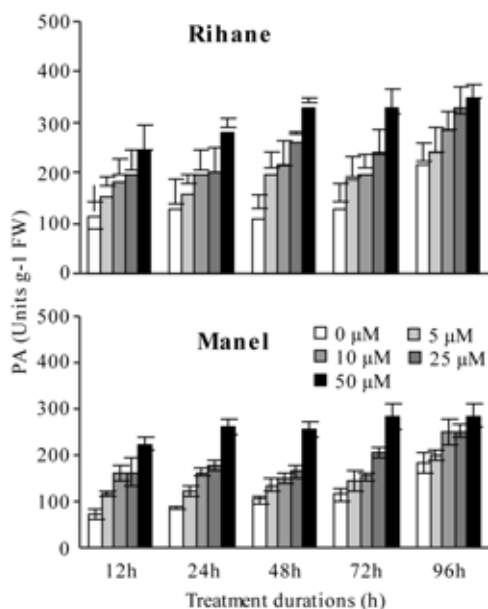


Figure 5. Changes of proteolytic activity (PA) in radicles of barley grains of Manel and Rihane exposed to increasing Cd concentration during 12, 24, 48, 72 and 96 hr of germination. Data are the means of five individual repetitions \pm SE at 0.05 significance level.

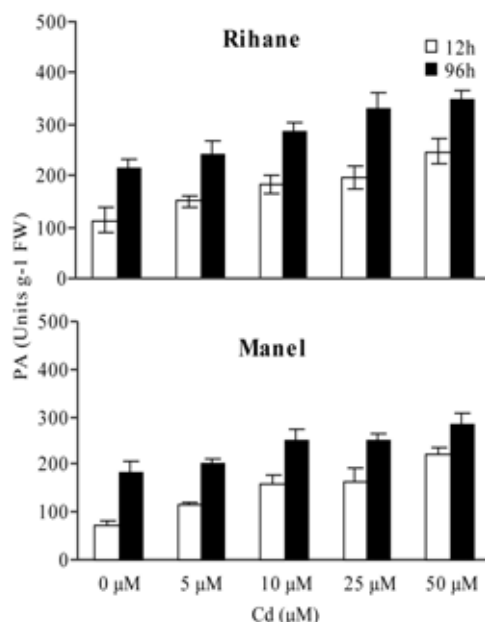


Figure 6. Changes of protease activity in radicles of barley grains of Manel and Rihane exposed to increasing Cd concentration (0, 5, 10, 25 and 50 μ M) applied for the extreme durations of 12 and 96 hr of germination. Data are the means of five individual repetitions \pm SE at 0.05 significance level.

growth reduction, an enhancement of protease activity in a concentration-dependant manner was recorded in Cd-treated radicles, especially in the long-term experiments. So the highest concentration of Cd stimulates the proteolytic activity of radicles (compared to the control), by about 55% and 63%, respectively, in Manel and Rihane at 96 hr (Figure 6).

Similar results have already been found in vegetable species (Palma *et al.*, 2002; Romero-Puertas *et al.*, 2002). However, and according to previous studies, it has been shown that protein degradation and proteolytic activity can be used as an appropriate index of oxidative stress and that an oxidative stress could be involved in Cd toxicity by either inducing oxygen free-radical production or

by decreasing enzymatic and non-enzymatic antioxidants (Romero-Puertas *et al.*, 2002; Pal *et al.*, 2006). This led to a concentration-dependant imbalance in the antioxidant status of pea plants (Romero-Puertas *et al.*, 2002).

Conclusion

Taken together, our findings suggest that the Cd stress affects more severely the variety Rihane, especially in the longer-term treatment combined with highest level of Cd, whereas, the shorter-term treatment with low amounts of Cd affect radicle growth and proteolytic activity only slightly. Together with the growth reduction, high concentrations of Cd enhanced the proteolytic activity in a concentration-dependant manner.

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Identification of a QTL on chromosome 7H for sodium exclusion from wild barley, *Hordeum spontaneum*

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Abstract

The purpose of this study was to accurately define the location of a QTL associated with sodium exclusion from wild barley (*Hordeum vulgare* L. subsp. *spontaneum* (C. Koch) Thell.), for use in improvement of salinity tolerance of cultivated barley (*H. vulgare* L.). The sodium exclusion locus on chromosome 7H was initially identified in a population of 72 F₁-derived DH lines made from a cross between cv. Barque and *H. spontaneum* accession CPI-71284-48, with the favorable (sodium exclusion) allele coming from *H. spontaneum*. Shoot sodium accumulation was measured in hydroponics and the data compared to scores for 1000 marker loci. The QTL had high significance (LRS = 45.6) and accounted for 51% of the total phenotypic variation. From an advanced-backcross (AB-QTL) population made using the same parents (recurrent parent: Barque), 21 DH lines with recombination in the 7H QTL region were used to further delimit the genetic interval. Twelve of the recombinant DH lines accumulated sodium to high concentrations, while nine lines accumulated significantly lower sodium. Comparison of the sodium accumulation phenotypes with the marker genotypes of the recombinants delimited the QTL to an interval of 18 cM bound by markers Bmag914 and EBmatc16. Links

to the related interval in the rice genome and candidate genes are currently under investigation.

Introduction

Salinity is one of the major abiotic stress affecting crop plants. While 20% of irrigated land is affected by high salinity, dry-land salinity is also becoming an increasingly important problem (Flowers and Yeo, 1995; Munns, 2007). The ability of plants to exclude toxic levels of sodium from the shoot is a major component of salinity tolerance (Munns and James, 2003; Tester and Davenport, 2003).

As a result of a long history of domestication and human selection, much of the available genetic diversity of wild barley has not been captured in cultivated barley. However, enrichment of the gene pool of current cultivars can be accomplished by tapping into the vast genetic resources that can be found in the wild relatives of the *Hordeum* genus (Forster *et al.*, 1997, 2000; Colmer *et al.*, 2005).

The strategy of using populations derived from hybrids between cultivated barley (*H. vulgare* L.) and land races or wild barley (*H. vulgare* L. subsp. *spontaneum* (C. Koch) Thell. has been effective for improving tolerance to boron toxicity (Jefferies *et al.*,

1999) and other agronomically important traits (Korff *et al.*, 2006). Using existing genetic maps, comprising thousands of molecular markers in doubled-haploid (DH) and advanced-backcross QTL (AB-QTL) populations in barley provides an effective means to rapidly localize other traits to particular chromosomal locations (Ellis *et al.*, 1997; Forster *et al.*, 2000; Wenzl *et al.*, 2006; Hearnden *et al.*, 2007).

This genetic approach requires several steps before putative candidate genes underlying the QTL can be identified:

- assessing phenotype plants from DH and AB-QTL populations originating from the same cross (Gorham *et al.*, 1990; Forster *et al.*, 1994). In the current study, this initial step allowed the recognition of individuals with either sodium exclusion or salinity tolerance; and
- assessing genotype plants using molecular markers within the QTL of interest (Ellis *et al.*, 1997; Forster 2001). Most molecular markers show co-dominant inheritance and are easily scored.

The aim of this study was to identify the QTL associated with sodium exclusion in a wild barley accession.

Materials and methods

Plant materials

Two populations, an F_1 -derived DH population and an AB-QTL (BC_2F_1 -derived DH) population were prepared from a cross between the Australian feed barley Barque-73 and *H. spontaneum* accession CPI-71284-48 (Eglinton *et al.*, 2000; Hearnden *et al.*, 2007). The initial DH population included 72 unique lines and the AB-QTL population included 325 lines. Seeds of DH lines from initial and AB-QTL populations, as well as parental forms (maternal, Barque-73 and paternal, CPI-71284-48), were provided by the Molecular Plant Breeding CRC, Adelaide.

Growth conditions, supported hydroponic system and ion determination

Seeds were germinated for 4 days at room temperature on moist filter paper before being transferred to a supported hydroponic setup. Seedlings were transplanted into buckets filled with 3 mm diameter polycarbonate fragments (Plastic's Granulating Services, Adelaide, Australia), to provide a solid substrate. Plants were supplied with irrigated growth solution as described previously (Shavrukov *et al.*, 2006) with pH maintained at 6.5–7.0. This was delivered to plants in a 20-min pump/20-min drain cycle, for 10 days, until the third leaf emerged. At third leaf emergence, NaCl was added twice daily in 25 mM steps until the final concentration reached 150 mM NaCl. Additional $CaCl_2$ was applied to the growth solution to maintain constant Ca^{2+} activity across NaCl treatments, as calculated using Visual MINTEQ (Department of Land and Water Resources Engineering, Stockholm, Sweden). After 10 days in elevated NaCl, the third leaf was harvested and fresh and dry weights were recorded before the leaf was digested in 10 ml of 1% HNO_3 at 85°C for 4 hrs using a Teflon hot-block (Environmental Express, USA). Concentrations of Na^+ and K^+ in the leaf were determined by flame photometry (Sherwood, UK, model 420, with a model 860 autosampler). Data are calculated and presented on a tissue water basis, as it is the concentration of Na^+ in the aqueous phase that is of most physiological relevance to the plant. This enabled a direct comparison to be made of the concentration of Na^+ in the leaf with that in the external solution.

Experimental design

Four biological replicates were used in the experiments with both the initial DH and the AB-QTL populations. Plants were grown in rows with equal distance within and between

rows. The order of the rows was randomized. Individuals from the F_2 population were grown in similar conditions until the third leaf was sampled, after which the plants were labelled and transplanted into 20 cm pots with soil for the harvesting of mature seeds. The phenotyping of the DH population (72 lines) was repeated twice, once without vernalization and once with a vernalization treatment. For vernalization treatment, 4-day-old seedlings were incubated at 4°C for six weeks before transplanting into hydroponics.

QTL analysis

Sodium concentration in the third leaf of 72 DH lines (average for four replicates in each DH line) was compared to scores for 1000 marker loci from the genetic map described earlier (Hearnden *et al.*, 2007). QTLs, permutation tests for significance, the relative contributions of parents, interval mapping, constrained additive regression with nearest molecular markers and percentages of phenotypic variation were determined using the MapManager QTX computer program (Manly *et al.*, 2001).

DNA extraction

Immediately after sampling the third leaf for flame-photometry, the fourth leaf was collected for DNA extraction using a freeze-drying method (Fox *et al.*, 2003), with some modifications:

- Two pieces of leaf from the seedling (each approximately 30 mm long) were placed into a 1.1 ml collection microtube in a 96-well plate (National Scientific, USA).
- The plates containing the tissue were frozen at -80°C for one hour and then freeze-dried at -60°C with vacuum overnight using a freeze-drier (Model Alpha 1-2/LD, Christ, Germany).
- Stainless-steel ball bearings were added to each well and the tissue ground in a vibration grinder (Model MM 300, Retsch Mill, Germany) for 5 min at a frequency of 25 times per sec.

- After removing the ball bearings, 600 µl of extraction buffer was added to each tube and the plates were sealed with caps and shaken thoroughly. The plates were incubated at 65°C for 30 min.
- The plates were incubated for 15 min at 4°C and 300 µl of cold (4°C) 6M ammonium acetate was added. Plates were shaken vigorously to mix-in the ammonium acetate and incubated for a further 15 min at 4°C.
- The plates were centrifuged for 15 min at 4000 rpm (Model 2-5, Sigma, USA) to pellet the precipitated proteins and plant tissue.
- 600 µl of the supernatant were recovered into new collection microtubes and 360 µl of iso-propanol was added to each well. The plates were mixed thoroughly and incubated at room temperature for 15 min to precipitate the DNA.
- The samples were centrifuged for 15 min at 4000 rpm in order to pellet the DNA and the supernatant was discarded. The remaining fluid was drained off the DNA pellet by inverting the tubes onto a piece of paper towel.
- The pellet was washed in 400 µl of 70% ethanol.
- The plates were centrifuged for 15 min at 4000 rpm and the supernatant was removed and discarded again.
- The pellet was re-suspended in 400 µl of milli-Q water and incubated overnight at 4°C.
- The plates were centrifuged for 20 min at 4000 rpm.
- 300 µl of the supernatant was transferred to a 96-well microtitre plate. This DNA was used directly as templates for PCR.

PCR amplification

Four SSR markers, Bmag914, GBM-1519, EBmatc16 and Bmag359 (Hearnden *et al.*, 2007) from the identified QTL were used for molecular analysis. For PCR, a reaction volume of 14 µl was used, which included

6 μ l of DNA and 8 μ l of reaction mix with 10 \times reaction buffer, 2 mM MgCl₂, 0.2 mM each of dNTPs, 0.25 μ M each of both forward and reverse primers and 0.75 units of Platinum Taq DNA polymerase (Invitrogen). Amplification was carried out in a PTC-100 (MJ Research) with the program protocol as follows: initial denaturation at 93°C for 2 min, 40 cycles of denaturation at 93°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, with a final extension at 72°C for 10 min. PCR products were checked on 2% agarose gels stained using ethidium bromide and visualized using a UV transilluminator.

Statistical treatment

Data were analyzed statistically to assess significant difference ($P > 0.05$) between means and presented as mean \pm standard error. Values are means of four replicates for initial DH and AB-QTL populations.

Results

The parental lines responded differently to 150 mM NaCl in hydroponics. In our experiments, Barque accumulated

252 \pm 7.9 mM sodium, while CPI-71284-48 had a lower sodium accumulation of 187 \pm 6.6 mM. This indicates that a difference in the genetic control of sodium exclusion exists between the two lines. Phenotypes in the mapping DH populations should therefore be easily scored.

QTL analysis of 72 DH lines derived from a cross between Barque and CPI-71284-48 was performed by comparing the phenotyping data for sodium accumulation with scores of 1000 molecular markers from the established genetic map (Figure 1). Only one QTL was identified. This was on chromosome 7H, at the interval 57.5–75.6 cM from the distal end and relatively close to the centromere. A permutation test indicated a highly significant LRS of 23.4, while the maximum of the QTL had an LRS of 45.6 (equal to an LOD of 9.9). The paternal parent (CPI-71284-48) contributed to the sodium exclusion trait in this QTL. The closest molecular marker was GBM-1519 (position 68.7 cM), with flanking markers Bmag914 and EBmatc16 defining the interval 57.5–75.6 cM (Hearnden *et al.*, 2007). The percentage of total phenotypic variation accounted for by this QTL was 51%.

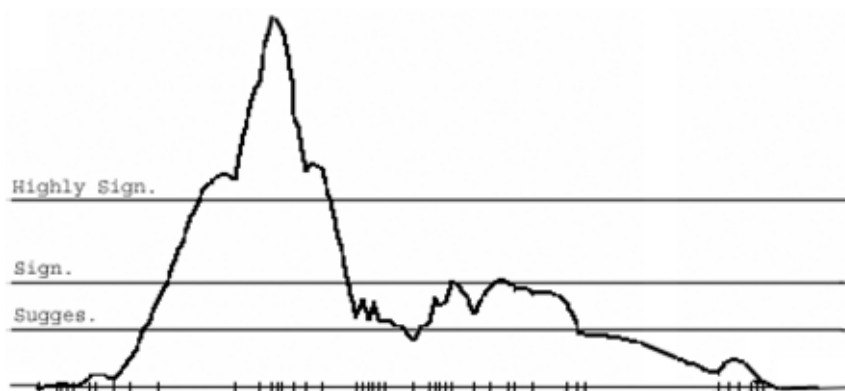


Figure 1. A QTL for sodium exclusion was identified by analysis of 72 DH lines originating from a cross between the barley cultivar Barque and accession CPI-71284-48 of wild barley, *H. spontaneum*. Seedlings were treated with 150 mM NaCl for 10 days, and sodium accumulation in the third leaf was measured. The QTL was localized on chromosome 7H, at the interval 57.5–75.6 cM, between SSR markers Bmag914 and EBmatc16 (Hearnden *et al.*, 2007).

An AB-QTL population (325 DH lines) was analyzed and 21 lines were identified with a recombination event occurring in the interval surrounding the identified QTL.

Four SSR markers were used for genotyping. The 21 DH lines selected from the AB-QTL population showed different recombination events between SSR markers and were organized into 5 groups according to the recombination events (Figure 2).

The 21 selected DH lines were phenotyped for sodium exclusion in hydroponics and the data for sodium accumulation are presented in Figure 3. The first twelve DH lines, from groups 1 to 3, accumulated significantly ($P > 0.05$) higher concentrations of sodium in leaf compared with the nine remaining DH lines from groups 4 and 5. This clearly indicated that the candidate gene responsible for sodium exclusion in this mapping population is located in the interval between SSR markers Bmag914 and EBmatc16. In this population, the exclusion locus co-segregates with the marker GBM-1519 (Figure 2). An example of PCR analysis of the DH lines using the SSR marker GBM-1519 is presented in Figure 4.

Discussion

Improvement of salinity tolerance in barley is limited by the size of the gene pool within cultivated barley, *Hordeum vulgare*. For this reason, the introduction of genes from the wild barley, *H. spontaneum*, may be a valuable strategy for further improving salinity tolerance. However, classical hybridization and recurrent selection between cultivated and wild barleys are accompanied by penalties in biomass or grain yield, or both, due to introgression of large numbers of deleterious alleles. Nevertheless, use of mapping DH populations from interspecific crosses between cultivated and wild barley can provide important information for the identification of QTL(s) and possible candidate gene(s) responsible for sodium exclusion as well as salinity tolerance.

In our study, only one QTL with high significance ($\text{LOD} = 9.9$) was identified on chromosome 7H during analysis of 72 DH lines from a cross between Barque and CPI-71284-48 (Figure 1). This suggests that there is only one major locus playing a crucial role in sodium exclusion in the mapping

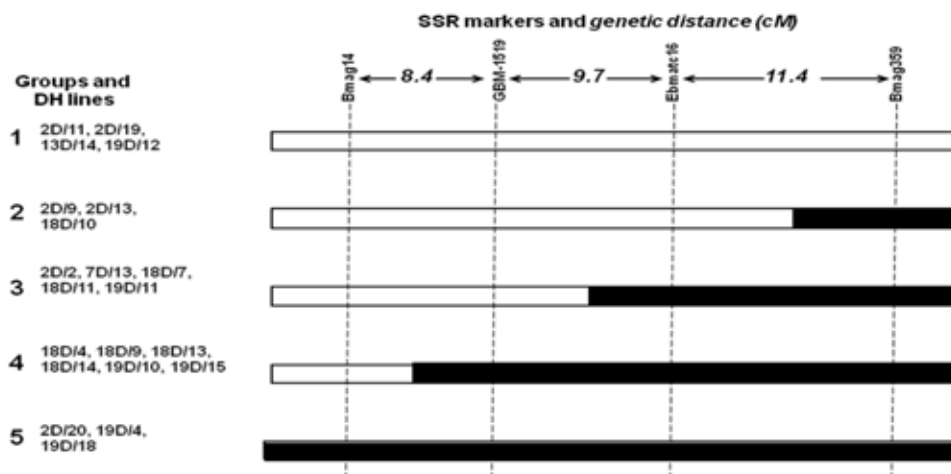


Figure 2. Molecular map of 21 DH lines from the AB-QTL population. Lines were organized into five groups representing similar types of recombinations within each group. Four SSR markers were used for fine mapping of the recombinations. White fragments of chromosome represent maternal allele A (Barque) and shaded fragments represent paternal allele B (CPI-71284-48).

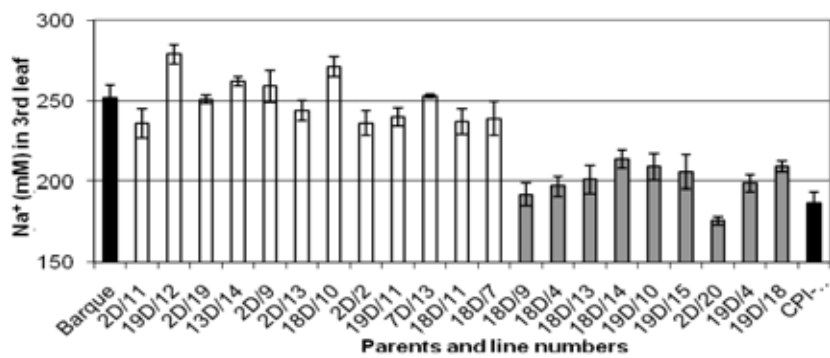


Figure 3. Comparison of average sodium accumulation (mM, in sap) in the third leaf of parents (black bars) and 21 DH lines from an AB-QTL population. White bars represent 12 DH lines from groups 1 to 3 and shaded bars represent nine DH lines from groups 4 and 5. Each value is the mean of four replicates \pm standard error.

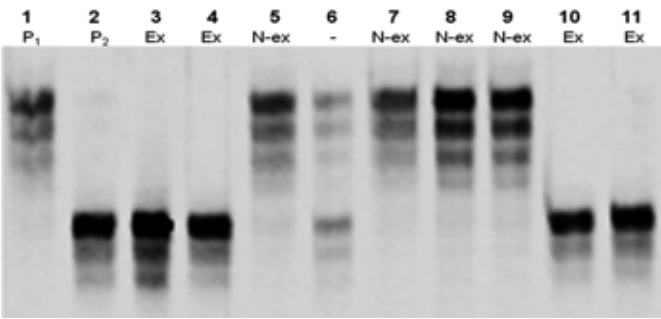


Figure 4. PCR analysis of barley lines using the SSR marker GBM-1519. Lanes 1 and 2 represent parents, Barque (P₁) and CPI-71284-48 (P₂), respectively. Other lanes represent different DH lines: Ex = excluder and N-ex = non-excluder. DNA from lane 6 was contaminated and eliminated from the analysis.

population. The CPI-71284-48 parent contributed the sodium exclusion QTL. The phenotyping score was repeated with a six-week vernalization of the seedlings at 4°C with similar results, indicating minimal influence of the vernalization response on the process of sodium exclusion.

The AB-QTL population contained 325 DH lines but only 21 DH lines were chosen with recombinations in the area of the QTL in the chromosome7H. Molecular analysis of these 21 DH lines with the four SSR markers showed that a number of DH lines had a recombination event in the same

interval and consequently they were organized into five groups (Figure 3). Ordering DH lines in a “step-ladder” arrangement according to their recombination may aid in the further localization of the QTL region.

Phenotypic analysis of 21 DH lines from AB-QTL population clearly demonstrated a difference in sodium accumulation or exclusion between Groups 1 to 3 (high sodium accumulation) and Groups 4 and 5 (low sodium accumulation), with consensus with the data for the parents (Figure 3).

Comparisons of the molecular map and phenotypic data of the 21 DH lines from the AB-QTL population indicated that the interval between SSR markers Bmag914 and EBmatc16 and surrounding the marker GBM-1519 is most likely to contain a candidate gene for sodium exclusion.

The corresponding interval in the rice genome includes 25 genes but contains no known genes responsible for ion transport, such as *HKT* (High-Affinity K⁺ Transporter), *NHX* (Na⁺-H⁺ Exchangers) or *SOS* (Salt

Overly Sensitive), previously identified to be involved in sodium exclusion and salinity tolerance (Tester and Davenport, 2003; Munns, 2005). Therefore, we can expect a new candidate gene in chromosome 7H for sodium exclusion to be identified from the wild barley *H. spontaneum* accession CPI-71284-48.

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Growing barley under Kuwait's harsh environmental conditions

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Abstract

Due to the desertic nature of Kuwait's environments and the combined effects of aridity and soil salinity, only a limited number of crops can be grown. Fresh water is used to irrigate crops, but this is an expensive approach and research is ongoing to exploit brackish water resources. Continuous irrigation with brackish water has resulted in widespread salinization of farmlands. Salt-tolerant barley lines selected in this study are useful in developing efficient agricultural systems for farmlands affected by salinity. Their use in combination with agronomic soil rejuvenation methods would allow the sustainable use of Kuwaiti resources and help bring into production areas previously considered unfit for cultivation.

A wide range of barley genotypes was studied under Kuwait field condition in Wafra. Results showed that sustainable barley cultivation is feasible in Kuwait by careful consideration of soil type, local groundwater, soil rejuvenation, water table control and genotype.

Introduction

Kuwait being an arid country has no surface waters and the average annual rainfall of 106 mm is insufficient to meet crop demand. Groundwater is the only natural water resource in Kuwait that can be used directly for agriculture without pre-treatment. The

total dissolved salts (TDS) of underground brackish water are 3 000–12 000 ppm. Improper use of brackish water will impair soil properties and plant growth. Since barley is one of the most salt-tolerant cereal crops, it was selected to study the effect of brackish-water irrigation in combination with agronomic soil rejuvenation methods.

Materials and methods

An experiment was conducted at the Agricultural Experiment Farm of the Public Authority for Agricultural Affairs and Fish Resources (PAAAFR) at Al-Wafra, Kuwait, using barley (*Hordeum vulgare* L.). The experiment included two treatments with three replications: in the first treatment the crop was irrigated with fresh water and in the other with brackish water (C₄S₁ grade). To avoid mixing of the two irrigation treatments, the fresh-water and brackish-water irrigated plots were 1.5 m distant. The experiment was set up as a trial series design. Seeds were sown in parallel rows and each replicate contained five seeds spaced at 25 cm intervals. After sowing, fertilizer was applied on the same day and manual weeding was done as and when necessary. The experimental plots were irrigated manually depending on the temperature and sunshine hours. When the plants attained maturity, the plants were covered with perforated plastic bags to prevent seed loss due to ear shattering, wind dispersal and bird damage.

Results

Observations were recorded from both treatments for traits including plant weight (g), no. of seeds per spike, no of seeds per plant, spike weight (g), survival, height of the main stem, no. of spikes and number and height of tillers. The data were subjected to analysis of variance (ANOVA) using the GenStat statistics program (GenStat 5, 1987). The ANOVA shows significant effects of treatment for all the nine traits. The brackish-water treatment reduced all the traits measured and the results are therefore in agreement with several reports on the negative effects of salinity on plant growth.

Discussion and conclusion

Generally, the growth parameters of crops will decrease under irrigation with saline water compared with fresh water. Since barley is a salt-tolerant crop, brackish-water irrigation did not have an adverse effect. However, the use of brackish water for irrigation might have changed soil physical and chemical characteristics, with changes in properties like soil structure, bulk density and infiltration rates. It might have increased the

pH, salinity and sodicity, and it might have affected the plant growth in the brackish-water experiment. Even then, barley can withstand such problems. Cultivated barley has tremendous potential for adaptation and it can grow well in Kuwaiti desert conditions. Hence improved stress resistance will be a crucial factor in increasing the growing area of barley in Kuwait.

Use of saline water for irrigating salt-tolerant strains would release high quality water for species that absolutely require it. If the C_4S_1 grade brackish irrigation water of Al-Wafra is not used and managed properly, greater salinization and soil degradation may result. However, C_4S_1 water has potential for irrigation in Kuwait, which merits continued investigation. The implication of this study is that the Wafra agricultural area can be considered for brackish-water irrigation, even though overall yields in brackish water irrigation were reduced by approximately 50%. It demonstrated great promise for future barley cultivation in Kuwait. Irrigation with brackish water had positive and negative effects on the soil, but analysis after each field trial showed that soil rejuvenation was possible by flushing out salts with fresh water.

Stability analysis and factors contributing to genotype by environment interactions in barley for low-moisture stress areas

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Abstract

Genotype \times environment (G \times E) interactions reduce progress from selection and make cultivar recommendation slower and difficult. To assess G \times E interactions in barley in low-moisture areas, ten genotypes, including one local variety, were evaluated over 10 environments (location \times year combinations). Different stability statistics and AMMI analysis were employed to study the performance of the genotypes and identify stable and superior varieties for recommendation. Entry EMBSN 5th 2/95-3-3-3 was characterized by small values for cultivar superiority measure, stability variance, ecovalence and IPCA1 score. It had non-significant regression coefficient and deviation from regression with high yielding ability. The yield stability (YS) statistic also proved high yielding and stable performance in the case of EMBSN 5th 2/95-3-3-3, which was released in 2006 under the name Bentu for cultivation in the low-moisture areas. The AMMI analysis revealed significant G \times E effects and the first three IPCA's accounted for 86% of the sum of square of G \times E interaction. The bi-plot analysis also showed EMBSN 5th 2/95-3-3-3 as stable and high yielding. Ecovalence and stability variance were equivalent in ranking genotypes. They were also positively rank-correlated with absolute value of IPCA1 and $1-b_j$, and S_{di}^2 . Thus, it may be sufficient to consider only some of these parameters in stability

analysis. The rains in June and September were more important for barley production in the low-moisture areas. Earliness is also an important genetic factor for the stability of some genotypes such as Bentu.

Introduction

Barley (*Hordeum vulgare* L.) is grown in Ethiopia at altitudes ranging from 1500 to 3500 masl. It is also an important cereal crop grown in the low-moisture areas of Ethiopia, where rainfall is frequently erratic. To develop varieties suitable for this environment, evaluating and testing adaptability and stability of the genotypes is crucial. Hence, breeders evaluate their material in a wide range of environments and seasons. Genotype \times Environment (G \times E) interactions are of major concern in plant breeding for two main reasons (Kang and Magari, 1996). First, it reduces progress from selection, and, second, it makes cultivar recommendation difficult as it is statistically impossible to interpret the main effects. Despite the problems associated with G \times E interactions, it is also possible to utilize the positive aspects of interactions. It is recommended to evaluate genotypes in various environments and apply different statistical techniques to make varietal recommendation depending upon varietal performance.

Among the statistical procedures, the regression-based models of Yates and Cochran (1938), Finlay and Wilkinson

(1963) and Eberhart and Russell (1966) were developed to aid selection when $G \times E$ interaction exists. Ecovalence (Wricke 1962), stability variance (Shukla, 1972) and cultivar superiority measure (Lin and Binns, 1988) are among the stability parameters to identify stable genotypes. Kang (1993) proposed a yield-stability (YS) statistic to select simultaneously for yield and stable performance. Statistical approaches such as AMMI (Additive Main Effect and Multiplicative Interaction) analysis and cluster analysis are also important in the analysis of $G \times E$ interactions (Gauch and Zobel, 1996). AMMI analysis is a multivariate approach, which depicts the value of genotypes, environments and their relationships in a bi-plot. The results of AMMI analysis are useful in supporting the program decision such as specific adaptation and selection of environments (Gauch and Zobel, 1996). The application of these statistical tools in yield trials improves the variety selection efforts.

Genotypes with high stability and high yield are the prime products required of any breeding program. So, analysis of stability of the genotypes by evaluating across locations and seasons is an important step in the breeding program, aided by various stability statistical methods. It is also important to study the relationships among these statistics in order

to exploit any complementary strengths. Thus, this study had the following objectives: (1) to identify genotypes with stable and superior yield; (2) to study the relationships among the different stability statistics; and (3) to identify the environmental and genetic variables contributing to the $G \times E$ interactions.

Materials and methods

Environments and genotypes

Ten environments (location \times year combinations) in Ethiopia were considered in this study. The locations included Dhera and Asasa in Arsi, and Arsi Negele in West Arsi. Data were available for 2001 to 2004 for all locations except Arsi Negele in 2001 and Dhera in 2002. The location \times year combinations are described in Table 1. In this study, nine barley genotypes introduced from ICARDA for low-moisture areas (both six-row and two-row types) were included, together with the local variety, Aruso.

Experimental procedures

The experiments were laid out as an RCBD design with 3 replications. The plot size was 3 m² (6 rows with 20 cm spacing and 2.5 m length). The central four rows from each plot were used to determine grain yield, hectolitre weight and 1000-kernel weight (TKW).

Table 1. Description of the environments (location \times year combinations).

Label	Environment	Site	Year	RF (June)	RF (Sept.)	RF (Oct.)	RF (June- Oct.)
A	E1	Asasa	2001	73.8	53.9	0.0	445.5
B	E2	Dhera	2001	85.9	88.8	17.2	664.9
C	E3	Asasa	2002	38.0	34.7	8.8	329.8
D	E4	Arsi Negele	2002	82.6	54.6	10.7	451.0
E	E5	Asasa	2003	61.2	95.0	6.7	330.5
F	E6	Dhera	2003	54.7	108.9	0.0	425.2
G	E7	Arsi Negele	2003	87.5	85.4	13.0	358.9
H	E8	Asasa	2004	73.4	30.7	5.3	328.0
I	E9	Dhera	2004	22.2	58.8	40.5	356.5
J	E10	Arsi Negele	2004	132.3	213.0	82.9	712.5

RF = rainfall in mm.

Grain yield was adjusted to 12.5% moisture and converted to t/ha. The data on days to heading and maturity were recorded on a plot basis, and plant height was averaged over five plants randomly selected from each plot.

Statistical procedures

Analysis of variance was done for each location and then the combined analysis of variance for grain yield was done after testing for homogeneity of error variance over locations using SAS statistical software. Parameters such as ecovalence (W_i) of Wricke (1962), stability parameters of Eberhart and Russell (1966), stability variance ($sh-\sigma^2_i$) of Shukla (1972) and cultivar superiority measure (P_i) of Lin and Binns (1988) were estimated to study stability of the genotypes. AMMI analysis was carried out using Agrobases 99 statistical software. In the AMMI analysis, the model for phenotypic performance of genotype g tested in environment e can be expressed as

$$Y_{ger} = \mu + a_g + b_e + SI_n g_{gn} d_{en} + r_{ge} + E_{ger}$$

where Y_{ger} is the yield of genotype g in environment e for replicate r , μ is the grand mean, a_g is the mean deviation of the genotype g (genotype mean minus grand mean), and b_e is the mean deviation of environmental mean; SI_n is the singular value for IPCA axis n ; g_{gn} is the genotype g eigenvector value for IPCA axis n ; d_{en} is the environment e eigenvector value for the IPCA axis n ; r_{ge} is the residual; and E_{ger} is the error.

The genotypes and environments were plotted on AMMI biplot using Agrobases 99 statistical software. The YS statistics were estimated following Kang (1993). Spearman's rank correlation coefficients among the different parameters were estimated to examine their relationship using SPSS v13.0 for Windows. Correlation coefficients were estimated for IPCA1 and mean grain yield of genotypes with days to heading, days to maturity, TKW and hectolitre weight. Similarly, correlation coefficients were

determined for IPCA1 and mean grain yield for environments with rainfall data.

Results and discussions

Analysis of variance

The combined analysis of variance indicated highly significant ($P < 0.01$) differences among genotypes and environments, as well as the $G \times E$ interaction effect (Table 2), indicating the contribution of the environmental factors to the total variability and the differential response of genotypes in different environments. The larger the size of the $G \times E$ interaction component relative to the genotypic component, the more complex the problem of identifying broadly adapted genotypes (DeLacy *et al.*, 1996). However, in this study, the $G \times E$ interaction component

Table 2. Analysis of Variance for combined, experiment-wise and AMMI analyses.

Combined Analysis		
Source	DF	MS
Genotype (G)	9	3.57**
Environment (E)	9	58.07**
G×E	81	0.62**
Error	180	0.15
Variance for genotype = 0.098; Variance for G×E interaction = 0.152		
AMMI Analysis		
Total	299	
Environments	9	58.1***
Rep(E)	20	0.21
Genotype	9	3.57***
G×E	81	0.62***
IPCA1	17	1.46***
IPCA2	15	0.71***
IPCA3	13	0.59***
IPCA Residuals	36	0.194
Residual	180	0.15
Grand Mean = 3.76	CV = 10.35 R ² = 0.96	

*, **, *** indicate significance at 0.05, 0.01 and 0.001 probability levels, respectively.

was only 1.55 times that of the genotypic component (Table 2). Thus, it is important to identify which genotypes and environments are contributing more to the total G×E interaction. This leads to further analysis of G×E interactions using different statistical tools to identify stable and high yielding genotypes.

Mean yield and stability statistics

Entries 3 (4.32 t/ha), 2 (4.09 t/ha) and 4 (4.08 t/ha) had significantly higher mean yield than the remaining genotypes (Table 3). Thus, these genotypes could be selected for high mean yield. The same genotypes were also superior according to the cultivar

superiority measure (P_i) (smaller P_i values of 0.13, 0.18 and 0.27 for entries 3, 4 and 2, respectively). According to Kang and Magari (1996), it may be difficult to select consistently high yielding genotypes based on mean grain yield alone if G×E exists. It is therefore essential to assess the stability of genotypes.

Stability variance (σ^2), however, revealed that the more stable genotypes were 5 (0.14), 6 (0.15) and 2 (0.18). Thus, entry 2 combines both stability and higher yield. Based on stability variance, entry 3 (1.14) was relatively less stable, but with a high mean yield and a good cultivar superiority measure. This genotype performed poorly in low-yielding environments, which include those

Table 3. Stability parameters for the 10 genotypes tested in 10 environments.

Entry	Label	Genotype	Mean (t/ha)	P_i	$sh-\sigma^2_i$	W_i	IPCA1 Score	IPCA2 Score	S_{di}^2	b_i
1	a	EMBSN 37/96-1-1-1	3.65	0.63	0.26	0.82	0.059	0.534	0.05	0.96
2	b	EMBSN 5th 2/95-3-3-3	4.09	0.21	0.18	0.62	-0.032	-0.193	0.01	1.08
3	c	EMBSN 5th 36/95-8-8-4	4.32	0.13	1.14	2.91	-0.811	-0.485	0.15**	1.28*
4	d	EMBSN 5th 46/95-9-9-5	4.08	0.18	0.60	1.62	-0.360	-0.744	0.12**	1.12
5	e	EMBSN 28/96-17-17-7	3.87	0.38	0.14	0.52	0.148	0.139	0.01	0.94
6	f	EMBSN 22/96-24-24-9	3.87	0.41	0.15	0.55	-0.026	0.403	0.02	1.00
7	g	EMBSN 1/96-41-41-13	3.52	0.80	0.51	1.42	-0.228	0.648	0.12**	1.04
8	h	EMBSN 43/96-42-42-14	3.63	0.68	0.56	1.53	-0.268	0.147	0.09**	1.15
9	i	EMBSN 9/96-43-43-15	3.28	1.05	0.27	0.84	0.136	-0.085	0.04	0.94
10	j	Local check	3.31	1.24	2.37	5.87	1.384	-0.369	0.14**	0.50*
Mean			3.76							
LSD (5%)			0.20							

* and ** indicate significance at 0.05 and 0.01 probability levels, respectively.

labeled as I, C and B. Entry 9 had a relatively small value for σ_i^2 (0.27) and large value for P_i (1.05), indicating poor performance in all or most environments. Since ecovalence (W_i) is estimated based on $G \times E$ values like that of the stability variance (Lin *et al.*, 1986), it ranked the genotypes in stability similar to stability variance (Table 3). Hence, the mean yield and P_i on one side and σ_i^2 and W_i on the other side, measure different characteristics of the genotypes.

The regression coefficient (b_i) and deviation from regression (S_{di}^2) must be non-significant for difference from 1 and 0, respectively, to consider genotypes as stable (Eberhart and Russell, 1966). For high stability to be desirable, it should also complement high mean yield. Entry 2 combined non-significance for b_i (1.08) and S_{di}^2 (0.01) with high mean yield of 4.09 (Table 3). This indicates stability and better yield performance of entry 2. Similarly, entry 9 was among the most stable genotypes, but with the lowest grain yield, which was in line with that discussed for cultivar superiority measures and stability variance. Entry 3 had significant value for b_i (1.28) and high mean yield, which was an indication of adaptation only to the most favorable environments. For the set of genotypes and environments considered in this study, entry 2 was best, based on YS statistic (Table 4). The result of YS for entry 2 was in line with that discussed above for other statistical tools considered in this study.

AMMI analysis

The AMMI analysis of variance indicated highly significant ($P < 0.001$) differences for genotype, environmental and $G \times E$ interaction effects, as well as the first three IPCAs (Table 2). Post-diction and prediction accuracies are used to determine the best fitting AMMI model for the data (Gauch and Zobel, 1988). The first three IPCAs accounted for about 86% of the $G \times E$ interaction sum of squares (Table 5). The first IPCA alone

contributed 49.4% of the interaction effect.

Based on the IPCA1 for genotypes, entry 6 (-0.026), entry 2 (-0.032) and entry 1 (0.056) were the genotypes that contributed little to the $G \times E$ interaction (Table 3). Entries 9, 5, 8 and 2 contributed less to the $G \times E$ interaction based on IPCA2 (Table 3). Entry 2 had also significantly higher grain yield (4.09 t/ha) compared with the grand mean yield (3.76 t/ha). The local variety (Aruso) and entry 3 (with IPCA1 = 1.384 and -0.811, respectively) contributed relatively more to the $G \times E$ interactions. These genotypes responded differentially in the varying environments. Among the environments, three of them (F, J and G) were associated with small values for IPCA1 scores (IPCA1 = 0.055, -0.065 and -0.086, respectively) and hence contributed less to the total $G \times E$ interaction (Table 6). In contrast, 3 environments (C, H and A) contributed most to the total $G \times E$ interactions. Entries 6, 2 and 1 (in the biplot indicated with f, b and a, respectively) were located near the origin of the biplot together with the environments F and G (Figure 1), indicating less contribution of these genotypes and environments to the $G \times E$ interaction.

The absolute value of IPCA1 for environments was negatively correlated with rainfall data for the environments (Table 7). However, only the rainfall in September was significantly correlated with absolute value of IPCA1 scores for environments ($r = -0.716^{**}$). Environments with little contribution to the $G \times E$ interactions (F, J and G) received more rainfall in September, while those contributing much to the $G \times E$ interactions (C, H and A) received low rainfall during this month (Tables 1 and 5). The rainfall in June was also important in determining yield ($r = 0.605^*$), while rainfall in September was also correlated positively with mean yield ($r = 0.222$). The rainfall in June was important for early seedling growth while that of September was essential for grain filling. The rainfall in these two

Table 4. Yield Stability (YS) statistics for the 10 genotypes evaluated.

Entry	Genotype	Mean yield (t/ha)	Rank (R)	Adjustment to rank (R')	Summed R + R'	$Sh-\sigma_i^2$	Stability rating (S)	YS
1	EMBSN 37/96- 1-1-1	3.65	5	-1	4	0.26	0	4a
2	EMBSN 5th 2/95-3-3-3	4.09	9	+2	11	0.18	0	11a
3	EMBSN 5th 36/95-8-8-4	4.32	10	+3	13	1.14**	-8	5a
4	EMBSN 5th 46/95-9-9-5	4.08	8	+2	10	0.60*	-4	6a
5	EMBSN 28/96- 17-17-7	3.87	7	+1	8	0.14	0	8a
6	EMBSN 22/96- 24-24-9	3.87	6	+1	7	0.15	0	7a
7	EMBSN 1/96- 41-41-13	3.52	3	-2	1	0.51*	-4	-3
8	EMBSN 43/96- 42-42-14	3.63	4	-1	3	0.56*	-4	-1
9	EMBSN 9/96- 43-43-15	3.28	1	-3	-2	0.27	0	-2
10	Local check	3.31	2	-3	-1	2.37**	-8	-9
	Mean	3.76						2.6
	LSD (5%)	0.20						

In YS: a = entries selected based on YS statistics > 2.6. * and ** indicate significance of approximate F test for $Sh-\sigma_i^2$ at 0.05 and 0.01 probability levels, respectively. Assignment of adjustment for rank (R'): +1 for mean yield > overall mean yield (OMY), +2 for mean yield ≥ 1 LSD over OMY, +3 for mean yield ≥ 2 LSD over OMY, -1 for mean yield < OMY, -2 for mean yield ≤ 1 LSD over OMY, and -3 for mean yield ≤ 2 LSD over OMY.

months is critical for barley production in low-moisture areas in Ethiopia. However, the total rainfall during the growing season did

not explain the difference in mean yield, nor the causes of G×E interactions.

Environmental mean yield and IPCA1 are correlated to rainfall data, while genotypic mean yield and IPCA1 are correlated to genetic factors.

With reference to Table 7, mean yield for genotypes was negatively correlated with TKW ($r = -0.579^*$). This negative correlation coefficient was primarily due to the composition of the genotypes. Both six-row and two-row types were included in the study. Those two-row types possessed heavy kernels, few kernels and low grain yield. The opposite was generally true for the six-row

Table 5. Contribution of each IPCA to the G×E interactions.

IPCA Axis	Eigen value	% G×E Explained	Cumulative %
1	8.247	49.4	49.4
2	3.547	21.3	70.7
3	2.563	15.4	86.1
Other IPCA's	2.326	13.9	100

Table 6. IPCA1 scores and mean grain yield for environments.

Label	Environment	IPCA1 Scores	IPCA2 Scores	Mean yield (t/ha)
A	Asasa in 2001	-0.699	0.518	4.5
B	Dhera in 2001	0.333	0.495	2.3
C	Asasa in 2002	1.122	0.391	1.7
D	Arsi Negele in 2002	-0.447	0.327	4.4
E	Asasa in 2003	-0.123	0.265	4.8
F	Dhera in 2003	0.055	-0.112	3.5
G	Arsi Negele in 2003	-0.086	-0.166	4.7
H	Asasa in 2004	-0.670	-0.483	5.4
I	Dhera in 2004	0.580	-0.556	1.7
J	Arsi Negele in 2004	-0.065	-0.677	4.7

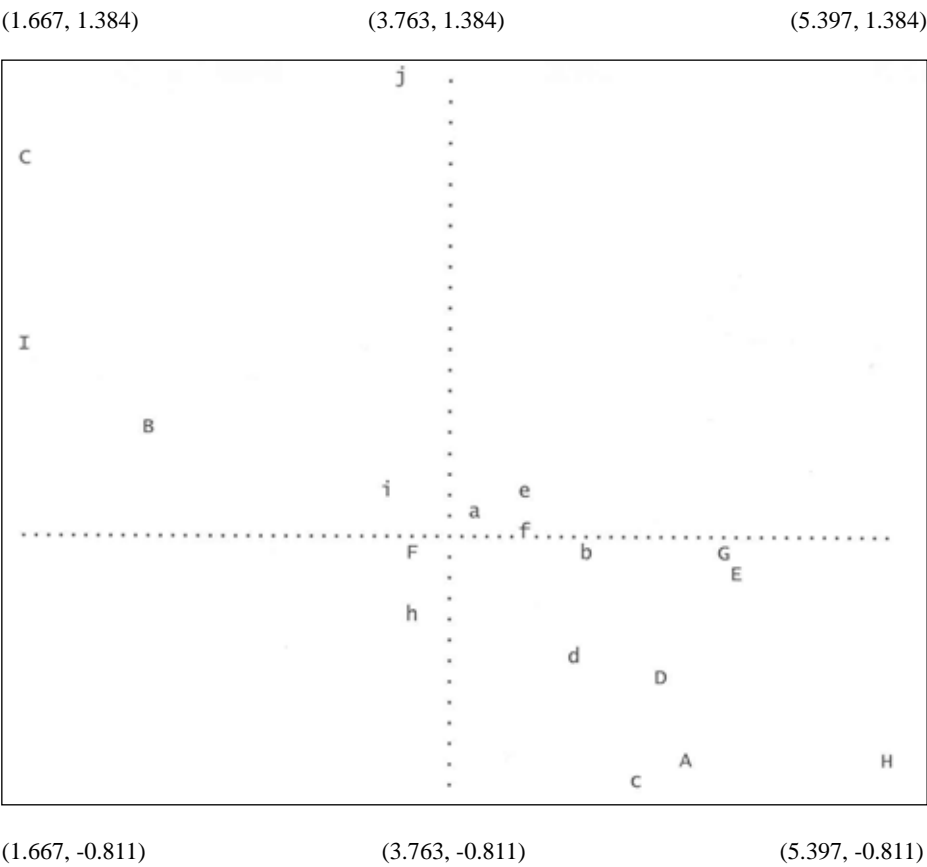


Figure 1. AMMI BI-PLOT (X-axis mean grain yield and Y-axis IPCA1 for genotypes and environments). Genotypes are plotted as a, b, c, d, e, f, g, h, i and j, and environments are plotted as A, B, C, D, E, F, G, H, I and J. Genotype (g) and environment (J) in place of others with similar means are not shown.

Table 7. Correlation of mean yield and IPCA1 for environments and genotypes with environmental and genetic factors.

Variable type	Variable Name	Mean yield	IPCA1
Environmental	Rainfall (June)	0.605*	-0.515
Environmental	Rainfall (September)	0.222	-0.716**
Environmental	Rainfall (October)	-0.159	-0.273
Environmental	Rainfall (June-October)	0.018	-0.382
Genetic	Days to heading	-0.400	0.741**
Genetic	Days to maturity	-0.051	0.723**
Genetic	Hectolitre weight	-0.513	0.070
Genetic	1000-kernel weight	-0.579*	0.747**

* and ** = significant at 0.05 and 0.01 probability levels, respectively.

types. The absolute value of IPCA1 score for genotypes was positively correlated with days to heading, days to maturity, and TKW ($r = 0.741^{**}$, 0.723^{**} and 0.747^{**} , respectively). According to Ashley (1999), earliness in genotypes is an important aspect of adaptation in low-moisture areas. These results also revealed the importance of earliness in determining mean grain yield and the G×E interaction of genotypes.

Relationship among the stability parameters

There was a perfect match between σ_i^2 and W_i ($r = 1.000^{**}$) in ranking genotypes (Table 8). These two parameters are grouped under type II stability concept (Lin *et al.*, 1986). Lin *et al.* (1986) and Kang *et al.* (1987) have reported that the two parameters are equivalent in ranking genotypes. Hence, it is sufficient to use one of these parameters. Both of these parameters were positively rank correlated with δ_{di}^2 ($r = 0.927^{**}$). Jalaluddin and Harrison (1993) and Mekbib (2003) have also reported similar results in wheat and common bean stability studies, respectively. The IPCA1 and b_i values are interpreted based on their absolute difference from 0 and 1, respectively. As shown in Table 8, the absolute value of IPCA1 ($|IPCA1|$) was positively correlated with σ_i^2 , W_i , S_{di}^2 and

$|1 - b_i|$ ($r = 0.879^{**}$, 0.879^{**} , 0.806^{**} and 0.818^{**} , respectively). The $|1 - b_i|$, in turn, was positively correlated with σ_i^2 and W_i ($r = 0.794^{**}$ and 0.794^{**} , respectively). The positive rank correlation among $|IPCA1|$, σ_i^2 , W_i , S_{di}^2 and $|1 - b_i|$ could indicate that they measured similar aspect of stability. AMMI analysis is important in understanding genotypes, environments and their interaction (Gauch and Zobel, 1996). It can produce graphs relevant to selection. The use of AMMI analysis could be recommended in combination with other stability statistical tools.

The regression coefficient (b_i) was positively correlated with all the parameters except cultivar superiority measure ($r = -0.673^*$). However, only the rank correlation coefficient between b_i with mean yield was significant ($r = 0.663^*$), which was in line with the results of Mekbib (2003). There was a high negative rank correlation coefficient between mean yield and cultivar superiority measure (-0.973^{**}), which coincides with that reported by Kang and Pham (1991). This indicates that the two parameters rank the genotypes differently. In practice, breeders select for high mean yield, whereas low values for cultivar superiority measure and the negative relationship between these two parameters was desirable.

Table 8. Spearman’s rank correlation coefficients among stability parameters.

	IPCA1	IPCA2	Mean yield	P_i	$sh-\sigma^2_i$	W_i	S_{di}^2	b_i	$1-b_i$
IPCA1	1.000	0.212	-0.079	0.055	0.879**	0.879**	0.806**	0.176	0.818**
IPCA2		1.000	0.310	-0.309	0.345	0.345	0.539	0.382	-0.115
Mean yield			1.000	-0.973**	-0.134	-0.134	-0.091	0.663*	0.073
P_i				1.000	0.139	0.139	0.103	-0.673*	-0.042
σ^2_i					1.000	1.000**	0.927**	0.248	0.794**
W_i						1.000	0.927**	0.248	0.794**
S_{di}^2							1.000	0.297	0.576
b_i								1.000	0.248
$1-b_i$									1.000

* and ** indicate significance at the 0.05 and 0.01 levels, respectively (2-tailed).

Conclusions

Generally, entry 2 (EMBSN 5th 2/95-3-3-3) was consistently identified as stable and high yielding by all the parameters considered in this study. It was released in 2006 with the name Bentu for cultivation in the low-moisture areas of Ethiopia. Bentu is an early variety in heading and maturity, which are key factors to escape drought in some years. However, the variety has small kernels indicating the need to improve this trait in the future.

It may be possible to use different combinations of stability parameters to identify the most stable variety. Most of the parameters considered in this study gave similar information on the stability of the genotypes tested. The absolute values of IPCA1 and $1-b_i$ were positively rank correlated with stability variance, deviation from regression and ecovalence. Thus, it may be sufficient to use some combinations of these parameters in the identification of stable genotypes. The use of AMMI analysis in combination with other stability statistical tools could be recommended due to its importance in understanding genotypes, environments and their interactions with the aid of bi-plots.

In the low-moisture areas considered in this study, the rainfall in June and September was important in explaining yield performance and was the cause of G×E interactions. The rainfall during these two months is important for early seedling establishment and grain filling, respectively. Earliness was also important in explaining G×E interactions.

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Detection of reaction types to drought stress in an international barley collection

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Abstract

Plants use several mechanisms to withstand drought. Our hypothesis is that with a multi-trait characterization it must be possible to describe the ‘whole plant’ reaction of barley accessions to drought using trials conducted for QTL analysis or association mapping. If this whole-plant behavior is under genetic control, it should be possible to detect comparably reacting genotypes and to describe them as reaction types.

A set of 104 barley accessions from an international barley collection were evaluated under irrigated and drought conditions, and phenotypic data were collected on 19 plant traits of shoots and roots. The dataset was standardized and analyzed with multivariate statistic cluster and discriminant analysis procedures.

With 12 of the 19 traits, we were able to detect 10 clusters of barley accessions. Both susceptible and adapted genotypes could be found. The first 5 clusters contain only a single genotype, which might be an indication that these clusters do not represent a reaction type with a general pattern. These genotypes could be outliers, but we cannot prove the existence of their pattern because in such trials for QTL analysis or association mapping there are no replications. Clusters 6 to 10 included several genotypes each, and we postulated that the respective genotypes show a different reaction pattern, which could be under genetic control, and we can characterize them as reaction types.

Introduction

Many plant stress experiments are described in the literature (Cattivelli *et al.*, 2008) that investigate the correlation between single-plant traits and their molecular basis. However, plants use several mechanisms to withstand drought. This must lead to a combination of different reactions and specific reaction patterns. In this sense, we postulated that barley genotypes show different reaction types due to drought stress. The objective of this study was to identify these reaction patterns and types using a population of 104 genotypes.

Material and methods

A set of 104 genotypes (accessions from the International Barley Core Collection (IBCC), the German regional Barley Core Collection, IPK, Gatersleben, and cv. Scarlett) were grown in spring season 2007 under a plastic tunnel at the Experimental Station Bonn-Poppelsdorf, Germany. Seeds were germinated in Petri dishes and 12 seedlings were planted in pots with 11.5 L of substrate. Substrate was a mixture of fine soil, sand, peat and lava. The two water regimes were “control”, = well watered, and “stress”, with a drought-stress-period of 20 days starting from mid-tillering (Figure 1). Water supply was through drip irrigation and adjusted to weather and transpiration conditions.

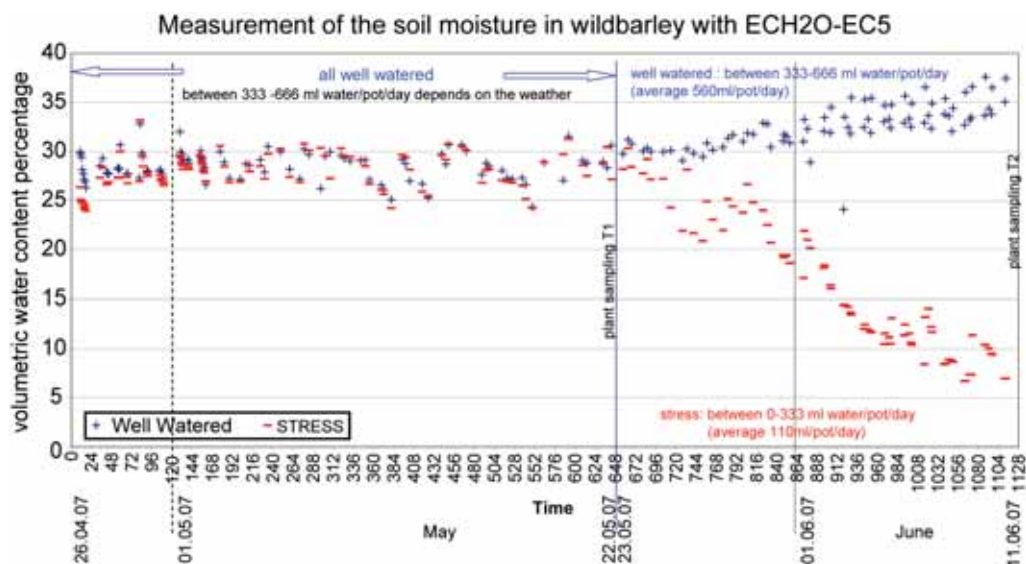


Figure 1. Water regimes for “control” (= well watered) and “stress” treatments.

Table 1. Traits recorded in both treatments

Trait	Plant part	Description	Unit	Source or method
SWC	Shoot	Relative Water Content in upper two leaves	%	Barrs and Weatherly, 1962
SFW	Shoot	Shoot Fresh Weight per plant	g	
SDW	Shoot	Shoot Dry Weight per plant	g	48 h @ 80°C
SMC	Shoot	Shoot Moisture Content per fresh mass	%	$(\text{SFW} - \text{SDW}) / \text{SFW} \times 100$
STI	Shoot	Tiller Number per plant	no.	
SEA	Shoot	Ear Number per plant	no.	
RLE	Root	Root Length per pot	cm	
RVO	Root	Root Volume in water per plant	cm ³	
RFW	Root	Root Fresh Weight per plant	g	
RDW	Root	Root Dry Weight per plant	g	48 h @ 80°C
RMC	Root	Root Moisture Content per fresh mass	%	$(\text{RFW} - \text{RDW}) / \text{RFW} \times 100$
RDA	Root	Root dry Weight 0–10 cm length per plant	%	relative to RDW
RDB	Root	Root dry Weight 0–20 cm length per plant	%	relative to RDW
RDC	Root	Root dry Weight >20 cm length per plant	%	relative to RDW
PFW	Plant	Plant Fresh Weight per plant	g	
PDW	Plant	Plant Dry Weight per plant	g	48 h @ 80°C
PMC	Plant	Plant Moisture Content per fresh mass	%	$(\text{PFW} - \text{PDW}) / \text{PFW} \times 100$
PSP	Plant	Shoot/Plant ratio on dry mass basis	%	$\text{SDW} / \text{PDW} \times 100$
PRP	Plant	Root/Plant ratio on dry mass basis	%	$\text{RDW} / \text{PDW} \times 100$

At the end of this 20-day period we terminated the experiment and evaluated the traits, as described in Table 1.

Roots were washed out and root volume was determined in a glass cylinder with

water, root length was measured with a ruler and parts of the root system from 0–10 cm (A), 10–20 cm (B) and >20 cm (C) depth were separated (Figure 2), weighed, dried and re-weighed.



Figure 2. Washed root system

The data were analyzed with the SAS Statistical Analysis System Package Version 8.2. For each accession we calculated the absolute differences between “stress” and “control” for determining the irrigation effects with SAS ANOVA (Table 2). Looking for the reaction types, we calculated the relative differences between “stress” and “control” for each trait and standardized them to a scale from 0% (= biggest negative difference) to 100% (= biggest positive difference), so that for all the traits the standardized differences lay in an interval from 0 to 100. With these standardized differences we conducted an Average Linkage Cluster Analysis (Figure 3) and SAS Discriminant Analysis using the procedure Stepdisc. The potential reaction types (the resulting clusters) were characterized by their mean standardized differences and their distances to the change point (Figure 4). This point on the scale 0 to 100 is the point where the relative differences were 0, the differences change from negative to positive, or, in terms of the plant trial, no

Table 2. Comparison of Control and Stress with Analysis of Variance and Means.

Trait	F-test	Mean (control)	Difference (stress)	Tukey
SWC	s	92.54	-19.92	s
SFW	s	8.82	-2.61	s
SDW		2.50	-0.11	
SMC	s	72.00	-10.58	s
STI		5.50	-0.06	
SEA		0.69	-0.08	
RLE	s	26.21	4.06	s
RVO	s	60.70	-1.94	s
RFW	s	7.78	-1.98	s
RDW		1.20	-0.14	
RMC	s	84.84	-3.28	s
RDA	s	74.21	-10.26	s
RDB	s	24.17	3.58	s
RDC	s	1.62	6.69	s
PFW	s	16.60	-4.59	s
PDW	s	3.70	-0.25	s
PMC	s	77.48	-6.51	s
PSP		67.16	1.32	
PRP		32.84	-1.32	

F-test ANOVA/Tukey test. s = significant at alpha = 0.05

reaction to the stress treatment was observed in relation to the control (For example: Figure 4 cluster 1 trait SDW “63/+14”: the averaged standardized difference of this cluster 1 is 63 and its distance to the change point “49” for SDW is +14, so SDW increased).

Results and discussion

Water stress caused in general a significant decrease in SWC, SFW, SMC, RVO, RFW, RMC, RDA, PFW, PDW and PMC (Table 2). SDW, STI, SEA, RDW, PSP and PRP showed no significant reaction, while RLE, RDB and RDC increased.

Using SAS Average Linkage Cluster Analysis with CCC, PSF and PTS2 criteria for determining the best number of clusters, we found 10 clusters with 1/1/1/1/15/62/13/5/4 accessions, respectively (Figure 3). With SAS

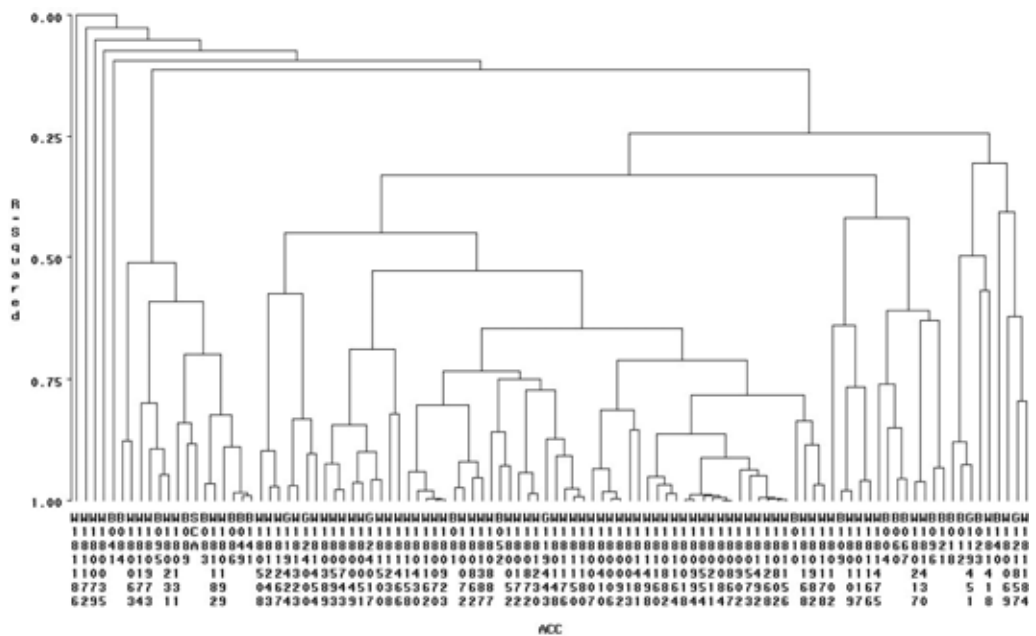


Figure 3. Cluster analysis of the 104 barley accessions

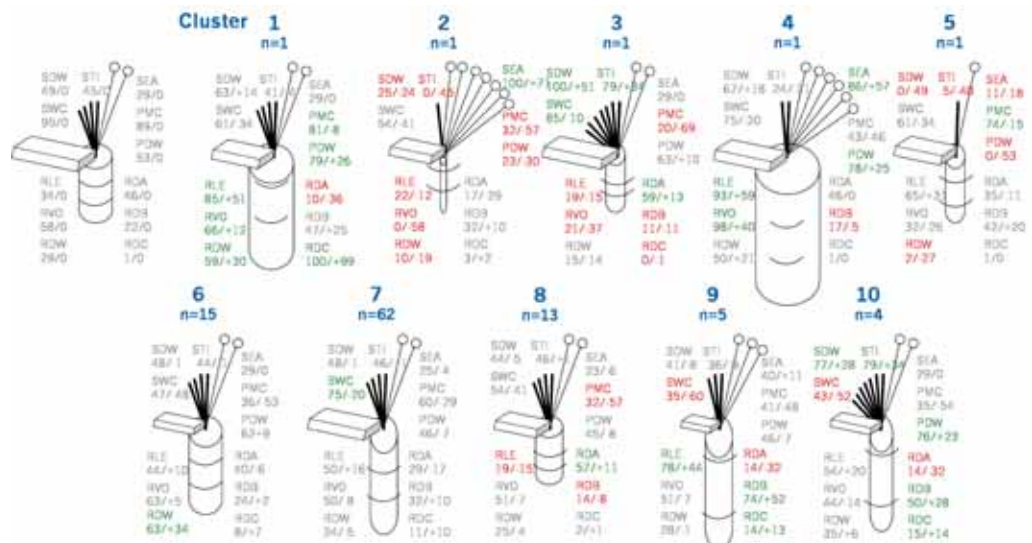


Figure 4. Mean standardized differences of the traits from the 10 clusters and their distance from the change point.

Discriminant Analysis procedure Stepdisc we identified the 12 traits (their standardized differences) which separated the 10 clusters best, namely SWC, STI, SEA, SDW, RLE, RVO, RDW, RDA, RDB, RDC, PMC and PDW.

The mean standardized differences of the 10 clusters are given in Figure 4. It shows all the 12 traits combined and gives us an impression of the reaction of the “whole plant system” to the stress treatment.

The first 5 clusters contain only a single genotype, which might be an indication that these clusters do not represent a reaction type with a general pattern. These genotypes could be outliers, but we cannot prove the existence of their pattern because there are no replications in such type of trials for QTL-analysis or association mapping. However, we found some interesting genotypes, such as cluster 1, with a larger root system (RLE+51, RVO+12, RDW+30, RDC+99), clusters 2 and 5 as the most susceptible to stress, and cluster 4 with strong increases in all traits as probably good adaptation to stress (SEA+57, PDW+25, RLE+59, RVO+40). Genotypes in cluster 4 need to be tested again to confirm its potential value.

Clusters 6 to 10 include several genotypes each, so we postulate that the respective genotypes show a different reaction pattern, which may result from genetic control, and we can characterize them as reaction types.

Clusters 6, 7, 8 and 9 do not show notable effects on the target trait SDW (shoot dry weight) due to the induced drought stress; in cluster 10, SDW actually increases (+28). So drought tolerance can be supposed. This reaction could be due to a good osmotic adaptation (cluster 7 – SWC only -20), a bigger root system in general (cluster 6 –

RDW +34), in the upper horizon (cluster 8 – RDA +11) or the shifting of the root architecture to longer and deeper roots (cluster 9 RDB +52, RDC +13 / cluster 10 RDB +28, RDC +14).

It is shown that the set of plant traits used and the multivariate statistical procedures produce a characterization of the barley accessions related to their susceptibility or tolerance to drought stress. The methods of cluster and discriminant analysis allow a statistically meaningful identification of groups. Also, extreme reactions can be detected.

The conclusions about the plant reactions of the accessions within the clusters seem to be comprehensible. With an additional set of physiological traits it should be possible to investigate which single reactions of the plants occur together and determine the “whole plant” behavior of the crop due to drought stress. In multi-environmental trials, the question of possible genotype \times environment interactions of the plant reactions will appear and its integration in the statistical analysis has to be discussed. With such multi-trait characterizations it might be possible to improve knowledge about the phenotypes of the populations used, helping to bridge the gap between genotyping and phenotyping.

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Session 5

Biotic stresses

The Barley-Puccinia rust system: a model to study the genetic factors that determine the host status of plants for specialized pathogens

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Abstract

Full non-host resistance can be defined as immunity, displayed by an entire plant species against all genotypes of a plant pathogen. The genetic basis of (non)host-status of plants is hard to study, since identification of the responsible genes would require interspecific crosses that suffer from sterility and abnormal segregation.

There are some plant+potential pathogen combinations where only 10% or less of the accessions are at most moderately susceptible. These may be regarded as marginal host or near-non-host, and can provide insights into the genes that determine whether a plant species is a host or a non-host to a would-be pathogen.

Barley (*Hordeum vulgare* L.) is an example of a near-non-host to several rusts (*Puccinia* spp.) of cereals and grasses. By crosses and selection, we accumulated susceptibility and developed an experimental line, SusPtrit, with high susceptibility to at least nine different heterologous rust taxa such as the wheat and *Agropyron* leaf rusts (*P. triticina* and *P. persistens*, respectively).

On the basis of SusPtrit and several regular, fully resistant barley accessions, we developed mapping populations. We established the polygenic inheritance (QTLs) of the near-non-host resistance to heterologous rusts. The QTLs have different and overlapping specificities. In addition, an occasional R-gene is involved. In each population, different sets of loci were

implicated in resistance. Very few genes were common between the populations, suggesting a high redundancy in barley for resistance factors. Selected QTLs are being introduced into near-isogenic lines to be fine-mapped. Our results show that the barley-*Puccinia* system is ideal to investigate the genetics of host-status for specialized plant pathogens.

Introduction

Plants are exposed to a huge number of potential pathogens that represent a very diverse array of micro-organisms. By far most plant species are non-host to by far most would-be pathogens. The term non-host has been defined as the resistant status of an entire plant species to all genotypes of a parasite or pathogen species (Heath, 2000). This definition is widely agreed upon, but implies that a non-host-status can never be proven. All genotypes of a plant species, even those in the past, present and future, cannot be tested against all past, present and future genotypes of a microbe species.

An important motive and justification for research into non-host resistance is the obviously high durability of this form of resistance. This is in contrast to the typically ephemeral effectiveness of R-genes that determine hypersensitive resistance. Exciting new insights are accumulating, and have been summarized and discussed in recent review papers (e.g. Jones and Dangl, 2006; Ingle *et al.*, 2006; Nürnberger and Lipka, 2005; Mysore and Ryu, 2004; O'Connell and

Panstruga, 2006; Thordal-Christensen, 2003; Schweizer, 2007). Most advances are in the understanding of perception of microbial intruders, and in the genes that play a role in signal transduction and defence. Many authors acknowledge that, despite these advances, still very little is known concerning the genes that determine the natural variation between plant species, making one a host, the other a non-host to a would-be (potential) pathogen.

In this contribution we will present the Barley-*Puccinia* rust system as a model to help in elucidating the specificity aspects of (non)host-status of a plant to a would-be pathogen.

Mechanisms of non-host resistance

The resistance of most plants to most would-be pathogens is based on a multilayered defence (Heath, 2001, 2003; da Cunha *et al.*, 2006), comprising physical and constitutive chemical features (preformed barriers) and induced defences. Would-be pathogens should be able to deal effectively with the defence that plant species use against maladapted microbial intruders (Heath, 1981). They may need to locate stomata on the plant leaf surface, and should tolerate or break down secondary metabolites. Since related plant species frequently have similar preformed barriers, it is reasonable to assume that, generally, preformed barriers are more likely to contribute to non-host resistance to pathogens of other plant families (like *Arabidopsis* to the wheat leaf rust), than to pathogens of plant species that are related to the non-host (like barley to the wheat leaf rust). Induced defences are effective against pathogens of plants of whatever close relationship with the non-host.

On non-host plant species, resistance against specialized maladapted fungal pathogens like rusts and powdery mildews commonly appears as defective haustorium formation, termed pre-haustorial or

penetration resistance (Heath, 1974; Elmhirst and Heath, 1987; Niks, 1987). This defence typically leads to the formation of cell wall reinforcements, also called cell wall appositions or papillae (O'Connell and Panstruga, 2006).

Frequently, the pre-haustorial non-host resistance is backed up by a hypersensitive post-penetration resistance for those infection units that still succeed in cell wall penetration (Heath, 2002; Lipka *et al.*, 2005). The process of this cell death in non-host plants is not necessarily the same as in hypersensitive resistance of the host species (Christopher-Kozjan and Heath, 2003).

Non-host resistance induced by MAMPs

It seems that plants are able to discriminate between self and non-self, and this ability is the basis for the activation of induced defences upon microbial infection. The perception of the non-self intruder in the plant tissue is probably mostly mediated by receptor-like kinases (RLKs). These recognize directly certain characteristics of microbe-associated molecular patterns (MAMPs). So, MAMP detection serves as an early warning system for the presence of non-self molecules. Also indirect perception of microbe-induced molecular patterns (MIMPs) occurs, where the product of the intrinsic activity of a microbe-derived compound will be recognized through the alteration of the functional state of a host molecule (Mackey and McFall, 2006). These recognition factors are also known as PAMPs (pathogen-associated molecular patterns) (Ingle *et al.*, 2006).

As a rule, MAMPs seem too much conserved to explain the difference between non-host and host pathogens. Indeed, host pathogens as well as related non-host pathogens contain very similar or identical MAMPs. As a consequence, adapted pathogens also trigger the basal defence reaction in their host, but are able

to suppress this reaction within hours (Li *et al.*, 2005; Truman *et al.*, 2006; Caldo *et al.*, 2006). Indeed, the failed or successful suppression of basal defence is presumed to be the key phenomenon determining a non-host or a host-status of the interaction (Heath, 1991; Panstruga, 2003; Caldo *et al.*, 2006; Nomura *et al.*, 2005). The organization of the plant defence to maladapted pathogens bears similarity to burglar alarm devices in buildings. The alarm is triggered by non-specific factors (motion and MAMPs, respectively) and should be suppressed in a very specific way (bypass code for authorized persons and a specific set of microbial factors, respectively).

Effectors mediate suppression of defence

Suppression of basal resistance is assumed to be mediated by so called effectors that are delivered into the apoplast or into plant cells (Kamoun, 2006). These have been particularly well studied in bacterial diseases (*Pseudomonas* spp. and *Xanthomonas* spp.) (Li *et al.*, 2005; Truman *et al.*, 2006; Ingle *et al.*, 2006; Gurlebeck *et al.*, 2006). In other pathogen classes, like rusts (Catanzariti *et al.*, 2006), oomycetes (Kamoun 2006) and other plant pathogenic fungi, effectors also have been implicated. Up to now, very little is known about the identity and biochemical function of effectors delivered by fungal and oomycete pathogens. Some of these effectors may act as transcription factors (Lahaye and Bonas, 2001); others may cleave specific cytoplasmic host proteins (Shao *et al.*, 2003; Coaker *et al.*, 2005).

What determines the specificity of pathogens?

Since related pathogens with different host ranges contain identical or very similar MAMPs, they all will activate the basal defence in any plant. However, the rye leaf rust fungus (*Puccinia recondita*), for example, is able to suppress the defence

in rye but not in barley, and the barley leaf rust (*P. hordei*) in barley but not in rye. So, the effectors of these rusts should differ, as should the targets of these effectors, making them only effective in the plant species to which their effectors have been adapted. This suggests that the effectors and their targets are important determinants of the (non)host-status of a certain plant-microbe combination (Niks and Marcel, 2009).

The next question is how we can find the plant targets that determine whether effectors of a would-be pathogen can or cannot suppress the basal defence. An obvious approach would be to study the inheritance of (non)host-status. This would, by definition, need an interspecific cross, namely between a host and a non-host plant species. The overriding difficulty of this approach is that in interspecific crosses, classical genetics are rarely feasible, due to hybrid sterility, abnormal segregation, lack of chromosome pairing and artefacts caused by odd plant morphology. This degree of incompatibility hampers the identification of individual genetic factors.

Exploitation of “near-non-host” status

There are several plant species that are only marginal hosts to a potential pathogen. For example, those plant species in which less than 10% of the accessions are, at most, moderately susceptible, and only at the seedling stage (Niks, 1987; Mains, 1933). This phenomenon is obvious in barley. Odd susceptible barley genotypes have been found to wheat stripe rust (*P. striiformis* f.sp. *tritici*) (Pahalawatta and Chen, 2005) and to at least nine other rust fungal species of cereals and grasses (Atienza *et al.*, 2004). This “near-non-host” status offers the possibility to analyze phenotypic segregations if rare susceptible barley accessions are crossed with common immune accessions. This circumvents the need for inter-specific host × non-host crosses. Still, it seems reasonable

to presume that genes that are responsible for the resistance or susceptibility to marginal pathogens will, by extrapolation, also teach us about principles that contribute to, or even determine, the full non-host-status to related pathogens.

We quantified the level of susceptibility of barley to several heterologous rust fungal species, all pathogenic on (other) cereals and grasses (Atienza *et al.*, 2004, and unpublished data). Some examples are presented in Figure 1. The high level of infection by *P. hordei* isolate 1.2.1. is as expected, since barley is the regular host species.

The other, heterologous, rusts were applied with 3× as much inoculum, but nevertheless several rusts produced no pustules (for

example the rye leaf rust *P. recondita*); others, like the wheat leaf rust (*P. triticina*) produced on fewer than 10% of the accessions more than 100 pustules per leaf. To the latter group of rusts, barley can be considered a “near-non-host”. As far as tested, adult plants were resistant to all rusts, except to *P. hordei* (Atienza *et al.*, 2004). The wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*) did not produce macroscopically visible colonies on any of the barley accessions.

Among the barley accessions with susceptibility to heterologous rusts, relatively many accessions had naked seeds or black seeds, and were African and Asian landraces, while relatively few were modern cultivars (Atienza *et al.*, 2004).

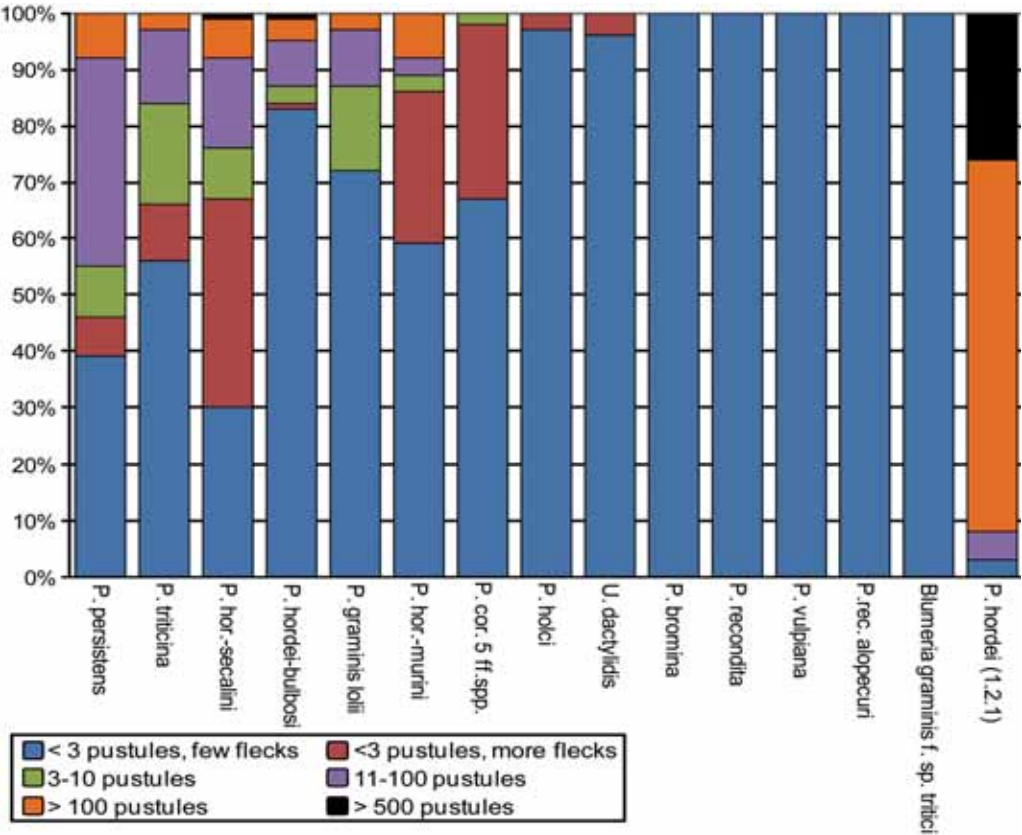


Figure 1. Percentage of barley accessions (n = 110) per susceptibility class for 13 heterologous rust fungi, wheat powdery mildew and barley leaf rust (*Puccinia hordei*), determined at the seedling stage. After Atienza *et al.* (2004).

Accumulation of susceptibility to heterologous rusts

None of the barley accessions was as highly susceptible as the regular host to the heterologous rusts. Therefore, Atienza *et al.*, (2004) accumulated susceptibility alleles by convergent crossing of accessions identified as fairly susceptible, selecting in their progeny for higher susceptibility to wheat leaf rust, *P. triticina*. This resulted in an experimental line, called SusPtrit, with, in the seedling stage, as high a susceptibility to *P. triticina* as a typical susceptible wheat accession. We followed the same procedure, starting from different parental material, to develop a line with very high susceptibility to the heterologous rust *P. hordei-murini*, called SusPmur (Atienza *et al.*, 2004).

Mapping populations

We developed two mapping populations by crossing SusPtrit, the susceptible parent, with the European cultivar Vada and the South American cultivar Cebada Capa, the parents that are regularly immune to the heterologous rust fungi. These mapping populations consisted of 152 recombinant inbred lines for Vada \times SusPtrit (V \times S), and 113 recombinant inbred lines for Cebada Capa \times SusPtrit (C \times S). We also found that the Oregon Wolfe Barley population (OWB; 94 DH lines; Costa *et al.*, 2001) segregated for susceptibility to the heterologous rusts for which barley was a near-non-host. Since the parents of the OWB population have been generated by convergent crosses of exotic barley accessions (Costa *et al.*, 2001), it was not entirely unexpected that this population would segregate for susceptibility to heterologous rust fungal species.

Research questions to be addressed

The availability of both the barley research lines with extreme susceptibility to heterologous rusts and the mapping populations enabled us to address the following research questions:

- Is host-status based on quantitative genes or on the stacking of several R-genes?
- Do non-hosts have specific genes for non-host-status, each effective against a different pathogen taxon, or do the genes have a broad spectrum of effectiveness?
- Is the resistance of members of a non-host species to a heterologous pathogen due to shared resistance genes, or are different members of the non-host species each resistant due to a different set of genes?
- Is non-host resistance due to variation in genes that have been identified as playing a key role in perception, signal transduction and defence?
- Are the genes that determine the (non) host-status to a heterologous pathogen also implicated in basal defence to the related adapted pathogen?

Quantitative genes or stacking of several R-genes?

The barley-rust model indicates clearly quantitative genes for (non)host-status. The mapping populations segregated quantitatively and continuously. Figure 2 presents two examples of such segregation. Typically QTL mapping led to the discovery of about two to five QTLs that explain the resistance of the resistant parent Vada or Cebada Capa to a particular rust species (Jafary 2006; Figure 3).

The quantitative, polygenic inheritance is also inferred from the fact that the accumulation of susceptibility by convergent crossing to produce SusPtrit and SusPmur (Atienza *et al.*, 2004) led to a gradual increase in the level of susceptibility.

It has been proposed that non-host resistance of plants may be due to *R*-genes of the NBS-LRR type that recognize pathogen-derived Avr factors (Heath, 1981; Schweizer, 2007; Jones and Dangl, 2006; Niks, 1988; discussed by Lu *et al.*, 2001). If several such *R*-genes occur in combination and at high allele frequency in the plant species, and if the cognate Avr factors occur also at high allele frequency in the microbe species, this would

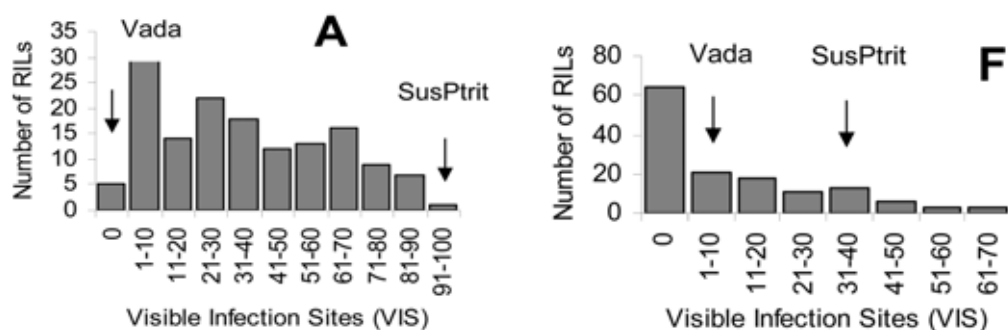


Figure 2. Frequency distribution of phenotypes for resistance to two heterologous rust fungi in the barley mapping population Vada \times SusPtrit. Values of the two parental lines are shown by arrows. A. *Puccinia triticina* isolate Flamingo; F. *P. graminis* f.sp. *lolii*.

lead to redundancy, and the resistance would be complete and durable. The resistance of barley to wheat stripe rust (*P. striiformis* f.sp. *tritici*) (Pahalawatta and Chen, 2005) and of wheat to barley stripe rust (*P. striiformis* f.sp. *hordei*) (Johnson and Lovell, 1994; Rodrigues *et al.*, 2004) seem indeed to be largely due to (a) major gene(s) for hypersensitivity resistance. However, in the crosses with SusPtrit and in the OWB we found only one locus (on chromosome 1H) carrying (a) major *R*-gene(s) for hypersensitive resistance to heterologous rusts: one contributed by Vada was effective against *P. hordei-secalini* (Jafary *et al.*, 2006), and the other, contributed by the Rec (the recessive parent of OWB), was effective against *P. hordei-secalini* and *P. hordei-murini* (Jafary, 2006). However, the *R*-gene was backed-up by quantitative resistance. We conclude that *R*-genes contribute occasionally to the resistance to heterologous rusts, but that quantitative resistance is a much more prominent mode of inheritance of the trait.

Pathogen specificity or broad spectrum effectiveness?

The QTLs found to underlay the near-non-host status of barley to heterologous rusts had overlapping rust specificity, i.e. they were typically effective against only one or two rusts, and only a few were effective against at least four heterologous rust species (Figure 3; Jafary *et al.*, 2006; Jafary 2006).

This combination of specificity and broader spectrum effectiveness was also found in the analysis of barley germplasm and the development of the experimental line SusPtrit (Atienza *et al.*, 2004). Accessions that were (moderately) susceptible to one heterologous rust had a high chance of also being somewhat susceptible to other heterologous rusts. The accession Trigo Biasa (from Indonesia) and L94 (from Ethiopia) were rather susceptible to most heterologous rusts to which barley is a near-non-host. The line SusPtrit, selected for susceptibility to *P. triticina*, was also very susceptible to the other heterologous rusts to which barley is a near-non-host, again suggesting a broad spectrum effectiveness of the resistance alleles that were selected against in SusPtrit. At the same time, some lines were susceptible to one heterologous rust pathogen and immune or completely resistant to others. In several cases, differential interaction occurred between barley accessions and heterologous rusts. This suggests that there are also genes with high rust species specificity.

High allele frequencies of the same resistance genes?

Is the resistance of members of a non-host species to a heterologous pathogen due to shared resistance genes, or are different members of the non-host species each resistant due to a different set of genes? One

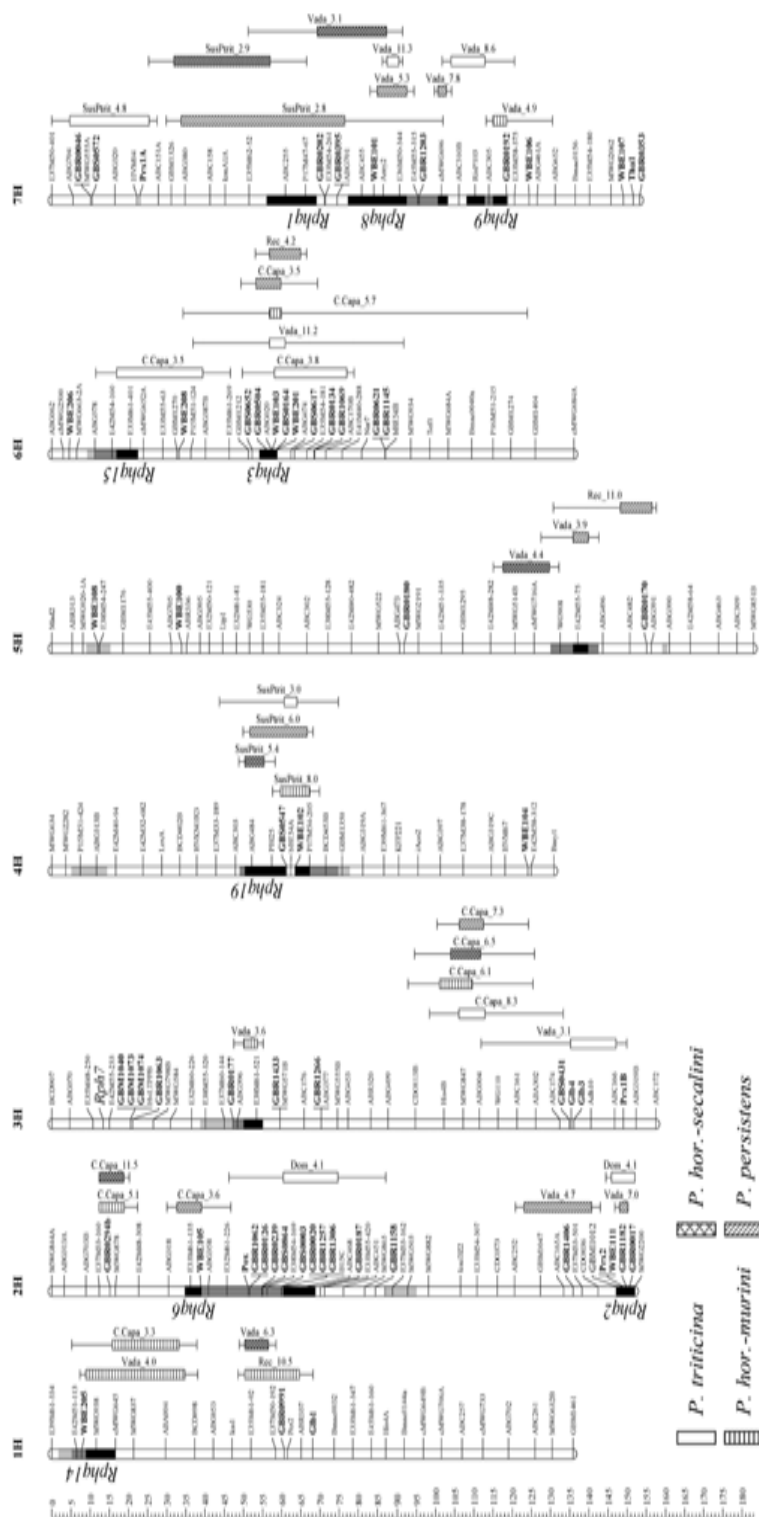


Figure 3. Locations of QTLs for non-host immunity to four heterologous rust species on a BIN map extracted from a high-density consensus map of barley (Marcel *et al.*, 2007a). The QTLs were originally mapped in three individual barley linkage maps. Length of QTL boxes (with pattern) correspond to the LOD-1 support interval (from peak marker) and QTL lines are extended to the LOD-2 support interval, based on results of rMQM. The parental line contributing the allele for resistance and the LOD value obtained by rMQM are indicated on the right side of the QTLs. Within chromosome bars, LOD-2 support intervals of QTLs for partial resistance to barley leaf rust (Marcel *et al.*, 2007a; Jafari, 2006) are indicated in black if overlapping with the LOD-1 support interval, in dark grey if overlapping with the LOD-2 support interval and in light grey if not overlapping with QTL(s) for non-host resistance presented in this study. The name of the QTL for partial resistance (*Rphq*-) is indicated on the left side of the chromosome bars when its peak marker(s) was within the LOD-1 support interval of QTL(s) for non-host resistance. The 63 loci in bold are defence gene homologue (DGH) based markers. The ruler on the left hand side of the figure indicates the distance in centiMorgans (according to Kosambi) from the top of each chromosome.

might expect that almost all immune barleys would carry the common, i.e. resistance, alleles for almost all loci on which SusPtrit carries the (probably rare) susceptibility allele. In that case, almost all susceptibility alleles should appear in any cross between SusPtrit with any immune barley. This was not what we found.

In each population, different sets of quantitative genes explain the resistance to a particular heterologous rust (Figure 3). For example, Vada and Cebada Capa share only one QTL from nine for resistance to *Phm* and *Phs* (Figure 3). Therefore, immunity to a heterologous rust may be due to many different sets of QTLs, indicating a high redundancy of genes for resistance in the barley species.

These observations also suggest that even immune barleys contain some susceptibility alleles on certain QTLs, and therefore will not segregate for the same QTLs when crossed with SusPtrit. This possibility is consistent with the observation that crossing exotic barley lines with slight susceptibility to heterologous rusts, results in transgression towards increased susceptibility, leading to the research line SusPtrit with extreme susceptibility (Atienza *et al.*, 2004).

Do the resistance QTLs represent defence-related genes?

At first thought, genes involved in plant defence are not likely candidates to determine the (non)host status of plants. These genes, such as peroxidases, MAPKinases, super-oxide dismutase and BAX inhibitor 1, in general are quite conserved and should be effective against a broad spectrum of the same class of pathogens. The barley lines SusPtrit and SusPmur (Atienza *et al.*, 2004) are very susceptible to some heterologous rusts, but they are fully resistant to several rusts to which barley is probably a full non-host. Therefore, it is unlikely that the susceptibility of these lines to some heterologous rusts is due to structurally defect key genes for

basal defence. However, an option is that such basal resistance genes contain minor sequence differences in promoter or coding sequences, or both, that would be the point of action of effectors to specifically reprogram such genes in order to suppress defence.

The placement of the QTLs that were found in the three mapping populations on a consensus map (Marcel *et al.*, 2007a) allowed us to compare the QTL positions with the position of EST-markers that have homology with genes implicated in plant defence. They were termed Defence Gene Homologues (DGHs) (Marcel *et al.*, 2007a). The BIN system of the barley consensus map was used to test by Chi-square test for a possible association between the distribution of loci for non-host resistance and the distribution of 63 mapped DGHs (Marcel *et al.*, 2007a). The null hypothesis was rejected (Table 1) with a very high probability ($P < 0.001$), suggesting that there is an association in the distribution of the QTLs and the DGHs over the consensus map of barley. We compared the positions of

Table 1. Chi-square values for the probability of independent distribution of barley QTLs for partial resistance to leaf rust (QTLpr), non-host resistance to heterologous cereal and grass rusts (QTLnh), and defence gene homologues (DGH), over BINs on a consensus genetic map of barley.

	QTLpr ^a	QTLnh	DGH
Marker nr. ^b	23	37	63
BIN nr. ^c	22	27	43
QTLpr	-	12.12 ^d	9.42
QTLnh	-	-	10.93

^a mapping data were obtained from Jafary (2006) for QTLpr and QTLnh; and from Marcel *et al.*, (2007a) for QTLpr and DGH. ^b number of (peak) markers mapped on the consensus map of barley (Marcel *et al.*, 2007a). ^c number of barley BINs (5 cM) occupied by the (peak) markers for the respective class. ^d in bold are the Chi-square values for which the null hypothesis of independent distribution is rejected with a probability $P < 0.01$ (with 1 df, $P = 0.01$ for $\chi^2 = 6.634$).

the DGH-derived markers with the positions of the QTLs for resistance to heterologous rusts, and found that 31 of the 63 DHG-based markers mapped within the LOD-1 confidence interval of the QTLs for non-host resistance. The DGHs that associated with the QTLs for non-host resistance include peroxidases, MAPKinases, superoxide dismutase and BAX inhibitor 1.

We conclude that indeed, QTLs for non-host resistance may represent allelic forms of certain defence-related genes.

Are genes that determine non-host resistance also implicated in basal defence to the related adapted pathogen?

In almost all plant-pathosystems there is variation within the susceptible class of plant accessions, ranging from moderately to extremely susceptible. Such variation is known as quantitative basal resistance. There is evidence that this type of resistance is part of the same system as non-host resistance:

- at least in powdery mildews and rust fungi, the quantitative basal resistance is typically predominantly pre-haustorial and associated with formation of cell wall reinforcements, also called cell wall appositions or papillae (O'Connell and Panstruga 2006), as in non-host resistance to these pathogen classes; and
- genetic segregation for resistance to rusts to which barley has a near-non-host status tends to be associated with segregation for levels of quantitative basal resistance to *P. hordei* in barley, indicating that in part the same genes are involved (Zhang *et al.*, 1994).

It would also make sense that non-host resistance to heterologous pathogens and quantitative basal resistance are associated. Assuming genetic factors that determine differences between plant species in (non) host status to a would-be pathogen, it may also be expected that such genetic factors differ within a host plant species to that

micro-organism, making one host plant genotype a more suitable host individual than an other genotype of the same species.

Indeed, mapping of the QTLs for resistance to heterologous rust fungi (Figure 3) confirms that many of those QTLs map to positions where in the same or different mapping populations QTLs for basal resistance to *P. hordei* are located. The chi-square test indicates that this association is statistically significant (probability $P < 0.01$) (Table 1).

Prospects

The near-non-host status of barley to several rust fungi that are adapted to grasses and other cereals (Atienza *et al.*, 2004) offers great prospects for understanding the genetics and specificity of resistance to unadapted specialized pathogens. Tools that have been developed are:

- hypersusceptible lines in which alleles for susceptibility to heterologous rusts have been accumulated (Atienza *et al.*, 2004);
- a large collection of rusts of different grasses and cereals;
- a large number of mapping populations segregating for their level of (non)host resistance to rust fungi of cereals and grasses;
- BAC libraries of Vada, SusPtrit (Marcel *et al.*, these proceedings), Cebada Capa (Isodore *et al.*, 2005) and Morex (Yu *et al.*, 2000);
- advances in the development of barley physical maps (Künzel *et al.*, 2000; Stephens *et al.*, 2004) and very dense marker linkage maps (e.g. Marcel *et al.*, 2007a; Stein *et al.*, 2007); and
- NIL-QTL lines for basal resistance have been developed in susceptible barley and barley with a high level of basal resistance (Marcel *et al.*, 2007b, 2008) that demonstrate that the QTLs are robust and most of them not obviously dependent on genetic background.

In this contribution, we presented data on the genetics of (near-)non-host resistance of

barley to heterologous rusts. These rusts enter through stomata to infect mainly the barley mesophyll cells. That infection style does not allow testing of candidate genes by reverse genetics through transient transformation by biolistics (Schweizer *et al.*, 1999), since that method only transforms epidermal cells. Such epidermal cells are relevant for powdery mildew fungi. Recently, we have started to accumulate genes in barley for unusual but natural susceptibility to wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*), and lines with very high and very low levels of quantitative basal resistance to the barley powdery mildew (*B. graminis* f.sp. *hordei*) (R. Aghnoum and R.E. Niks, unpublished data). That material will allow similar studies and comparisons to those reported here for rust fungi, but with the additional advantage of the amenability to reverse genetics approaches.

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Markers for resistance to three foliar diseases in barley

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Abstract

Three foliar fungal diseases—net form of net blotch (caused by *Pyrenophora teres* f. *teres*), covered smut (*Ustilago hordei* (Pers.) Lagerh) and spot blotch (*Bipolaris sorokiniana*)—commonly occur in the northern production regions of Australia and significantly reduce yields and quality of harvested grain. Many Australian commercial varieties are susceptible to one or more of these diseases. We have investigated quantitative trait loci (QTLs) associated with resistance to these diseases in a number of Australian doubled-haploid (DH) barley populations. The three DH populations, Alexis × Sloop, WI2875-1 × Alexis and Arapiles × Franklin were inoculated with *P. teres* f. *teres* to identify QTL associated with NFNB field (adult plant) resistance. The most significant QTL were identified on chromosomes 2H and 3H in both Alexis × Sloop and WI2875-1 × Alexis, and on chromosomes 1H and 2H in Arapiles × Franklin. The resistance was contributed by Sloop, WI2875-1 and Arapiles, respectively. The Alexis × Sloop population was also inoculated with *U. hordei* to investigate covered smut resistance. A resistance locus from Sloop was identified on chromosome 7HS and explained 50% of the phenotypic variance (LOD score of 19.7). A major QTL conferring spot blotch seedling resistance against all Australian isolates tested was identified on chromosome 7HS in the

North Dakota line ND11231-12. This QTL explained 60% of the phenotypic variance in a ND11231-12 × VB9524 DH population. Adult plant resistance to spot blotch was mapped to this same region, where the QTL explained up to 44% of the phenotypic variance. A second QTL on chromosome 3HS was also detected, explaining up to 19% of the phenotypic variance. These loci were confirmed in the closely related population ND11231-11 × WI2875*17.

Introduction

Three foliar fungal diseases—net form of net blotch (NFNB; caused by *Pyrenophora teres* f. *teres*), covered smut (*Ustilago hordei* (Pers.) Lagerh) and spot blotch (*Bipolaris sorokiniana*)—commonly occur in the northeastern barley production regions of Australia and significantly reduce yields and quality of harvested grain. Many Australian commercial barley (*Hordeum vulgare* L.) varieties are susceptible to one or more of these diseases.

P. teres f. *teres* is a highly variable pathogen and at least thirteen different pathotypes have been identified in Australia (Platz *et al.*, 2000). This variability, combined with the adoption of reduced- or zero-tillage practices, has increased the incidence of NFNB significantly in recent years. A major objective of the Australian barley breeding program is to increase resistance to this

disease in new barley varieties. Cultivated barley lines that are resistant to NFNB at both the seedling and adult growth stages have been identified (Gupta *et al.*, 2003). Since seedling assays fail to detect adult plant resistance (APR, i.e. resistance that is manifested in mature plants but not in the younger, seedling stages), trials to assess adult plant resistance (APR) are sown in the field with ratings taken after heading. We have investigated the genetic control of APR to NFNB in three Australian barley populations. The same three populations had previously been investigated for NFNB seedling resistance (Raman *et al.*, 2003). Our goal was to determine which quantitative trait loci (QTLs) were the major contributors to APR in order to discover which independent genetic regions might be combined with seedling resistance (SLR) loci in a marker assisted selection program, seeking to provide stable resistance against the NFNB pathogen.

Growers with covered-smut-infected grain face both yield loss and marketing problems since grain contaminated with covered smut is unacceptable for malting and there is a nil tolerance imposed by handling authorities. Fortunately, this disease can be effectively controlled by fungicidal seed treatments (Wildermuth, 1988) and resistant lines are available. Nevertheless, molecular marker-assisted selection for covered smut is desirable, as screening for this disease is space-, labour- and time-consuming, with affected plants usually not showing symptoms until ear emergence (Willits and Sherwood, 1999; Grewal *et al.*, 2008). We have identified a major gene for covered smut resistance in an Alexis \times Sloop doubled-haploid (DH) population. Expressed sequence tag (EST) markers closely linked to this gene have been identified by successful application of the high resolution melting (HRM) technique to map single nucleotide polymorphisms (SNPs). HRM is a post-polymerase chain reaction (PCR) technique, which involves a standard PCR

reaction and the use of a double stranded DNA binding dye. When melted, each PCR product will exhibit a characteristic melting or disassociation behavior (Montgomery *et al.*, 2007). Specialized HRM instruments can plot the change in fluorescence that occurs when double-stranded DNA amplicons melt to form single-stranded DNA.

Bipolaris sorokiniana is found worldwide in nearly every region where barley is grown, but is especially prevalent in more humid and higher rainfall areas. Work conducted in the USA by Steffenson *et al.* (1996) on the DH population Steptoe \times Morex revealed that SLR to spot blotch was monogenetically inherited and was governed by the gene *Rcs5* on the short arm of chromosome 7HS, and resistance in adult stages by two QTLs: one with major effect on chromosome 1HC and another with minor effect on 7HS (*Rcs5*). The SLR gene on 7HS was confirmed in a Harrington \times Morex population, although the APR gene on 1HC could not be detected and the gene on 7HS explained a major percentage of the phenotypic variance (Bilgic *et al.*, 2005). Bilgic *et al.* (2005) also studied spot blotch resistance in the Dicktoo \times Morex population and identified three regions conferring seedling resistance; a major QTL was identified near *Rcs5* and two minor QTLs on chromosomes 7HC and 3HS, respectively. APR QTL were identified on 3HS, 3HL and 7HS (*Rcs5*). In Australia, spot blotch predominantly occurs in sub-tropical northern New South Wales (NSW) and Queensland (QLD), where localized yield losses of up to 70% have been reported (G Platz, pers. comm.). The genetics of resistance to spot blotch in barley, particularly in Australia, is not well understood. Malting cultivars bred in the upper mid-west of the USA from the breeding line NDB112 have provided durable spot blotch resistance for over 4 decades (Bilgic *et al.*, 2005; Steffenson *et al.*, 1996). We have tested the robustness of this resistance to spot blotch under Australian environmental conditions.

Materials and methods

Plant material and linkage maps

Two DH populations—Alexis \times Sloop (N = 111) and Arapiles \times Franklin (N = 225)—and one population of recombinant inbred lines established by single seed descent, WI2875-1 \times Alexis (N = 153), were screened for resistance in field plots for reaction to NFNB. The Alexis \times Sloop and WI2875-1 \times Alexis populations were also used for the identification and validation of the covered smut resistance locus, respectively. All three populations were developed by the Australian National Barley Molecular Marker program (NBMMMP; Barr *et al.*, 2003; D.B. Moody *et al.*, unpublished data). Sloop (breeding line WI2875-22) and WI2875-1 were reselected in the F₆ from the F₂-derived breeders' line WI2875 (Barr *et al.*, 2003). Linkage maps for all three populations had previously been constructed (Barr *et al.*, 2003; Willsmore *et al.*, 2006).

The population ND11231-12 \times VB9524 consisting of 180 DH lines was used to identify the spot blotch QTL. A molecular map of this population was produced by Emebiri *et al.* (2005). The spot blotch resistant parent ND11231-12 originated from the two-row breeding program at North Dakota University, Fargo, USA, and VB9524 from the Dept of Primary Industries, Victoria, Australia. QTLs were validated in the DH population ND11231-11 \times WI2875*17. ND11231-12 and ND11231-11 are sister lines.

NFNB screening

All parents were assessed for seedling resistance (SLR) by inoculating them with isolate NB330 when they were 23–24 days old. This indicated that all parents were susceptible at the seedling stage. APR screening of the three populations was conducted in the field at the Hermitage

Research Station, QLD. Spreader rows were inoculated with field-collected conidia of isolates NB329 (2003), NB329 and NB333 (2004) and diseased straw of NB330 (2005). These isolates were of the same pathotype. For further details of the methodology see Lehmensiek *et al.* (2007).

Covered smut screening

Each line was inoculated using the spore suspension method (Tapke and Bever, 1942). Race 5 spores were obtained from the previous year's smutted heads. Fungus-inoculated seeds of 81 and 50 lines of the Alexis \times Sloop population were planted in the field at Wellcamp, QLD, and screened for covered smut in 2001 and 2002, respectively. A further 20 lines of this population (which had not been rated in previous years) were screened in 2003 and again in 2004, together with 28 lines of the WI2875-1 \times Alexis population. After harvest, plants were rated for percentage incidence of plants with smutted heads per experimental plot.

Spot blotch screening

The population ND11231-12 \times VB9524 was screened for resistance in seedling glasshouse trials (2005, 2006) at the Hermitage Research Station, QLD, and in the field at Redlands Research Station, QLD (2004, 2006). The ND11231-11 \times WI2875*17 population was screened in 2006 in seedling glasshouse trials and in the field at Redlands Research Station using isolate SB61. The seedlings were rated 13 days post-inoculation, based on a 1–9 scale for increasing susceptibility (Fetch and Steffenson, 1999). In the field trials, infection response was determined at growth stage Z75 (Zadoks *et al.*, 1974) on a similar scale.

QTL analysis

Cartographer Version 2.5 (Wang *et al.*, 2006) was used for the QTL analyses. QTL effects were considered to be significant if the log-likelihood (LOD) score was ≥ 3 .

Development of EST markers and HRM analysis

EST markers were added to the region containing the covered smut gene. Thirteen loci on the distal end of 7HS with potential to contain single nucleotide polymorphisms (SNPs) were identified by applying a comparative genomics approach using rice sequence data. For further details of the methodology see Lehmensiek *et al.* (2008). EST amplification and HRM analysis was performed with a Rotor-Gene6000™ (Corbett Life Science, Sydney, Australia). A standard PCR protocol was used to amplify the ESTs, detailed in Lehmensiek *et al.* (2008). The melt analysis was performed once amplification was completed by ramping the temperature from 75°C to 95°C, raising by 0.1°C each step with continuous acquisition of fluorescence. For the HRM analysis the fluorescence versus temperature graphs were normalized to 100 to allow all the curves to be compared, thus having the same starting and ending fluorescent signal level. The raw data graph was used to adjust the regions of normalization. This was done according to the protocol provided by the supplier (Corbett Life Science, Sydney, Australia).

Results

The three populations—Alexis \times Sloop, WI2875-1 \times Alexis and Arapiles \times Franklin—were inoculated with *P. teres* f. *teres* to identify QTLs associated with NFNB field (adult plant) resistance (Figure 1). The most significant QTLs were identified on chromosomes 2HC and 3HL in both Alexis \times Sloop and WI2875-1 \times Alexis, and on chromosomes 1HS and 2HS in Arapiles \times Franklin. The resistance was contributed by Sloop, WI2875-1 and Arapiles, respectively. Only the QTL on 7HS was contributed by Alexis. Detailed results can be obtained from Lehmensiek *et al.* (2007).

The Alexis \times Sloop population was also inoculated with *U. hordei* to investigate covered smut resistance. A single region,

contributed by Sloop, was located on chromosome 7HS (LOD score 16.7) and explained 51% of the phenotypic variance (Figure 1). This major locus for covered smut resistance was confirmed in the validation population WI2875-1 \times Alexis and was designated *CS.A/S-7HS*. The QTL peak was located at the most distal marker on the map (abg704) and it was therefore possible that *CS.A/S-7HS* was located closer to the telomere. Polymorphic microsatellites or other PCR-based markers known to map to this region could not be found. The colinearity of the barley and rice genomes was therefore investigated to identify potential EST markers from this region. Sequences of twelve barley EST markers in the telomeric 7H region were obtained and primers designed. A derived cleaved amplified polymorphic sequence (dCAPS) marker (BV078160) obtained from Bulgarelli *et al.* (2004) was also tested. Seven of the 13 EST markers produced a single fragment of the same size in both parents, while one marker (AV836787) was co-dominant. The 7 non-polymorphic markers were amplified with the Rotor-Gene™ 6000 and HRM analysis was subsequently performed. A difference in T_m indicating a SNP could be identified in 2 of the 7 ESTs, CK123008 and BV078160. The normalized HRM graphs for both markers are illustrated in Figure 2.

The SNP markers CK123008 and BV078160 and the co-dominant marker AV836787 were mapped on the Alexis \times Sloop map. Marker AV836787 was located 2.7 cM distal to marker abg704 and CK123008 was located 4.6 cM distal to AV836787 (Figure 3). Marker BV078160 mapped 4.2 cM proximal to abg704. Re-analysis of the covered smut data indicated that *CS.A/S-7HS* was located closest to marker AV836787 with an increase in the LOD score from 16.7 to 23.6 (Figures 1 and 3). The average phenotypic variance explained increased from 51% to 57.2%.

A highly significant single major QTL conferring spot blotch seedling resistance

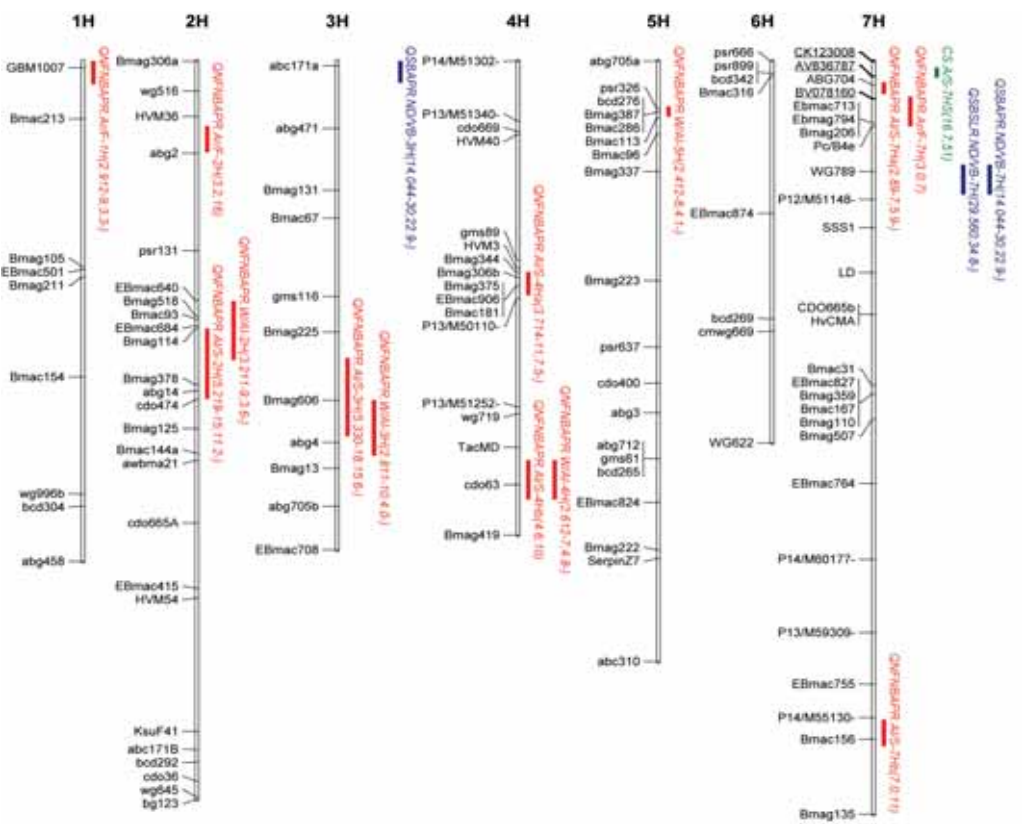


Figure 1. Approximate chromosomal locations of NFNB, covered smut (CS) and spot blotch (SB) QTLs. Map construction was based on segregating markers across the three populations, Alexis \times Sloop (Al/S), WI2875-1 \times Alexis (W/Al) and Arapiles \times Franklin (Ar/F). QTL locations for the ND11231-12 \times VB9524 (ND/VB) population were estimated based on common markers. Map distances are not given as marker positions are approximations. The naming convention for the identified QTL uses the format ‘*QFNBAAPR.AL/S-2H*’ indicating a QTL for resistance to NFNB, followed by ‘APR’ or ‘SLR’ indicating whether it is an adult plant or seedling resistance QTL. This is followed by the cross in which the QTL was identified and the chromosome on which the QTL was mapped. The lowest and highest LOD scores and phenotypic variances explained across years are given in brackets. The underlined EST markers on chromosome 7H were added to the map.

against all Australian isolates tested was identified on chromosome 7HS in the ND11231-12 \times VB9524 population (Figure 1). While field trial results confirmed the major QTL on chromosome 7HS, they identified a second major QTL on chromosome 3HS, also inherited from ND11231-12 (Figure 1). These loci were

confirmed in the closely related population ND11231-11 \times WI2875*17.

Discussion

We have investigated the location of resistance genes for three important Australian barley foliar diseases and have identified markers

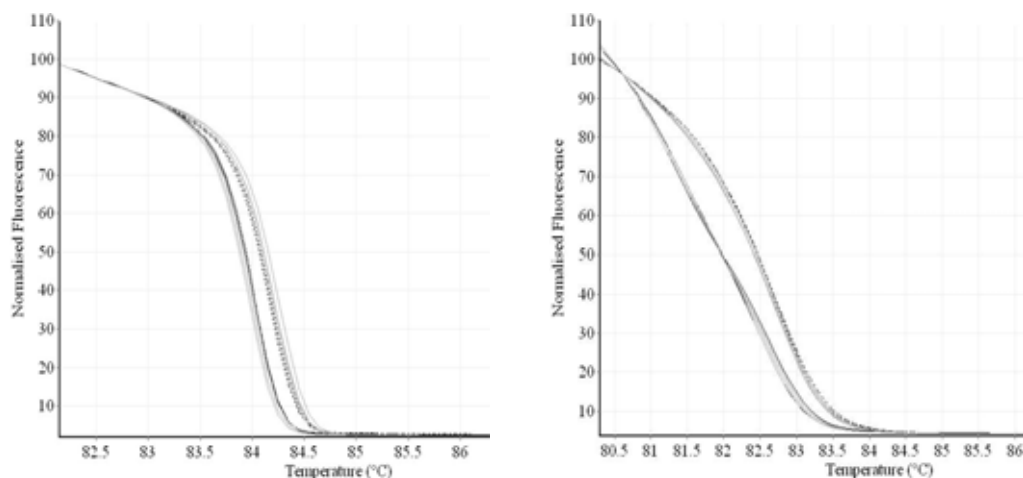


Figure 2. Normalized HRM curves for marker CK123008 (left) and marker BV078160 (right); two independent samples of parent Alexis (black solid lines) and Sloop (black dashed lines) and 8 progeny (gray) are shown.

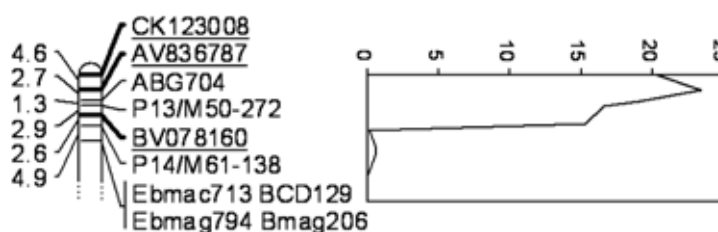


Figure 3. Location of the covered smut (CS) gene on chromosome 7H of the barley cross Alexis × Sloop showing the result for the average taken across four years. Map distances (cM) are given on the left and the EST markers have been underlined.

that could be used in breeding programs.

Differences were observed between APR QTL for NFNB and SLR QTL previously published by Raman *et al.* (2003). Even though some genomic regions involved in APR were similar to those for SLR, the resistance was contributed by the other parent. These differences almost certainly result from the use of isolates from different pathotypes in the two studies. For example, isolate NB34 in the SLR study (Raman *et al.*, 2003) is avirulent on Franklin (rating = 3) and moderately virulent on Alexis (rating = 6.5),

while isolate NB330, used in this study, is virulent on seedlings of both these lines (rating = 10). Pathogen variability and the use of different pathogen isolates by different research groups make the comparison of independent studies difficult. Given that a race structure is recognized but poorly characterized in *P. teres*, thorough testing of promising resistant materials against a wide range of isolates is essential. Attempts are underway to establish an international differential set of host lines for determination of NFNB pathogenic races, coupled with an

international naming convention for each race identified. Such a differential set would aid in the identification of individual isolates and enable researchers to identify race-specific and race-non-specific (if they exist) QTLs for both seedling and adult plant resistance.

The NFNQ QTL contributed by Alexis on 7HS was located in the same region as the major covered smut gene, *CS.A/S-7HS*. The addition of two EST markers distal to marker *abg704* indicated that the covered smut gene was located closest to EST AV836787. Re-analysis of the NFNQ data, after the addition of the two EST markers, indicated that *abg704* is still the closest marker to the NFNQ gene, suggesting that this locus is located a few cM proximal to *CS.A/S-7HS*. The SLR and APR spot blotch QTL identified on 7HS in the ND11231-12 × VB9524 population are located a few cM proximal to the NFNQ QTL. The spot blotch QTLs are in the region of the spot blotch gene, *Rcs5*, identified in other studies (Steffenson *et al.*, 1996; Bilgic *et al.*, 2005). The APR spot blotch QTL identified on 3HS in the ND11231-12 × VB9524 population is in a similar region to the APR QTL identified in the Dicktoo × Morex population (Bilgic *et al.*, 2005).

HRM analysis provides a quick way of mapping SNPs without sequence knowledge or electrophoresis and is also useful as an alternative method for scoring known CAPS markers. The dCAPS marker BV078160, mapped by Bulgarelli *et al.* (2004), was examined in our study. dCAPS markers are normally amplified by standard PCR, the product digested with a restriction enzyme overnight and the fragments compared on an agarose gel. Use of HRM analysis immediately after the PCR step eliminates the need for restriction enzyme digestion and gel electrophoresis, and results are obtained within two hours of commencing PCR. The BV078160 marker was mapped 4.2 cM proximal to marker *abg704* in our study, which is similar to the estimate of 5.6 cM for this interval indicated by Bulgarelli *et*

al. (2004) using 93 recombinant lines of a Thibaut × Micro population. The HRM technique clearly has applications for trait mapping across a wide range of crops.

From this study it is clear that a cluster of resistance genes is present on the 7HS chromosome. Other disease resistance genes have been mapped to this 7HS region, including the leaf and stem rust resistance genes, *Rdg2a* and *Rpg1* (Ayliffe *et al.*, 2000; Brueggeman *et al.*, 2002, 2006; Bulgarelli *et al.*, 2004). Fine mapping of this region is continuing, to determine whether there are one or more genes conditioning multiple resistances, the effective recombination distances between them, and the ease with which any linkage in repulsion can be overcome.

Acknowledgements

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An association-genetic approach to durable powdery mildew resistance in barley

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Abstract

Resistance of barley to the powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) is often mediated by natural or induced loss-of-function alleles of the *Mlo* gene that give broad-spectrum resistance, or by nucleotide - binding - site/leucine - rich - repeat (NBS - LRR) - type genes that confer race-specific resistance. Both types of resistance depend on an arsenal of signaling and downstream defence-related genes that—by themselves—may be responsible for complex patterns of quantitative resistance loci (QTLs). The aims of this paper are the estimation of haplotype diversity of QTL candidate genes and their association with strong race-nonspecific resistance to *Bgh* in a set of barley (*Hordeum vulgare* subsp. *vulgare* L.) accessions from the IPK *ex situ* collection that include exotic genotypes and landraces. A set of 35 accessions that exhibited strong race-nonspecific resistance at the seedling stage, most carrying the *Mlo* allele, plus 30 extremely susceptible accessions was identified and selected for genotyping. Re-sequencing of 88 selected candidate genes is in progress and revealed a number of significant associations of single-nucleotide polymorphisms with resistance in the presence of the *Mlo* gene, by taking into account population structure that has been determined using 46 SSR markers. Promising haplotypes will be functionally tested by transient expression in epidermal cells that interact with the fungus. These data

may shed light on molecular mechanisms underlying race-nonspecific basal defence and allow gene-haplotype introgression in order to improve durable disease resistance.

Introduction

Resistance of barley to the powdery-mildew fungus *Blumeria* [syn *Erysiphe*] *graminis* f.sp. *hordei* (*Bgh*) is often mediated by natural or induced loss-of-function alleles of the *Mlo*-gene that give broad-spectrum resistance, or by nucleotide-binding-site/leucine-rich-repeat (NBS-LRR)-type genes that confer race-specific resistance. Both types of resistance depend on an arsenal of signaling and downstream defence-related genes that—by themselves—may be responsible for complex patterns of quantitative resistance loci (QTLs). In a phenomics approach based on transient-induced gene silencing (TIGS; Douchkov *et al.*, 2005) we have identified approximately 40 barley mRNAs that affect quantitative, or basal, host resistance. The corresponding genes plus approximately 40 additional candidate genes known to be relevant for general defence responses in other plants were used for a re-sequencing approach. The aims of the project are the estimation of haplotype diversity of QTL candidate genes and their association with strong race-nonspecific resistance to *Bgh* in a set of barley accessions from the IPK *ex situ* collection that include exotic genotypes and landraces.

Materials and methods

Re-assessment of resistance to *Bgh* was done in a detached-leaf assay of 2- to 3-week-old seedlings. Disease severity was scored as described by Schweizer *et al.* (1995). The genetic diversity present in this customized barley collection was estimated using 45 microsatellite markers and revealed no indication of grouping according to the resistance phenotype (software STRUCTURE). Two DNA fragments of approximately 200–500 bp were re-sequenced per candidate gene. Associations of SNPs and/or haplotypes with resistance were calculated by using TASSEL software (general and mixed-linear models; Yu *et al.*, 2006). Rare SNPs or haplotypes occurring in less than 5% of the population were eliminated from the analysis.

Results and discussion

We tested 112 spring barley accessions in a detached leaf assay for the verification

of previously determined race-nonspecific resistance to *Bgh* and could confirm 36 resistant accessions (Nover and Lehmann, 1972, 1973; Nover and Mansfeld, 1955). By using a transient complementation assay with a *Mlo*-containing BAC clone we could classify the accessions into non-complementing, partially and fully complementing groups. A final set of 62 accessions (32 resistant carrying not the naturally occurring *mlo-11* allele and 30 susceptible) have been selected for the production of single seed descent lines and for genotyping (Figure 1). The population structure of the customized collection appears to be very pronounced and roughly corresponds to the geographical origin of the selected genotypes. In order to test whether this population is suitable for revealing true SNP and haplotype-trait associations, we re-sequenced part of the *Vrs-1* gene controlling row number (Komatsuda *et al.*, 2007). As shown in Figure 2, three SNPs including the known 1 bp indel of haplotype *vrs-1.a1* were identified as significantly associated with row number.

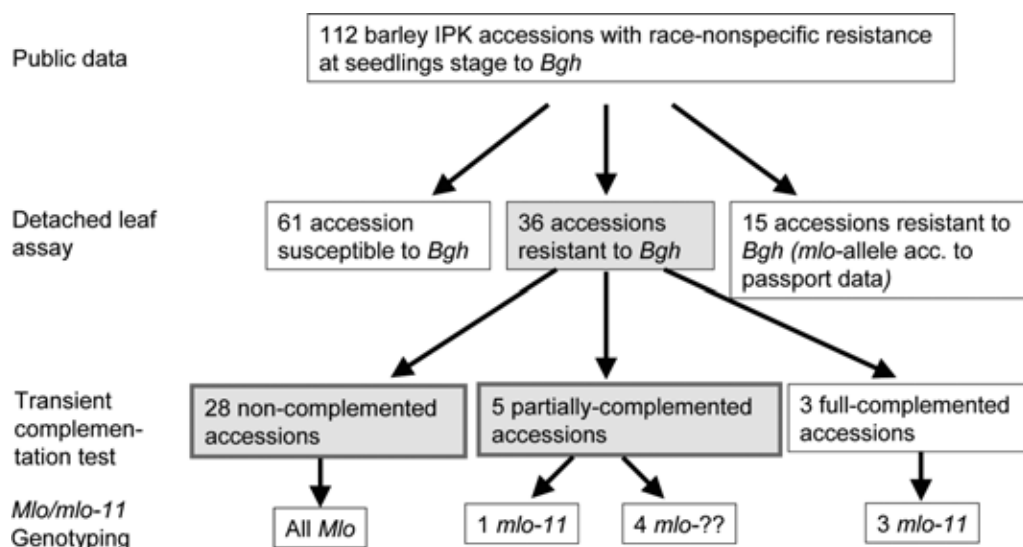


Figure 1. Flow chart for the selection of barley accessions exhibiting race-nonspecific, basal resistance against *B. graminis*. Accessions highlighted in yellow were selected for the customized collection.

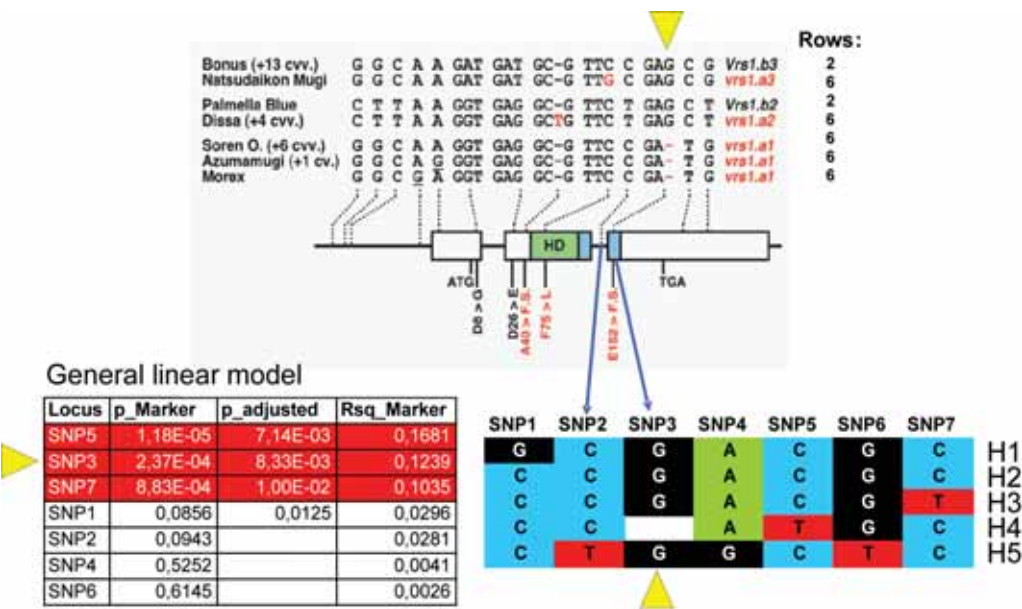


Figure 2. Proof of concept for the structured association approach in the selected barley collection. Haplotypes of the *Vrs-1* gene were identified and associated with the row number of the genotypes. Three significant SNPs out of seven detected are highlighted in red ($P < 0.05$ Bonferroni-corrected = “p_adjusted”). A total of eight haplotypes were identified (not all shown). The upper part of the figure was modified from Komatsuda *et al.*, 2007.

Re-sequencing of 77 selected candidate genes is in progress and has so far revealed 16 genes with significant, structured association of SNPs or haplotypes with resistance in accessions carrying the wild type *Mlo* gene (Table 1). In eight genes, SNPs as well as haplotypes were associated with resistance. The selection of model (general linear *versus* mixed linear) did not strongly influence the results. Associated haplotypes will be entirely sequenced and functionally tested by transient expression in epidermal cells that interact with the fungus.

Conclusion

A considerable number of selected, defence-related candidate genes of barley exhibited significant structured association with race-nonspecific resistance to *Bgh*. These data may reveal host genes underlying race-nonspecific, basal defence.

Table 1. Summary of the current state of the re-sequencing approach of defence-related candidate genes of barley.

Genes re-sequenced	60 out of 77
Total sequence (Mbp)	7.4
Total number of SNPs	647
1 SNP/x bp	59.7
Total number of haplotypes	398
Haplotypes/gene	6.6
Genes significantly associated with resistance	16

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Asymptomatic infection of winter and spring barley by *Rhynchosporium secalis*: effects and implications for epidemiology and host resistance

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Abstract

Asymptomatic infection of barley by *Rhynchosporium secalis* has been reported and appears to be widespread. Its importance is not known but it could have significant implications for resistance breeding and control strategies. To assess this, trials of resistant and susceptible winter and spring barley cultivars were grown at sites across the UK. To gain an insight into potential inoculum sources, Burkard spore samplers were operated at two sites to investigate the concentration of air-borne *R. secalis* particles, compared to those of a pathogen known to have air-borne ascospores (*Leptosphaeria maculans*). Quantitative PCR (qPCR) results showed that *R. secalis* colonized both susceptible and resistant cultivars extensively and spread onto the grain without the development of visual symptoms during the growing season. Amounts of *R. secalis* DNA in winter barley were greater than in spring barley. qPCR detected *R. secalis* early in the growing season (GS 13–14) and distinguished susceptible from resistant cultivars. Amounts of *R. secalis* DNA on harvested grain of both resistant and susceptible cultivars were greater than on grain at time of sowing, indicating that *R. secalis* could colonize and spread throughout the growing season and be transmitted to subsequent crops on infected seed. Visual assessments of the disease for

determining resistance, therefore, need to be supplemented with quantitative molecular assessments for more reliable evaluation of disease threat.

Introduction

Leaf scald caused by *Rhynchosporium secalis* (Oudem.) J.J. Davis, is one of the most economically important diseases of barley. Yield losses of 10 to 40% are not uncommon (Shipton *et al.*, 1974) and in severe epidemics 100% losses in susceptible cultivars have been reported (Yahyaoui, 2004). Control of *R. secalis* by the use of resistant cultivars, cultural practices and fungicide application has proved unsustainable (Shipton *et al.*, 1974; Xi *et al.*, 2000).

Leaf scald is a polycyclic disease, normally involving several pathogen generations during the growing season, and secondary disease spread by splash-dispersed conidia (Fitt *et al.*, 1989; Zhan *et al.*, 2008). The significance of this splash-dispersal of *R. secalis* in the colonization of grain is not yet known, but symptomless infection of barley seed by *R. secalis* has been shown to be important in the dissemination of the pathogen (Lee *et al.*, 2001). Primary inoculum is thought to be via splash-dispersed conidia or mycelium from infected plants or crop debris. While no sexual stage has been discovered, a wide pathogenic

variation exists in most populations of *R. secalis* (Abang *et al.*, 2006). Furthermore, the considerable genetic variation for neutral markers in *R. secalis* populations (Salamanti *et al.*, 2000) and the presence of both mating types (Foster and Fitt, 2003) on the same lesion (Linde *et al.*, 2003) are consistent with the presence of a teleomorph.

The fungus is able to grow under the leaf cuticle and produce new conidia without the development of visual symptoms (Jorgensen *et al.*, 1993; Zhan *et al.*, 2008). Infection by *R. secalis* in UK winter barley generally produces few visual symptoms before January–February, although *R. secalis* has been detected by PCR before then (Fountaine, 2005). Due to the polycyclic nature of the disease, several pathogen generations may occur before symptom development, during which time *R. secalis* may interact with both major-gene and partial resistance in barley cultivars (Zhan *et al.*, 2008). Symptom development has been demonstrated to be a plant host response to the presence of secreted fungal proteins such as NIP1 in barley cultivars carrying the *R* gene *Rrs1* (Steiner-Lange *et al.*, 2003; Slot *et al.*, 2007). To improve guidelines for growers and breeders it is important to understand the symptomless phase of *R. secalis*. For example, such understanding would help to establish when best it is to apply fungicides to control *R. secalis*.

Guides are available (e.g. HGCA London, Recommended Lists (RL): www.hgca.com); they are produced after a series of experimental trials at different sites during different seasons where winter and spring barley cultivars are assessed for development of common diseases affecting crop yield and quality. Fountaine *et al.* (2007) demonstrated that there is a poor correlation between RL resistance ratings and severity of disease assessed by visual symptoms or quantitative PCR (qPCR) data. Combining both visual assessment and qPCR data to assess amounts of *R. secalis* in leaf and grain samples of different cultivars could greatly benefit growers and breeders.

The aims of this study was to monitor the development of *R. secalis* in winter and spring barley crops using visual assessment of symptoms and qPCR (to assess amounts of *R. secalis* DNA), and to investigate the presence of airborne inoculum of *R. secalis* by comparison with levels of the ascospore producing plant pathogen *Leptosphaeria maculans* (Guo and Fernando, 2005).

Material and methods

Trial sites

Winter (2006/07) and spring barley (2007) trials were established at sites throughout the UK (Table 1). In each trial, there were six randomized blocks, each block included plots of two resistant (winter barley: Manitou [9] and Flagon [8]; spring barley: Doyen [8] and

Table 1. Location of spring barley (2007) and winter barley (2006/07) trials in UK.

Spring Barley		Winter Barley	
Site	Location	Site	Location
Blairnathort farm	Aberdeen, Scotland	Skeddoway farm	Fife, Scotland
Tipperty	Aberdeenshire, Scotland	Manor Hill farm	Borders, Scotland
Skeddoway farm,	Fife, Scotland	Tipperty	Aberdeenshire, Scotland
Manor Hill farm	Borders, Scotland	Throws field	Essex, England
Fowlmere	Cambridgeshire, England	Fowlmere	Cambridgeshire, England
High Mowthorpe	Yorkshire, England	High Mowthorpe	Yorkshire, England
Rothamsted	Hertfordshire, England	Rothamsted	Hertfordshire, England
		Terrington	Yorkshire, England

Oxbridge [7]) and two susceptible (winter barley: Sumo [5] and Saffron [6]; spring barley: Optic [6] and Troon [7]) cultivars [figures in brackets represent the cultivar RL rating]. Half of the plots were sprayed with a fungicide regime recommended by the HGCA RL protocol (www.hgca.com) to control *R. secalis*. Visual assessment of disease symptoms was done at growth stages (GS) 13, 26, 39 and 75 for winter barley and GS 26, 39 and 75 for spring barley. Samples for DNA extraction were taken from the sown seed and from plants sampled from plots at GS 13 (winter barley only), 26, 39 and harvested grain.

Burkard spore samplers

Burkard spore samplers were set up following the guidelines set out by Lacey and West (2006). The samplers were set up at winter barley trials sites at Rothamsted Research (Hertfordshire, England) and Scottish Agronomy (Arlary, Perth and Kinross, Scotland) during the trials in 2006/07. Tapes were periodically collected from the spore samplers. Once removed, tapes were cut into lengths corresponding to 24-hour periods. Each of these pieces of tape was cut lengthways, along the centre line in the direction of rotation, into two halves. One half was stored at -20°C and DNA was extracted from the remaining tape sections according to the protocol outlined by Graham *et al.* (1994). DNA was screened (2.5 μl from a 50 μl preparation) for *R. secalis* using the qPCR assay as described by Fountaine *et al.* (2007; Cytochrome b LAN probe and primer set). DNA levels were converted to spores per m^3 for *R. secalis* by converting the DNA levels to per spore (each *R. secalis* spore contained 0.15 pg DNA) then using the calculations described by McCartney *et al.* (1997).

Spores per m^3 for *R. secalis* were compared with that of the known ascospore producer *L. maculans* on spore tapes taken from a Burkard spore sampler where barley and oil seed rape stubble had been stacked

at Rothamsted Research. DNA levels, and subsequent spores per m^3 , were calculated for *R. secalis* as above, whereas ascospores of *L. maculans* were counted under a microscope from the other half of tape and converted to spores per m^3 using the calculations outlined in McCartney *et al.* (1997).

DNA extraction from leaf and grain material

DNA was extracted from leaf samples (each with 10 leaves) or grain samples (5 g) in accordance with the protocol of Fraaije *et al.* (1999), with the modification outlined by Fountaine *et al.* (2007). The protocol for the pre-extraction stage was amended, with the leaf samples being frozen in liquid nitrogen before crushing in a pestle and mortar rather than put through a leaf crusher, and grain samples ground in a coffee grinder (CG100 model, Kenwood, UK). DNA was quantified using a nano-drop spectrophotometer ((ND-1000, Labtech International Ltd, Sussex, UK) and the liquid was diluted into 20 ng μl^{-1} aliquots. A total of 50 ng DNA was used for subsequent qPCR reactions. The qPCR reactions were performed as above (Fountaine *et al.*, 2007).

Results

The presence of *R. secalis* DNA was detected in most of the samples of grain sown for the spring and winter barley trials (Figure 1). The amount of *R. secalis* DNA detected in grain harvested was generally greater than the amount on grain sown for most of the cultivars (Figure 1), even for sites where there were no visual symptoms of disease on the upper two leaves at GS 39 or 75 (Tables 2 and 3). The two susceptible winter barley cultivars, Sumo and Saffron, had greater amounts of *R. secalis* DNA than the two resistant winter barley cultivars, Manitou and Flagon.

R. secalis DNA was detected at an earlier growth stage in the winter barley trials than in spring barley cultivar trials (Tables 2, 3;

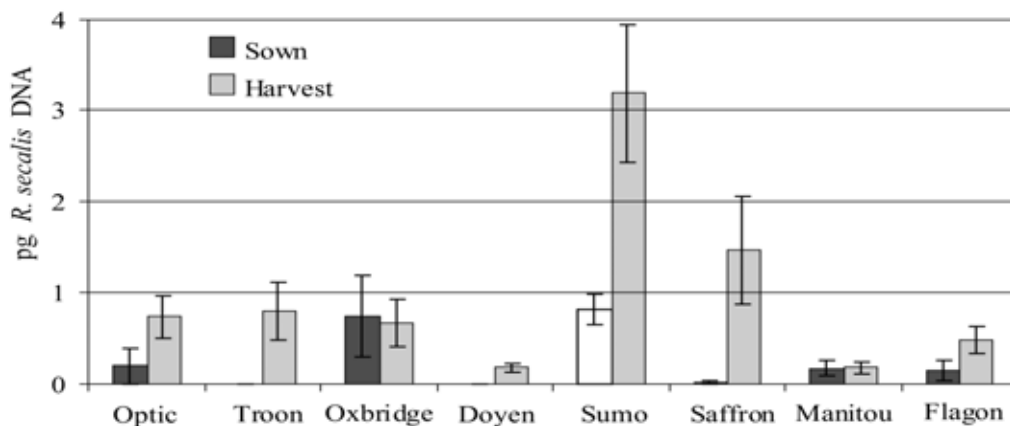


Figure 1. Amount of *Rhynchosporium secalis* DNA in grain at time of sowing and at harvest. Spring barley cultivars sown in 2007 (Optic; Troon; Oxbridge; Doyen); mean of eight sites in UK. Winter barley cultivars sown in 2006 (Sumo; Saffron; Manitou; Flagon); mean of eight sites in UK.

Figure 2). Greater amounts of *R. secalis* DNA were detected at GS 26 in the winter barley trials than in the spring barley trials, irrespective of RL resistance rating (Figure 2). Detection of large amounts of

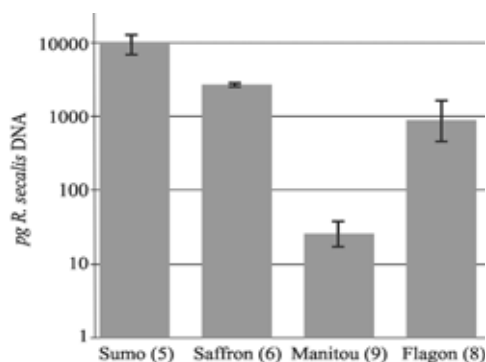


Figure 2. Amount of *Rhynchosporium secalis* DNA in leaves of winter barley cultivars at GS 26. Data are mean of eight sites in UK. Numbers in brackets are Recommended List (RL-HGCA) ratings for resistance to *R. secalis* for that cultivar (1 = most susceptible, 9 = most resistant). No *R. secalis* DNA detected at GS 26 in spring barley cultivars at trial sites in 2007. RL ratings for spring barley cultivars are Optic (6); Troon (4); Oxbridge (7) and Doyen (8).

R. secalis DNA in winter barley cultivars early in the season was not associated with visual symptom development (Table 2).

Amounts of *R. secalis* DNA detected in the upper two leaves at GS 39 in the winter barley were small (Table 2). The time between sampling points (GS 26 and GS 39) corresponded with a dry April and May in 2007 (data not shown). Risk of disease pressure was small for the spring barley trials in 2007 as indicated by the low levels of *R. secalis* visual symptom development and *R. secalis* DNA amounts in samples taken (Table 3).

The presence of *R. secalis* DNA in the winter barley was greater in Scotland than on sites within England (Figure 3), but only one site (Tipperty) had severe visual symptoms, compared with two sites in England (Throws Field and Fowlmere – Table 2). These two sites had severe visual symptoms but low amounts of *R. secalis* DNA.

Detection of *R. secalis* DNA in air samples at both the Arlary and Rothamsted Research site during the winter barley trials (2006/07) was low (Figure 4), especially when compared with that of a known ascospore producer (Figure 5).

Table 2. Development of *Rhynchosporium secalis* on winter barley trials (2006/07) in the UK, showing severity of disease visual symptoms (VS = % leaf area affected) and qPCR data (pg = picogram of *R. secalis* DNA in 50 ng aliquot \pm SE of mean) at specified growth stages (GS) during season. Data shown are only for cultivar Sumo.

Site	GS 13		GS 26		GS 39 – Leaf 2		GS 75 – Leaf 2		Grain (harvest)
	VS	qPCR	VS	qPCR	VS	qPCR	VS	qPCR	qPCR
Skeddoway farm	0	1.2 \pm 0.6	0.2	8504 \pm 2521	0	0.2 \pm 0.1	0	X ^a	0.8 \pm 0.2
Manor Hill farm	0	0.2 \pm 0.1	2.2	7604 \pm 2201	5	5 \pm 2.9	4	X	5.7 \pm 2.8
Tipperty	0	3.8 \pm 0.9	0.3	12320 \pm 1971	17	33.8 \pm 19.5	dead	X	5.7 \pm 2.8
Throws field	0	4.8 \pm 2.1	0	250 \pm 30	35	1.44 \pm 0.5	0	X	3.6 \pm 1.2
Fowlmere	0	0.2 \pm 0.1	0	160 \pm 43	35	0.2 \pm 0.1	0	X	0.2 \pm 0.1
High Mowthorpe	0	9.76 \pm 0.5	3.9	8.8 \pm 2.4	0.03	4.2 \pm 1.3	0.2	X	1.9 \pm 0.4
Rothamsted	0	5.4 \pm 1.4	5	9619 \pm 2932	0	3.3 \pm 2	1	X	6.5 \pm 3.2
Terrington	0.4	1.3 \pm 0.6	0.5	115 \pm 23.4	0	65.8 \pm 41.7	0	X	6.2 \pm 3.8

a = not done

Table 3. Development of *Rhynchosporium secalis* on spring barley trials (2007) in the UK, showing severity of disease visual symptoms (VS = % leaf area affected) and qPCR data (pg = picogram of *R. secalis* DNA in 50 ng aliquot \pm SE of mean) at specified growth stages (GS) during season. Data shown are only for cultivar Optic.

Site	GS 26		GS 39 – Leaf 2		GS 75 – Leaf 2		Grain – (harvest)
	VS	qPCR	VS	qPCR	VS	qPCR	qPCR
Blairnathort farm	0	0	0	0	0	X ^a	0.4 \pm 0.1
Tipperty	0	0.1 \pm 0.07	0	0	trace	X	1.1 \pm 0.2
Skeddoway farm,	0	0	0	0.02 \pm 0.01	0	X	0.2 \pm 0.1
Manor Hill farm	0	0	0	0	0	X	0.05 \pm 0.03
Fowlmere	0	0.02 \pm 0.01	0	18.5 \pm 11.8	0	X	0.4 \pm 0.2
High Mowthorpe	0	0.06 \pm 0.02	0	0.04 \pm 0.01	0	X	0.1 \pm 0.05
Rothamsted	0	0.3 \pm 0.07	0	77.7 \pm 47	0	X	3.5 \pm 1.4

a = not done.

Discussion

This study has demonstrated that further research is necessary to understand how *R. secalis* interacts with the host plant. Results have demonstrated that *R. secalis* is able to colonize the host plant extensively without causing visual symptoms of disease, thus providing a potential inoculum source for later epidemics, and confirms other

published data sets (Davis and Fitt, 1993; Fountaine *et al.*, 2007; Zhan *et al.*, 2008).

A high level of *R. secalis* DNA early in the growing season, as seen in the winter barley cultivars, did not necessarily lead to an epidemic. Spread of the pathogen to upper leaves later in the season may have been limited by the warm, dry April and May in 2007, indicating that environmental conditions may be important in the spread of

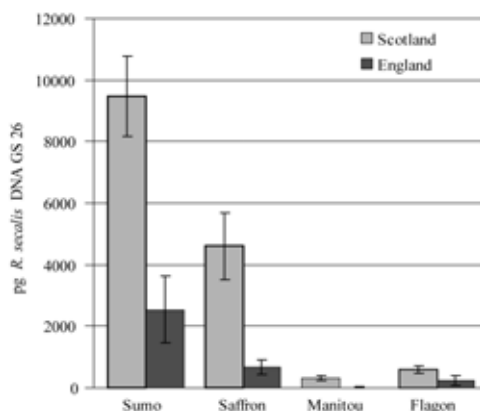


Figure 3. Amounts of *Rhynchosporium secalis* DNA (pg) in leaves of winter barley cultivars at GS 26 in trials in Scotland and England during 2006/07. Data are means of three Scottish sites and five English sites.

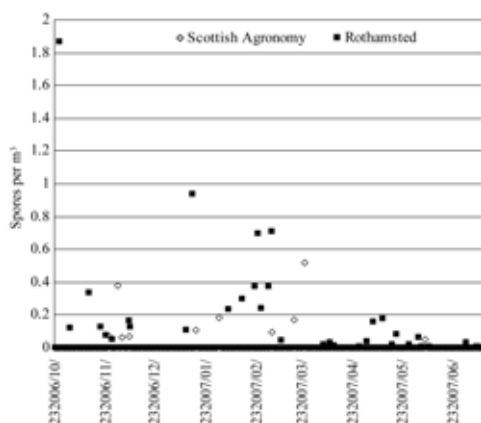


Figure 4. Detection of *Rhynchosporium secalis* DNA on Burkard spore samplers sited at Arlary and Rothamsted Research (October 2006 – June 2007). DNA levels converted to spores per m³ air sampled.

R. secalis, as seen in other trials (reviewed by Zhan *et al.*, 2008). This may indicate a disease escape by the host rather than true resistance. The qPCR data demonstrated that *R. secalis* was more prevalent in Scotland compared with trials in England, indicating that a wetter, colder growing season is more conducive for fungus development. The Recommended

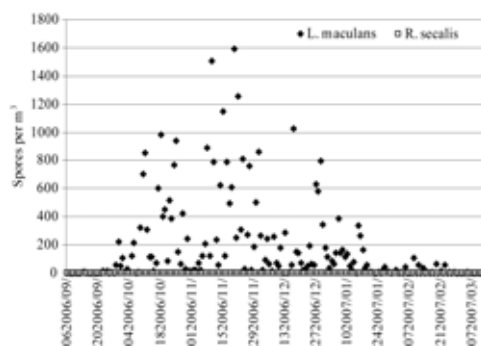


Figure 5. Detection of *Rhynchosporium secalis* DNA and *Leptosphaeria maculans* ascospores on a Burkard spore sampler sited at a barley and oil seed rape stubble site at Rothamsted Research (September 2006 – March 2007). DNA levels and spore counts converted to spores per m³ air sampled.

List (www.hgca.com) suggests that there is a difference in resistance between spring and winter barley cultivars, with the winter cultivars having greater resistance. Spring barley cultivars grown in the winter season are highly susceptible to *R. secalis* infection (Newton *et al.*, 2004). The low level of *R. secalis* DNA but high level of visual symptom development (Table 2) at Throws field and Fowlmere in the winter barley trials indicate that the *R. secalis* population present at the site may be important in epidemic development. More pathogenic populations may have greater impact on barley cultivars, even when levels are low, compared with a larger population of less pathogenic isolates. Visual symptoms (necrotic lesions) do not develop when populations of *R. secalis* expressing NIP1 isoform types III and IV are present on *Rrs1* expressing barley cultivars, compared with visual symptom development when *R. secalis* population expressing NIP1 isoforms I or II are present (Fiegen and Knogge, 2002, 2003; Slot *et al.*, 2007).

Combining qPCR data with visual assessment could provide greater information on host pathogen interactions, particularly

during the symptomless phase of the fungus. The qPCR data presented in Figures 1 to 3 show that cultivar resistance affects *R. secalis* DNA levels, with the most susceptible cultivars having more pathogen DNA than the resistant cultivars. Figure 1 and Tables 2 and 3 demonstrate that spread of the fungus onto the grain is possible, even when visual symptoms of the disease are not detected. Given this evidence, and other published data (Lee *et al.* 2002), fungicide seed treatment may be important in decreasing the impact of early season *Rhynchosporium* epidemics.

Application of fungicides to trial plots also decreased *R. secalis* DNA levels (data not shown) and the qPCR data could provide information on the effect of early fungicide sprays on pathogen levels. The high level of *R. secalis* DNA at GS 26 in the winter barley plots demonstrates that an autumn fungicide application may have been advantageous, although no epidemic developed during the growing season, but this may have been due to environmental conditions rather than inoculum. Data presented by Young *et al.* (2006) indicates that optimal fungicide application is around GS 31–32.

The use of qPCR data on its own may not be useful as a predictive tool for *R. secalis* epidemics and needs to be combined with environmental conditions and other information, such as cultivar and local climate. The development of a model is necessary to predict epidemic development, similar to that outlined by Evans *et al.* (2007) for phoma stem canker on oil seed rape. Disease pressure was low for the spring barley trials and little information could be gained from these trials at present.

Data presented by Foster and Fitt (2003) and Linde *et al.* (2003) suggest that *R. secalis* has a teleomorph that is similar to that of the closely related *Pyrenopeziza brassicae* and *Oculimacula yallundae* (Foster and Fitt, 2003). Detection of airborne *R. secalis* DNA was low at the sites tested, especially when compared with a known ascospore producer,

L. maculans (Figure 5), and would indicate that primary inoculum of *R. secalis* epidemics is not by an airborne sexual stage but rather by splash-borne dispersal of asexual conidia and mycelium from infected plants or stubble. Zaffarano *et al.* (2006) suggests that the centre of origin for *R. secalis* is northern Europe and that the pathogen may have originally evolved on another graminaceous host. This suggests that completion of the sexual stage may not take place on barley but may on another grass host.

International trade in seed has been implicated in the spread of the pathogen (Zaffarano *et al.*, 2006) and evidence presented here on spread of the pathogen to grain, even when colonization and spread was symptomless in the field, indicates a high potential for spread via this route.

The use of qPCR to accurately estimate *R. secalis* DNA may provide a more reliable assessment of cultivar resistance than visual assessment of disease symptoms, particularly when assessments during the symptomless colonization of the host plant can be performed. There is a need to construct a predictive model for *R. secalis* epidemics that takes into account environmental, pathogen population, cultivar and agronomic factors, and to determine inoculum sources for *R. secalis* so that more sustainable control strategies can be designed.

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Fusarium head blight evaluation and genetic diversity assessment by simple sequence repeats in 88 barley cultivars and landraces

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Abstract

Fusarium head blight (FHB) is a destructive disease of barley. A better understanding of the genetic variation in barley with different levels of FHB should help in the development of effective and efficient breeding strategies for FHB-resistant cultivars. The objectives of the study were to evaluate FHB severity and estimate the genetic diversity among 88 accessions and to identify new sources of high resistance to FHB. Simple sequence repeats (SSR) were used to characterize the genetic diversity. A total of 207 alleles were detected by 33 SSR markers distributed throughout the genome, and polymorphic fragments with a mean of 6.3 alleles per locus were amplified. Genetic similarities calculated from SSR data ranged from 0.12 to 0.99. A dendrogram was generated on the basis of a similarity matrix using the UPGMA algorithm, showing that the 88 accessions fell into two groups, which corresponded well with the results of principal component analysis. Thirteen accessions were found to be resistant to FHB. These resistant accessions could be useful in the identification of suitable parents for the development of mapping populations for tagging the FHB resistance genes. Five SSR markers, Bmag0125, EBmac0415, Bmag0807, HVR CABG and EBmac0679, were significantly associated with FHB severity. These results suggest that the molecular markers linked to an

economically important quantitative trait like FHB resistance could be identified from a collection of unrelated genotypes.

Introduction

Knowledge regarding the genetic variation in cultivated crops is important for plant breeding, germplasm enhancement and utilization of germplasm resources. Such knowledge is useful for breeders to design optimal plant breeding programs and select parents for hybridization, which will maximize gain from selection and maintain genetic diversity. Understanding the genetic relationships between various accessions may eliminate the possibility of elite germplasm becoming too genetically uniform and increase the efficiency of breeding efforts. In barley, breeders have made crosses between highly selected genotypes and the genetic base for breeding has become quite narrow. More knowledge regarding the genetic structure of breeding materials could help to maintain genetic diversity (Graner *et al.*, 1994; Troyer *et al.*, 1998). Assessment of germplasm diversity can be obtained in terms of morphology, pedigree, isozyme analysis and DNA analysis. DNA analysis is currently the most feasible strategy for characterizing genetic diversity. Many types of DNA markers have been used to characterize germplasm and their usefulness may depend on the nature of the marker, the map location

and the population under investigation. SSRs are co-dominant, abundant and a highly informative marker system (Saghai-Maroo *et al.*, 1994; Dávila *et al.*, 1999). They are powerful markers for characterization and evaluation of genetic diversity, construction of linkage maps and marker assisted selection (Powell *et al.*, 1996; Tsuru *et al.*, 2005).

Fusarium head blight (FHB) is a destructive disease of barley (*Hordeum vulgare*) in many warm, humid growing regions of the world. The disease is caused primarily by *Fusarium graminearum* and can result in reduction of yield and quality (McMullen *et al.*, 1997). It has been estimated that in some years in China, 30% of total barley production has been lost (Wang *et al.*, 2005). The most serious threat associated with FHB is the possible accumulation in the kernels of mycotoxins produced by the fungi. These compounds are toxic to humans and other animals and can cause over-foaming or gushing in beer (Schwarz *et al.*, 1997). The most effective strategy for controlling FHB in barley is the deployment of resistant cultivars, and many breeders have realized the need for incorporating FHB resistance into their breeding material. Unfortunately, breeding for FHB resistance has been difficult, because resistance to FHB is conditioned by many genes distributed throughout the genome (de la Pena *et al.*, 1999; Zhu *et al.*, 1999; Ma *et al.*, 2000; Kolb *et al.*, 2001; Mesfin *et al.*, 2003). To date, quantitative trait loci (QTLs) associated with FHB resistance have been identified on all seven chromosomes (de la Pena *et al.*, 1999; Ma *et al.*, 2000; Zhu *et al.*, 1999; Mesfin *et al.*, 2003; Dahleen *et al.*, 2003; Canci *et al.*, 2004; Horsley *et al.*, 2006). Most of them are often inconsistently detected among different mapping populations and environments, and are usually associated with morphological traits such as late heading, tall height, two-row spike and lax spike. Belina *et al.* (2002) examined the genetic diversity of a set of spring six-row barley accessions with partial

resistance to FHB and found that they were a relatively diverse group that is genetically distinct from current USA midwest six-row cultivars. The objectives of the study reported here are: (1) to estimate the genetic diversity among 88 barley cultivars and landraces with different levels of FHB resistance used by the Chinese Barley Improvement Center in Hangzhou, China, and (2) to identify new source of high resistance to FHB.

Materials and methods

Plant materials

From our field evaluation of more than 2000 barley accessions for FHB resistance in 2002, 2003 and 2004, we selected 88 accessions of different types and origins for use in this study (Table 1), comprising 60 accessions from 9 provinces of China, 21 cultivars from different breeding institutes in China, and 7 cultivars introduced from abroad. All of these accessions were collected by the Chinese Barley Improvement Center.

FHB evaluation in the greenhouse

All the materials were planted and evaluated in the greenhouse of the Chinese Barley Improvement Center in a completely randomized design with three replications, with a seeding date of 21 November 2005. Each replicate consisted of a single pot with four plants, and five heads injected with spore suspension were scored per replicate. The greenhouse temperature averaged 22°C during the day, with a range of 18–25°C, and 19°C at night with a range of 17–21°C. At anthesis, plants were inoculated with 10 µL of a macroconidial suspension (concentration of 10 000 macroconidia/mL) of *F. graminearum*. Inoculum was placed in a single central floret using a syringe. The inoculum of *F. graminearum* was a field isolate (Hang43) originating from Hangzhou (Zhejiang). Inoculated spikes were misted with water for three consecutive days and

Table1. Fusarium head blight severity of 88 barley cultivars and landraces used in this study

Landrace / Cultivar	Origin	Spike type	FHB severity ^a (%)	Resistance group
Chinese Landraces				
Yuhuang-zhuweiba	Zhejiang	2-Row	5	R
Jiangshan-erleng	Zhejiang	2-Row	6	R
Huangyan-ye	Zhejiang	2-Row	7	R
Yueqing-bian	Zhejiang	2-Row	7	R
Shangyu-hong	Zhejiang	2-Row	9	R
Yuyao-yang	Zhejiang	2-Row	10	R
Shouchang-wugongmai	Zhejiang	6-Row	11	R
Zhenhai-hunzazhong	Zhejiang	2-Row	13	R
Sixian-sanyuehuang	Anhui	6-Row	13	R
Yuyao-hong	Zhejiang	2-Row	14	R
Tiantai-erleng2	Zhejiang	2-Row	16	MR
Gaochunmang	Jiangsu	6-Row	16	MR
Yuhuan-liulengmimai	Zhejiang	6-Row	16	MR
Yongkang-erleng	Zhejiang	2-Row	16	MR
Tiantai-erleng	Zhejiang	2-Row	17	MR
Qinghe-siyuehuang	Hebei	6-Row	17	MR
Dulihuang	Gansu	6-Row	17	MR
Dongtou-baimang	Zhejiang	6-Row	17	MR
Fenghua-erleng	Zhejiang	2-Row	18	MR
Huangchangguang	Yunnan	2-Row	18	MR
Sanshizhun	Shanxi	6-Row	19	MR
Jingjiang	Jiangsu	6-Row	21	MR
Kuanyinluomai	Yunnan	2-Row	21	MR
Aijiao' erlengluomai	Zhejiang	2-Row	21	MR
Pujiang-sanyuehuang	Zhejiang	6-Row	21	MR
Quzhou	Zhejiang	6-Row	22	MR
Aibaiyang	Zhejiang	2-Row	23	MR
Wenchenghuangmai	Zhejiang	6-Row	25	MR
Zhuji-zao	Zhejiang	6-Row	25	MR
Yunhe-silengbaimai	Zhejiang	6-Row	25	MR
Yiwu-erleng	Zhejiang	2-Row	26	MR
Hangzhoushi-sanyuehuang	Zhejiang	6-Row	27	MR
Pujiang-chaotianmai	Zhejiang	6-Row	27	MR
Pan'an-nuomai	Zhejiang	6-Row	28	MR
Jizaoshu	Fujian	6-Row	28	MR
Kaihua-sileng	Zhejiang	6-Row	28	MR
Jinyun	Zhejiang	6-Row	31	MS
Lanxi-chi	Zhejiang	6-Row	31	MS
Nantong-daobadan	Jiangsu	6-Row	34	MS
Rudong-sanyuehuang	Jiangsu	6-Row	34	MS
Yueqing-yang	Zhejiang	2-Row	34	MS
Huai'an-sanyuehuang	Jiangsu	6-Row	35	MS
Xinchang-xiaojiang	Zhejiang	6-Row	35	MS
Fenglinlao	Hubei	6-Row	36	MS
Taishun-bendi	Zhejiang	6-Row	38	MS
Chun'an-bendi	Zhejiang	6-Row	41	S
Langyihunzao'ai	Fujian	6-Row	41	S
Longyou-mimai	Zhejiang	6-Row	41	S
Xiaoshan-erleng	Zhejiang	2-Row	42	S
Yinxian-erleng	Zhejiang	2-Row	46	S
Yueqing-zhongshu	Zhejiang	6-Row	53	S
Songyang-silengmimai	Zhejiang	6-Row	53	S
Jingning-zaomai	Zhejiang	6-Row	57	S
Rui'an-huaguchui	Zhejiang	6-Row	57	S
Chibahuang	Hebei	6-Row	61	S
Yongkang-bianmai	Zhejiang	6-Row	62	S
Yongjia-bai	Zhejiang	6-Row	71	S
Lanhua	Hebei	6-Row	83	S
Lanxi-jinhuazhong	Zhejiang	6-Row	84	S

Landrace / Cultivar	Origin	Spike type	FHB severity ^a (%)	Resistance group
Chinese Cultivars				
Erdamai6	Hubei	2-Row	12	R
Sunong7249	Jiangsu	6-Row	13	R
Zhepi8	Zhejiang	2-Row	14	R
Xiumai3	Zhejiang	2-Row	20	MR
Pu808047	Fujian	2-Row	20	MR
Zhepi33	Zhejiang	2-Row	21	MR
Zhenongda6	Zhejiang	2-Row	22	MR
Zhepi1	Zhejiang	2-Row	23	MR
Pudamai4	Fujian	2-Row	23	MR
Zhe0419	Zhejiang	2-Row	23	MR
Erpi1	Hubei	2-Row	23	MR
Zheyuan18	Zhejiang	2-Row	24	MR
Zhexiu12	Zhejiang	2-Row	25	MR
Zhenongda3	Zhejiang	2-Row	26	MR
Pudamai7	Fujian	2-Row	26	MR
Zhenongda7	Zhejiang	2-Row	29	MR
Hua30	Zhejiang	2-Row	29	MR
Zhepi2	Zhejiang	2-Row	35	MS
Zhe04153	Zhejiang	6-Row	36	MS
Zhepi3	Zhejiang	2-Row	37	MS
Dan2	Jiangsu	2-Row	52	S
Non-Chinese Cultivars				
Hedist	Denmark	6-Row	19	MR
HDE84194-622-1	France	2-Row	22	MR
Suyin21	Japan	2-Row	23	MR
Meilihuangjin	Japan	2-Row	23	MR
Siguoluo87	Japan	6-Row	31	MS
Zaoshu3	Japan	2-Row	36	MS
longjiangzao	Korea	6-Row	54	S

a = The LSD at the 0.05 level for FHB severity was 20.4.

covered with a glassine bag to provide the humidity required for infection and to prevent movement of isolates to other spikes. Infected and total kernels in a spike were counted on the 21st day after inoculation. FHB severity was calculated as the average percentage of infected kernels per spike (Table 1). The degree of resistance was classified into four groups based on the value of FHB severity: resistant (R) 0–15%; moderately resistant (MR) 15–30%, moderately susceptible (MS) 30–40%; and susceptible (S) 40–100%. This study measured Type II resistance, i.e. resistance to the spread of *F. graminearum* in a spike.

Morphological traits

Morphological traits were also recorded on these 88 accessions in the greenhouse.

Heading date was recorded as the number of days from seeding to the date when approximately 50% of the heads were completely emerged from the boot. Spike morphology was recorded as two-row or six-row, which refers to the number of fertile florets per rachis node. Spike density was the number of rachis nodes per cm in the spike. Plant height was determined at maturity as the distance from the soil surface to the tip of the spike, excluding awns.

SSR analysis

Young leaves from each accession were collected, lyophilized and ground to powder. DNA extraction followed the CTAB (cetyltrimethyl ammonium bromide) method described by Doyle and Doyle (1987), with minor modification. The purified total DNA

was quantified by gel electrophoresis and its quality verified by spectrophotometry.

DNA samples were stored at -20°C. The isolation of the microsatellite markers and their location on barley genetic map has been described previously in detail (Becker and Heun, 1995; Ramsay *et al.*, 2000). To select the best SSRs, primers were screened across 6 accessions (Shangyu-hong, Zhenhai-hunzazhong, Yuyao-hong, Siguoluo87, Zaoshu3 and Sunyin21). A total of 321 microsatellites were tested, and classified according to amplification quality and polymorphism. From the 321 microsatellites tested, 33 primers showing high quality and polymorphic SSR pattern were chosen for the diversity analysis (Table 2). PCR was carried out in a PTC-200 thermocycler (MJ Research Inc., USA). The program used was identical to the PCR conditions of Becker and Heun (1995) and Ramsay *et al.* (2000). The PCR products were separated on PAGE gels and

detected by silver staining as described by Bassam *et al.* (1991).

Data analysis

Only the unambiguous bands were scored on the polyacrylamide gels as present (1) or absent (0), generating two binary matrices. Jaccard's coefficient (Jaccard, 1908) of similarity was calculated for all pair-wise comparisons between accessions and a dendrogram was created by cluster analysis using the unweighted pair group method based on arithmetic average (UPGMA) of a computer program called NTSYS-pc package (Rohlf, 1990). Principal Component Analysis (PCA) was performed using the SAS System version 8 for Windows. Trait associations were calculated using SPSS 10.0 for windows. The significance of the Pearson correlations was determined. Differences in frequencies for SSR markers between the two selected groups were tested by a χ^2 test.

Table 2. Chromosome location, map position (according to Becker and Heun, 1995, and Ramsay *et al.*, 2000) and number of alleles for SSR loci assayed in 88 barley accessions.

SSR	Chromosome	No. of alleles	SSR	Chromosome	No. of alleles
Bmac0090	1H	5	HVM40	4H	8
HVWXYG	1H	11	EBmac0679	4H	4
Bmac0032	1H	6	GMS089	4H	4
HVM20	1H	6	Bmag0808	4H	4
Bmac0213	1H	8	EBmac0775	4H	5
WMC1E8	1H	4	Bmag0337	5H	4
EBmac0501	1H	6	Bmag0751	5H	9
Bmag0125	2H	12	EBmac0824	5H	2
EBmac0415	2H	9	Bmac0316	6H	7
HVM54	2H	6	Bmag0496	6H	6
Bmag0720	2H	4	Bmag0870	6H	5
Bmag0813	2H	18	Bmac0047	4H/6H/7H	8
Bmag0603	3H	2	Bmac0064	7H	5
Bmac0209	3H	4	Bmag0011	7H	4
EBmac0705	3H	6	EBmac0827	7H	3
EBmacc0009	4H	10	GMS002	nd	3
HVRCABG	4H	9			

nd = not determined

Results

FHB severity and trait based analysis

A large variation for FHB severity (range 5%~83%) was found among the 88 accessions (Table 1). Two landraces from Zhejiang Province of China—Yuhuan-zhuweiba and Lanxi-jinhuazhong—were found to be the most resistant and susceptible accession, giving the lowest (5%) and the highest (84%) severity score, respectively. Thirteen accessions, including 10 landraces, all native to Zhejiang and Anhui Province, and 3 Chinese cultivars were identified as resistant. Ten of them were the two-row type. Sixteen accessions, including 14 landraces, 1 Chinese cultivar and 1 Korean cultivar, were identified as susceptible, and most of them were the six-row type. Half of the accessions (26 landraces, 14 Chinese cultivars and 4 non-Chinese cultivars) were moderately resistant to FHB. The remaining 10 landraces, 3 Chinese cultivars and 2 cultivars introduced from Japan were classified as moderately FHB susceptible. These results indicated that FHB resistance was present in the landraces, but more frequently in the two-row types than in the six-row.

The correlation between FHB severity and plant height was not significant. FHB severity was positively correlated with spike type and spike density and was negatively correlated with heading date (Table 3). Spike type showed a negative correlation with heading date. In general, later heading lines exhibited lower levels of FHB severity. We

also found that two-row accessions headed later and were more resistant to FHB than six-row accessions. Most of the accessions in the resistant group were two-row and were generally later heading. In contrast, most of the susceptible accessions were six-row and generally early heading.

Allelic variation of SSR

The fragments in the 88 accessions were amplified by 33 primer pairs. A total of 207 alleles were obtained, and polymorphic fragments ranged from 2 to 18, with a mean of 6.3 alleles per locus. The SSR marker Bmag0813 produced the greatest number of alleles per locus (Table 2).

Genetic similarity

Based on the SSR data, genetic similarity was calculated with NTSYS-pc using Jaccard's coefficients. The similarity matrix is available upon request. The genetic similarity coefficient among the source germplasm ranged from 0.12 to 0.99, with an average value of 0.47. The highest similarity was 0.99 between Quzhou and Lanxi-chi, which were landraces from Zhejiang Province; and the lowest similarity was 0.12 between Meilihuangjin and Siguoluo87. We also calculated the genetic similarity coefficient between the FHB resistance sources and found that the closest germplasm were Yueqing-bian and Yuyao-yang, while the two farthest germplasm were Zhenhai-hunzazhong and Sunong7249. The lowest similarity between the FHB resistance

Table 3. Correlation coefficients among spike type, heading date, plant height, spike density and FHB severity in 88 barley accessions.

Traits	Spike type	Heading date	Plant height	Spike density
Heading date	-0.48**			
Plant height	0.19	0.15		
Spike density	0.89**	-0.20	-0.03	
FHB severity	0.39**	-0.30**	-0.18	0.51**

** significantly different at $P = 0.01$.

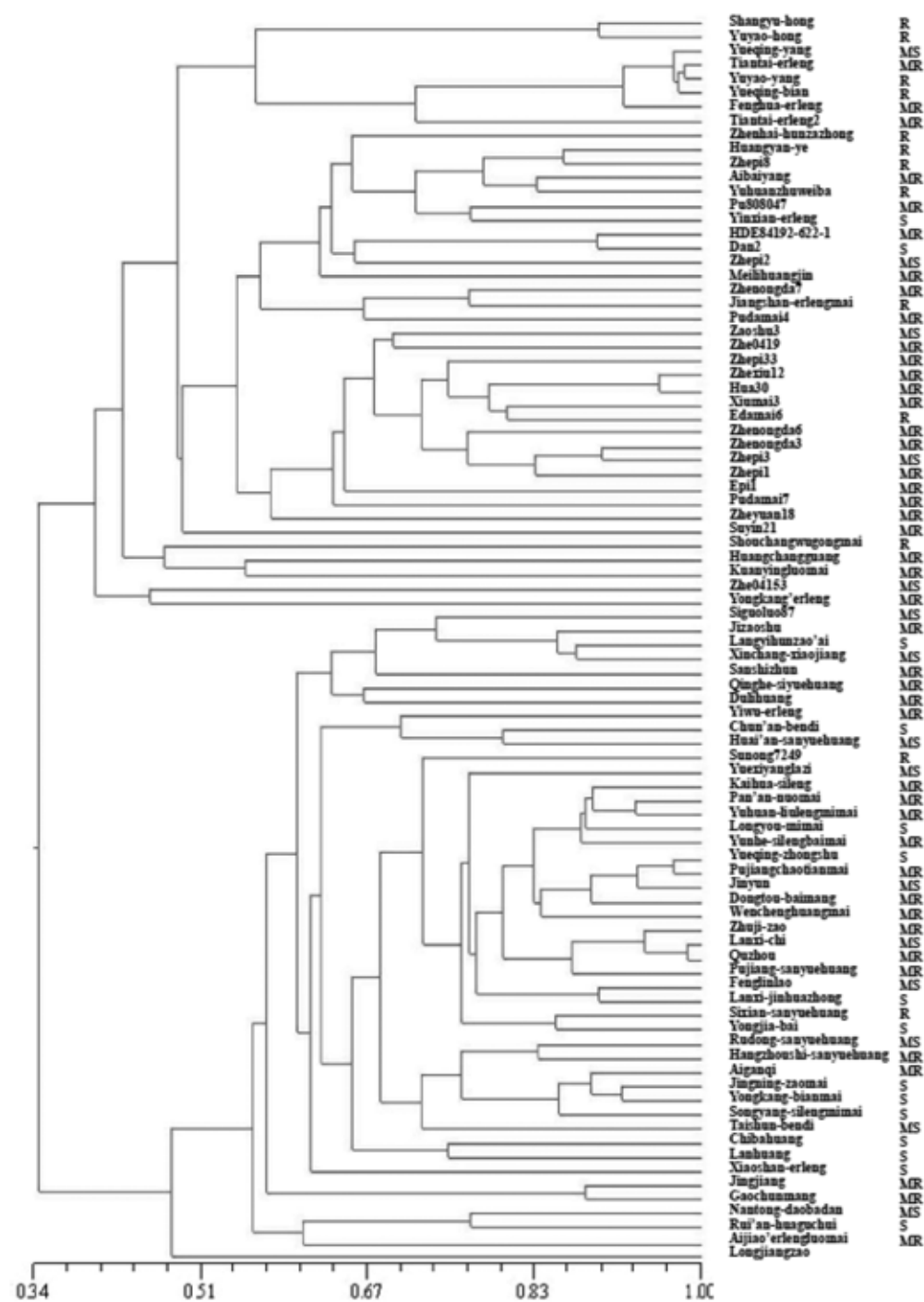


Figure 1. UPGMA dendrogram showing genetic similarity among the 88 barley accessions used in this study. The dendrogram was constructed based on Jaccard's coefficients. Key: ○ Chinese Landrace, ▲ Chinese Cultivar, ■ Non-Chinese Cultivar.

landraces were Zhenhai-hunzazhong and Sixian-sanyuehuang.

UPGMA cluster analysis

All 88 barley accessions were discriminated by the 33 SSR markers (Figure 1). Some cultivars clustered closely together according to their geographic origin. For example, two landraces—Jingjiang and Gaochunmang—originating in Jiangsu Province clustered closely together; similarly, Quzhou and Lanxi-chi, both native to the Jinhua region of Zhejiang Province, were also closely related. Other cultivars, for instance, Zhexiu12, Hua30 and Xiumai3, Zhepi33 and Zhe0419, were ancestrally related, and grouped together (Figure 1), showing a certain degree of correlation between DNA data and pedigree.

However, most of them did not follow this pattern. For example, some landraces from Shanxi, Hebei, Gansu and Hubei were mingled with those of Zhejiang Province. The French accession HDE84194-622-1 had a close affinity to Dan2, which was developed by the Institute of Genetics, Chinese Academy of Sciences, and the Institute of Agricultural Sciences in the Coastal Area Jiangsu. The accessions are divided into two distinctive clusters (Figure 1). Cluster I consists of 40 two-row and 2 six-row accessions, and cluster II contains 3 two-row and 43 six-row. Spike density and FHB severity in cluster I are lower than those in cluster II and the differences between them are highly significant ($P < 0.01$). We also found that most of the resistant accessions were in cluster I, while most of the susceptible ones were in cluster II. Cluster I could be further divided into eight subgroups using the similarity coefficient of 0.55 (Figure 1), with 9 of the 13 resistant accessions clustered into I-1 and I-2. Most Chinese cultivars were moderately resistant to FHB and concentrated in subgroup I-3, along with Zaoshu3 (Figure 1). Suyin21, derived from Japan, became the fourth subgroup.

The FHB resistant landrace Shouchang-wugongmai from Zhejiang Province, as the least genetically related to other resistant genotypes in cluster I, formed subgroup I-5. Subgroup I-6 consisted of two landraces, Huangchangguang and Kuanyinluomai, from Yunnan Province. Zhe04153 and Yongkang-erlengmai formed the seventh and eighth subgroups, respectively. In cluster II, more than 90% of the accessions were landraces, with 2 resistant accessions (Sunong7249 and Sixian-sanyuehuang) and 14 susceptible accessions scattered into different subgroups. A non-Chinese cultivar, Longjiangzao from Korea, was unique in cluster II, showing 0.48 similarity to the other accessions in cluster II. As to the moderately resistant and moderately susceptible accessions, no obvious relationship was evident.

Principal Component Analysis

The first and the second principal components explained 43.9% and 13.5% of the total variation, respectively (Figure 2). From the PCA, two distinct groups were found among the accessions. The landraces divided into two clusters, with most of the cultivars from China clustered together, while non-Chinese cultivars separated from each other.

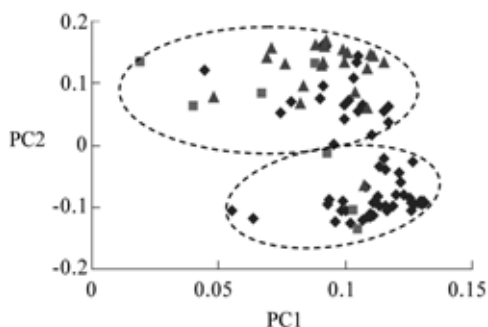


Figure 2. Associations between 88 accessions on the basis of the first two principal components (PC1, PC2) obtained from principal component analysis of Jaccard similarity coefficients based on the combined SSR data. Chinese Landrace, ▲ Chinese Cultivar, ■ Non-Chinese Cultivar.

Markers associated with FHB resistance

The χ^2 test showed that Bmag0125, Bmag0807, HVRCABG and EBmac0679 were significantly associated with FHB severity at $P < 0.01$, and that the association between EBmac0415 and FHB severity

was significant at $P < 0.05$ level (Table 4). We also found that the five SSR markers banding pattern of Sunong7249 and Sixian-sanyuehuang in the resistant group and of Yinxian-erleng and Dan2 in the susceptible group were unique (Table 4).

Table 4. SSR markers whose frequencies differed significantly between the resistant (FHB severity <15%) and susceptible (FHB severity >40%) groups.

Landrace or cultivar	FHB severity (%)	Markers				
		Bmag 0125-1	EBmac 0415-1	Bmag 0870-1	HVRCABG-1	EBmac 0679-1
Yuhuan-zhuweiba	5	1	1	1	1	1
Jiangshan-erlengmai	6	1	1	0	1	1
Huangyan-ye	7	1	1	1	1	1
Yueqing-bian	7	0	1	1	1	1
Shangyu-hong	9	1	1	1	1	1
Yuyao-yang	10	0	1	1	1	1
Shouchang-wugongmai	11	1	0	1	1	1
Erdamai6	12	1	1	1	1	1
Sunong7249 ^a	13	0	0	1	0	0
Zhenhai-hunzazhong	13	1	1	1	1	1
Sixian-sanyuehuang ^a	13	1	0	0	0	0
Yuyao-hong	14	1	1	1	1	1
Zhepi8	14	1	1	1	1	1
Chun'an-bendi	41	0	0	0	0	0
Langyihunzao'ai	41	0	0	0	0	0
Longyou-mimai	41	0	0	0	0	0
Xiaoshan-erlen	42	0	1	0	0	0
Yinxian-erleng ^a	46	1	1	1	1	1
Dan2 ^a	52	1	1	1	0	1
Yueqing-zhongshu	53	0	0	0	0	0
Songyang-silengmimai	53	0	0	0	0	0
Longjiangzao	54	0	0	0	0	1
Jingning-zaomai	57	0	0	0	0	0
Rui'an-huaguchui	57	1	0	0	0	0
Chibahuang	61	0	0	0	0	0
Yongkang-bianmai	62	0	0	0	0	0
Yongjia-bai	71	0	0	0	0	0
Lanhua	83	0	1	0	0	0
Lanxi-jinhuazhong	84	0	1	0	0	0
Marker frequency	<15	0.769	0.769	0.846	0.846	0.846
	>40	0.125	0.313	0.125	0.063	0.188
Difference		0.644**	0.456*	0.721**	0.783**	0.658**

(a) = accessions showing a unique pattern of 5 SSR markers; * and ** significant at the 0.05 and 0.01 probability levels, respectively

Discussion

There were several correlations between morphological traits and FHB severity. FHB severity was negatively correlated with heading date or plant height, possibly because late heading lines might have escaped the most favorable period for the infection in the field and tall lines were distant from *Fusarium* inoculum on the ground and remote from the soil moisture (de la Pena *et al.*, 1999; Ma *et al.*, 2000; Urrea *et al.*, 2002; Dahleen *et al.*, 2003; Mesfin *et al.*, 2003; Horsley *et al.*, 2006). Steffenson (2003) suggested that the association between lower FHB severity and late heading might be due to shorter inoculum exposure (escape) or tight linkage of separate genes for flowering time and disease resistance. Nduulu *et al.* (2007) suggested that the relationship between FHB and heading date at the Qrgz-2H-8 region was probably due to tight linkage rather than pleiotropy. In this study, FHB severity was negatively correlated with heading date. The correlation between FHB severity and heading date indicated that there might be linkage between genes for FHB resistance and heading date. Note that it was impossible for the host plant to escape the pathogen in our experiment. The correlation between FHB resistance and plant height was not significant, which differs from previous studies (de la Pena *et al.*, 1999; Ma *et al.*, 2000; Urrea *et al.*, 2002). It is possible that our inoculation procedure completely removes the effects of plant height and other environmental factors on FHB severity. Hilton *et al.* (1999) suggested that there were independent genes affecting FHB and plant height in wheat that may allow plant breeders to select resistant cultivars of any height. Our results also showed that FHB severity was positively correlated with spike type. The two-row type was more resistant to FHB than the six-row type, probably because two-row spikes dry faster and it was more difficult for the pathogens to spread upward and downward (Chelkowski *et al.*, 2000; Choo *et al.*, 2004). Dense spikes were observed to have greater FHB susceptibility,

which was consistent with the observation of Zhu *et al.* (1999). All these results imply that development of lines with late heading and lax, two-row spikes could result in lower levels of FHB severity.

To date, many investigators have reported the level of SSR polymorphism within barley itself, with the average numbers of alleles per locus ranging from 2.1 to 18. Becker and Heun (1995) reported that 10 of the 15 microsatellites gave fewer polymorphic bands, with 2.1 alleles per locus in 11 barleys. The most polymorphic alleles, with an average of 18 alleles per locus, were reported by Saghai-Maroo *et al.* (1994), based on four SSRs assayed in 207 genotypes. Matus and Hayes (2002) assayed 42 SSRs on 147 barley accessions and obtained 687 alleles, with an average of 16.3 alleles per locus. In our study, we also found a low number of alleles per locus, which might be due to the fact that only the genome regions conferring the SSR polymorphism among six cultivars were tagged.

The results of genetic diversity based on the dendrogram derived using genetic similarity values and the PCA conducted directly from binary matrix data were in agreement to a great extent (Figures 1 and 2). The highest genetic similarity was detected between Quzhou and Lanxi-chi (0.99) and the lowest similarity between Meilihuangjin and Siguoluo87 (0.12). This indicated that the non-Chinese cultivars had the lowest affinities and that genetic diversity at the genomic level of the selected germplasm was rich.

Most of the cultivars from China clustered together with Zaoshu3. These corresponded well with their positions in the dendrogram as well as in the PC plot (Figure 2) and indicated that the genetic background of the improved cultivars from China was narrow. There are two reasons for this. Firstly, Zaoshu3 (originating from Japan) was introduced into China in 1966, and was a cultivar with early maturity, high yielding and wide adaptability.

It was very popular in the Yangtze River Valley, and had become a parent for crossing in the late 1960s. Secondly, some genetic variability was lost in the process of selection for high quality, high yield and wide adaptability in the breeding program, and thus resulted in a narrow genetic base for breeding.

Landraces are old local strains selected by farmers from natural variation occurring in production fields; their pedigree information is therefore unknown. SSRs may be the best way to determine their genetic relatedness. In this study, landraces separated into two distinct groups. Most of the FHB-resistant landraces clustered together; therefore the genetic relationship among these landrace is close. This may be due to the fact that these landraces were grown geographically close to each other and subject to similar natural selection, or originated from a common ancestor. Fortunately, three FHB-resistant landraces, Jiangshan-erlengmai, Shouchang-wugongmai and Sixian-sanyuehuang, were not genetically closely related to other resistant landraces (Figure 1). This indicates that new resistant genes might be present in these Chinese landraces. In our previous study, we also found that Shouchang-wugongmai had good resistance to DON (Deoxynivalenol) accumulation (Jia *et al.*, 2007). Previously, Chen *et al.* (1991) reported that Shangyu-hong and Yuyao-hong were resistant to FHB. There were three FHB-resistant cultivars, namely Zhepi8, Erdamai6 and Sunong7249, and their genetic relationship, based on microsatellite markers, was not close (Figure 1). Researchers have reported that some of the cultivars from China showed some resistance to FHB and had a different origin from the other sources of FHB resistance previously mapped, such as CIho4196, Zhedar1 and Zhedar2 (Prom *et al.*, 1996, 1997; Rudd *et al.*, 2001; Dahleen *et al.*, 2003; Horsley *et al.*, 2006). We also found that Sunong7249 and Sixian-sanyuehuang were uniquely identified in the resistant

group, while Yinxian-erleng and Dan2 were uniquely identified in the susceptible group using the five SSR makers (Table 4). These unique alleles are one explanation why these accessions are distinct from the other resistant or susceptible accessions in the dendrogram (Figure 1). We conclude that there is some FHB-resistant germplasm among Chinese genetic resources. These results are useful in the identification of suitable parents for the development of mapping populations for tagging the FHB-resistance genes.

Resistant cultivars are the most cost effective means of controlling the disease. However, breeding for FHB resistance is difficult for various reasons: (1) the most resistant germplasm is of exotic origin and has poor agronomic traits; (2) the inheritance is oligogenic to polygenic; and (3) screening for FHB resistance is environmentally biased, tedious and expensive (Buerstmayr *et al.*, 2002). These problems encourage identification of molecular markers linked to QTL for FHB resistance that can be used in marker-assisted selection. Only a few results concerning the development and application of molecular markers in improving disease resistance in barley have been reported. Two major FHB QTL regions (*Ebmac0521a-Bmag0140* and *Vrs1-Bmag0125*) detected in the field experiments were successful validated in an independent population with Fredrickson and Stander as parents (Mesfin *et al.*, 2003). Four FHB QTLs that were detected in the original mapping population of Chevron \times M69 were also detected in either Stander \times MNS93 or M92-299 \times M81 populations in one environment (Canci *et al.*, 2004). Wingbermuehle *et al.* (2004) validated six known FHB QTLs in nine populations and found that markers linked to four of the six QTLs were associated with FHB severity in at least one of the populations. Our study showed that five SSR markers were significantly associated with the FHB-resistant groups. Among the five SSR markers, three of them linked to

the previously identified FHB QTL regions. The loci Bmag0125 and EBmac0415 were found to be associated with QTLs for FHB resistance and low DON accumulation on chromosome 2H (Mesfin *et al.*, 2003); marker Bmag0870 on chromosome 6H was close to an FHB QTL (Canci *et al.*, 2004). This suggested that the molecular markers linked to economically important quantitative trait like FHB resistance could be identified from a collection of unrelated genotypes. To our knowledge, the other two markers have not been previously reported to be associated with the QTLs for FHB resistance. So it is likely that HVR CABG and EBmac0679 can be used as candidate markers of FHB QTL regions for further investigations. Our study is analogous to the study by Sun *et al.* (2003), who identified 3 RAPD markers significantly associated with FHB-resistant spring wheat cultivars. The advantage of using a collection of accessions rather than a population to identify markers for traits is that the markers are more likely to be applicable to a large number of breeding programs. This method of identifying SSR loci will be useful for marker-assistant selection and can be used as candidate markers for further gene mapping and cloning.

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QTL analysis of spot blotch and leaf rust resistance in the BCD47 × Baronesse barley mapping population

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Abstract

Leaf rust (LR) (caused by *Puccinia hordei*) and spot blotch (SB) (caused by *Cochliobolus sativus*) are two of the main diseases of barley in Uruguay. We studied the genetics of the resistance to both diseases present in a doubled-haploid (DH) population derived from the cross BCD47 × Baronesse. BCD47 has low SB severity and high susceptibility to LR, while Baronesse is susceptible to SB and has low susceptibility to LR. Both resistances were expressed at the adult plant stage. The population was phenotyped in 9 environments for each disease. Four QTLs were detected for SB, on chromosomes 1H, 3H, 6H and 7H. In three of them, BCD47 contributed the resistant alleles. The QTLs on chromosome 1H (located in the Bmac213-Bmag770 interval) and chromosome 3H (located in the Bmag225-Bmag013 interval) were the most consistent across environments. Two main QTLs were detected for LR on chromosomes 6H (Baronesse contributing the resistant allele) and 7H (BCD47 contributing the resistant allele), coincident with SB resistance QTLs. The QTL on chromosome 6H (linked to the SSR Bmag173) was the most consistent across environments.

Introduction

Barley spot blotch (SB), caused by *Cochliobolus sativus* (Ito and Kurib.) Drechsl. ex Dastur [anamorph *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem., syn. *Helminthosporium sativum* Pamm., King and Bakke], is one of the most important barley diseases in Uruguay. Grain yield losses in epidemic years have been estimated at between 7 and 30% (Pereyra, 2005). Spot blotch is also considered important because it reduces malting quality (Steffenson *et al.*, 1996). The growing use of no-tillage systems has caused an increase in its incidence in the last fifteen years (Stewart *et al.*, 2001; Pereyra *et al.*, 2003). Barley leaf rust (caused by *Puccinia hordei*) (LR) is one of the most important barley disease worldwide. In Uruguay is also an important disease, which had presented low incidence level for more than 20 years, but in the last two seasons appeared in epidemic proportions, causing important losses in susceptible cultivars (German, 2007). Losses in susceptible cultivars estimated during 2006 were as high as 60% in yield and 65% in the percentage of plump grains (Castro *et al.*, 2006).

Genetic resistance is one key objective for barley plant breeding programs, and QTL analysis is very useful tool for the analysis of the genetic basis of disease resistance, in particular in the case of quantitative resistance (Williams, 2003). QTL analysis has also been used to accelerate the introgression of disease resistance QTLs into different genetic backgrounds using marker assisted selection (Toojinda *et al.*, 1998) and to develop pyramids of disease resistance QTLs and genes (Castro *et al.*, 2003a, b).

In this research we use QTL analysis to study the genetic basis of the resistance to SB and LR segregating in a doubled-haploid (DH) mapping population derived from the cross between the varieties BCD47 and Baronesse.

Materials and methods

BCD47 and Baronesse are the parents of the mapping population. BCD47 (Orca × Harrington*2//D-1-72) is a two-row spring habit DH line developed via marker-assisted selection for barley stripe rust (BSR)

resistance alleles at Oregon State University. Baronesse (343-6/V34-6//T-427/3/ORIOL/6153-P40) is a two-row spring habit European variety. The mapping population was developed using the *Hordeum bulbosum* method.

The full population consists of 409 DH lines. In this study we use the full population in some experiments and a representative sample of 100 lines in the rest of the experiments. The whole population was genotyped and a linkage map was constructed using a total of 59 molecular markers, as described in Vales *et al.* (2005). The map construction was performed using JoinMap 3 (Van Ooijen and Voorrips, 2001).

The population was tested in fourteen field experiments, planted from 2002 to 2006 in three locations: “Dr Mario A. Cassinoni” Experimental Station (EEMAC) at Paysandú, Ombues de Lavalle, and La Estanzuela (Table 1). Two rows (20 cm between rows), 1-m length plots were used, except for 05A and 05B (six-row, 15 cm between rows, 4-m length plots), and 106/07 (1-row, 1-m length plots).

Table 1. List of experiments included in the present study.

Code	Location	Planting date	Population size (n)	Diseases assessed
02	Paysandú	17 July 2002	100	SB
03	Paysandú	15 Aug. 2003	100	SB
04A	Paysandú	30 July 2004	100	SB
04B	Paysandú	25 Aug. 2004	100	SB, LR
04C	Paysandú	1 Sept. 2004	100	LR
05A	Paysandú	12 July 2005	100	LR
C05	Paysandú	3 Aug. 2005	409	LR
M05	Ombúes de Lavalle	9 Aug. 2005	100	LR
05B	Paysandú	31 Aug. 2005	100	LR
I05/06	La Estanzuela	26 Dec. 2005	100	SB
C06	Paysandú	25 July 2006	409	SB, LR
I06	La Estanzuela	19 Aug. 2006	100	LR
06B	Paysandú	28 Aug. 2006	100	SB, LR
I06/07	La Estanzuela	28 Dec. 2006	409	SB

The sites were Paysandú: Estación Experimental “Dr. Mario A. Cassinoni” (EEMAC); La Estanzuela: Estación Experimental La Estanzuela, EELE; Ombúes de Lavalle.

At Paysandú, artificial inoculation was used, except in 02, 05A and 05B, which had good natural infection. Plots were sprayed at growth stage Z49 (Zadoks *et al.*, 1974) with a mixture of four *C. sativus* isolates with wide virulence, using 48 mL/m² with a concentration of 8×10^3 conidia/mL. In 105/06 and 106/07, plants were inoculated at Z45 with a mixture of 8 *C. sativus* monospore isolates from different barley growing areas within Uruguay, using 50 mL/m² with a concentration of 8×10^3 conidia/mL. Disease severity was measured based on visual assessment of the percentage of diseased area in the top three leaves, on a plot basis, at the end of the growing cycle. In 105/06, SB severity was measured starting at flowering.

SB at the seedling stage was measured in the greenhouse at La Estanzuela. Seedlings were inoculated when the second leaf was fully deployed, using a conidial suspension similar to the one used in the field experiments. The inoculum concentration was 6×10^3 conidia/mL. After inoculation, seedlings were incubated in controlled conditions (20–22°C, 12-hour photoperiod, 100% relative humidity) for 24 h. Seedling reaction was measured on the second leaf, 10–12 days after inoculation, using a 1–9 scale (1 = no symptoms; 9 = fully susceptible) (Fetch and Steffenson, 1999).

Inoculum from three *Puccinia hordei* races (UPh1, UPh2 and UPh3) was used to evaluate seedling reaction in the population parents. Plants were inoculated with a spore suspension in mineral oil in the greenhouse at an age of approx. 7 days (first leaf fully expanded). Plants were incubated for 14 h at 100% relative humidity and then moved back to the greenhouse (temperature: 20–24°C). Infection type (Stakman *et al.*, 1962) was measured 12 days after inoculation.

The QTL analysis was performed using the composite interval mapping (CIM) procedure (Zeng, 1994) implemented in Windows QTL Cartographer 2.5 (Wang *et al.*, 2005). Up to 10 cofactors for CIM were

chosen using a stepwise regression procedure with a significance threshold of 0.05. Walk speed was set to 2 cM and the scan window to 10 cM beyond the markers flanking the interval tested. A LR threshold of 11.5 was used. Epistatic interactions between QTLs were evaluated with the Multiple Interval Mapping (MIM; Kao *et al.*, 1999) tool implemented in Windows QTL Cartographer using Bayesian Information Criteria (BIC-M0).

QTLs detected through QTL Cartographer were confirmed using an approach analogous to candidate gene analysis, where the genotypes at the QTL region (determined through the genotype of the markers at that region) are used as independent variables. The treatment design was a $2 \times n$ factorial, where n is the number of genome regions considered, based on QTLs detected by the QTL analysis. Statistical analyses were performed using the GLM procedure of SAS 9.0 (SAS Inst., 2004).

Results and discussion

BCD47 and Baronesse exhibited a differential response to both diseases. Regarding SB, BCD47 had intermediate severity values at the adult plant stage (Table 2), which were always lower than those of Baronesse (except from 04B and 105/06, where both varieties had similar SB severity). In contrast, at the seedling stage, both varieties have closer values, although BCD47 had better scores. Regarding LR at the adult plant stage, Baronesse had intermediate to low severity, while BCD47 was highly susceptible (Table 3). At the seedling stage, both varieties had a susceptible response to the prevalent LR race in 2004–2006 (UPh3). All factors considered, Baronesse showed intermediate adult plant resistance to LR.

Frequency distribution of SB severity (Figure 1) was continuous, with variations between experiments reflecting differences in mean severity, suggesting a quantitative inheritance of SB resistance. Of the five

Table 2. Mean spot blotch severity at adult plant stage in each experiment and seedling infection for BCD47 and Baronesse.

Experiment	Baronesse	BCD47
Adult plant ¹		
02	25	19
03	8	3
04A	13	4
04B	34	36
I05/06	46	47
C06	27	5
06B	50	8
I06/07	66	37
Seedling stage ²		
	6–7	5

¹ Adult stage mean severity, measured as percentage of leaf area (three top leaves) infected.

² Assessed using scale of Fetch and Steffenson (1999): 0–9 where 0 = fully resistant, 9 = fully susceptible.

experiments included in the figure, only in the one with the lowest mean severity (C06) were there were plots without disease. This suggests that the segregating resistance is possibly not complete, or partial, allowing some disease development under favorable conditions.

A total of five SB adult plant resistance QTLs, located on chromosomes 1H, 2H, 3H, 6H and 7H were identified, with BCD47 contributing the resistance alleles in three of them and Baronesse contributing the resistance alleles in the other two (Table 4). The QTL detected on 5H was significant in 3 out of 4 years, while the rest were significant in only two years (2004 and 2006). No SB seedling resistance QTL was identified.

The SB adult plant resistance QTL on 1H is located close to the mapping location of *Rcs6*, an SB resistance gene mapped by Bilgic *et al.* (2006) in the Calicuchima-sib × Bowman-BC mapping population, with Calicuchima-sib contributing the resistance gene. Orca, one of BCD47 parents, came from that mapping population, opening the possibility that *Rcs6* could be the candidate gene for the QTL on 1H, but we have not confirmed the allele correspondence yet. The QTLs on 2H and 3H are coincident with the two main QTLs controlling flowering time in the population (Castro *et al.*, 2007). According to the consensus map of Varshney *et al.* (2007), the QTL on 3H was located in the same region as one SB seedling resistance QTL mapped in Steptoe × Morex and one SB adult plant resistance QTL mapped in Dicktoo × Morex (Bilgic *et al.*, 2005). No SB

Table 3. Summary of BCD47 and Baronesse reactions to LR at adult plant stage (natural infection under field conditions) and at seedling stage (controlled conditions and inoculated with three races of *Puccinia hordei*: UPh1, UPh2 y UPh3) in 2004–2006.

	2004		2005		2006	
	Baronesse	BCD47	Baronesse	BCD47	Baronesse	BCD47
Adult Plant ¹	30MR	80MSS	10RMR	25MS	5R	80S
Seedling ²						
Race UPh1	3	-	3	2	3+	1
Race UPh2	3	3	2	3	2-	-
Race UPh3	3	-	3	3	2+3	3

¹ Adult plant severity (% leaf area infected according to Cobb's scale, Peterson *et al.*, 1948) and reaction (Stakman *et al.*, 1962), where R = resistant; MR = moderately resistant; MS = moderately susceptible; S = susceptible. ² Seedling stage infection type (IT) assessed using a 0–4 scale (Stakman *et al.*, 1962), 0–2 resistant, 3–4 susceptible.

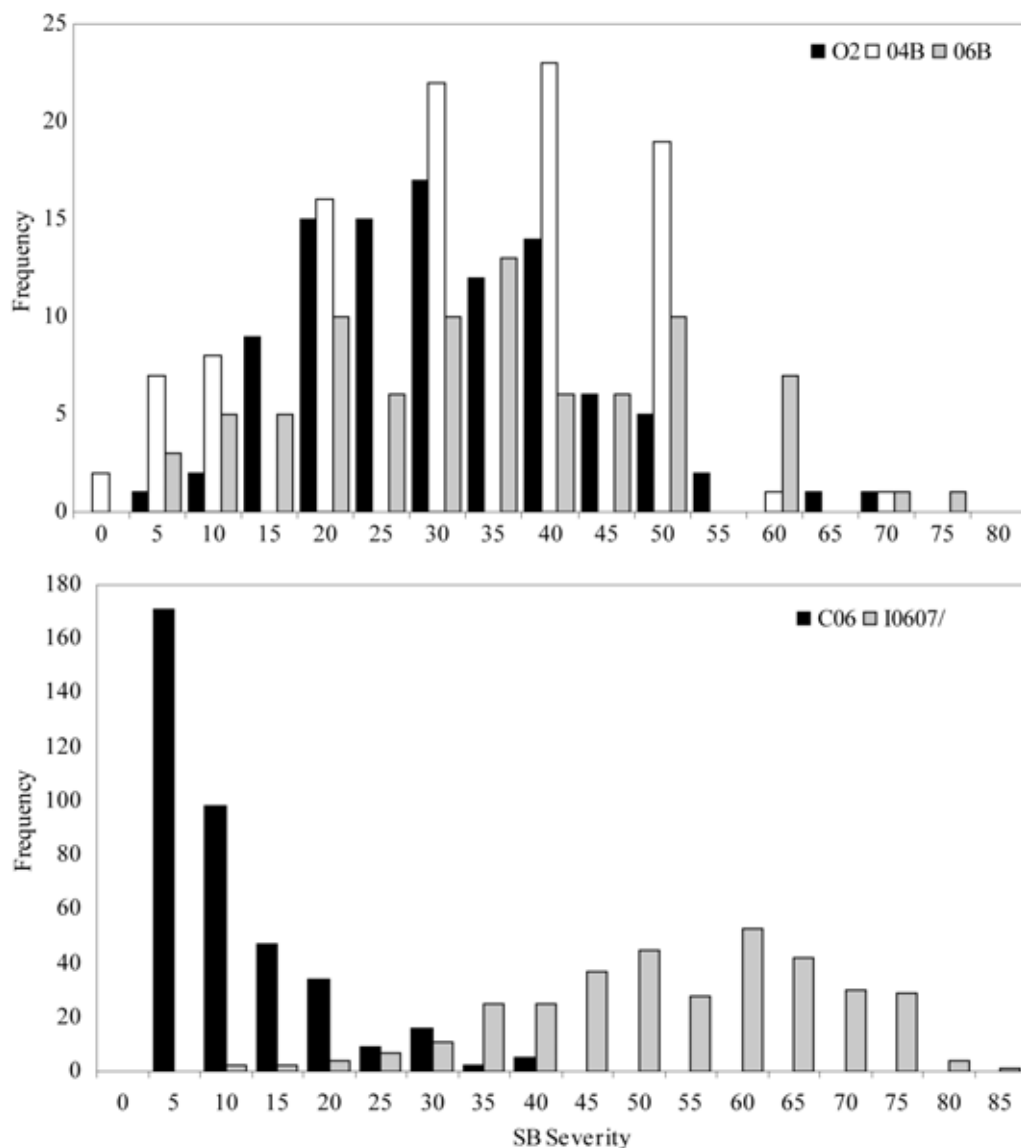


Figure 1. Frequency distributions for the average SB severity on adult plants of lines from the BCD47 \times Baronesse population in three experiments where a 100-line sample was tested (top figure) and two experiments where the full population was tested (bottom figure).

resistance gene or QTL are reported in the genomic regions of the QTLs on 6H and 7H.

Frequency distributions of LR severity (Figure 2) suggest a possible quantitative inheritance for the adult plant resistance present in Baronesse. As we already mentioned for SB, there were very few lines

without disease, suggesting that the LR segregating resistance was also incomplete.

We detected significant QTL effects for LR severity in nine experiments, and identified two adult plant resistance QTLs, one in chromosome 6H with Baronesse contributing the resistance alleles and the

Our results indicate that SB adult plant resistance segregating in the BCD47 \times Baronesse population presented a relatively complex expression, and was controlled by five QTLs on chromosomes 1H, 2H, 3H, 6H and 7H. The QTL in 5H was the one with most consistent effect within experiments and had a relatively complex expression. In two of the three experiments with *C. sativus* infection but not with *P. hordei* infection, this QTL was the only one detected. The QTLs on 2H and 3H are coincident with QTLs affecting flowering time, and the resistance alleles are coincident with alleles determining late

Marker interval		Resistance allele	r ² by experiment								
			04A	04B	04C	05A	M05	C05	C06	06B	I06
6H	<i>Bmag009-Bmag173</i>	Baronesse	(2.8)	9.6	8.7	19.6	10.9	6.5	15.0	12.6	7.9
7H	<i>Ris44-Bmag135</i>	BCD47	4.1	14.0	17.5			1.2			
	6H × 7H		5.4								

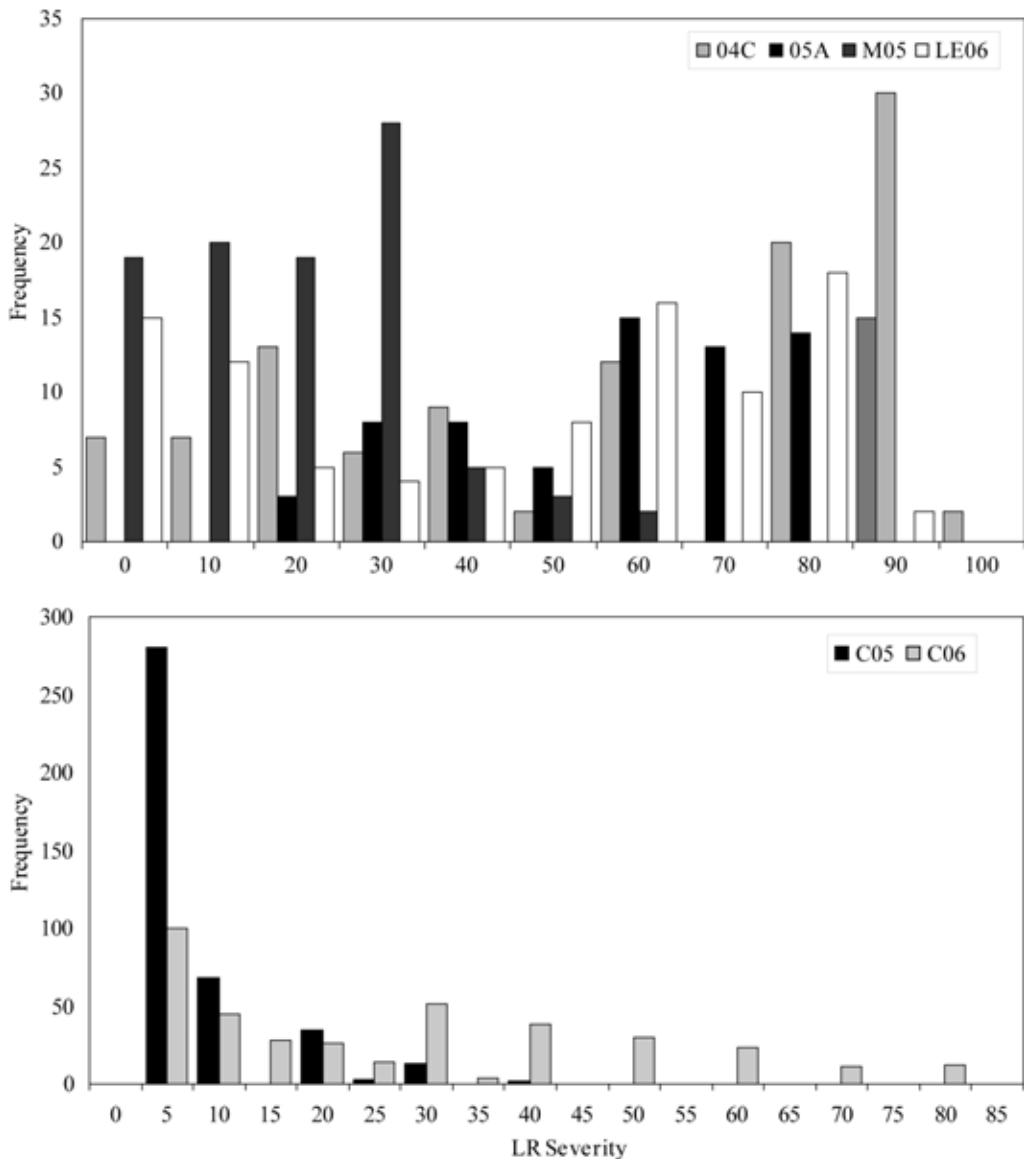


Figure 2. Frequency distributions for the average LR severity on adult plants of lines from the BCD47 \times Baronesse population in three experiments where a 100-line sample was tested (top figure) and two experiments where the full population was tested (bottom figure).

flowering. The effects of these QTLs on SB severity are probably a pleiotropic effect of the flowering time and not a proper resistance effect.

The two remaining SB resistance QTLs, on 6H and 7H, are coincident with the two

LR resistance QTLs, but the allele phases were in repulsion, with alleles associated with lower values for one disease associated with higher values for the other one. One explanation is that the QTLs affected directly only one disease, and the associated effect on

the other disease was due to the extra green tissue left because of the limited development of the first disease. Based on a comparison of Tables 4 and 5, and the situation in experiments with only one disease present, our hypothesis is that the QTLs on 6H and 7H are LR disease resistance QTLs, and their effect on SB severity is a consequence of the effects on LR development.

Regarding the LR resistance QTLs identified the relationship between the QTL on 6H and *Rphq3* can not be analyzed with the available information and genetic stocks. There is a strong possibility that the QTL on 7H was the same as reported by Rossi *et al.* (2007). Its relationship to other genes reported in the literature requires more information and appropriate genetic stocks in order to be resolved.

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Mapping of quantitative trait loci for net blotch resistance in Tunisian barley

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Abstract

Net blotch, caused by *Pyrenophora teres*, is a prevalent foliar disease of barley (*Hordeum vulgare* L.) in Tunisia. The identification in local germplasm of QTLs conferring resistance has not yet been studied. A barley population of 59 doubled-haploid lines derived from a cross between Roho, a Tunisian cultivar derived from an ICARDA nursery, and the local line '90' was used for mapping potential QTLs associated with net blotch resistance at seedling and adult plant growth stages using simple sequence repeat markers. The population was evaluated with three isolates at the seedling growth stage and with one at the adult growth stage. At the seedling growth stage, three to six QTLs were identified depending on the isolate used and the form of *P. teres*. For the two isolates of *P. teres* f. *maculata*, comparable QTLs were mapped on chromosomes 3H, 4H and 7H. These QTLs explained 5 to 20% of the phenotypic disease variation. For *P. teres* f. *teres*, other QTLs located on chromosomes 4H and 6H, explaining 10 to 31% of the phenotypic variation, were mapped. At the adult growth stage, three QTLs located on chromosomes 3H and 6H contributed 4 to 17% of the disease severity. This investigation indicated that two QTLs conferring resistance to *P. teres* f. *teres* are expressed at both seedling and adult plant growth stages. These two QTLs are located on chromosome 6H and are linked to Bmac0500 for the former QTL and to both HVM31 and EBmac0806

for the latter QTL. Further investigations are needed to use this tool in marker assistant selection for field resistance to net blotch.

Introduction

Net blotch of barley (*Hordeum vulgare* L.), caused by *Pyrenophora teres* Drechs., is a major disease in many growing areas around the world. In Tunisia, Cherif *et al.* (1994) reported that net blotch is the most common disease of barley and it occurs at high severity levels (70–80%) in some regions. They also reported that the two forms of the disease, the net form caused by *P. teres* f. *teres*, and the spot form caused by *P. teres* f. *maculata*, were identified.

Many studies identified sources of resistance to *P. teres* and explored the inheritance of resistance to this disease. They showed that resistance is due to either one or several genes, depending on the source of resistance, the plant development stage (seedling or adult), and the pathotype used for testing (Steffenson *et al.*, 1996; Afanasenko *et al.*, 2004). From the six different genes conditioning resistance to net blotch (Chelkowski *et al.*, 2003), at least four have been mapped (Williams *et al.*, 1999; Manninen *et al.*, 2000; Gupta *et al.*, 2004; Ma *et al.*, 2004; Williams *et al.*, 2004). QTLs for *P. teres* f. *teres* resistance, in seedling and adult growth stage have been reported on chromosomes 1H, 2H, 3H, 4H and 6H (Steffenson *et al.*, 1996; Ma *et al.*, 2004; Yun *et al.*, 2005); and on chromosomes 2H,

3H, 4H and 6H (Cakir *et al.*, 2003; Gupta *et al.*, 2004), respectively. Those for *P. teres* f. *maculata* resistance have been reported on chromosomes 2H, 3H, 4H and 7H (Molnar *et al.*, 2000; Williams *et al.*, 2004); and on chromosomes 4H, 5H and 7H (Williams *et al.*, 2004) in seedling and adult growth stage, respectively.

In Tunisia, most barley cultivars are susceptible or moderately susceptible to *P. teres*. Quantitative or partial resistance as determined by a reduction in the percentage of affected leaf tissue and a reduction in the rate of disease development in the fields, but with compatible infection response, was previously reported for some local lines of barley (Cherif, pers. comm.). From these, line '90' was selected from a Tunisian national breeding program for its resistance to net blotch in the field. The objective of this study was to determine the number and chromosomal location of loci controlling net and spot form of *P. teres* resistance in barley at both seedling and adult plant stages of development.

Materials and methods

Mapping population and genetic map

Fifty-nine doubled-haploid (DH) barley lines were obtained from F_1 plants of the cross between Roho and the local line '90'. Roho is a widely grown two-row barley cultivar susceptible to net blotch, whereas line '90' is a six-row line resistant to net blotch in the field. The original cross was made at the Institut National Agronomique de Tunisie (INAT) and the DH lines were produced at Florimond Desprez, using anther culture and the *Hordeum bulbosum* method (Devaux and Pickering, 2005). The DH lines were scored for 40 SSR markers. Of these markers, 34 were assigned to map positions on the seven barley chromosomes according to the consensus map of Varshney *et al.* (2007). The SSR map length was around 540 cM (Kosambi function) with an average interval

of 10 cM between markers (unpublished data). These DH lines were also used for net blotch evaluation.

Disease response tests

Seedling evaluations. For seedling evaluations, the three isolates PT.2/2003, PT.11/2003 and PT.30/2003 of *Pyrenophora teres* were chosen to screen the DH population because Roho and line '90' exhibited differential reactions to these isolates in a previous study (Cherif, unpublished data). PT.2/2003 and PT.11/2003 (from Tebourba and Sejnane, respectively) produced spot symptoms, whereas PT.30/2003 (from Bir M'cherga) produced net symptoms. Single conidial cultures from each strain were used for inoculum preparation.

Five seeds of each parent and DH line were sown in replicated trials in alveolar plates. Plants were grown in a growth chamber at 20–24°C with a 12-hour photoperiod. After about 14 days, when seedlings were at the 2-leaf stage, they were inoculated with aqueous suspensions containing 10^4 conidia/mL of the target isolate. About 200 mL of suspension was applied for each plate. Inoculated seedlings were immediately placed in a fogging chamber and held at 100% relative humidity for 36 h. They were then returned to the growth chamber for disease development. Notes on response infection (RI) were scored 9 days after inoculation using a scale developed by Tekauz (1985).

Adult evaluations. DH lines and the parents were sown in plastic pots (25 cm diameter) filled with loamy-clay soil and grown in a greenhouse at INAT. A completely randomized design with three replications was used. Plants were inoculated three times every 15 days from the mid-tillering stage of growth (GS 22–26) by spraying a mono-conidial suspension of PT.30/2003 (from Bir M'cherga) isolate of *P. teres* f. *teres* adjusted to 10^4 conidia/mL. Pathogen culture and inoculum preparations were made

as described by Steffenson *et al.* (1996). Inoculated plants were then covered for 48 h with plastic sheets to ensure a high level of humidity. Disease reactions were recorded three times at weekly intervals, starting after symptom appearance from the ear emergence stage (GS 53–58) to the milk stage (GS 72–76) using the percent net blotch severity scale as devised by Burleigh and Loubane (1984).

Statistical analysis

The parameters used in this study were the response infection (RI), in the seedling growth stage; and the disease severity (S), the area under the disease progress curve (AUDPC) and the apparent infection rate (r), in the adult growth stage. AUDPC was calculated using the equation proposed by Steffenson and Webster (1992):

$$\text{AUDPC} = \sum_{i=1}^n [(Y_{i+1} + Y_i) \times 0.5] [T_{i+1} - T_i] \quad (\text{Eq. 1})$$

where Y_i is the disease severity at the i th observation, T_i the time (in days) at the i th observation, and n the total number of observations. The apparent infection rate (r) (Vanderplank, 1963) was estimated using a linear regression of the natural logarithm (\ln) of the percent diseased tissue divided by the percent of non-diseased tissue ($S/(100-S)$) over time (T). The slope of the regression line was then taken as an estimate of r .

Single-marker analysis of QTL was used in individual environments to determine the effect of each molecular marker on each trait using SAS software (SAS Institute, 1988). The significance of the QTL on disease reaction was determined by an F -test. An association was considered to be significant only when $p \leq 0.005$. The phenotypic variation for disease reaction explained by the QTL-linked marker(s) was estimated by the R^2 value. According to this model the main additive effects were estimated by:

$$(m_{22} - m_{11})/2, \quad (\text{Eq. 2})$$

where, m_{22} is the phenotypic mean of line '90' at a specified marker and m_{11} is the phenotypic mean of Roho at the same marker.

Results and discussion

Seedling resistance

The parental lines differed widely in their response to pathotypes PT.2/2003, PT.11/2003 and PT.30/2003. Frequency distributions of RI against the three isolates seem to be bimodal with presence of DH lines that exhibited intermediate RI. For all the pathotypes used, transgressive segregation was observed. The presence of transgressive segregants and DH lines with intermediate RI suggest the presence of minor genes for resistance in the parents. Cakir *et al.* (2003) and Raman *et al.* (2003) also observed transgressive segregation for net blotch reaction in several DH populations.

Response infection to pathotype PT2/2003 was affected by five significant QTLs, which explained from 5 to 20% of the phenotypic variation (Table 1). These QTLs were detected on chromosomes 3H (two QTLs), 4H (one QTL) and 7H (two QTLs) (Table 1 and Figure 1). At *RI.3.1*, *RI.3.3* and *RI.4.1* QTLs, the resistance alleles were contributed by line '90'. At *RI.7.1* and *RI.7.2* loci, Roho contributed the resistance alleles.

The most significant QTL associated with RI to pathotype PT.2/2003 is *RI.7.2*, which explained 20% of the phenotypic variation. For response infection to PT.11/2003, six significant QTLs were detected. These were the five QTLs identified for response infection to PT.2/2003, plus another one: *RI.3.2* associated with HVM27 on chromosome 3H. The phenotypic variation explained by each QTL ranged from 5%, for *RI.3.3* and *RI.4.1*, to 16% for *RI.7.2*. At *RI.3.1*, *RI.3.2*, *RI.3.3* and *RI.3.4* loci, the resistance alleles were contributed by line '90', and at *RI.7.1* and *RI.7.2* loci, the resistance alleles were contributed by Roho. The most significant loci conferring resistance to PT.11/2003 (*RI.7.2* loci) explained 16% of the phenotypic variation. The results obtained indicate that effective seedling resistance to *P. teres* f. *maculata* is conditioned by loci

Table 1. Putative QTLs detected for net blotch resistance in a doubled-haploid barley population (Roho \times line '90') using single-marker analysis.

Trait	Experiment	QTL	Associated Marker	Mean square	R^2 (%)	Additive effect
RI	PT.2/2003	<i>RI.3.1</i>	HvLTPPB	50.61***	7.36	-0.53***
		<i>RI.3.3</i>	EBmac0708	31.41***	4.62	-0.46***
		<i>RI.4.1</i>	HVM67	44.28***	6.44	-0.50***
		<i>RI.7.1</i>	Bmag0121	78.70***	11.57	+0.73***
		<i>RI.7.2</i>	HVM11	138.02***	20.07	+0.87***
	PT.11/2003	<i>RI.3.1</i>	HvLTPPB	64.85***	14.34	-0.61***
		<i>RI.3.3</i>	EBmac0708	21.99***	4.97	-0.38***
		<i>RI.3.2</i>	HVM27	40.03***	8.85	-0.49***
		<i>RI.4.1</i>	HVM67	22.26***	4.92	-0.35***
		<i>RI.7.1</i>	Bmag0121	40.91***	9.19	+0.53***
	PT.30/2003	<i>RI.7.2</i>	HVM11	73.16***	16.18	+0.63***
		<i>RI.4.2</i>	HVM68	68.87***	10.18	-0.63***
		<i>RI.6.1</i>	Bmag0500	203.45***	31.35	-1.12***
		<i>RI.6.2</i>	HVM31	176.16***	26.00	-0.99***
S (%)	Tunis 04	<i>S.3.1</i>	HvLTPPB	293.78***	4.40	-1.29***
		<i>S.6.1</i>	Bmag0500	1086.64***	16.75	-2.59***
		<i>S.6.2</i>	EBmac0806	615.29***	9.22	-1.91***
AUDPC	Tunis 04	<i>AUDPC.3.1</i>	HvLTPPB	$0.19 \times 10^{6***}$	8.03	-32.83***
		<i>AUDPC.3.2</i>	Bmag0225	$0.16 \times 10^{6***}$	6.85	-30.78***
		<i>AUDPC.6.1</i>	Bmag0500	$0.30 \times 10^{6***}$	14.29	-43.17***
r	Tunis 04	<i>r.3.1</i>	HvLTPPB	$20.06 \times 10^{-4***}$	8.34	-0.0034***
		<i>r.3.2</i>	HVM27	$13.50 \times 10^{-4***}$	5.61	-0.0028***
		<i>r.6.1</i>	Bmag0500	$43.90 \times 10^{-4***}$	19.39	-0.0052***
		<i>r.6.2</i>	HVM14	$19.25 \times 10^{-4***}$	9.38	-0.0035***

RI = response infection; S = disease severity; AUDPC = area under disease progress curve; r = apparent infection rate. *** = Significant at $P < 0.005$.

on chromosomes 3H, 4H and 7H, and that loci on the 7H are the most significant one. In a previous study, Williams *et al.* (1999) found that resistance to the spot form of net blotch was conferred by a single gene, that is located on the long arm of chromosome 7H. However, in the absence of common markers among these studies, it cannot be concluded that these QTL regions represent the same resistance genes. Moreover, it appears that recombination of alleles on 3H and 4H from line '90' with the alleles on 7H from Roho may have given the transgressive lines higher resistance than line '90'. Raman *et al.* (2003) obtained similar results.

Three QTLs conferring resistance to PT.30/2003 (a net form isolate) were consistently detected on chromosomes 4H (one QTL) and 6H (two QTLs) (Table 1 and Figure 1). The chromosome 4H QTL *RI.4.2* associated with HVM68 explained about 10% of the phenotypic variation. The chromosome 6H QTL *RI.6.1* associated with Bmag0500 explained more than 31% of the phenotypic variation. The chromosome 6H QTL *RI.6.2* associated with HVM31 explained 26% of the phenotypic variation. At these three loci, resistance alleles were contributed by line '90'. In several previous investigations, significant putative QTLs for net form of

6H, respectively. The only QTL that did not overlap with QTLs for low net blotch severity was on chromosome 3H near Bmag0225. The phenotypic variance explained by each QTL ranged from 6.85 to 14.29%. Line '90' was the parental source of the alleles for lower AUDPC on chromosomes 3H and 6H. Four QTLs for lower apparent infection rate (*r*) were detected on chromosomes 3H and 6H (two QTLs in each chromosome). All four overlapped with QTLs for either disease severity or AUDPC, or both (Figure 1). They explained from 5.61 to 19.39% of the phenotypic variation. The QTLs associated with HvLTPPB and Bmag0500 were detected for the three evaluation parameters used in the adult growth stage. Several QTLs conferring net form of net blotch resistance at the adult growth stage have been previously reported on 3H and 6H (Cakir *et al.*, 2003; Gupta *et al.*, 2004). From the four putative QTLs identified in this study, two on 3H and 6H have been previously mapped by Gupta *et al.* (2004) in Pompadour, WPG8412 and WA4794. Bmag0603 and HVM74 have been found the closest SSR markers associated to QTLs on the 3H and the 6H, respectively.

Furthermore, this investigation indicated that two QTLs conferring resistance to *P. teres* f. *teres* are expressed at both seedling and adult plant growth stage. These QTLs were located on chromosome 6H: the first linked to Bmac0500 and the second to HVM31 and to EBmac0806. The QTL associated with HVM68, on 4H, was detected only at seedling stage; and the QTLs associated with HvLTPPB and HVM27 (or Bmag0225) on 3H were detected only at the adult growth stage. Further investigations are needed to use this tool in marker assistant selection for field resistance to net blotch.

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An overview of current investigation within the barley and powdery mildew relationship

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Abstract

In the Czech Republic, barley is the second most widely grown species and on it powdery mildew is the most frequent disease. To limit the damage to barley, genetic resistance is an effective, economically sound and safe alternative to fungicide application. In spring barley cultivars, the fully effective resistance gene *mlo* predominates. However, the resistance of winter barley is still insufficient; therefore, we recommend using new resources specifically for this crop. To make breeding easier, we determined the number of genes encoding the resistance from selected sources, location of genes, as well as their type of inheritance; we also tried to develop DNA markers for the corresponding genes. Practical effectiveness of each resistance is limited by adaptability of the pathogen population, which is therefore monitored to obtain information on the effectiveness of resistance genes, the structure and adaptability of the given population, and the effect of evolutionary forces responsible for the loss of resistance gene effectiveness. Because the pathogen is very adaptable, at least two fully effective genes should be combined in a cultivar for more durable resistance.

Introduction

In the Czech Republic (Central Europe), barley is the second most widely grown crop after wheat, and its harvested area averaged

505 000 ha from 2001 to 2005, with 75% percent of the area under two-row spring type. Winter type comprises both two- and six-row varieties, but the latter predominate. Powdery mildew caused by the ascomycete fungus *Blumeria* [syn *Erysiphe*] *graminis* f.sp. *hordei*, is a widespread disease of barley, causing up to 40% of yield loss.

Importance of diseases

We analyzed the disease infection in barley in the Czech official trials conducted in 1989–2000 (Dreiseitl, 2003a). Out of 320 trials with spring barley, heavy infection by one of the four major barley diseases (powdery mildew, leaf rust, net blotch and leaf blotch) was found in 210 trials (65.6%) and of these, 105 trials (50%) were strongly infected by powdery mildew. Winter barley was infected by more diseases. However, heavy infection was caused only by the four main diseases, and in 99 of 145 official trials (68.3%), strong infection by powdery mildew was recorded in 39 trials (40%). Among all epidemics caused by the diseases, the powdery mildew infection was found in 50% and 40% of the spring and winter barley trials, respectively.

The severity of powdery mildew was analysed in greater detail. We used again the data of the Czech official trials, i.e. the data from 923 trials (1971–2000) with spring barley (Dreiseitl and Jurecka, 2003) and from 392 trials (1976–2005) with winter barley (Dreiseitl, 2007a). More than 33% of the trials with spring barley exhibited strong and 43%

moderate infection. In winter barley, 21% and 35% of the trials were characterized by heavy and moderate infection, respectively. It indicates that a minimum of 33% of fields planted to spring barley (126 000 ha) and 21% of fields planted to winter barley (27 000 ha) have to be treated with fungicides against powdery mildew every year.

Protection conception

To limit the damage to barley, genetic resistance is an effective, economically sound and safe alternative to fungicide application. In spring barley varieties, a fully effective gene of non-specific (non-host) resistance, *mlo*, is now dominating (Dreiseitl, 2005, 2006a), which ensures durable powdery mildew resistance. The resistance of winter barley varieties, however, is still insufficient (Dreiseitl, 2006b, 2007b) and at present the crop plays a key role in the overwintering of the pathogen, for its reproduction in spring and also for its adaptability. Therefore, new efficient resistance resources need to be used primarily for breeding resistant varieties to limit the damage to winter barley and transmission of the pathogen to the spring barley crop. The pathogen of powdery mildew is very adaptable (Dreiseitl, 2003b), so at least two fully effective genes should be combined in varieties to ensure more durable resistance.

Resistance resources

New resistance resources are intended mostly for breeding winter barley. Since it is difficult to find them within cultivated barley (Dreiseitl and Yang, 2007), we focused on wild barley, *Hordeum vulgare* subsp. *spontaneum*. A large number of accessions fully resistant to both the local population and all isolates maintained in our pathogen genebank were detected in one of the collections of wild barley, and the testing with local isolates from Israel revealed wide diversity for this trait (Dreiseitl and Bockelman, 2003; Dreiseitl and

Dinoor, 2004). Searching for new resistance resources has been continuing. Currently, we are investigating part of the wild barley collection held in the ICARDA genebank. We examine the resistance of the accessions at the adult plant stage (in the field) and their resistance at the seedling stage (in a greenhouse). At the same time, however, we strive to obtain resistance patterns for all the accessions with detected specific resistance and to postulate the genetic background of this trait.

Resistance genes

The selected fully effective resistance resources are screened for the number of genes (the resistance is often controlled by two or three genes), the mode of inheritance of the genes (Dreiseitl *et al.*, 2007) and their location in the barley genome (Repkova *et al.*, 2006). Our aim is to detect suitable DNA markers for the selected resistance genes (Repkova *et al.*, 2009) in order to ease the selection of resistant progenies by breeders and allow combining fully effective resistance genes in one variety.

Pathogen population

Practical effectiveness of each resistance gene is limited by the pathogen population's adaptability. Therefore, our effort, which is focused on the use of genetic resistance against powdery mildew, is accompanied by monitoring a respective pathogen population not only within the given territory (Dreiseitl, 2004; Dreiseitl *et al.*, 2006), but also in other regions of the world (Dreiseitl *et al.*, 2006; Dreiseitl and Wang, 2007). The objective is to obtain information on the effectiveness of the selected resistance genes, the structure and adaptability of the given population, and the effects of evolutionary forces responsible for loss in the effectiveness of resistance genes (Dreiseitl, 2008).

Varietal resistance

We carefully monitor the resistance of current varieties and varieties in the registration process, as well as the resistance of varieties in pre-registration trials. We identify genes of specific resistance and their combinations, and examine the effectiveness of these genes in both the field and against isolates obtained nationally. Also, in the case of the host, we monitor the resistance of varieties grown more widely (Dreiseitl and Rashal, 2004; Dreiseitl, 2007c).

Conclusions

In the Czech Republic, barley is the second most important crop and both spring and winter varieties are grown. Powdery mildew is the most widespread disease on barley. Growing resistant varieties is a crucial alternative for protection purposes. Our research is based on immediate practical needs. However, we are not limited to only these problems and are ready to join wider international collaboration.

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Wheat dwarf virus (WDV) on barley in Germany – importance and screening for resistance

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Abstract

Since its first detection in Germany in 1990, the leafhopper-transmitted Wheat dwarf virus (WDV) has gained evident importance. In 1994, in the period 1998 to 2000, and in 2004, WDV was the predominant insect-transmitted virus in winter barley in Germany, and in 2006 similar incidences of WDV and of the aphid-transmitted Barley yellow dwarf virus (BYDV), i.e. 18% for WDV and 24%, for BYDV, were observed. Taking into account global warming, breeding for resistance or tolerance to insect-transmitted viruses will become more important in the future, because longer and warmer periods in autumn will result in longer flight activities of the vectors, leading to an increased risk of winter barley becoming infected with these viruses. In contrast to BYDV, nothing was yet known about tolerance and genetics of tolerance against WDV, so 300 winter barley accessions were evaluated for their reaction to WDV by artificial WDV inoculation in the field using viruliferous leafhoppers of the species *Psammotettix alienus*. Of these, only cv. Post, which is also tolerant to BYDV, revealed a higher level of tolerance to WDV. To obtain information on the genetics of tolerance of cv. Post to WDV, a QTL analysis was carried out on the doubled-haploid (DH) population Post × Vixen, which has already been used for mapping QTLs for BYDV tolerance. Respective QTL flanking markers

will offer the opportunity to simultaneously incorporate tolerance to BYDV and WDV derived from cv. Post into adapted high yielding cultivars.

Introduction

Due to global warming, insect-transmitted viruses, such as the aphid-transmitted Barley yellow dwarf virus (BYDV) and the leafhopper-transmitted wheat dwarf virus (WDV) will become more important in the future, because longer and warmer periods in autumn will result in longer flight activities of the vectors, leading to an increased risk of winter barley becoming infected by these viruses. Because of the high mobility of leafhoppers, chemical measures to control these virus vectors are less promising. Therefore, growing tolerant cultivars has to be considered as the most environmental sound and effective method to control WDV. In contrast to BYDV, so far only small quantitative differences in the degree of virus attack (Vacke and Cibulka, 2000, 2001; Bartos *et al.*, 2002; Lindblad and Waern, 2002; Širlová *et al.*, 2005) have been reported. The aims of this study were, on the one hand, to investigate the incidence of BYDV and WDV in the central part of Germany, and, on the other hand, to evaluate winter barley accessions for their reaction to WDV, including genetic analyses of tolerance.

Material and methods

The incidence of BYDV and WDV was monitored in 10 to 15 winter barley and winter wheat fields in the period 1998 to 2007. In early spring, 150 leaf samples per field (30 samples taken randomly from each field) were analyzed by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) using polyclonal BYDV- and WDV-specific antibodies. DAS-ELISA was conducted according to Clark and Adams (1977) and extinction at 405 nm was estimated on a microtitre plate reader (DYNATECH MR 5000).

From 2002 to 2006, 248 winter barley genotypes (accessions from the genebank of the Institute of Plant Genetics and Crop Plant Research at Gatersleben (IPK), breeding lines and cultivars) were evaluated for their reaction to WDV by artificial inoculation in the field using viruliferous leafhoppers of the species *Psammotettix alienus*. In mid-September each year, 12 seeds per accession were sown in two replications in an inoculated (I) and an uninoculated (Control, C) treatment in the field. To increase the infection pressure, one WDV-infected barley plant was planted between each row of the I-treatment shortly before inoculation. At the 1- to 2-leaf stage, the plots of the I-treatment were covered with a tunnel made of cotton, and viruliferous leafhoppers were distributed in the tunnel in a density of approximately 1 leafhopper per plant. To keep the C-treatment virus-free, the plots were treated regularly with an insecticide. After 4 weeks, the cover was removed and the whole test was sprayed with an insecticide. Scoring of symptom expression was conducted at heading stage using a 1–9 scale, from score 1 = without symptoms to 9 = plant died. On the basis of these data the degree of attack (DA) was calculated as follows:

$$DA = \frac{\sum_{s=2}^9 n^*(s-1)}{N*8}$$

where: n = number of plants per scoring class; s = scoring class; and N = number of plants with symptoms

DAS-ELISA was used to determine the virus concentration of selected genotypes as described above. At harvest, plant height, number of ears per plant, kernel weight per plant and 1000-kernel weight of both treatments were determined.

To obtain information on the genetics of the WDV-tolerance detected in cv. Post in more detail, two independent populations of doubled-haploid (DH) lines of the cross Post \times Vixen, comprising 86 (I) and 79 (II) lines were phenotypically analyzed in gauze house tests in 2006 and 2007, as described above. The observed frequency distributions for the investigated traits were analyzed for the fit to a Gaussian distribution by the Kolmogorov-Smirnov-Test. On the basis of the existing genetic map developed by Scheurer *et al.* (2001) for mapping QTL for BYDV-tolerance in the DH-population Post \times Vixen (II), and the phenotypic data (relative values) scored for this population, first preliminary QTL analyses concerning WDV tolerance were carried out (using MapQTL®5 software, Plant Research International BV and Kyazma BV, Benelux and USA).

Results and discussion

Since its first detection in Germany in 1990 (Vacke, pers. comm.) the leafhopper-transmitted Wheat dwarf virus (WDV) has gained importance in winter barley as well as in winter wheat (Figure 1). In the period 1998 to 2000, and in 2004, WDV was the predominant insect-transmitted virus in winter barley in Germany. In 2006, there were similar incidences of WDV and of the aphid-transmitted Barley yellow dwarf virus (BYDV), i.e. 18% for WDV and 24% for BYDV.

Out of the 248 barley accessions tested, only cv. Post—which is also tolerant to BYDV—and 3 breeding lines having this accession in their pedigree revealed a higher

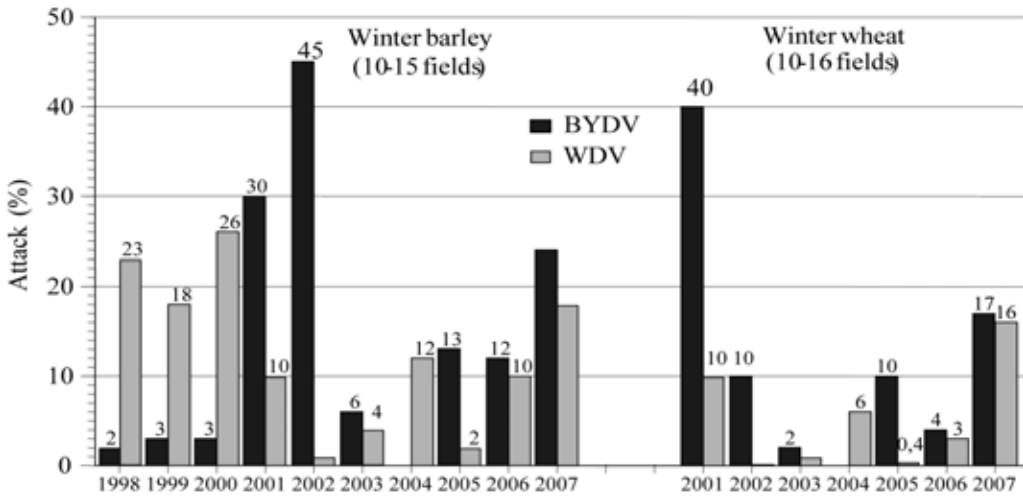


Figure 1. Virus attack in winter barley and winter wheat fields in spring in Saxony-Anhalt (central Germany)

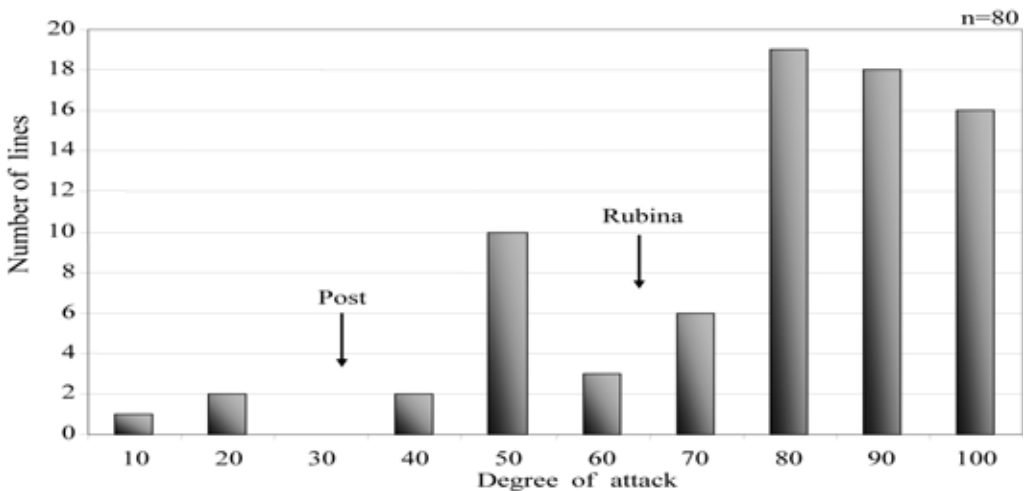


Figure 2. Degree of attack in winter barley lines in comparison with cvs. Rubina and Post in the field 2006.

level of tolerance to WDV (Figure 2). However, no reduction in virus concentration was detected in these genotypes (data not shown).

Concerning all the characters investigated, cv. Post showed the highest relative values, i.e. the smallest reduction of these traits after WDV-infection. For example the kernel weight per plant was approx. 50% of control

in cv. Post, but less than 10% in the other cultivars (Figure 3).

In 2006, the DH-population (I) of the cross Post \times Vixen was analyzed for reaction to WDV-inoculation in a gauze house. Concerning the frequency distributions, a good fit to a Gaussian distribution was detected for the characters investigated, e.g. for the degree of attack ($P > 0.15$) (Figure 4.)

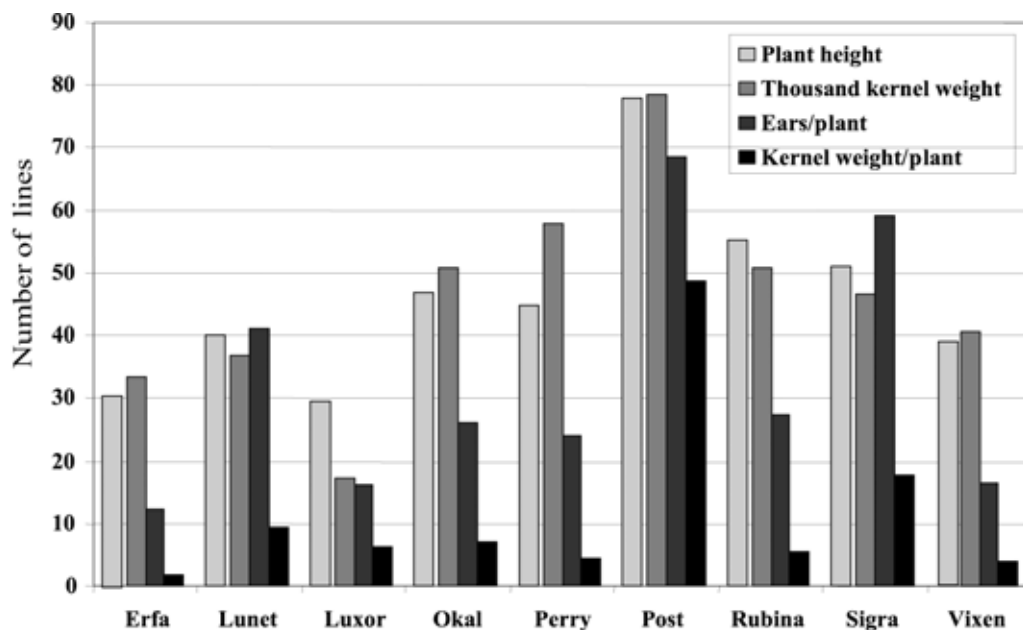


Figure 3. Relative performance of cv. Post in comparison with other cultivars in the field in 2006 regarding plant height, 1000-kernel weight, ears per plant, and kernel weight per plant.

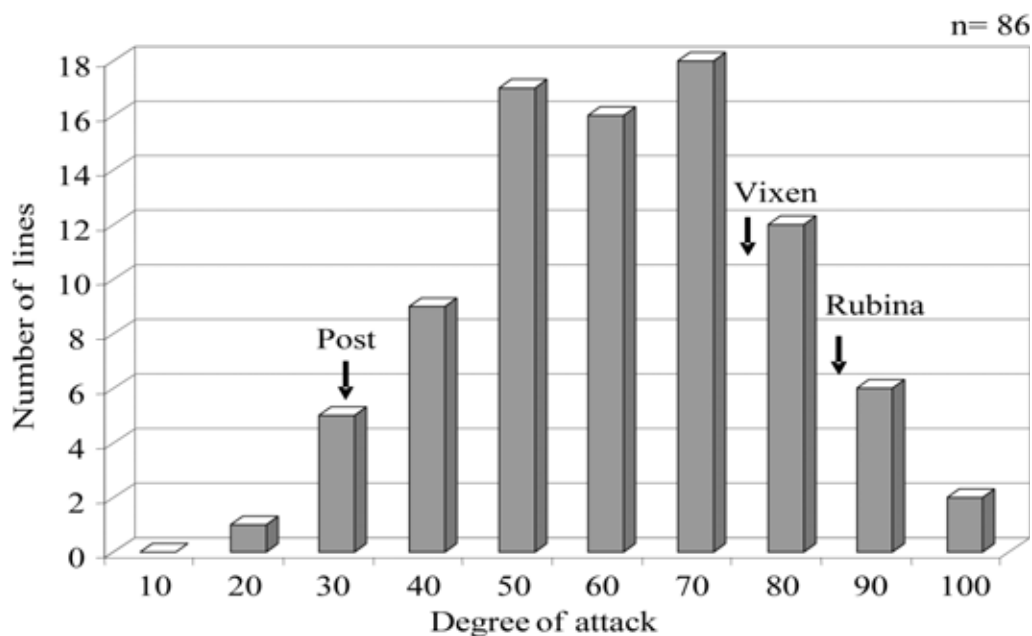


Figure 4. Reaction of DH-population (I) of Post × Vixen to WDV-infection in comparison with their parents and the susceptible cv. Rubina in gauze-house tests in 2006.

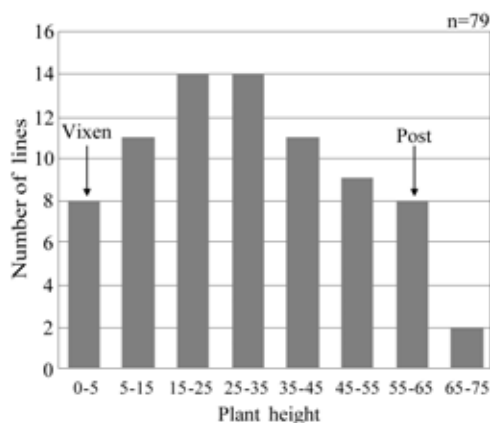


Figure 5. Relative expression of plant height in DH-population (II) of Post \times Vixen after artificial WDV-inoculation in 2007.

These observations indicate a polygenic inheritance of the WDV tolerance detected in cv. Post.

In the test of the second DH-population (II) in 2007, the high level of tolerance of cv. Post was confirmed and again cv. Vixen turned out to be highly susceptible (Figure 5).

Concerning the relative plant height after WDV infection, a good fit to a Gaussian distribution was observed ($P > 0.15$). On the basis of these first phenotypic data of the 79 DH-lines and the available genetic map for this population (Scheurer *et al.*, 2001), so far one QTL for relative plant height after WDV infection could be detected on chromosome 4H (LOD 4.76), explaining about 26% of the phenotypic variance.

The phenotypic characterization of the DH-population (II) will be repeated during the next years in order to get the reliable phenotypic data that are a prerequisite for QTL analyses. Respective QTL flanking

markers will offer the opportunity to simultaneously incorporate tolerance to BYDV and WDV derived from cv. Post into adapted high yielding cultivars.

Acknowledgement

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Identification of differentially expressed genes in barley after infection with Barley yellow dwarf virus (BYDV) using cDNA-AFLPs

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Abstract

On a global level, the aphid-transmitted Barley yellow dwarf virus (BYDV) is the most important virus disease of barley. In order to get information on genes differentially expressed after BYDV infection in general and between tolerant and non-tolerant genotypes, cultivars Vixen (*Ryd2*) and Post (QTL on 2HL) and DH-lines derived from a cross of these cultivars carrying all possible combinations of positive and negative alleles at respective loci (*Ryd2*, QTL on 2HL) as well as the non-tolerant cultivar Nixe were artificially infected at the one-leaf stage with *Rhopalosiphum padi* carrying the BYDV-PAVASL-Isolate. The success of artificial infection was checked at 15 days post-inoculation by analyzing the tip of the third leaf by DAS-ELISA, and the rest of this leaf was used for RNA extraction. The mRNA of infected and healthy control plants was translated to double stranded cDNA and subsequently analyzed by 256 EcoRI+2/MseI+2 primer combinations. Using this approach, 45 differentiating fragments were identified and sequenced. By BLASTN analysis, these fragments revealed homology to genes such as those involved in the synthesis of membranes and oxidative stress.

Currently, these fragments are integrated in the extant map of Post \times Vixen to identify whether they are located in genomic regions contributing to BYDV tolerance.

Introduction

Globally, the aphid-transmitted Barley yellow dwarf virus (BYDV) is the most important virus disease of barley. The disease is caused by several serotypes, of which BYDV-PAV is the most prevalent in Europe. Symptoms of BYDV infection are stunted growth, chlorosis and discoloration of leaves, and late heading. Several loci conferring tolerance against BYDV in barley (*Hordeum vulgare* L.) are described. A major gene, named *Ryd2*, was first described in Ethiopian barley accessions (Rasmusson and Schaller, 1959) and mapped on chromosome 3HL (Schaller *et al.*, 1964; Collins *et al.*, 1996). Two QTLs explaining together about 50% of the relative yield after BYDV infection were mapped on chromosome 3HL in the vicinity of the *Ryd2* gene and on chromosome 2HL in the doubled-haploid (DH) population Post \times Vixen (Scheurer *et al.*, 2001). Besides this, *Ryd3*, also derived from an Ethiopian landrace, was mapped on chromosome 6H (Niks *et al.*, 2004).

Material and methods

In order to gain information on genes differentially expressed after BYDV infection in general and between tolerant and non-tolerant genotypes, cultivars Vixen (*Ryd2*) and Post (QTL on 2HL) and DH lines derived from a cross between these cultivars carrying all possible combinations of positive and negative alleles at respective loci (*Ryd2*, QTL on 2HL), together with the non-tolerant cultivar Nixe, were raised in growth chambers at 18°C, 70% relative humidity in a 16-hour light regime with an illumination of 10^4 lm cm⁻¹. The artificial infection took place at the one-leaf stage with *Rhopalosiphum padi* carrying the isolate BYDV-PAV-ASL. Two days post-inoculation the aphids were removed by spraying the systemic insecticide Pirimor (Syngenta Agro, Maintal, Germany), and at 15 days post-inoculation the success of artificial infection was checked by analyzing the tip of the third leaf by DAS-ELISA, with the rest of this leaf used for RNA extraction based on a method using guanidinium thiocyanate, as described by Chomczynski and Sacchi (1987). Selective isolation of mRNA was carried out with magnetic beads (Invitrogen, CA, USA) and the mRNA of infected and healthy control plants was translated to double stranded cDNA based on a protocol of Okayama and Berg (1982). For cDNA-AFLP reactions (Bachem *et al.*, 1996) the AFLP Core Kit (Invitrogen) was used, and subsequently 256 *EcoRI*+2/*MseI*+2 primer combinations were analyzed. Differentiating fragments were cut out of the gel and re-amplified by using the appropriate +2 primer combinations. Re-amplified fragments were cloned in the PCR 2.1-TOPO vector (Invitrogen) and sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit on a 3130xl Genetic Analyzer (Applied Biosystems, Warrington, UK). Sequences were compared to the EST and nucleotide databases of the National Center for Biotechnology Information (NCBI) using the BLASTN algorithm (Altschul *et al.*, 1997).

Sequence tagged sites (STS) and primers for allele specific sequencing were deduced using Primer 3 (Rozen and Skaletsky, 2000). STS based on deletions were amplified with a M13 tailed forward primer for the binding of a Cy5-labeled sequence and detected on the CEQ 8000 Genetic Analysis System (Beckman Coulter, Krefeld, Germany). Single SNPs were examined either by using Pyro Gold Reagents on the Pyro Mark ID System (Biotage, Uppsala, Sweden) or as cleaved amplified polymorphic sequences (CAPS). The later were separated on 1% agarose gels (Seakem LE, BioWhittaker Molecular App., Rockland, USA) in Tris Acetate EDTA buffer (TAE) with ethidium bromide staining, at 90 V for 60 min. Respective differing fragments were integrated into the Post × Vixen map of Scheurer *et al.* (2001) using the Joinmap 4 software (Kyazma BV, Wageningen, Netherlands) applying the Haldane transformation (Haldane, 1919).

Results and discussion

Using this approach, 45 differentiating fragments were identified and sequenced. By BLASTN analysis, these fragments revealed homology to genes involved in the synthesis of membranes (e.g. UDP-D-glucuronate decarboxylase) and oxidative stress (e.g. diphosphonucleoside phosphatase, glutathione peroxidase, O-acetylserine(thiol) lyase). Out of the 3' non-translated region of the sequence homologous to the UDP-D-glucuronate decarboxylase, an STS marker with the primers (forward 5'-M13-gtgcacgacgattctggatg-3' and reverse 5'-acgaacccacccaactatga-3') was derived, including an indel. Cv. Post showed a fragment length of 336 bp whereas cv. Vixen lacked 3 bp. In the DH population Post × Vixen (Scheurer *et al.*, 2001), the STS for UDP-D-glucuronate decarboxylase maps proximal on chromosome 4H. SNP analysis using the pyrosequencing technique was carried out for a fragment which differed

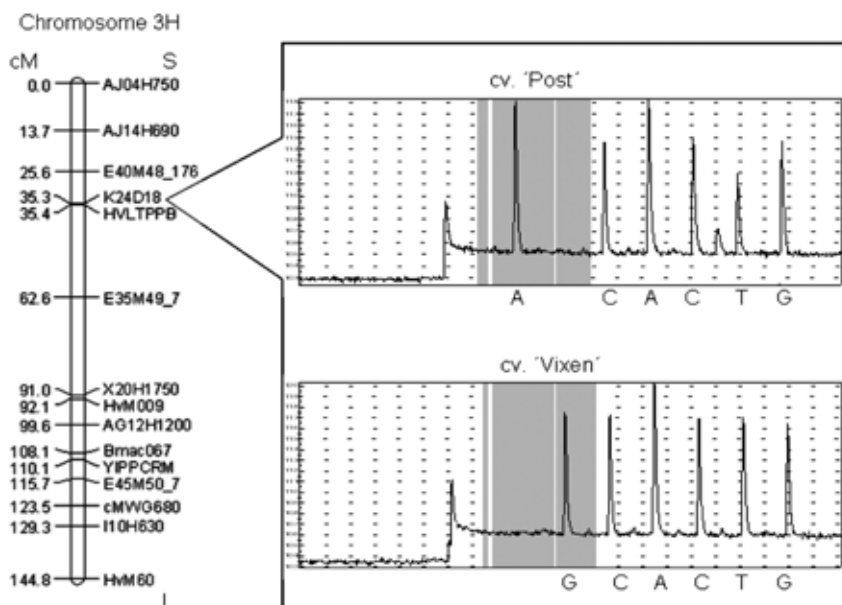


Figure 1. Pyrograms of the SNP K24D18 with the differentiating nucleotides A and G, respectively, and its chromosomal location on the short arm of barley chromosome 3H.

for a constitutive expression in Post and DH lines carrying the Post allele at the QTL on 2HL showing no significant similarities in the database (K12D08) and at the same time for a sequence matching to a cDNA from barley (8e¹²⁶) which is upregulated in infected plants (K24D18). Mapping integrated the constitutively expressed K12D08 on chromosome 7H, and K24D18 upregulated after infection with BYDV, on chromosome 3HS (Figure 1). Up to now, no fragments have been mapped in regions of known QTL, but mapping of additional fragments is in progress.

The enzyme UDP-D-glucuronate decarboxylase is involved in the synthesis of arabinoxylan which is part of the cell wall of the Poaceae and downregulated after a BYDV-infection. Therefore it could be involved in the phloem degradation observed after infection with BYDV. Enzymes like glutathione peroxidase are often expressed at an increased level in plant tissues undergoing

stress, e.g. infection by bacterial or viral pathogens (Levine *et al.*, 1994). Comparable to our studies, an increased peroxidase activity had already been reported in BYDV-infected plants by Orlob and Arny (1961).

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Scald resistance and malting quality relationships in Canadian two-row barley

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Abstract

For the past fifteen years in our two-row barley-breeding program at the Field Crop Development Centre, we have been trying to incorporate durable or multi-gene resistance to scald (*Rhynchosporium secalis*) into lines with good malting quality. It has proven to be elusive. This study was undertaken to investigate the relationship between the scald resistance from cv. Seebe two-row feed barley and malting quality. While Seebe has proven to have durable resistance (released in 1992, it still gets 'resistant' ratings in field evaluations), it does not modify well and has high beta-glucan levels in the malt. Four crosses were made between Seebe and lines with better malting quality. Lines from these four crosses were advanced to the F₅ using single seed descent. In 2006, up to one hundred lines from each cross were assessed for seedling resistance to a common *R. secalis* strain isolated from the susceptible cultivar Harrington and markers for the Seebe scald resistance genes. The F₆ generation of these lines and up to an additional 100 lines per cross were grown out in 2007 for marking of Seebe scald resistance genes, assessment of field resistance to scald, and malting quality using NIRS. Results from these two years of study showing the relationship of seedling and field response to *R. secalis*, Seebe scald resistance markers, and malting quality are presented in this paper.

Introduction

Scald, caused by *Rhynchosporium secalis* (Oudem.) J.J. Davis, is one of the major foliar

diseases affecting barley in its production area in Alberta, Canada (Xi *et al.*, 2003). Because of the variability in the pathogen, breeding for durable resistance in barley for this area has been challenging. Combining this with breeding for malting quality and the challenge has increased exponentially, such that no currently registered cultivars currently classed as malting types have even intermediate or moderate resistance to the pathogen.

It has been the long-term goal of the two-row breeding program at Field Crop Development Centre (FCDC) to incorporate durable scald resistance into malting barley. When the malting program began at the center in 1993, crosses were made between cv. Harrington and several cultivars that had been identified with scald resistance, including CDC Guardian. The gene for resistance in CDC Guardian was short lived (Xi *et al.*, 2000) and the efforts to incorporate this resistance, although successful, were fruitless in obtaining a malting line with durable scald resistance. The next round of crosses involved Seebe, a feed barley released by FCDC in 1992. The resistance in this line has proven to be very durable under Alberta conditions (Xi *et al.*, 2003).

Unfortunately, in crosses of Seebe with cultivars with excellent malting quality, such as Harrington, Manley and AC Metcalfe, selection for malting quality has led to the loss of the scald resistance levels found in Seebe, and vice versa, the selection for scald resistance as found in Seebe has led to poor quality parameters the most obvious being high beta-glucan levels of the malt. These results have led us to this study to determine if the scald gene(s) in Seebe are

in close association with poor malting traits (especially reduced beta-glucanase activity, as indicated by high beta-glucan levels of the malt) or if the genes being expressed in Seebe actually preclude the expression of good malting traits. For this paper, only the relationship of scald reactions with malting quality traits will be discussed, and the relationships with the Seebe molecular markers will be discussed in a later paper.

Materials and methods

Four crosses were initiated in 2004 between advanced lines with good malting quality and with Seebe. These crosses were grown as F₂ bulk populations in the field in 2005. Approximately 400 heads were selected at random to advance using single seed descent in the growth room during the winter of 2005–2006. In the summer of 2006, approximately 250 F₅ lines were grown per population. One hundred of these lines were also tested as seedlings for their scald reaction (method to follow) and DNA was collected for marking for the Seebe gene for scald resistance and Harrington genes for susceptibility. Each line was harvested (although some were lost to stress) and advanced to the next generation. The F₆ generation of each line was grown in the field in 2007 as a two-row plot under conditions that would be conducive to good malting quality (approximately 100 available N through soil and applied N). Malting quality was assessed using NIRS calibrations developed by FCDC (Oatway and Helm, 2002). Hill plot assessment of scald (see below) and DNA marking was also made on the F₆ lines.

Seedling method

Barley seeds were planted in plastic trays containing a soilless potting mix that consisted of a slow-release 14-14-14 (N-P-K) fertilizer at 350 g with approximately 30 kg of PRO-MIX ‘BX’ soilless nutrient medium (Premier Horticulture Inc. Quakertown, PA, USA). Plants were grown in a growth chamber programmed at 17°C and a 14:10 hour light:dark photoperiod. A *R. secalis* isolate (H#2) derived from the susceptible cultivar Harrington was used for inoculation. The seedlings were inoculated at Zadoks growth stage (GS) 13 (Zadoks *et al.*, 1997) or approximately 14 to 16 days after seeding. Inoculum was applied at 1×10⁶ spores/mL and 30 mL/tray with a De Vibliss atomizer fitted to an air pump. Within the growth chamber, inoculated plants were kept for 48 h in a mist chamber in darkness to maintain relative humidity at 100%. Plants were watered twice weekly from the base to prevent cross contamination. Disease assessment was made on the second and third leaves of each plant at 14 days after inoculation using a 0–3 reaction scale, as described by Jorgensen and Smedegaard-Petersen (1995). An average rating was obtained based on 8 leaves per line for most lines, but in some cases fewer leaves were assessed due to missing plants.

Adult plant method

Hill plots were established at Edmonton (53°43’ N, 113°31’ W) and Lacombe (52°28’ N, 113°44’ W), Alberta, Canada, in the growing season of 2007. Plots were set up on land where scald of barley had

Table 1. Pedigrees of the four crosses used to study the relationship of scald reaction to malting quality

Population	Female parent	Male parent
J04064	TR04665 = H92014003 [(Manley/Leo)F ₁ //AC Metcalfe]	Seebe
J04070	TR04666 = H92014014 [(Manley/Leo)F ₁ //AC Metcalfe]	Seebe
J04075	TR04667 = H93016013 [(Manley/Leo)F ₁ //TR238]	Seebe
J04079	TR04668 = H93102002 (I92124/TR238) where I92124 = Bowman*2/DWS1008//ND0232	Seebe

occurred in the previous season. Seeding was done by planting approximately 30 seeds to a hill plot with 0.5 m spacing between hills and 1 m between replicates. Hill plots were inoculated with a suspension of isolate H#2 at approximately 1×10^5 spores/mL at GS 22–24, using a backpack sprayer until run off. At the same growth stage, dried straw with *R. secalis* infestation from the same field of the previous season was spread on the plots. At GS 85, disease severity was assessed visually using a 0–9 scale described by Couture (1980) and Saari and Prescott (1975).

Data analysis

Simple correlations were calculated between malting quality traits and the scald ratings and markers using SAS systems PROC CORR software. Because of reaction variability within the fields, a code was assigned to scald reactions such that 0 = susceptible reaction, 1 = intermediate or moderate reaction, and 2 = resistant reaction. These assignments were based on a visual assessment of the tests and ranking in relationship to check cultivars in different parts of the field. Scoring was applied also to the seedling data. A malting score was developed by assigning points when traits were within the range as given on Table 2. These ranges were based on the quality of Harrington grown in plots within

the populations and the desired range as supported by the registration system of the Prairie Recommending Committee on Oat and Barley (PRCOB) and the Brewing and Malting Barley Research Institute (BMBRI; <http://www.bmbri.ca/index.html>).

Results and discussion

In 2007 at the Edmonton site, there was insufficient development of scald, with the susceptible Harrington having scores of 4–5 and many plots getting ratings of 0. In 2007 at the Lacombe nursery, scald development was excellent, with the susceptible Harrington having scores of about 8. The correlations between the two sites were not significant and this lack of correlations was not unexpected. Lack of correlation of scald reaction between the Lacombe site and the seedling rating was also not unexpected, as in multiple years of seedling testing using races 837 and 1493, it was rated as being susceptible, moderately susceptible or resistant (1989, 1990 and 1991 Western 2-row Barley Coop Reports). However, three of these populations may also carry scald resistance genes from Leo and some of our advanced lines with Leo in their parentage (TR04665, TR04666 and TR04667) received moderate resistance ratings to the seedling test with race 1493c (2004 Western 2-row Barley Coop Report). None of the correlations found were very

Table 2. Quality levels assigned for good malting quality based on measurement of the grain using NIRS.

Trait	J04064	J04070	J04075	J04079
Protein (grain) (%)	<14	<14	<14	<14
Fine extract (%)	>77	>77	>77	>77
Diastatic power (°)	100–170	100–170	100–170	100–170
Alpha-amylase (DU)	60–80	60–80	60–80	60–80
Soluble malt protein (%)	4–6	4–6	4–6	4–6
Kobach Index (%)	35–45	35–45	35–45	35–45
Malt wort beta-glucan (ppm)	<680	<600	<550	<520
Friability (%)	>40	>40	>40	>40
Viscosity (cP)	<1.55	<1.55	<1.55	<1.55
Homogeneity (%)	>80	>80	>80	>80

tight ($P > 0.5$), although some were highly significant. Only those correlations that were consistent between the raw scores and the coded scores will be discussed.

Of the 199 lines screened in 2007 from the J04064 population, 10 lines had a malting score of 18 or higher and a resistance score of 1 or 2 at both 2007 locations (data not shown). For the J04064 population, based on scald reaction at Lacombe, grain protein, diastatic power and alpha amylase were found to be negatively correlated with scald resistance, while viscosity was positively correlated with resistance (Table 3). For the seedling reactions, malt wort beta-glucan and viscosity were negatively correlated with resistance (Table 3).

Of the 156 lines screened in 2007 from the J04070 population, 15 lines had malting scores of 18 or higher and a resistance score of 1 or 2 at both 2007 locations. Fewer significant correlations were found between scald reaction and malting quality for this sister line of J04064 (Table 4). As found in the J04064 population, for the J04070 population diastatic power was negatively correlated with scald resistance and viscosity was

positively correlated with scald resistance. In this population, malt wort beta-glucan was correlated positively with scald resistance.

Of the 192 lines screened in 2007 from the J04075 population, 15 lines had malting scores of 18 or higher and a resistance score of 1 or 2 at both 2007 locations. In this population, no significant correlations between the adult plant scald rating at Lacombe and malting quality were found (Table 5), except for a negative correlation between malt wort beta-glucan and scald rating that was not significant when expressed as a code. The seedling rating was correlated negatively to scald resistance, and positively correlated to soluble malt protein. Adult plant resistance at Edmonton was correlated positively to malt wort beta-glucan.

Of the 129 lines screened in 2007 from the J04079 population, 41 lines had malting scores of 18 or higher and a resistance score of 1 or 2 at both 2007 locations. No significant correlations between malting quality traits and scald resistance were found for this population (Table 6). This lack of correlation may have been due to the smaller population size tested, as the scald ratings at Lacombe

Table 3. Correlation of malting quality traits with adult plant and seedling scald reactions for the J04064 population (where EDM refers to the nursery results from Edmonton, and LAC refers to those from Lacombe).

Trait	Adult plant (EDM) n = 122	Adult plant (LAC) n = 169	Seedling n = 96	Code (EDM)	Code (LAC)	Code (Seedling)
Protein(grain)	-0.084	0.163*	-0.148	-0.111	-0.176*	0.063
Fine extract	0.075	-0.099	0.097	0.072	0.067	-0.141
Diastatic power	-0.121	0.173*	-0.255*	-0.123	-0.194*	0.086
Alpha amylase	-0.039	0.241**	-0.210*	-0.122	-0.250**	0.015
Soluble malt protein	-0.096	0.145	-0.027	0.007	-0.160*	-0.111
Kobach Index	-0.064	0.094	-0.032	0.047	-0.112	-0.114
Malt wort beta-glucan	0.218*	-0.085	0.347***	0.007	0.097	-0.226*
Friability	-0.078	0.041	-0.269**	-0.073	-0.073	0.083
Viscosity	0.152	-0.210**	0.393***	0.122	0.209**	-0.214*
Homogeneity	-0.201*	0.042	-0.137	0.050	-0.044	-0.031
Malt score	-0.098	-0.023	-0.103	0.041	0.014	-0.043

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Table 4. Correlation of malting quality traits with adult plant and seedling scald reactions for the J04070 population (where EDM refers to the nursery results from Edmonton, and LAC refers to those from Lacombe).

Trait	Adult plant (EDM) n = 97	Adult plant (LAC) n = 140	Seedling n = 54	Code (EDM)	Code (LAC)	Code (Seedling)
Protein (grain)	0.029	0.095	-0.189	-0.099	-0.070	0.161
Fine extract	-0.076	-0.120	0.198	0.152	0.110	-0.160
Diastatic pPower	0.040	0.176*	0.042	-0.039	-0.173*	-0.019
Alpha amylase	0.084	0.081	0.105	0.001	-0.109	-0.055
Soluble malt protein	0.079	0.007	0.043	0.065	-0.021	-0.041
Kobach Index	0.050	-0.059	0.098	0.144	0.033	-0.070
Malt wort beta-glucan	-0.093	-0.217*	-0.082	-0.044	0.213*	0.042
Friability	0.064	0.033	0.195	0.098	-0.076	-0.125
Viscosity	-0.133	-0.226**	-0.027	0.080	0.236**	-0.034
Homogeneity	0.052	0.075	0.217	0.061	-0.070	-0.116
Malt score	0.007	0.001	0.134	0.115	-0.001	-0.055

* = $P < 0.05$; ** = $P < 0.01$.

Table 5. Correlation of malting quality traits with adult plant and seedling scald reactions for the J04075 population (where EDM refers to the nursery results from Edmonton, and LAC refers to those from Lacombe).

Trait	Adult plant (EDM) n = 153	Adult plant (LAC) n = 152	Seedling n = 68	Code (EDM)	Code (LAC)	Code (Seedling)
Protein (grain)	-0.095	-0.066	0.259*	0.011	0.088	-0.289*
Fine extract	0.114	0.103	-0.226	-0.091	-0.116	0.211
Diastatic power	0.039	0.001	0.203	-0.221	0.025	-0.244*
Alpha amylase	0.135	0.045	0.157	-0.305***	-0.027	-0.168
Soluble malt protein	-0.018	0.021	0.306*	-0.153	-0.012	-0.318**
Kobach Index	0.033	0.083	0.137	-0.208*	-0.079	-0.144
Malt wort beta-glucan	-0.212**	-0.169*	0.112	0.361***	0.145	-0.039
Friability	0.091	0.142	0.015	-0.236**	-0.132	-0.022
Viscosity	-0.092	-0.052	-0.146	0.299***	0.044	0.165
Homogeneity	0.088	0.123	-0.062	-0.189*	-0.132	0.039
Malt score	0.102	0.084	-0.058	-0.204*	-0.079	0.045

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Table 6. Correlation of malting quality traits with adult plant and seedling scald reactions for the J04079 population (where EDM refers to the nursery results from Edmonton, and LAC refers to those from Lacombe).

Trait	Adult plant (EDM) n = 129	Adult plant (LAC) n = 129	Seedling n = 51	Code (EDM)	Code (LAC)	Code (Seedling)
Protein (grain)	0.064	-0.066	0.077	-0.060	0.041	-0.058
Fine extract	-0.137	0.087	0.043	0.121	-0.077	-0.026
Diastatic power	0.008	0.059	0.011	-0.023	-0.081	-0.021
Alpha amylase	-0.056	0.047	-0.006	0.088	-0.089	0.005
Soluble malt protein	-0.054	0.074	0.069	0.083	-0.103	-0.044
Kobach Index	-0.120	0.077	0.051	0.137	-0.095	-0.033
Malt wort beta-glucan	0.105	-0.166	-0.069	-0.097	0.151	0.119
Friability	-0.077	0.028	-0.187	0.045	-0.044	0.089
Viscosity	-0.003	-0.066	0.065	-0.022	0.088	-0.048
Homogeneity	-0.014	0.170	-0.072	-0.011	-0.165	0.046
Malt score	-0.123	0.094	0.005	0.127	-0.085	0.024

with malt wort beta-glucan and homogeneity were just slightly over $P = 0.05$.

Our hypothesis that scald resistance is conferred by the resistance gene(s) from Seebe was not strongly supported by these data. However, there was a significant correlation between scald resistance and high malt wort beta-glucan in two of the populations, with the third population being at the edge of significance. The resistance that may be in these populations from Leo may have confounded the results, and the work with the Seebe markers may help confirm whether the Seebe gene can be used in breeding malting barley with its level of resistance.

Based on concurrent selection for scald resistance and malting quality, it may be possible to select for both traits. However, when agronomic merit was also added to the equation very few of these lines had been selected based on yield and good visual ratings in the field.

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Genetic analysis of BaYMV and BaMMV resistance *rym1* and *rym5* to local strains in Korea

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Abstract

About 40% of barley fields are known to be infected by Barley yellow mosaic virus (BaYMV) and the yield loss caused by Barley mild mosaic virus (BaMMV) is very serious in southern regions of Korea. Molecular marker systems were applied to facilitate selection and pyramiding of major resistance genes, and to overcome phenotypic variation of field resistance. In order to trace the gene action of *rym1* and *rym5* derived from Mokusekko3, which showed resistance to local strains in Korea, a total of 130 lines of a mapping population (F_{5:6}) were developed from its cross with Jinyangbori, a susceptible 2-row malting barley cultivar, and were evaluated for BaYMV or BaMMV, or both, resistance at Jinju and Suwon in 2005 and 2006, respectively. The number of resistant lines was nearly twice that of susceptible lines in Jinju, but almost the same in Suwon, suggesting that segregation of resistance was controlled by more than one gene in Jinju. Individual lines were genotyped at each marker locus on chromosomes 3H and 4H to verify the effect of *rym1* and *rym5* of Mokusekko3. Association analysis, based on linkage disequilibrium between DNA markers and disease resistance, identified two significant regions of the genome, HVM70-CDO474c on chromosome 3H and HVM68-Bmac0186 on chromosome 4H, for *rym5* and *rym1*, respectively. The region of *rym5* was not significant for resistance to the Suwon strain.

Introduction

It has been quite often observed in the early spring that new leaves of overwintered barley plants become yellow and wither in the southern regions of Korea. It was found that these symptoms were mainly due to infection by Barley yellow mosaic virus (BaYMV), even if some symptoms in plants were related to other factors, such as waterlogging. Park (2004) reported that BaYMV and Barley mild mosaic virus (BaMMV) were relatively dominant among the soil-borne viruses in barley fields where disease symptoms were serious in Korea. About 40–56% yield reduction was observed, depending on the resistance level of barley cultivars in the infected fields (Park, 2004).

It is not effective to control BaYMV or BaMMV by chemicals because these viruses are transmitted by the soil-borne fungus *Polymyxa graminis*. Development of resistant cultivars is the only way to solve the disease problems in the consistently infected fields. In particular, DNA-marker assisted selection is strongly recommended as a powerful strategy facilitating the early and rapid introduction and pyramiding of resistance genes in breeding programs.

So far, sixteen resistance genes (*rym1* – *rym16^{hb}*) have been reported on the basis of their reactions to BaYMV and BaMMV strains in Germany and Japan. Extensive mapping studies have been conducted for these genes since molecular marker systems

were introduced in order to efficiently manipulate them in resistance breeding programs in the 1990s. Most resistance genes could be assigned to individual genomic locations over the seven chromosomes by using flanked DNA markers as well as morphological and isozyme markers (Werner *et al.*, 2003). So far, the resistance genes associated with BaYMV and BaMMV have been mapped to six of the seven chromosomes (nothing on chromosome 2H) (Gouis *et al.*, 2004; Ruge *et al.*, 2003; Werner *et al.*, 2003). In particular, four and six resistance genes mapped together on chromosomes 3H and 4H, respectively. Although *rym4* and *rym5* were closely linked on the same chromosome, analysis of their gene action could be effected with the aid of DNA markers associated with these resistance genes. Significant differentiation of BaYMV strains and their pathogenicity was observed in the southern regions of barley cultivation in Korea. Mokusekko 3 and Ishuku Shirazu showed moderate or high tolerance to BaYMV among the resistant genetic stocks, based on field evaluation in Korea (Kim *et al.*, 2005).

The objective of this study was to characterize the resistance of *rym1* and *rym5* genes of Mokusekko 3 against different strains of the BaYMV and BaMMV complex and to identify the closely linked PCR-based DNA markers, aiming for the efficient marker-based selection in the backcrossing procedure to accelerate pyramiding of resistance genes.

Materials and methods

The Chinese landrace Mokusekko 3 was crossed to the Korean susceptible two-row malting barley cultivar, Jinyangbori. A total of 130 lines of a mapping population ($F_{5,6}$ and $F_{6,7}$) were developed, and their reactions to BaYMV and BaMMV were assessed in consistently infected fields at two separate locations, Jinju in 2005 and Suwon in 2006.

A line was considered to be resistant when all the evaluated plants had no disease symptom consistently; otherwise it was considered susceptible.

DNA was isolated from seedlings of the two parents and the bulked plants of individual lines by a modified SDS procedure (Anderson *et al.*, 1992). About eighty markers chosen to represent chromosomes 4H and 3H where *rym1* and *rym5* were mapped, respectively, were tested for polymorphism between two parents. Polymerase chain reactions (PCR) were carried out in a T1 thermocyclers (Biometra, Germany). The reaction mixture contained 30 ng genomic DNA, 1 × PCR Buffer, 1.5 mM $MgCl_2$, 1U *Taq* polymerase (Bioneer Corp., Korea), 0.2 mM of dNTPs, and 0.2 μ M of each primer. The PCR conditions of each primer set were given by Liu *et al.* (1996) and Ramsay *et al.* (2000). PCR products were separated on 4% polyacrylamide gel, and were silver-stained for detection of fragments with different sizes (Bioneer Corp., Korea).

Segregation of the genotypes for field resistance or susceptibility was checked by a chi-square test against the expected 1:1 ratio in the $F_{5,6}$ and $F_{6,7}$ generations. Linkage analysis was performed using the MAPMAKER software (Lander *et al.*, 1987). Recombination values were calculated by use of the Kosambi function for genetic map distance (cM). Test of association between haplotypes of DNA markers and phenotypes of disease resistance in Case-Control studies were conducted for one marker at a time to detect genomic location by using the Power Marker software (V3.25; Liu and Muse, 2005) and the Proc PSMOOTH routine in SAS/Genetics™ software was then applied to output data sets of probability values (*P*-values) calculated from the Case-Control studies.

Results and discussion

Mokusekko 3 has been considered an important breeding source since it gives broad range of resistance against BaYMV

and BaMMV strains in Europe (Götz and Friedt, 1993). Our study was conducted to investigate how the resistance derived from Mokusekko 3 reacted to the complex strains in the consistently infected fields in Korea. Segregation of the reaction to BaYMV and BaMMV in $F_{5:6}$ & $F_{6:7}$ progenies of Mokusekko3 \times Jinyangbori was examined (Figure 1). Those lines segregating susceptible plants were recorded as susceptible (S) as opposed to resistant (R). The virus infection of barley plants grown in the field was effective so that resistant lines were easily distinguished from the susceptible ones.

Table 1 shows the segregation ratio for resistance and susceptibility at two locations, Jinju and Suwon. The number of lines for

resistance or susceptibility in Jinju were 80 and 42, respectively. Eight lines could not be evaluated due to poor germination in the field. The segregation ratio for BaYMV did not approach a 1R:1S ratio ($\chi^2 = 11.8$, $P < 0.005$), and the number of resistant lines were almost twice as many as the susceptible lines, suggesting that segregation of resistance was controlled by more than one gene.

As evaluated for BaYMV resistance to Jinju-strain in 2005, the same population with one more advanced generation ($F_{6:7}$) was evaluated at Suwon in 2006. The numbers of lines for resistance or susceptibility were 72 and 56, respectively (two lines were lost). The segregation ratio approached a 1R:1S ratio ($\chi^2 = 2.0$, $0.1 < P < 0.5$). This suggested that the segregation of field resistance in the



Figure 1. Infection by BaYMV in barley seedlings of Mokusekko3 (resistant) and Jinyangbori (susceptible) grown in the field in Jinju (left), and evaluation of a mapping population ($F_{5:6}$) derived from their crosses for BaYMV resistance (right).

Table 1. Segregation of $F_{5:6}$ and $F_{6:7}$ progenies of Mokusekko3 \times Jinyangbori for resistance to BaYMV/BaMMV in Jinju and Suwon.

Year	Location	Generation	No. of lines	No. of resistant lines	No. of susceptible lines	x2	P	Note
2005	Jinju	$F_{6:7}$	130	80	42	11.8	< 0.005	8 lines lost
2006	Suwon	$F_{7:8}$	130	72	56	2.0	0.1-0.5	2 lines lost

population against the Suwon strain was controlled by a single gene.

When considered that Mokusekko3 carries at least two resistance genes, *rym1* and *rym5*, our results showed that the effects of these two genes might be different on the infection of local strain types. DNA marker-based molecular mapping was conducted to decide which genomic region conferred resistance against different strains in our study. Individual lines were genotyped at the DNA marker loci on the chromosomes 3H and 4H to verify that *rym1* and *rym5* of Mokusekko 3 might be involved in the field resistance.

As an initial step to map the genes, we examined polymorphisms between the parents, Mokusekko3 and Jinyangbori, using 39 DNA markers (RAPD, STS, and SSR) selected as polymorphic markers between parents and used for linkage analysis. Seven linkage groups were constructed from DNA marker analysis for $F_{5,6}$ progenies of Mokusekko3 \times Jinyangbori, as shown in Figure 2. Since more than one gene was involved in the resistance against the Jinju strain, we could not do fine mapping of resistance genes by using a simple linkage analysis. Instead, correlations caused by linkage disequilibrium between markers and disease resistance were analyzed in the case-control studies, and (-) logarithm of *P*-values adjusted by Proc Psmooth in SAS/Genetics™ was plotted.

Finally, association analysis based on the linkage disequilibrium between DNA markers and disease resistance in Jinju revealed two highly significant genomic regions: HVM70 - CDO474c on chromosome 3H and HVM68 - Bmac0186 on chromosome 4H (Figure 3). These regions were traced to almost the same or close to map positions of *rym5* and *rym1*, respectively, which were previously reported with RFLP and STS markers (Graner *et al.*, 1999; Okada *et al.*, 2004).

At the same time, only one region of the genome was statistically significant in the association analysis with disease resistance

data of $F_{6,7}$ progenies of Mokusekko3 \times Jinyangbori against the Suwon strain (Figure 3). It was between HVM68 - Bmac0186 on chromosome 4H, where *rym1* was presumably located. However, the region of HVM70 - CDO474c on chromosome 3H was not significant against the Suwon strain where *rym5* was presumed to be located and activated against the Jinju strain.

In the late 1990s, some BaYMV and BaMMV resistance genes were extensively mapped with RFLP-based markers. These markers provided the necessary genetic information on target loci regarding disease resistance, but could not be used as a practical tool for marker assisted selection (MAS) due to their complex and high-cost procedures. In our studies, Mokusekko3-originated *rym1* and *rym5* were mapped with closely linked PCR-based markers such as SSR and STS. These markers were typically user-friendly and were presumed to be very useful for MAS. In particular, since most BaYMV and BaMMV resistance was inherited as recessive, at least two generations were required to obtain recessive homozygotes after crossing homozygote parents in the conventional selection schemes. With the aid of PCR-based DNA markers, recessive genes could be efficiently selected in the heterozygote status during the backcross-based introgression process.

Different reactions of resistance genes derived from Mokusekko3 suggests that multiple infection of various strains may exist in the barley field and that the virus strains are pathologically different between local regions, or both. In fact, both BaYMV and BaMMV were detected in the ELISA test of infected plants grown in Suwon, but only BaYMV was found in Jinju (data not shown). So far, it is not known if BaYMV strains are similar or not. If the BaYMV strains were the same in both regions, *rym5* of Mokusekko 3 might be susceptible to BaMMV in Suwon.

According to Park (2004), BaYMV strains could be classified into 4 regional

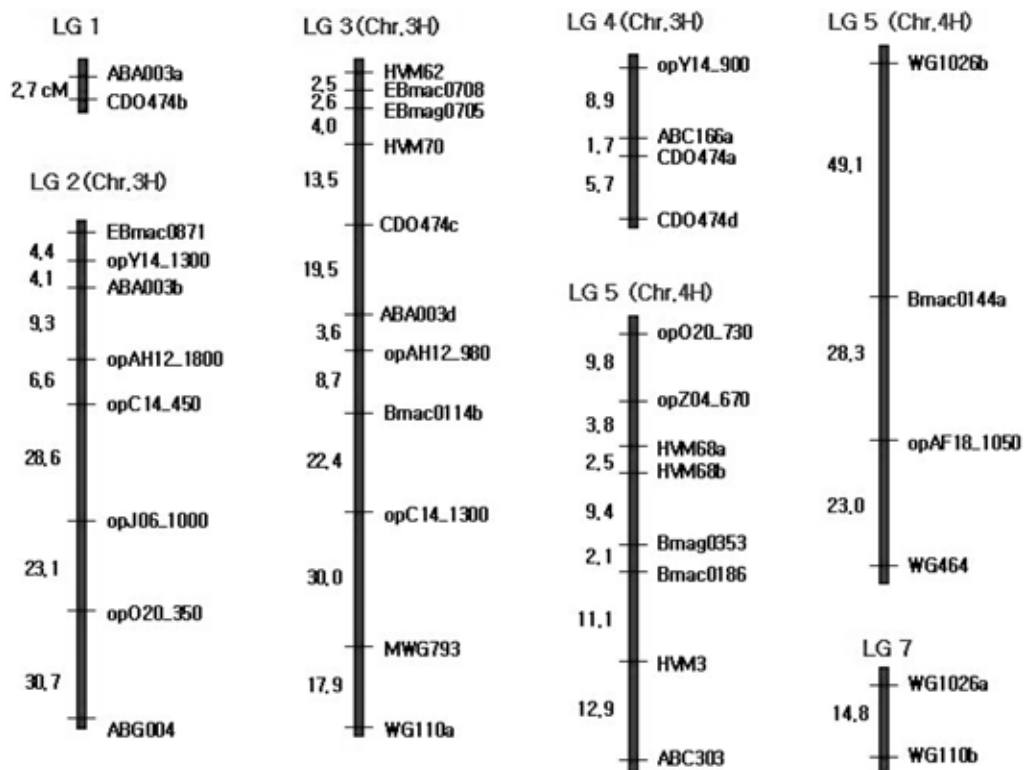


Figure 2. Linkage groups consisting of 39 DNA markers assigned to barley chromosomes 3H and 4H. A total of seven linkage groups were constructed from DNA marker analysis for $F_{5,6}$ progenies of Mokusekko3 \times Jinyangbori

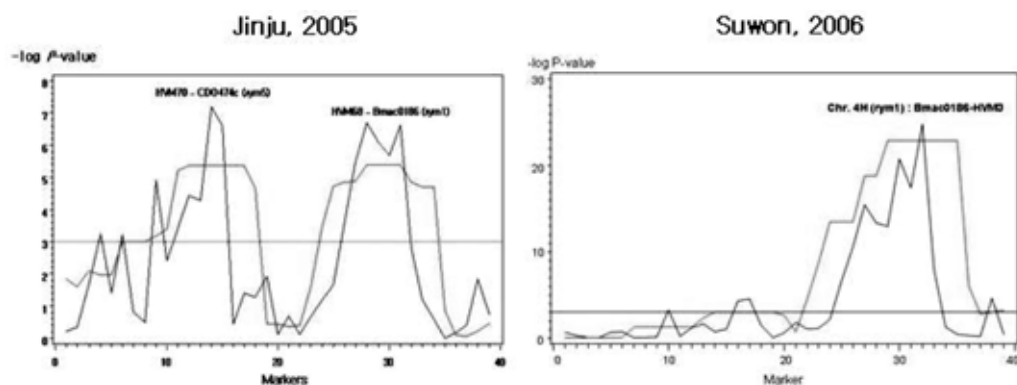


Figure 3. Plots of -logarithm of p-values calculated from association analysis between haplotypes of DNA markers and phenotypes of disease resistance in case-control studies. Two regions of the genome were highly associated with BaYMV resistance in Jinju, HVM70 - CDO474c on chromosome 3H in which *ym5* was located and HVM68 - Bmac0186 on chromosome 4H in which *ym1* was located (left). Only one region of HVM68 - Bmac0186 on chromosome 4H revealed resistance against the Suwon strain in 2006 (right).

groups based on the clustering analysis of RNA1 sequence homology. This indicates that development of barley cultivars with a single resistance source is not enough to control BaYMV and BaMMV. It is therefore important to combine more than two different genes in a single genotype through gene pyramiding. We expect that DNA marker-based molecular breeding tool should help the identification of most suitable resistance genes and their introgression into the current barley varieties.

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Genome-wide and *Mla* locus-specific characterization of Latvian barley varieties

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Abstract

Genetic diversity present in locally adapted germplasm forms the basis for crop improvement and selection. While single loci have been routinely used for studies of genetic diversity, the high-throughput genotyping platforms that have recently become available for large-genome crop plants offer an unbiased view on genetic diversity on a genome-wide scale. We assessed genetic diversity in Latvian barley varieties and some progenitors based on DArT markers, and compared these results with genetic diversity at two loci flanking the barley powdery mildew *Mla* locus conferring race-specific resistance. The *Mla* locus encompasses several closely related resistance gene homologues with a complex evolutionary history, which complicates the design of molecular markers for different *Mla* genes. DNA sequence haplotypes at the two loci ABC15612 and 538P8 flanking the *Mla* locus were largely in agreement with known phenotypic data and thus may be potentially diagnostic for *Mla* resistance genes in hybrids.

Introduction

Genetic diversity provides the material for natural selection and ensures that species as a whole survives changing environmental conditions (von Bothmer *et al.*, 2003). In domesticated crop plants, genetic diversity provides the raw material for plant breeders to develop new varieties with

novel uses or better adaptation to the local environment. Knowledge of genetic diversity available genome-wide and at specific loci responsible for key traits is essential for crop improvement.

Genetic diversity is significantly reduced in domesticated crop plants in comparison with their wild relatives (Tanksley and McCouch, 1997). Studies in barley have found significantly reduced genetic diversity in modern cultivars compared with wild barley accessions and landraces (Russell *et al.*, 2004), traditionally explained by a ‘founder’ effect. Further reduction in diversity may have occurred during the transition to modern breeding, when only a limited number of genotypes were used for development of modern varieties, although this may be in part compensated for by introgression of specific traits from wild relatives and landraces. For instance, even though most of the European cultivars bred in the 20th century have a narrow germplasm base due to a small number of varieties that served as breeding material (Fischbeck, 1992), there appeared to be little reduction in genetic diversity in the case of Baltic and Nordic barleys, or even an increase in allele diversity in modern varieties compared with those bred in the middle of the 20th century (Kolodinska-Brantestam *et al.*, 2004, 2007).

Genome-wide diversity studies allow characterization of the overall diversity of germplasm, as well as establishing the relatedness of certain genotypes. It can also be used to roughly identify genome regions

subjected to selective sweeps during the domestication and breeding process. Various high-throughput genotyping technologies are available for whole-genome analysis of crop plants. Recently, a high-throughput single nucleotide polymorphism (SNP) genotyping platform based on Illumina GoldenGate technology was developed for barley that allowed studying genetic diversity in ca. 100 European barley varieties using over 1000 SNPs (Rostoks *et al.*, 2006). Alternatively, there are genotyping platforms that do not require prior knowledge of gene sequences, such as DArT (Jaccoud *et al.*, 2001). The DArT platform has been used extensively in barley, both for studies of genetic diversity and for linkage mapping (Wenzl *et al.*, 2004). Most of the barley DArT markers are mapped on a consensus map including RFLP and SSR loci, making the DArT platform useful for diversity studies (Wenzl *et al.*, 2006).

Powdery mildew is an economically important barley disease, caused by a fungal pathogen *Blumeria* [syn *Erysiphe*] *graminis* f.sp. *hordei*. While the pathogen is relatively easily controlled by fungicides, it may represent a serious threat for barley produced in low input and organic agriculture. Alternatively, disease can be controlled using resistance genes that are either specific for certain fungal pathotypes or confer resistance to a broad range of pathotypes. In barley, the complex, race-specific powdery mildew resistance *Mla* locus is located on the short arm of barley chromosome 5(1H) and consists of several NBS-LRR type disease resistance genes providing resistance to different fungal pathotypes (Wei *et al.*, 1999). The locus has been completely sequenced from the susceptible cultivar Morex, and resistance genes *Mla1* (Zhou *et al.*, 2001), *Mla6* (Halterman *et al.*, 2001), *Mla12* (Shen *et al.*, 2003) and *Mla13* (Halterman *et al.*, 2003) have been characterized (Mejlhede *et al.*, 2006). Because of the close sequence homology of different *Mla* genes, it is complicated to design molecular markers

for specific *Mla* genes. However, because modern barley varieties have originated from a limited germplasm pool and relatively recently (Fischbeck, 1992), it is reasonable to expect that linkage disequilibrium across the *Mla* locus is maintained. Thus, sequence analysis of closely linked loci could be used both to assess the sequence diversity at the *Mla* locus and to predict the disease resistance specificity of the *Mla* locus. There are over 30 known *Mla* alleles, although only a few of them are extensively used in European barley breeding (Weibull *et al.*, 2003). Transfer of novel *Mla* genes in adapted germplasm necessitates development of efficient tools for introgression, and this is essential as the pathogen evolves virulence against the currently used disease-resistance genes.

In this study we used genome-wide DArT genotyping to assess genetic diversity in Latvian barley varieties and compare it with other European varieties. Subsequently, we characterized DNA sequence diversity at the race-specific disease resistance locus *Mla* in locally adapted Latvian barley varieties.

Materials and methods

DNA was extracted from a single barley plant using a modified procedure of Edwards *et al.* (1991). Plant tissue was collected either from a field-grown plant or a seedling germinated on a Petri dish in the laboratory. Seeds were obtained from State Priekuli Plant Breeding Institute and State Stende Cereal Breeding Institute, both in Latvia. Seeds of European barley varieties were obtained from the IPK Gene Bank, Germany, and from the Nordic Gene Bank (now NordGen Plants), Alnarp, Sweden.

DArT genotyping of the Latvian and foreign barley varieties (44 altogether) was carried out by Triticarte Pty Ltd (North Ryde, NSW, Australia). DArT genotype analysis was done as described by Jaccoud *et al.* (2001) and Wenzl *et al.* (2004). Principal Coordinate Analysis (PCoA) of the genotype

data was performed with DARwin5 software (<http://darwin.cirad.fr/darwin>) using the Manhattan dissimilarity index. An UPGMA dendrogram of the DArT genotype data was constructed using distances derived by Restdist software from the Phylip software package, using modified neighbor joining algorithm. *Mla* loci diversity was analyzed for Latvian varieties only. Primer sequences for the ABC15612 and 538P8 loci flanking the *Mla* locus were obtained from Dr Luke Ramsay (SCRI, Dundee, Scotland, UK). PCR was performed in 20 µl reactions consisting of ca. 50 ng DNA, 10 µM primers, 1 × reaction buffer (Fermentas, Vilnius, Lithuania), 2.5 mM MgCl₂, 0.2 mM dNTPs and 1 unit of Hot Start Taq polymerase (Fermentas, Vilnius, Lithuania). Sequencing was done using BigDye 3.1 terminator mix (Applied Biosystems, Foster City, CA, USA) on an ABI3730 sequencer according to manufacturer's recommendations. Base calling and sequence assembly was done using the Staden software package (Staden, 1996). Sequence alignments were done with Clustal 1.83 software (Thompson *et al.*, 1997). Sequence alignments were used to calculate haplotype number, haplotype diversity and nucleotide diversity at each locus using DNASP 4.20 (Rozas *et al.*, 2003). The sequence haplotype distance matrix based on ABC15612 and 538P8 loci and neighbor program from the Phylip package (Felsenstein, 1989) were used to construct an UPGMA (Unweighted Pair Group Method with Arithmetic means) dendrogram.

Results and discussion

Twenty three Latvian barley varieties, as well as some progenitors and several other European barley accessions were genotyped using the barley DArT genotyping platform (Wenzl *et al.*, 2004). Of the ca. 1100 polymorphic loci, 326 were selected with quality (Q) value greater than 80 and as having no missing data among the 44 barley

accessions. The average polymorphism information content (PIC) value of selected DArT markers in this germplasm set was 0.36. Principal Coordinate Analysis (PCoA) was used to study the partitioning of the germplasm (Figure 1). There was no obvious differentiation between Latvian varieties and the rest of the European germplasm, although the germplasm was strongly partitioned along the PCo1 axis.

The UPGMA dendrogram was constructed from the genotype data to study the relationships among Latvian and other European germplasm (Figure 2). The two North American varieties, Parkland and Bowman, clustered separately, while the European germplasm, including Latvian varieties, was partitioned into two clusters. Three varieties, Emir, Kenia and Proctor, clustered together and the genotype data of these varieties appeared to be identical. Similarly, varieties Mari and Rasa differed only at three DArT loci. Because these are well known as distinct varieties, it is possible that seed, tissue or DNA mixture had occurred. Data from these varieties will not be used in further analysis. While at the moment there is no obvious explanation for the partitioning of the germplasm (excluding North American varieties) into two groups, there are several apparent agreements with known pedigrees, e.g. Otra and Agra are clustered together (Agra is Priekulu 1 × Otra) and Abava and Domen are grouped together (Abava is from a cross involving Mari, Elsa and Domen). All the varieties included in analysis are of spring growth habit and most of them are two-row, with the exception of Agra, Druvis, Priekulu 1, Vairogs, Dzintars, Parkland and Otra. These major phenotypic traits often differentiate the germplasm because of breeding traditions (Fischbeck, 1992). However, growth habit could not have affected the structure observed in our germplasm set, and there was no apparent clustering based on row type. Further studies involving comparison of agronomic qualities

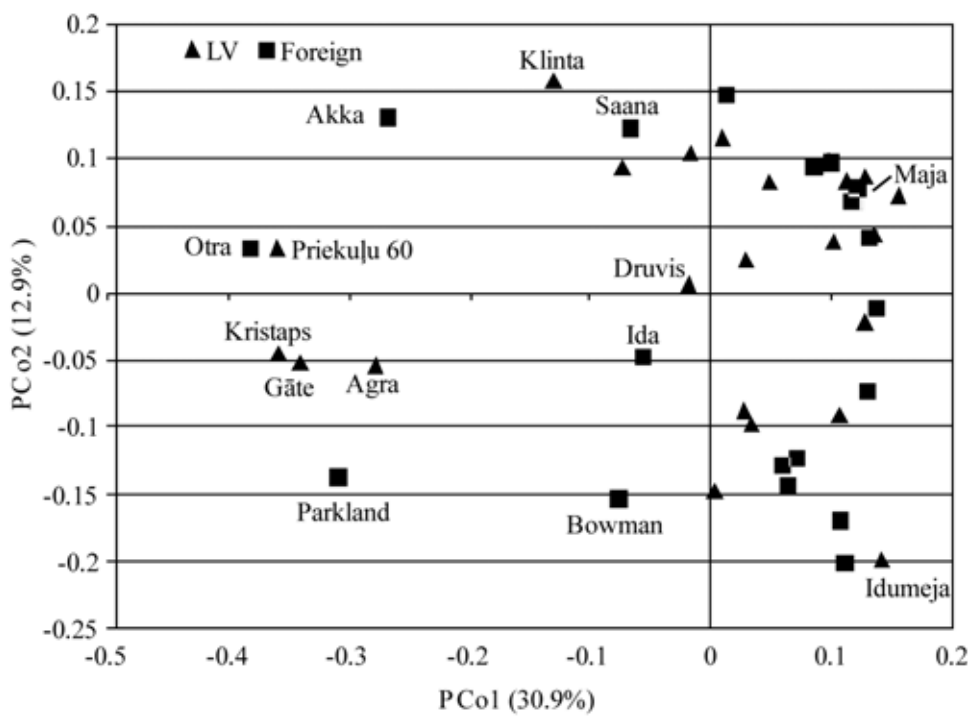


Figure 1. Principal Coordinate Analysis (PCoA) of DArT genotype data performed with DARwin5. PCoA is based on data from 44 varieties and 326 loci. PCo1 and PCo2 explain 30.9% and 12.9% variation in genotype data, respectively. Triangles and squares indicate Latvian varieties, respectively and foreign.

and pedigrees will be needed to explain the observed partitioning.

The *Mla* locus has been completely sequenced from susceptible variety Morex (Wei *et al.*, 2002) and several *Mla* genes that provide race-specific mildew resistance have been isolated. Complexity of the locus, high homology between *Mla* genes and putative paralogues elsewhere in the barley genome (Mejlhede *et al.*, 2006) have so far precluded design of molecular markers for different *Mla* resistance genes. Linkage disequilibrium (non-random association of alleles) in cultivated spring barley was recently reported to extend to centiMorgan distances (Caldwell *et al.*, 2006; Rostoks *et al.*, 2006). Therefore, the allelic state of the loci flanking *Mla* genes could be used to predict the resistance specificity at the *Mla*

locus. The two loci, ABC15612 and 538P8, flanking the *Mla* region (Figure 3A) on barley chromosome 5(1H) were amplified and sequenced from 23 Latvian barley varieties. Resulting sequences were used to identify over 40 SNP and several indel polymorphisms among the Latvian barley varieties (data not shown). DNA polymorphisms were used to group sequences into twelve haplotypes across both loci and those, in turn, were used to construct an UPGMA dendrogram (Figure 3B; Table 1). Latvijas Vieteje (H8 in Figure 3), a Latvian landrace, appeared to be very distant from other Latvian varieties. However, this was due to high polymorphism at the ABC15612 locus, while at the 538P8 locus, Latvijas Vieteje was identical to Abava, Imula, Klinta, Kombainieris, Ruja and Priekule 60.

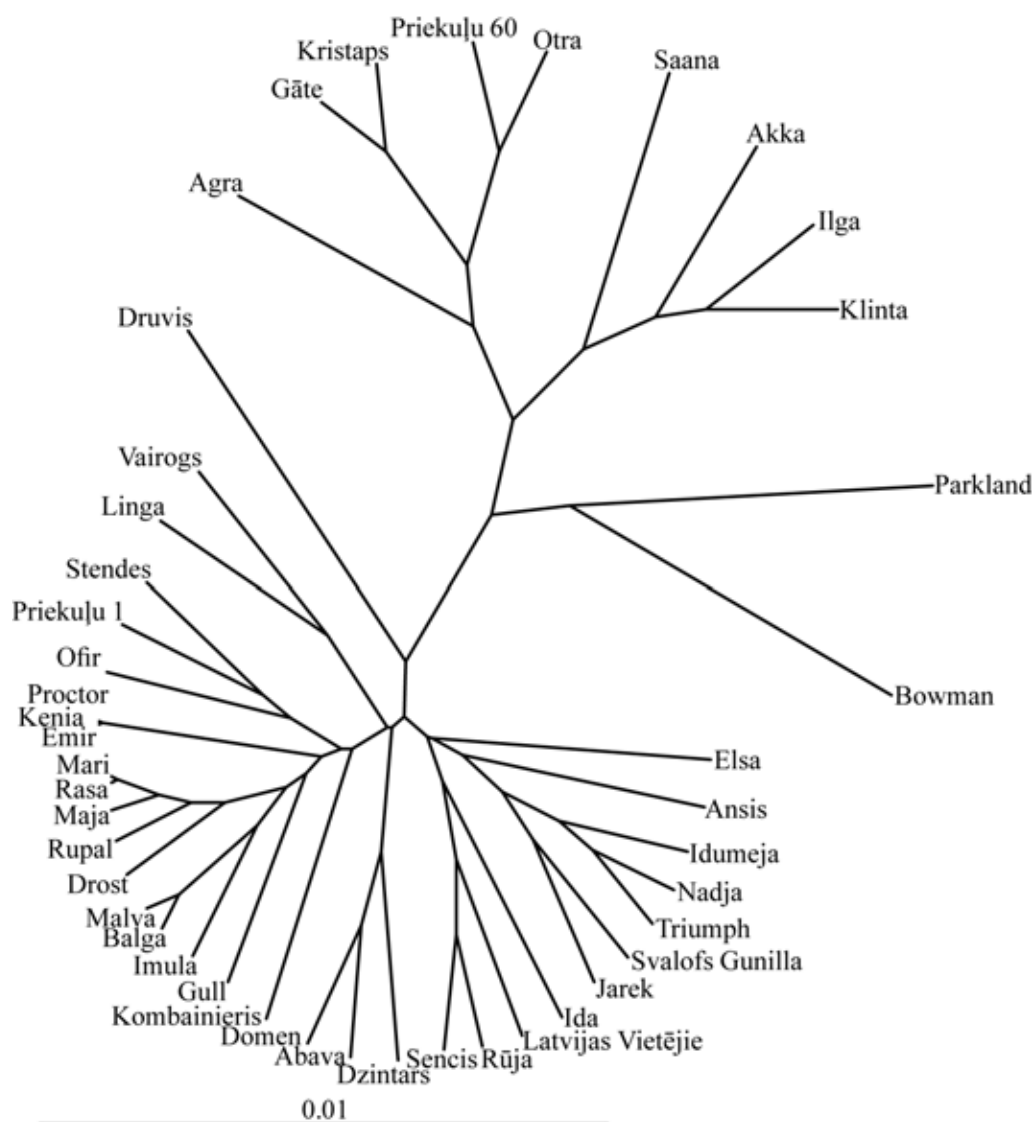
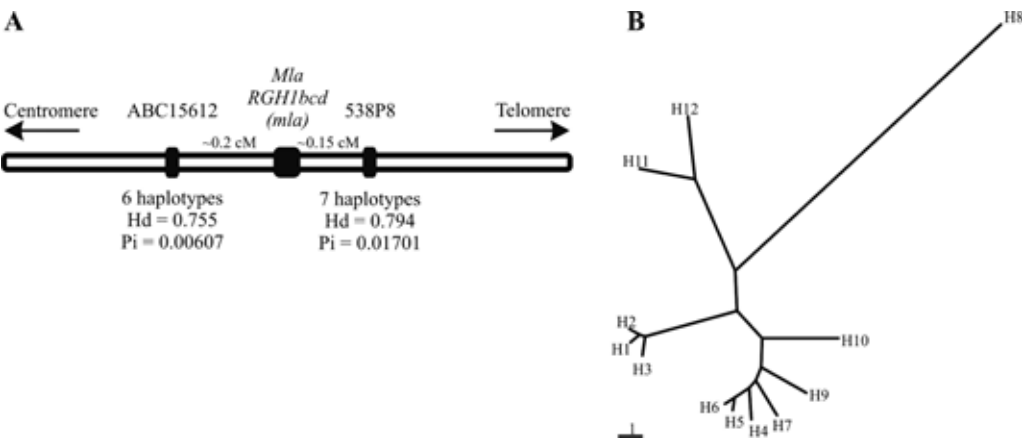


Figure 2. Unrooted UPGMA dendrogram of DArT genotypes based on data from 44 varieties and 326 loci. The distance matrix was derived using the fragment option in the Restdist program from the Phylip package using modified Nei distances (Felsenstein, 1989). The dendrogram was constructed using the UPGMA option in the neighbor program.

Sequence haplotypes were compared to published mildew resistance specificities of Latvian barley varieties (Dreiseitl and Rashal, 2004; Tueryapina *et al.*, 1996) (Table 1). In general, good agreement was found between sequence haplotype data and experimentally determined *Mla* resistance genes in Latvian

barley varieties (Dreiseitl and Rashal, 2004; Tueryapina *et al.*, 1996) (Table 1), while the existing discrepancies can be explained by differences in methodology. While we sequenced marker loci from a single plant for each variety, mildew resistance tests were carried out on pools of plants



(Dreiseitl and Rashal, 2004). The same study identified the majority of Latvian barley Figure 3. Structure and sequence analyses of the *Mla* locus in Latvian barley varieties. A. Structure of the *Mla* locus with molecular marker loci ABC15612 and 538P8 indicated (Wei *et al.*, 1999). Sequence haplotype number, haplotype diversity and nucleotide diversity at each locus are indicated below. B. UPGMA dendrogram of the sequence haplotypes identified at both ABC15612 and 538P8 loci.

Key: H1 = Gate, Kristaps, Rasa; H2 = Ansis, Balga, Ilga, Linga; H3 = Stendes; H4 = Malva, Sencis; H5 = Vairogs; H6 = Dzintars; H7 = Abava, Imula, Klinta, Kombainieris, Priekulu 60; H8 = Latvijas Vietējie; H9 = Priekulu 1; H10 = Druvis; H11 = Idumeja; and H12 = Agra. Branch length indicates number of nucleotide differences between haplotypes.

Table 1. Classification of 23 Latvian barley varieties into haplotypes based on sequence polymorphisms at the ABC15612 and 538P8 loci.

Variety	Sequence haplotype	<i>Mla</i> resistance ¹	Variety	Sequence haplotype	<i>Mla</i> resistance ¹
Gāte	H1	<i>Mla</i> 7	Abava	H7	<i>Mla</i> 8
Kristaps	H1	<i>Mla</i> 7	Imula	H7	<i>Mla</i> 8
Rasa	H1	<i>Mla</i> 7	Klinta	H7	<i>Mla</i> 6+ <i>Mla</i> 8
Ansis	H2	<i>m1a</i>	Kombainieris	H7	<i>Mla</i> 8
Balga	H2	<i>m1a</i>	Priekulu 60	H7	<i>Mla</i> 8
Ilga	H2	<i>Mla</i> 8	Rūja	H7	<i>Mla</i> 8
Linga	H2	<i>m1a</i>	Latvijas vietējie	H8	<i>Mla</i> 8
Stendes	H3	<i>Mla</i> 8	Priekulu 1	H9	<i>Mla</i> 8
Malva	H4	<i>Mla</i> 8+ <i>Mla</i> 13	Druvis	H10	<i>Mla</i> 8+ <i>Mla</i> 12
Sencis	H4	<i>Mla</i> 13	Idumeja	H11	<i>Mla</i> 9
Vairogs	H5	<i>Mla</i> 8	Agra	H12	<i>Mla</i> 9
Dzintars	H6	<i>Mla</i> ?			

¹ *Mla* resistance according to Dreiseitl and Rashal, 2004, and Tueryapina *et al.*, 1996.

cultivars as heterogeneous with respect to *Mla* resistance genes. Similar heterogeneity has also been observed with molecular markers (Dr Kolodinska-Brantestam, pers. comm.). For instance, variety Klinta, which was identified as carrying *Mla6* and *Mla8* genes, in the sequence analysis clustered with the *Mla8* group, probably indicating that the individual plant under study was *Mla8* resistant. In addition, the *Mla8* allele is very difficult to phenotype, because only a few pathogen isolates have a compatible avirulence gene (Jensen, 1995). Thus, the varieties identified as *Mla8* may in fact possess a different sequence allele. Overall, the agreement was good and we are currently working on extending sequence analysis on some mildew resistance donors with a goal to establish molecular markers that can be used to pyramid mildew resistance genes.

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The use of microsatellites to screen barley genotypes for resistance to net blotch

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Abstract

Barley (*Hordeum vulgare* L.) is an important crop species, grown especially for malting and for feed. One of the diseases that threaten the yield and the quality of barley is net blotch caused by the fungus *Pyrenophora teres* Drechs. Although fungicides are commonly used, the most worthwhile way to cope with the problem is to breed new lines resistant to the disease. This study examines the use of neutral genetic markers (microsatellites) to screen barley lines and varieties that could be introduced into net blotch resistance breeding programs. Forty microsatellite loci localized on all barley chromosomes were chosen. The barley cultivars originated from countries worldwide, including commonly used cultivars, old cultivars, landraces and genotypes resistant or very susceptible to net blotch. For 43 barley microsatellite loci and 266 barley genotypes, 640 alleles were detected. The average number of alleles per locus was 14.9. Cluster analysis based on microsatellites data showed that diversity was not randomly distributed. Generally, it can be explained mainly by temporal impact of the breeding processes in different countries during the last century. The question of allele distribution between net blotch resistant and susceptible genotypes is very interesting and it is further discussed.

Introduction

Barley (*Hordeum vulgare* L.) is an important crop species, and the Research Institute of Crop Production genebank

holds 1865 accessions of winter barley and 2707 accessions of spring barley. Ancient cultivars or landraces and wild relatives of domesticated species can be a good source of interesting and agriculturally valuable features. Currently, a large number of accessions from major crop species and their wild relatives are stored in genebanks, at *in situ* conservation sites and in on-farm programs of conservation. The large size of some of these collections and limited funding limit the characterization of the material available and hinder their use in breeding programs (Brown, 1995). Therefore, an increasingly popular proposal for germplasm management is to assemble smaller 'core collections' from these larger collections. Ideally, core collections should be chosen to represent the maximum of the genetic diversity contained in the larger collection. Construction of such a collection usually starts by stratifying the larger collection into a series of groups according to their genetic distances (Bataillon *et al.*, 1996). Then, sub-core collections should be evaluated taking into account specific characteristics, such as yield, resistance, quality parameters and so on.

Every year, a complex of leaf spots threatens the yield and quality of barley grains. Net blotch is one of the leaf spot diseases and it is caused by *Pyrenophora teres* (Smedegaard-Peterson, 1971). Identification of sources of resistance to net blotch and an understanding of their genetics are very important to develop resistant varieties. Resistance to net blotch is polygenic and it is also dependent on plant development stage

(seedling or adult). Mapping of quantitative trait loci (QTLs) associated with resistance to net blotch has been done with several mapping populations (Raman *et al.*, 2003; Williams *et al.*, 2003; Cakir *et al.*, 2003; and many others). Except for RFLP and AFLP, that are very popular techniques for QTL mapping, the analysis of microsatellites are commonly used to study genetic relationships among genotypes within species because of their high level of polymorphism (Devos *et al.*, 1995; Plaschke *et al.*, 1995; Korzun *et al.*, 1997). In addition, microsatellites exhibit co-dominant inheritance (Hernandez *et al.*, 2002), which is essential for effective discrimination between closely related lines (Akkaya *et al.*, 1992). Microsatellite markers are currently used to identify genotypes, quantitative trait loci (QTLs) and genetic diversity (Leisova and Ovesna, 2001; Roussel *et al.*, 2005; Medini *et al.*, 2005).

The aim of this study was to evaluate the genetic diversity of a barley collection, using microsatellite markers, with a special

emphasis on preparing a barley sub-core collection from the perspective of resistance to net blotch disease.

Materials and methods

Plant material

We worked with 266 barley accessions chosen from 27 different countries (Table 1). A part of the barley accessions were obtained from the Prague-Ruzyně genebank; the rest from Vera Minarikova of the Agricultural Research Institute Kromeriz and Lenka Stemberkova of the Selgen Stupice. Plants were grown in greenhouse conditions and about 30 plants per accession were pooled and frozen at -80°C. Genomic DNA was extracted using a CTAB method according to Saghai-Marooof *et al.* (1984).

Microsatellite analysis

To study the barley collection, a set of 43 microsatellite markers were chosen from several publications (Liu *et al.*, 1996; Russell *et al.*, 1997; Ramsay *et al.*, 2000). They are listed in Table 2, including repeat motif, annealing temperature and number of detected alleles per each microsatellite locus. PCRs with fluorescently labeled primers (6-fam, vic, ned and pet) were performed in a reaction volume of 15 µL according to the optimized protocol. Reactions were run using a UNO II (Biometra) cyclor. The analysis of PCR products was performed using capillary electrophoresis on an ABI PRISM 3130 (Applied Biosystems) sequencer. A multiplexed configuration of four reactions was used in one analysis. For internal size standardization, a LIZ500 (Applied Biosystems) was used. Electrophoretograms were processed by GeneMapper software.

Data analysis

For each locus, the presence or absence of bands in each size category through all genotypes was scored. Data were set in a binary matrix. Genetic similarities were

Table 1. Summary of barley accessions and their country of origin.

State	No.	State	No.
Australia	1	Italy	2
Austria	7	Japan	4
Bulgaria	7	Netherlands	5
Canada	3	Poland	7
Czech Republic	101	Romania	5
Denmark	1	Spain	1
Ethiopia	1	Sweden	2
France	11	Turkey	2
Germany	42	Former USSR	27
Greece	1	United Kingdom	4
Hungary	7	USA	15
China	2	Yugoslavia	3
Ireland	1	Algeria	1
		Korea	3
TOTAL			266

Table 2. List of barley microsatellites used in this study.

SSR primer-label	Chromosome	Motif type	Annealing temp. (°C)	Product size (bp)	No. of alleles
BMS02-6FAM	1H	(CA)21	60	190 – 232	15
BMS32-VIC	1H	(AT)5(CA)22	60	192 – 276	29
BMS90-NED	1H	(GA)8(AT)9(CA)20	60	187 – 237	24
HVM36-PET	2H	(GA)13	60	102 – 140	12
HVM54-6FAM	2H	(GA)14	60	107 – 165	15
HVBKASI-VIC	2H	(C)10(A)11	60	178 – 200	6
Bmag0136-NED	3H	(AG)6-(AG)10-(AG)6	60	183 – 201	5
HVM60-PET	3H	(AG)11,(GA)14	60	103 – 120	14
HVM62-6FAM	3H	(GA)11	60	235 – 267	11
HVM40-VIC	4H	(GA)6(GT)4(GA)7	55	131 – 161	11
HVM67-NED	4H	(GA)11	60	99 – 119	10
BLYRCAB-PET	4H	(AT)29	55	160 – 218	26
EBmac0906-6FAM	4H		55	131 – 157	7
Bmac0181-VIC	4H	(AC)20	55	158 – 184	10
Bmag0222-NED	5H	(AC)9(AG)17	50	148 – 184	11
Bmag0394-PET	5H	(AG)9CG(AG)4CG(AG)4	55	161 – 179	8
HVM30-6FAM	5H	(CA)8	60	135 – 155	8
Bmag0500-VIC	6H	(AG)7C(AG)30-(AG)6	60	115 – 209	32
BMS18-NED	6H	(CT)9(CA)12	60	123 – 141	11
BMS40-PET	6H	(CA)21(GA)21	60	157 – 237	28
Bmag0021-6FAM	7H	(CA)10AA(GA)28	55	99 – 169	23
Bmag0135-VIC	7H	(AG)10GG(AG)12	60	117 – 157	10
HVCMA-NED	7H	(AT)9	60	119 – 139	8
Bmag0496-6FAM	6H	(CT)20	60	170 – 227	24
Bmag0807-VIC	6H	(TC)18	55	95 – 113	9
HVM74-NED	6H	(GA)13	55	168 – 246	26
HvTUB-PET	2H	(AGC)8	60	131 – 151	3
Bmag0112-6FAM	3H	(AG)18	55	153 – 243	29
Bmag0120-VIC	7H	(AG)15	60	214 – 264	13
Bmag0173-NED	6H	(CT)29	60	104 – 162	21
Bmag0381-PET	2H	(CT)7(ATCT)6(CT)14	50	107 – 151	19
EBmac0874-6FAM	6H		60	147 – 211	19
HvBARE3-VIC	xH	(CTT)6	60	246 – 260	4
HvChit-NED	xH	(AGAT)4	60	91 – 121	8
HvJas-PET	xH	(AAAT)4	60	120 – 156	8
HVM05-6FAM	7H	(GT)6(AT)16	60	159 – 247	30
HVM11-VIC	6H	(GGA)3(GGA)(GAA)2	60	161 – 203	7
HvMyb-NED	xH	(AACCC)4	60	224	2
HVPRP1B	7H	(AV)6(AT)16	60	145 – 199	24
Bmac0067-6FAM	3H	(AC)18	60	149 – 189	13
Bmac0310-VIC	4H	(CT)11(AC)20	55	127 – 181	19
Bmag0306-NED	4H	(AG)16	55	131 – 171	17
HVM43-PET	1H	(CA)9	55	219 – 243	11
Total					640

calculated using the Hamman coefficient and dendrogram as well as PCA (Principal Component Analysis) obtained by clustering according to the UPGMA method using Statistica for Windows software (StatSoft, Inc, CR). Allele frequency was assessed using MS Excel software.

Results and discussion

The cultivars were chosen to represent core collections from genebank accessions so all distinct genotypes of barley were represented. In all, 22 modern cultivars, 6 potential sources of resistance to the net blotch and other diseases and 56 breeding lines were added to the screening. Leaves from about 30 plants at the two-leaf stage were used for DNA extraction and microsatellite analysis. At least five microsatellite markers were chosen per chromosome. The list also included microsatellites found to be related to the barley resistance genes for *Pyrenophora teres*. Most amplification products revealed only one allele per genotype. When two or three alleles were present at a single locus and genotype, they all were taken into account, especially in the case of landraces and old varieties, which are populations of several different genotypes and which could carry agronomically interesting alleles. In total, 640 alleles were detected from the 43 amplified loci in barley. The total number of alleles per locus ranged from 2 to 32, with an average of 14.9 alleles per locus.

The cluster analysis could clearly identify the genetic relationship of barley genotypes and demonstrates the potential and ability of microsatellite markers for genome analysis in the crop. Cluster analysis based on microsatellite data showed that diversity in these accessions is not randomly distributed. Country of origin, age of variety, botanical characters and level of resistance to net blotch influenced to a large degree the grouping of the barley varieties into clusters. From all 6 clusters, one is formed of only

two-row varieties. The remaining two-row varieties occur in another cluster. Czech modern varieties grouped together in two large clusters with breeding lines, while old Czech varieties and landraces were in two other clusters with other foreign landraces. Varieties from America, Australia and Asia were in the left part of the dendrogram, mainly in two clusters together with genotypes from the former Soviet Union. The other Russian varieties occur in one cluster, together with varieties from Germany and from south Europe. Varieties from Germany are spread in all clusters. No cluster is formed solely by genotypes originating from the same country, or even from the same continent. Struss and Plieske (1998) obtained the same results: they observed no correlation between geographical origin of cultivars or landraces and their classification. The intensive exchange of seed material might have caused a mis-alignment of accessions to a particular origin of cultivars or advanced landraces. The line CI739 showing resistance to net blotch occurs together in a cluster with more modern foreign varieties in the right part of dendrogram. Breeding lines form two clusters together with other sources of resistance to several fungal diseases and varieties from Asia. To clarify the relationship between genotypes, a PCA analysis was conducted.

PCA analysis based on microsatellite data of barley genotypes showed two basic clusters (Figure 1). In the smaller one, mainly spring varieties are present, both modern registered cultivars and breeding lines. The cluster showed a narrow gene pool. The larger cluster is formed mainly by winter barley accessions from the core collection and by five genotypes considered as sources of resistances. Two of them (Sanalta and Tifang) are spring barley. Two genotypes considered as winter barley (Frolac and Martha) grouped close to the spring barley cluster; they could be alternative types.

In total, 640 alleles were found. The analysis of allele frequency showed that some

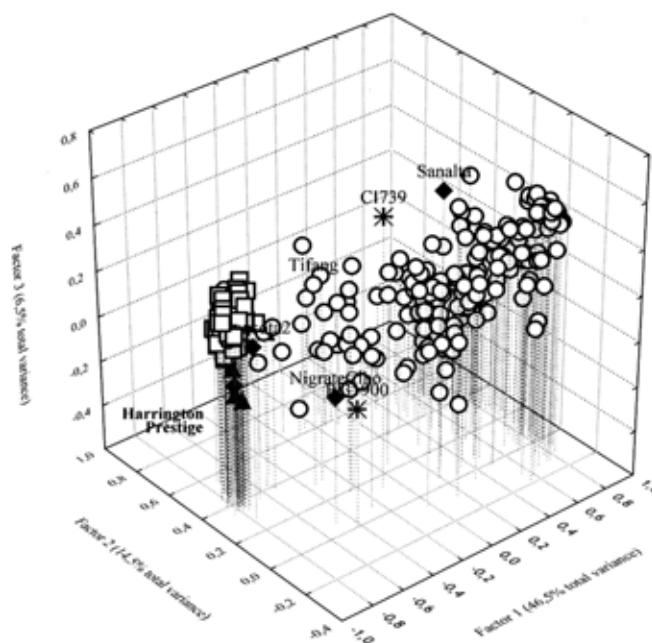


Figure 1. Three-dimensional principle component analysis (PCA) plot of barley genotypes based on unweighted pair-group method (UPGMA) clustering analysis of microsatellite data. KEY: ○ = barley core collection; * = source of resistance to net blotch disease; ◆ = source of resistance to other diseases; ▲ = modern registered cultivar; □ = breeding line.

of them are widely spread throughout all genotypes studied, such as allele 212 of the BMS02 locus or allele 252 of the HvBARE locus. Other alleles are specific to only several genotypes. We focused on alleles that were found in lines or varieties resistant to net blotch or other diseases. In several loci, resistant and susceptible genotypes have very different alleles – for example in SSR locus

Bmag0222 (Figure 2): alleles 148 and 178 were dominant in Beate and other susceptible varieties, and allele 158 was found mainly in resistant varieties. Similar distribution was found in microsatellite Bmag0381 (Figure 5). In microsatellites BMS18 (Figure 3) and Bmag0173 (Figure 4) the situation is different: alleles found in resistant genotypes neighbor those found in susceptible varieties.

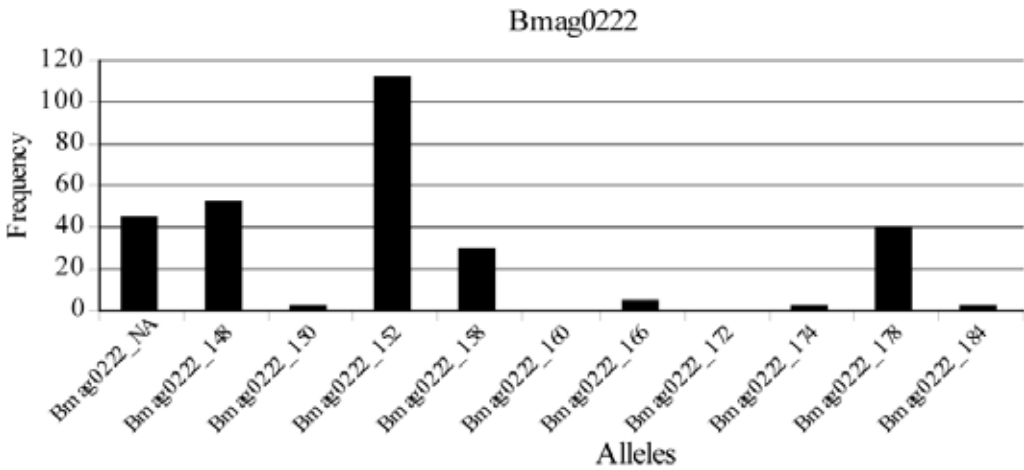


Figure 2. The allele frequency of microsatellite locus Bmag0222.

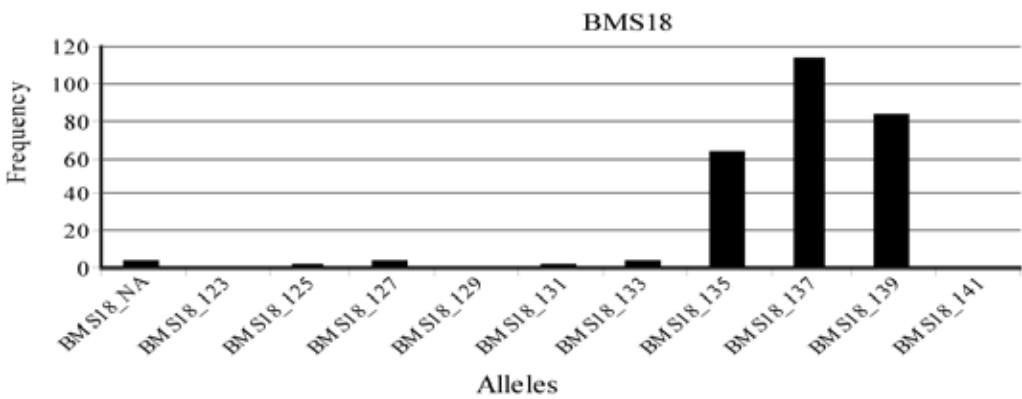


Figure 3. The allele frequency of microsatellite locus BMS18.

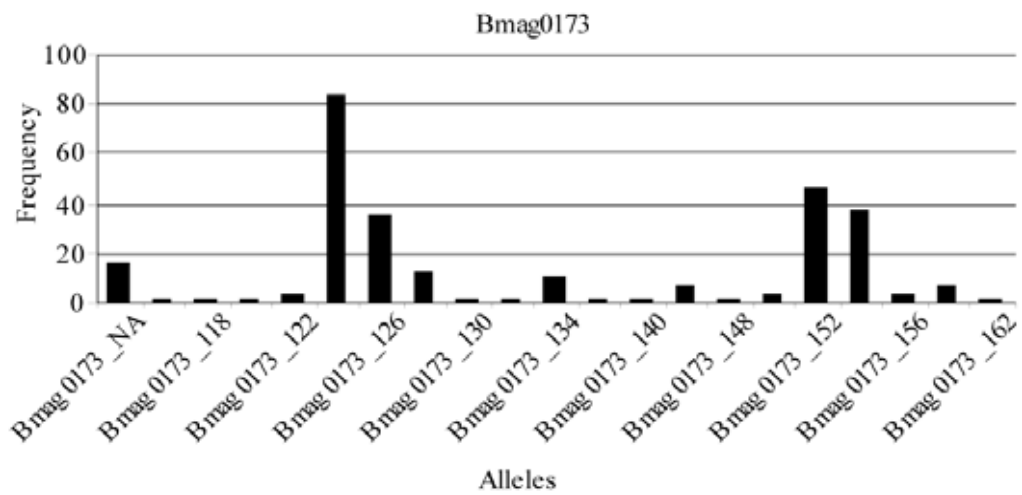


Figure 4. The allele frequency of microsatellite locus Bmag0173.

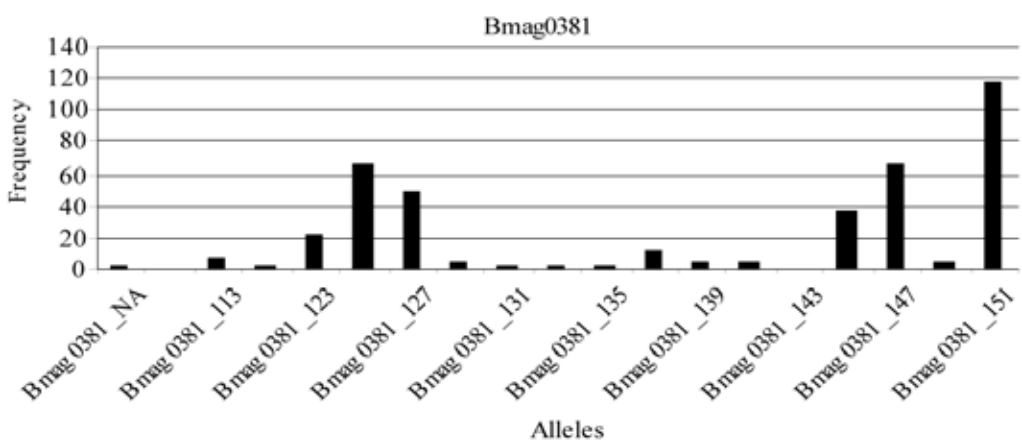


Figure 5. The allele frequency of microsatellite locus Bmag0381.

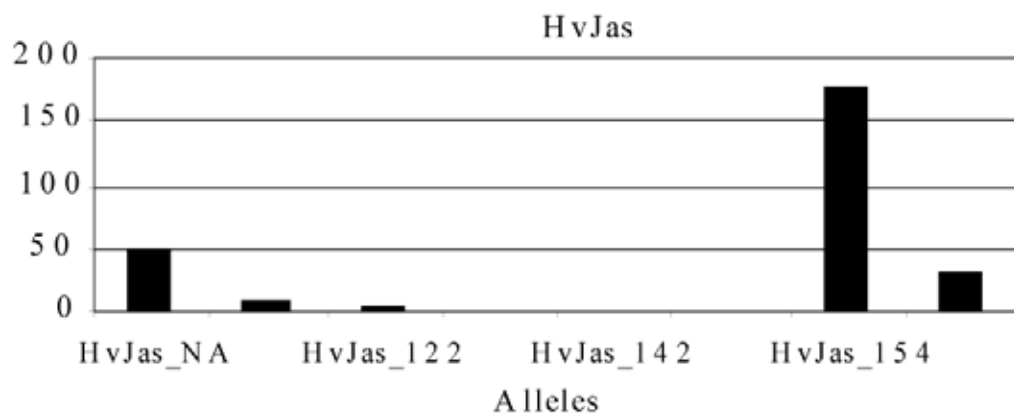


Figure 6. The allele frequency of microsatellite locus HvJas.

It is known that most genotypes are susceptible or moderately resistant to net blotch (Raman *et al.*, 2003). This situation is represented in Figure 6, where the allele distribution of microsatellite HvJas is shown. While about 180 genotypes contain allele 154, less than 30 genotypes contain the same allele (156) as was found in resistant line CI739.

The distribution of alleles differs from a normal distribution. The creation of a core collection on the basis of random sampling may lead to considerable change in character of an alleles' frequency distribution. A lot of unique and rare alleles may be lost. Therefore it is necessary to take into account this fact and choose a strategy that conserves as much as possible of the unique or very rare alleles. Especially, alleles that could mark the presence of resistance to net blotch should not be omitted from the barley core collection.

Two classes of alleles—those specific to some region with high frequency; and those widespread with low frequency—are of most interest, especially as the former may be a source of local adaptation (Bataillon *et al.*, 1996). In spite of the fact that breeding processes in the past led to the loss of alleles, as shown by Roussel *et al.* (2005), breeders currently need new, interesting alleles as sources of disease resistance.

For the development of core collections and their sub-core collections for breeding

purposes, it is necessary to take into account all these facts. As genebank collections are increasingly searched for specific genotypes, or even structural features of a specific gene, the information about allele structure and frequency is very important. That is why molecular markers, including microsatellites, are powerful tools to help in the development of a really valuable barley core collection.

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Two non-gridded BAC libraries of barley for the identification of genes involved in basal resistance to cereal rusts

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Abstract

The construction of a bacterial artificial chromosome (BAC) library is an essential step towards the map-based cloning of a gene of interest. Although BAC libraries have already been developed for most cultivated crops like barley, the presence of the target gene can only be guaranteed in a BAC library from its specific genotype. This is *a fortiori* true for genes involved in disease resistance that undergo rapid duplication and diversification. Many major genes for hypersensitive resistance have already been cloned in plants. However, we still do not know what genes or gene families are responsible for the quantitative effect of minor genes, identified as quantitative trait loci (QTL), that are involved in basal (host or non-host) resistance to pathogens. Such genes could be responsible for an increased resistance (resistance factor) or for an increased susceptibility (susceptibility factor) to the attacking pathogen. We constructed two BAC libraries, one from cv. Vada that contains the resistance alleles of at least 18 QTLs for basal resistances to cereal rusts, and one from cv. SusPtrit that contains the susceptibility alleles of those QTLs. We adopted a non-gridded, or pooled, BAC library strategy that allows rapid, and low-cost generation and screening of genomic libraries. The pooled BAC library of Vada contains approximately 161 000 recombinant clones with an average insert size of 81 kbp,

and the library of SusPtrit with approximately 173 000 recombinant clones with an average insert size of 108 kbp. Giving a barley genome size estimated at 4551 Mbp and the representation of 14 microsatellite markers in the BAC pools, the genomic coverage of our BAC libraries was estimated at 2.9–5.1 and 4.1–6.9 genome-equivalents for Vada and SusPtrit, respectively. The two libraries are being screened with markers closely linked to *Rphq2*, a QTL for partial resistance to barley leaf rust.

Introduction

Cultivated barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world after wheat, maize and rice. Barley is also well-known as an extensively studied plant species in the field of genetics because it is a self-pollinated diploid crop ($2n = 2x = 14$ chromosomes), with a relatively short life cycle (100–120 days), simple growth requirements and exceptional adaptation to abiotic stresses (e.g. drought and salinity). Furthermore, important research resources have become available in recent years, such as thousands of morphological and quantitative traits and molecular markers mapped on the barley genome (Marcel *et al.*, 2007a; Rostoks *et al.*, 2005), a large number of expressed sequence tags (ESTs) (Thiel *et al.*, 2003; Varshney *et al.*, 2006; Nasuda *et al.*, 2005; Stein *et al.*, 2007), the Affymetrix 22K Barley1 GenChip probe array (Close

et al., 2004), and three bacterial artificial chromosome (BAC) libraries of genotypes Morex, Cebada Capa and Haruna Nijo (Yu *et al.*, 2000; Isidore *et al.*, 2005; Saisho *et al.*, 2007). Nevertheless, construction and organization of BAC libraries remains laborious and costly, especially for organisms with large and complex genomes like barley (4550–5300 Mbp, depending on the author) (Bennett and Smith, 1976; Arumuganthan and Earle, 1991; Jones and Pašakinskiene, 2005). In barley, about 200 000 clones with an average insert size of 120 kbp would be required to achieve a genome coverage of five genome-equivalents, which is needed for a more than 99% probability of recovering any specific sequence of interest. The inconveniences linked to the gridding, storage and maintenance of such a quantity of clones can be circumvented by the pooled library approach described by Ma *et al.* (2000) for wheat and Isidore *et al.* (2005) for barley. This approach consists of pooling several hundreds of clones together without the need for picking and storing individual clones. The pooled BAC library of barley cv. Cebada Capa was successfully used to establish a single contig of six BAC clones spanning 230 kbp at the *Rph7* locus on Chromosome 3H (Isidore *et al.*, 2005). This BAC library could be used as well to help in the construction of physical maps around any other target gene, but to isolate genes of interest in plants, it is often essential to construct BAC libraries from specific genotypes. Therefore, we decided to use the pooled strategy to develop two barley BAC libraries from cultivars Vada and SusPtrit, carrying the resistance and the susceptibility alleles, respectively, of quantitative trait loci (QTLs) for basal resistance to several cereal rust species.

Basal resistance refers to the early response of plants to a pathogen attack. Evidence suggests basal resistance to be a weak form of non-host resistance, resulting from the partial success of the microbe to

deal effectively with the defence that plant species mount against maladapted microbial intruders. At Wageningen University, we use the interaction between barley and cereal leaf rusts (*Puccinia* spp.) as a model plant-pathosystem to understand basal resistance mechanisms (Niks *et al.*, these proceedings). We pursued a positional cloning strategy to identify the coding sequence of *Rphq2*, a minor gene for quantitative resistance to barley leaf rust detected in the biparental mapping populations L94 × Vada and Vada × SusPtrit (Qi *et al.*, 1998; Jafary *et al.*, 2006), and in an association mapping population of 146 barley genotypes (Kraakman *et al.*, 2006). By substitution mapping, we could pinpoint gene *Rphq2* to an interval of about 0.1 cM (Marcel *et al.*, 2007b). The construction of two BAC libraries from genotypes Vada and SusPtrit will allow the construction of contigs containing the resistant and the susceptible alleles of the *Rphq2* leaf rust resistance locus that will lead us to the identification of candidate gene(s) to explain the effect of this QTL. This achievement will represent a significant step in our understanding of quantitative and durable disease resistance and will speed up the positional cloning of additional QTLs segregating between Vada and SusPtrit.

Material and methods

The methods for BAC library construction are essentially described in the manual prepared by Peterson *et al.*, (2000), with modifications proposed in subsequent publications (Allouis *et al.*, 2003; Chalhoub *et al.*, 2004; Isidore *et al.*, 2005).

Plant material

Two BAC libraries were constructed for the barley cv. Vada and a recently developed experimental barley line SusPtrit. Vada is an obsolete Dutch cultivar developed from the cross *Hordeum laevigatum* × Gold (Dros, 1957), which has a high level of

partial resistance to *P. hordei* (Neervoort and Parlevliet, 1978; Niks, 1982) and is immune to the wheat leaf rust fungus *P. triticina* (Niks, 1987; Niks *et al.*, 2000). SusPtrit was developed by a selection program for increased susceptibility to the non-host pathogen *P. triticina* (Atienza *et al.*, 2004). Interestingly, SusPtrit is highly susceptible to *P. hordei* and to *P. triticina*, but is partially or highly susceptible to some other heterologous rust fungal pathogens as well. QTL mapping in Vada \times SusPtrit identified at least 18 QTL regions involved in host and non-host resistances to eight rust species and isolates (Jafary *et al.*, 2006; Jafary, 2006).

Preparation of high-molecular-weight DNA

About 500 seeds each from SusPtrit and from Vada were sown in plastic trays and placed in a greenhouse compartment. Leaves were harvested twice from the same plants between two and four weeks after sowing, flash-frozen in liquid nitrogen and stored at -80°C . Before each harvest the plants were kept in the dark for 48 hours to promote the burst of chloroplasts and limit the presence of chloroplast DNA in the BAC libraries. About 40–50 g of leaves were used to extract nuclei and prepare high-molecular-weight (HMW) DNA following the protocol described by Peterson *et al.* (2000), with modifications (Allouis *et al.*, 2003; Chalhoub *et al.*, 2004). The modifications consisted mainly in the omission of polyvinyl pyrrolidone 40000 (PVP-40) and ascorbic acid from the sucrose-based extraction buffer (SEB) and from the lysis buffer. The quantity of PVP-40 was also lowered to 0.25% instead of 2% in the wash buffers (WB-A, -B, -C).

Partial digestion and size fractionation

Plugs of HMW DNA were prepared in 0.75% InCert® agarose (BMA) as described by Peterson *et al.* (2000). Twelve plugs macerated in the *Hind*III modified restriction

(H3M) buffer were digested at different enzyme concentrations: 0.2 – 0.5 – 1.0 – 2.0 – 5.0 – 7.5 – 10.0 – 15.0 – 20.0 and 40.0 units/mL. The partial digestion was performed in a 37°C water bath for exactly 20 minutes. The plugs were then migrated together on a 1.0% SeaKem® Gold agarose gel (Cambrex) in $0.25\times$ TBE in a CHEF-Mapper apparatus (Bio-Rad) with the following conditions: pulse linear ramping from 1 to 40 sec, angle 120° , potential 6.0 V/cm and 21 hr run time at 14°C . After electrophoresis, the partially digested DNA was subjected to a single size selection. The flanking lanes loaded with the lambda ladder PFGE marker (New England Biolabs) were removed from the gel and colored with ethidium bromide to indicate the location of the size ranges. For each library, five slices of agarose-containing DNA in the ranges 50–100 kbp (H0 fraction), 100–150 kbp (H1 fraction), 150–200 kbp (H2 fraction), 200–250 kbp (H3 fraction) and 250–300 kbp (H4 fraction) were excised from the gel and stored at 4°C in $1\times$ TAE buffer.

The HMW DNA was isolated by electroelution using a BioRad Electroelution system run for 1 hr at 60 mA and 90 V. From each agarose slice, 40 to 80 μL were recovered with a wide-bored tip.

Ligation and transformation

Depending on the fraction, the insert DNA was ligated into the pIndigoBAC vector (CalTech) prepared for high efficiency cloning with *Hind*III as described by Chalhoub *et al.* (2004) or into the commercial pIndigoBAC-5 vector (Epicentre Biotechnologies). Ligations were performed in a 50 μL reaction volume with 33 μL insert DNA (50–100 ng), 50 ng of vector DNA, 10 μL of $5\times$ reaction buffer and 5 units of T4 DNA ligase (Invitrogen). Ligation mixtures were incubated at 16°C overnight and dialysed for 90 min at 4°C , as described by Chalhoub *et al.* (2004), then 16 μL of de-salted ligation was mixed with 110 μL ElectroMax DH10B

electrocompetent cells (Invitrogen), and 17 μ L of the mixture were electroporated at 330 V, and the electroporations were pooled in a tube containing 2 mL SOC medium (Sambrook *et al.*, 1989) with 0.3 mL of 2M glucose.

Pooling of the BAC clones

Transformed cells diluted with SOC were incubated at 37°C under gentle agitation (220 rpm) for 60 min and plated on a selective Luria-Bertani (LB) medium with 12.5 μ g chloramphenicol, 0.55 mM IPTG (isopropylthio- β -D-galactoside) and 80 μ g/mL X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) (Sambrook *et al.*, 1989). A test plating of each transformation was performed in order to allow an average of about 1500 colonies per plate, as suggested by Isidore *et al.* (2005). The plates were incubated at 37°C for 20 hr. The clones were collected from each plate in 3 mL storage buffer (LB medium supplemented with 50% glycerol) and homogenized for 30 min under gentle agitation (220 rpm). Each of the resulting 3 mL cultures represents a pool. The pools were then aliquoted into four tubes, each corresponding to one copy of the library (copies A, B, C and D). Copies A and B are stored in a -80°C freezer at URGV (Evry, France) and copies C and D in another -80°C freezer at WAGUR Plant Breeding (Wageningen, the Netherlands).

Characterization of the BAC libraries

Twenty-four BAC clones were randomly selected from the fractions H1, H2 and H3 of each library (i.e. 72 BAC clones per library) and grown for 24 hr at 37°C in 1.5 mL LB medium containing 12.5 μ g chloramphenicol. The BAC DNA was extracted following an alkaline lysis procedure (Sambrook *et al.*, 1989) with ready-to-use buffers P1, P2 and P3 (QIAGEN) and digested overnight with *NorI* (New England Biolabs). Digested products were separated on a 1% SeaKem®

LE agarose gel (BMA) in 0.5 \times TBE in a CHEF-DR™ II apparatus (Bio-Rad) with the following pulsed field gel electrophoresis parameters: 200 V, 5–15 sec switch time, for 14.3 hr at 10°C. The insert sizes of selected BAC clones were estimated after comparison with a CHEF DNA size standard lambda ladder (Bio-Rad) run in the same gel.

BAC-pool DNA was isolated from a 250 μ L aliquot per pool from copy D of the libraries as described previously. The two barley BAC libraries were characterized for genome representation by PCR-screening of 46 pools per library with one microsatellite marker from each of the 14 barley chromosome arms. The markers were selected from the barley microsatellite consensus map of Varshney *et al.* (2007). The reverse primer of each microsatellite was labeled with IRDye-700 or IRDye-800 and the PCR-product visualized on a LICOR 4200 DNA sequencer (LICOR® Biosciences).

Results and discussion

We have constructed two non-gridded BAC libraries of barley from cv. Vada and from the experimental line SusPtrit. Vada contains the resistance allele of our target QTL for map-based cloning, *Rphq2*, but also for many other QTLs for partial and non-host resistances to homologous and heterologous rust species, while SusPtrit contains the susceptibility allele of those QTLs.

Construction and characterization of BAC libraries

The Vada BAC library derived from 6 different ligation reactions with pIndigoBAC vector and 8 different ligation reactions with pIndigoBAC-5 vector. The Vada library was organized in 116 pools named V1 to V116, each pool containing an average of 1435 clones (Table 1). The percentage of recombinant clones was estimated to be 96.8%, based on the count of blue and white

Table 1. Composition of the Vada BAC library.

Size selection range (kbp)	Pool number	White colonies ¹	Blue colonies ¹	Insert size ² (kbp)	Coverage ³ (Mbp)
H0: 50–100	3	225	0	—*	—
H1: 100–150	41	1636	45	98.3	6593.6
H2: 150–200	35	1724	79	67.4	4066.9
H3: 200–250	34	963	20	71.9	2354.1
H4: 250–300	3	97	1	—*	—
Total	116	1389	46	81.2	13014.6

¹ Estimation of the average number of colonies per pool. ² Average insert size estimated with 24 randomly selected BAC clones per fraction. * = because of their low number, the clones from fractions H0 and H4 had to be ignored. ³ Calculated with the estimated number of white colonies and their average insert size.

colonies per plate. Thus, the library consists of approximately 161 000 recombinant clones. Surprisingly, the average size of inserts decreased from 98.3 kbp for selected fraction H1 to 71.9 kbp for selected fraction H3 (Table 1). This could be due to the fact that only one size selection was performed instead of two or three as recommended by previous protocols (Peterson *et al.*, 2000; Chalhoub *et al.*, 2004). Indeed, if the first size selection gets rid of most of the DNA of less than 100 kbp, some small DNA molecules do get trapped by the longer DNA molecules, and this is especially true when the DNA concentration in the plugs is relatively high (Peterson *et al.*, 2000). Repeated sizing cycles improve the average insert size but may also be followed by a severe drop in transformation efficiency (Cai *et al.*, 1995; Chalhoub *et al.*, 2004). The finding of a balance between those two parameters depends on the final use of the BAC library. If a BAC library is to be used for genome-wide physical mapping and genome sequencing, then maximizing the average size of inserts is essential to limit walking. However, if a BAC library is to be used for positional cloning of genes that have already been confined to a very small interval, then having a large number of clones is more important in order to increase the chance of finding the gene of interest.

The principal aim of our BAC libraries is to isolate genes involved in basal resistance to cereal rusts after their high-resolution genetic mapping. Consequently, our priority was to obtain a large number of clones at low cost. Nonetheless, the average insert size over the complete Vada BAC library is of 81 kbp, with individual clones ranging from 18 to 209 kbp (Figure 1), which is comparable with many plant BAC libraries successfully used for the positional cloning of genes of interest (Yang *et al.*, 2001; Yoo *et al.*, 2001).

The SusPtrit BAC library derived from 6 different ligation reactions with pIndigoBAC vector and 7 different ligation reactions with pIndigoBAC-5 vector. The SusPtrit library was organized in 110 pools named S1 to S110, each pool containing an average of 1606 clones (Table 2). The percentage of recombinant clones was estimated to be 97.9%, based on the count of blue and white colonies per plate. Thus, the library consists of approximately 173 000 recombinants clones. In contrast to the Vada library, the average size of SusPtrit inserts increased from 106.7 kbp for selected fraction H1 to 140.6 kbp for selected fraction H3. It may be due to a lower DNA concentration in SusPtrit than it was for Vada in the size fractionation gel, resulting in a lower amount of small DNA molecules trapped by the longer ones. For

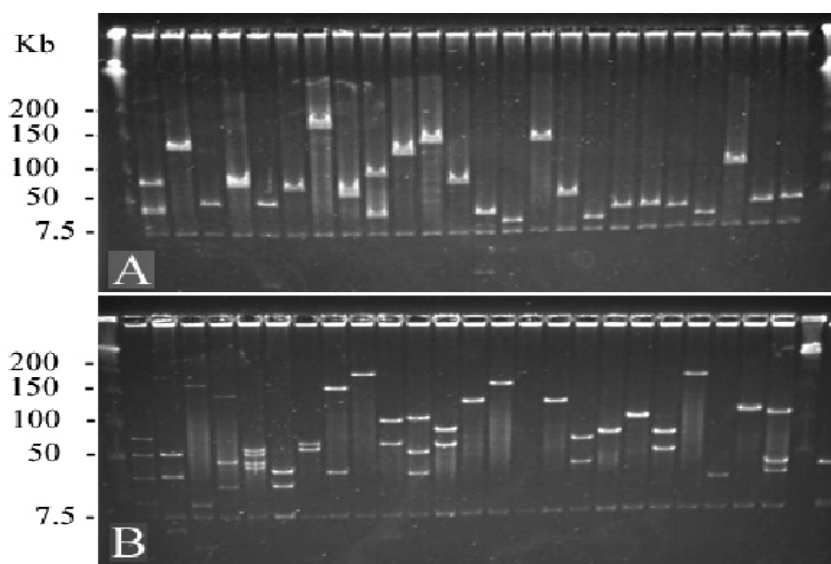


Figure 1. Analysis of 24 randomly selected barley BAC clones from Vada, fraction VH2 (A), and from SusPtrit, fraction SH3 (B), on an ethidium bromide-stained CHEF gel (5–15 sec switch time, 14.3 hr) showing insert DNA above and below the common 7.5 kbp pIndigoBAC or pIndigoBAC-5 vector bands.

Table 2. Composition of the SusPtrit BAC library.

Size selection range (kbp)	Pool number	White colonies ¹	Blue colonies ¹	Insert size ² (kbp)	Coverage ³ (Mbp)
H0: 50–100	9	3316	3	83.0*	2477.1
H1: 100–150	37	1716	43	106.7	6775.8
H2: 150–200	38	1704	48	110.5	7152.9
H3: 200–250	26	571	12	140.6	2087.7
H4: 250–300	–	–	–	–	–
Total	110	1572	34	108.0	18493.5

¹ Estimation of the average number of colonies per pool. ² Average insert size estimated with 24 randomly selected BAC clones per fraction, and * corresponds to the insert size of a single BAC clone isolated from fraction H0. ³ Calculated with the estimated number of white colonies and their average insert size.

this reason, the average insert size over the complete SusPtrit BAC library was 108 kbp, with individual clones ranging from 33 to 274 kbp (Figure 1; Table 2). This is 27 kbp more in average for SusPtrit inserts than for Vada inserts, making the SusPtrit BAC library comparable to previously published

barley BAC libraries (Yu *et al.*, 2000; Saisho *et al.*, 2007).

In general, DNA fractions H1, H2 and H3 have been used to construct the libraries. The percentage of non-recombinant clones (blue colonies) was lower with pIndigoBAC vector than with pIndigoBAC-5 vector in

both libraries, but remain in all cases below 4%. Such a high proportion of recombinant clones over the non-recombinant ones reduces the problem of a possible bias in the libraries caused by faster growth of non-recombinant clones during the short amplification step (Isidore *et al.*, 2005). More revealing, the average size of inserts ligated into pIndigoBAC vector was always higher than the average size of inserts ligated into pIndigoBAC-5 vector. In fractions H1, H2 and H3 of the Vada BAC library, the inserts were respectively 13, 52 and 8 kbp longer in pIndigoBAC. Similarly, in fractions H1, H2 and H3 of the SusPtrit BAC library, the inserts were respectively 4, 12 and 31 kbp longer in pIndigoBAC. This observation demonstrates the quality of the pIndigoBAC vector prepared following the 'single tube vector' method described by Chalhoub *et al.* (2004). Indeed, another barley BAC library, of cv. Cebada Capa, constructed with vectors prepared following the same method, reached an average insert size of 140 kbp covering 6 genome-equivalents (Isidore *et al.*, 2005). Based on a haploid barley genome size of 4551 Mbp (Jones and Pašakinskienė 2005) and on the genome coverage of each fraction of the libraries (Tables 1 and 2), we estimated that the coverage of the Vada and SusPtrit BAC libraries are approximately 2.9 and 4.1 genome-equivalents, respectively. Together, the libraries cover 6.9 genome-equivalents that allow for a probability greater than 99% of recovering any specific sequence from the barley genome (Clarke and Carbon, 1976).

Genome representation of the BAC libraries

To check the genome representation of the libraries, we screened 46 pools of Vada (V1 to V46) and 46 pools of SusPtrit (S1 to S46), corresponding approximately to 1.5 and 2.1 genome-equivalents, respectively, with 14 microsatellite markers, each representing a chromosome arm of barley (Table 3). The microsatellite markers were mapped on a

consensus map of barley (Varshney *et al.*, 2007). The priority was to select the most robust markers that were also polymorphic between Vada and SusPtrit. Vada and SusPtrit had the same allele for only two microsatellites, GBMS062 and GBM1482 (Table 3). The number of positive pools was determined (Table 3) by counting the number of pools displaying a band of similar size to the one of the parental genomic DNA run on the same gel. None of the bands amplified in a BAC pool from one genotype had the size of the allele from the other genotype, indicating that contamination of one library with clones from the other library is unlikely.

An average of 2.7 positive pools per microsatellite marker was obtained for the Vada library and an average of 3.5 positive pools for the SusPtrit library (Table 3). All markers were represented at least once in the 46 pools of the SusPtrit library and only two markers were not represented in the 46 pools of the Vada library, indicating that the overall barley genome is well represented in our BAC libraries. Based on the average representation of the 14 microsatellite markers in 46 pools per library, we estimated that the total coverage of the Vada and SusPtrit BAC libraries are 5.1 and 6.9 genome-equivalents, respectively. The discrepancy between barley genome coverage estimated by BAC clones insert sizes (i.e. 2.9× and 4.1×) and by microsatellite markers (i.e. 5.1× and 6.9×) may be due to an underestimation of the average size of the BAC clones or to an overestimation of the number of positive pools per microsatellite. As is often observed in monocots (Peterson *et al.*, 2000), several bands were obtained after *NotI* restriction of BAC clones (Figure 1), making possible the underestimation of the insert size from some clones. At the same time, half of the microsatellite markers used for screening the BAC pools derived from barley ESTs and genes (i.e. EST-SSR markers), implying that a marker amplifying a member from a gene family may in some cases also amplify

Table 3. PCR-based screening of the Vada and SusPtrit barley BAC libraries with microsatellite markers representing each chromosome arm of barley on a subset of 46 pools per library (representing approximately 1.5 and 2.1 barley genome-equivalents).

Barley chrom.	Position (cM) ¹	Microsatellite	Vada allele ² (bp)	SusPtrit allele ² (bp)	No. pools Vada ³	No. pools SusPtrit ³
1HS	20.5	GBMS062	127	127	2	8
1HL	73.7	Bmac0032	–	220	1	2
2HS	31.0	HVM36	126	108	6	4
2HL	95.7	GBM1062	215	219	3	2
3HS	23.3	scssr10559	210	216	3	4
3HL	139.4	HVM62	260	250	2	4
4HS	36.8	GBM1482	210	210	12	5
4HL	115.9	GBM1015	232	220	0	1
5HS	18.6	GBM1176	–	300	1	4
5HL	86.9	Bmag0223	174	170	1	5
6HS	7.2	Bmac0316	169	165	1	3
6HL	127.7	GBM1087	201	198	4	2
7HS	20.6	Bmag0007	200	204	2	4
7HL	85.8	GBM1359	151	145	0	1

¹ Position of the corresponding microsatellite marker on a consensus linkage map of barley (Varshney *et al.*, 2007). ² Approximate size of the allele amplified on Vada and SusPtrit genomic DNA. ³ Number of positive pools per library.

other genes from the same family. Indeed, the pressure of a primer to anneal on a similar but not identical sequence is much stronger on BAC DNA than it is on full genomic DNA. One example might be the marker GBM1482 that is overrepresented in the Vada library compared with the other markers. GBM1482 was developed on a glutamine synthetase gene that is a member of a small gene family in plants.

Conclusion

The large number of clones necessary for a barley BAC library makes it more difficult to exploit usual BAC techniques in this species. The main reasons are due to the gridding of the library, which is time consuming and expensive, and requires the use of robotics and large freezer spaces. Following a pooled

BAC library strategy (Ma *et al.*, 2000; Isidore *et al.*, 2005) and the technical improvements proposed by Chalhoub *et al.* (2004), it has been possible for a single person working for six weeks to construct the two libraries of Vada and SusPtrit (from the isolation of nuclei to the obtaining of ready-to-screen BAC pools). One copy of the 216 BAC pools constructed in this study occupies a space of 3 dm³ (three standard boxes) in a -80 °C freezer, while if gridded, one copy of the 334 000 clones constituting those BAC pools would occupy a space of 203 dm³ (870×384-well microtitre plates) in one or more -80°C freezers.

A peculiarity of our BAC libraries is that all the BAC pools are characterized by the size fraction from which the HMW DNA is derived, the vector used in the ligation reaction and an estimated number of clones.

Then, if several pools are positive while screening the library with PCR markers linked to a gene of interest, it is possible to select the pool having larger inserts or fewer clones in order to increase the chance of picking the right one. Our BAC libraries have been screened with PCR-markers linked to our target QTL for positional cloning, *Rphq2* (Marcel *et al.*, 2007b). Seven positive BAC pools were picked with an average 1.5-fold coverage (22 265 clones picked) and 8 single clones were recovered and successfully used to construct a contig encompassing our QTL-region. Those results demonstrate the effectiveness of our BAC libraries in the identification of genes involved in basal resistance to cereal rusts. Pools and clones of each library are available upon request to the authors (R.E. Niks at Wageningen University and B. Chalhoub at URGV-INRA).

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Search for resistance against *Blumeria graminis* f.sp. *hordei* in barley landraces from Palestinian Territories

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Abstract

Eleven winter barley cultivars were screened for resistance to powdery mildew in the field and under controlled conditions and two types of resistance were identified. Three accessions showed low infection type (IT) at seedling stage and low disease severity (DS) in the adult plant stage. Six other lines showed low DS and low infection frequency (IF) with no macroscopically visible necrosis. These resistant accessions were selected and selfed for future studies.

Introduction

Powdery mildew caused by *Blumeria* [syn *Erysiphe*] *graminis* f.sp. *hordei* is one of the most important and highly variable foliar diseases of barley (*Hordeum vulgare* L.), causing severe losses and quality reduction, especially for production of malting barley (Balkema-Boomstra and Mastebroek, 1995; Czembor, 2001). Yield losses due to powdery mildew can reach 30%. Early infection reduces tillering, while late infection of the upper leaves and of the spike can heavily reduce grain yield (Scott and Griffiths, 1980).

Powdery mildew on barley can be controlled by the use of fungicides and resistant cultivars. Breeding for resistance is a cheap alternative to reduce the loss in yield caused by powdery mildew. Based on the gene-for-gene hypothesis of Flor (1955), many race-specific powdery mildew resistance genes from different origins have

been recognized in barley landraces and from wild relatives (Xu and Kasha, 1992; Jahoor and Fischbeck, 1993; Jørgensen and Jensen, 1997; Czembor and Czembor, 2000), but most of them have already been overcome by new virulent strains (Dreiseitl and Jorgensen, 2000; Dreiseitl and Bockelman, 2003), thus reinforcing the need to continuously search for new sources of resistance.

Many sources of resistance to powdery mildew in cultivated varieties and in their wild relatives have been reported in the past (Xu and Kasha, 1992; Mastebroek and Balkema-Boomstra, 1995; Schonfeld *et al.*, 1996; Czembor and Johnson, 1999).

Powdery mildew has been present in barley fields in Palestine for many years. There is limited information on the genetics of powdery mildew in Palestinian lines and cultivars. The objectives of the present study were to evaluate the resistance of some Palestinian barley cultivars in the greenhouse and the field.

Materials and methods

Plant material

Seed samples of 11 barley landraces from the West Bank, Palestine, were kindly provided by the National Agricultural Research Centre (NARC), Jenin, Palestine.

Inoculum

Isolate (TU-07) of *B. graminis* f. sp. *hordei* (virulence/avirulence factors *Mla1*, *a3*, *a7*, *a8*,

a9, a10, a12, a22, a23, k, p, g, La, h/a6, a14, a13, at, o5) collected at Tulkarm, Palestine, was used in the experiment. The isolate was maintained and increased on young seedlings of the cultivar Pallas. The virulence spectrum of the isolate was determined using the Pallas isolines differential set of powdery mildew (Kolster *et al.*, 1986).

Seedlings studies

About 15 seeds per accession were sown in 7×7×11 cm boxes. Eleven days after sowing, when the primary leaf was fully expanded, 40 mm of a central leaf segment was excised from each seedling, and placed adaxial surface up in plastic trays filled with 0.6% agar and 125 ppm benzimidazole. In each tray, segments of 11 accessions were randomly fixed, (4 segments per line), in three replicates. One day before using the inoculum, heavily infected plants were shaken to remove ageing conidia to ensure a supply of vigorous young spores. Inoculation was made by blowing spores from the infected plants over the leaf segments. A glass slide was placed between trays to monitor inoculum density, which was adjusted to give approximately 20 conidia per mm² (Haugaard *et al.*, 2002). After inoculation, Petri dishes were transferred to a growth chamber at 18–20°C and incubated in darkness for 12 h. They were then transferred to a growth chamber with fluorescent lighting (12 h light/12 h dark) and 18–20°C (Edwards, 1993).

Infection type (IT) was recorded five days after inoculation, following the 0–4 scale of Mains and Dietz (1930) where: 0 = no visible signs of infection; 1 = brown necrotic lesions with little or no mycelial development; 2 = some necrosis and chlorosis with slight to moderate mycelial development; 3 = chlorosis with moderate mycelial development; and 4 = abundant mycelial development with little or no necrosis or chlorosis. This scale was broadened by including an additional symbol 0(4) for IT characterized by sparse

small colonies originating from the stomatal subsidiary cells (Czembor, 2002). Infection frequency (IF) was calculated as number of powdery mildew colonies per cm².

Field experiment

Field testing was performed at the experimental farm of the faculty of agriculture at Tulkarm, during the 2006–2007 growing season. Accessions were sown in November 2006 using a randomized block design with three replications. Each accession was represented by 25–30 seeds in a single row, 1 m long, per replicate. A spreader row of the very susceptible line Pallas was sown in the alleyways, perpendicular to the accessions.

A powdery mildew epidemic was initiated by artificial inoculation of the spreader rows at growth stage GS 43 (Zadoks *et al.*, 1974) by shaking heavily infected Pallas plants. Disease severity (DS) was estimated three times during the growing season as the percentage of leaf area covered by the powdery mildew colonies.

Data analysis

Analysis of variance (ANOVA) was calculated by using PROC GLM in SAS software (SAS Institute, 1988). Comparisons between lines were made using the Duncan test.

Results

Table 1 shows the reaction of the 11 accessions under controlled conditions and in the field. The collection showed reactions ranging from completely resistance to completely susceptible (IT 0 to 4), which frequently differed between the cultivars. In the seedling tests, 8 of the accessions (72.7%) displayed compatible interaction (IT ≥ 3), whereas the remaining 3 accessions (27.3%) showed complete resistance (IT = 0–0(4)). Six accessions (54.5%) showed low disease severity.

Table 1. Macroscopic components of resistance to powdery mildew (*Blumeria graminis* f.sp. *hordei*) isolate TU-07 in selected accessions of barley landraces from the Palestinian Territories at the seedling stage and in the field.

Cultivar or Line	Seedlings in growth chamber			Adult plants in the field
	IT ¹	DS ²	IF ³	DS ²
PAL001	3	25.0bc ⁴	46.7b	55.6ab
PAL002	4	73.7a	81.7a	50.3abc
PAL003	0(4)	0.10d	3.0c	9.8de
PAL004	3	21.7c	48.3b	40.3bc
PAL005	4	30.0bc	38.3b	45.8abc
PAL006	4	27.7bc	43.3b	41.7bc
PAL007	4	23.3bc	35.7b	31.4cd
PAL008	4	76.7a	77.3a	49.6abc
PAL009	4	31.7b	49.0b	46.9abc
PAL010	0(4)	0.10d	2.0c	12.3de
PAL011	0	0.0d	0.0c	5.2e
Pallas	4	75.0a	91.0a	67.8a

¹ IT = Infection type on a scale of 0–4 (Mains and Dietz, 1930, as modified by Czembor, 2002). ² DS = Disease Severity estimated as leaf area covered by powdery mildew colonies. ³ IF = Infection frequency calculated as the number of uredia/cm². ⁴ Data with the same letter per column are not statistically different (Duncan-test, $P \leq 0.05$).

Under field conditions, susceptibility was common in the collection. Five accessions (45.5%) showed high levels of DS (as high as the check Pallas). The remaining six accessions (54.5%) showed a lower DS, which was particularly low in three accessions (PAL003, PAL010 and PAL011).

Discussion

Barley breeders are seeking gene pools from which new genes can be introduced into existing cultivars in order to improve their resistance to powdery mildew. Barley landraces, especially those originating from centers of origin for cultivated barley,

constitute such a gene pool (Jørgensen and Jensen, 1997; Czembor and Johnston, 1999).

Among the 11 Palestinian accessions tested, 3 were completely resistant to powdery mildew in the growth chamber and they showed high levels of partial resistance in the field (Table 1). The different resistant ITs of the cultivars may be explained by the presence of different resistance genes.

Seedling tests do not necessary predict adult plant resistance and field performance of the selected resistant accessions, but are considered effective and sufficient to postulate race-specific resistance genes and the identification of levels of partial resistance (Dreiseitl and Jørgensen, 2000; Backes *et al.*, 1996).

The results of IT at the seedling stage in the growth chamber and also the DS level in the field varied from almost completely resistant to completely susceptible. This may be due to different virulence spectrum of the mildew between the growth chamber and the field, or to differences in growth stage (Masterbroek *et al.*, 1995). This study showed that barley landraces from Palestinian Territories are a valuable source of resistance to powdery mildew. However, the presence of landraces with resistance to powdery mildew is less than observed in other studies (Czembor, 2002).

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Identification of molecular markers associated with powdery mildew and barley grass stripe rust resistance

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Abstract

Powdery mildew (PM) is an important foliar pathogen of barley. Significant yield losses have been recorded worldwide. Barley grass stripe rust (BGYR) is a potential threat to barley production, as it can cause significant rusting on certain barley lines, such as Skiff and its derivatives, and some Chinese lines. The discovery of new resistance genes and linked molecular markers can be a great help to plant breeders in improving the resistance of barley to these diseases. A doubled-haploid (DH) population produced from Yerong and Franklin was used in this study. PM tolerance was evaluated in both field and glasshouse, and BGYR resistance was evaluated in the field. Both parents showed medium tolerance to medium susceptibility to PM, and resistance to BGYR. However, the DH lines showed a much broader range of resistance to both diseases, from very tolerant to very susceptible. One major QTL was found for PM on 1H, which contributed to more than 56% of the variation. Another significant QTL was found on 4H, but contributing only 4% of the variation. For BGYR, the DH population showed the segregation of 140 non-affected and 36 affected lines, which is close to 3:1. Three QTLs were found for BGYR resistance, with two on 5H and one on 7H. The molecular markers identified in this study have potential for use in marker-assisted selection and pyramiding of genes for resistance to PM and BGYR.

Introduction

Powdery mildew (PM) is an important foliar pathogen of barley. The yield reduction can reach 26% when severely infected (Mathre, 1997). The use of resistant barley varieties has proved a most effective and economical means to reduce disease severity and to prevent yield losses. It was proven that resistance in the seedling stage was determined largely by one or two major genes (Dreiseitl, 2007) but some quantitative trait loci (QTLs) are also found to have PM resistance (Yun *et al.*, 2005). Most of the major resistance genes deployed in commercially grown cultivars had a short effective life, with the exception of the *mlo* resistance gene (Hovmøller *et al.*, 2000). The discovery of new genes is one of the major objectives in germplasm development.

Barley grass stripe rust (BGYR) is a potential threat to barley production, as it can cause significant rusting on certain barley lines, such as Skiff and its derivatives, and some Chinese lines. In the past there have been several investigations on barley stripe rust, but very few reports on the genetics of the resistance to BGYR.

In the present study, we attempt to identify new gene(s) for resistance to powdery mildew and stripe rust in a population of 177 doubled-haploid (DH) lines derived from a Franklin \times Yerong cross, to identify closely linked molecular markers that can be used for pyramiding and combining resistance genes.

Material and methods

Plant material

The population used for mapping consisted of 177 doubled-haploid lines from a barley cross between Yerong and Franklin. Yerong is an Australian six-row feed variety and Franklin is a two-row malting barley.

Phenotypic evaluation of disease symptoms

Both powdery mildew and barley grass stripe rust resistances were evaluated in the 2006–2007 growing season in the birdcage at Mt Pleasant laboratories in Launceston, Tasmania, Australia. For powdery mildew, we used a 0–5 scoring scale, where 0 = very tolerant and 5 = very susceptible. For BGYR the score was 0 = very tolerant and 3 = very susceptible. Each line or variety was grown in a 2-m row with 0.4 m between rows. All agronomic management methods, including fertilization and weed control, were as in local practice. The experiments were arranged as a randomized complete block design with two replications.

QTL analysis

A genetic linkage map was constructed using 496 DArT and 28 microsatellite markers (Wenzl *et al.*, 2006). QTL analysis was performed using the software package MapQTL5.0 (Van Ooijen and Kyazma, 2004).

Results and discussion

PM resistance of the DH population

Both parents showed medium tolerance to PM (rated 2–3). However, the DH lines showed vast segregation for PM tolerance, from very tolerant to very susceptible (Figure 1). More tolerant lines were found. The numbers of lines of tolerant (0–0.5), medium tolerance (1–1.5), medium susceptible (2–2.5) and susceptible (3–5) are 62, 42, 31 and 41, respectively. It is suggested that at least two major genes control the tolerance. The ratio between the tolerant

and the medium to susceptible lines is close to 3:1 ($\chi^2 = 0.27$, $P > 0.05$). Very tolerant lines contain both tolerance genes while medium tolerance and medium susceptible lines contain only one of the major genes.

BGYR resistance of the DH population

Both parent showed very good resistance to BGYR. The DH lines derived from these two parents showed wide segregation in resistance. Unlike the segregation ratio for PM, a relatively small number of DH lines were susceptible (Figure 2). Of the 176 lines, 140 were resistant. The ratio of resistant lines to susceptible was close to 3:1 ($\chi^2 = 1.70$, $P > 0.05$), indicating two major genes involved in the resistance. Both genes have similar effect (both very resistant). Among the susceptible lines, segregation was found for susceptibility, possibly indicating that another minor gene for tolerance existed.

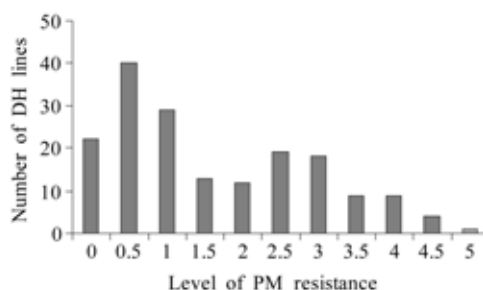


Figure 1. Distribution of DH lines with different PM infection levels.

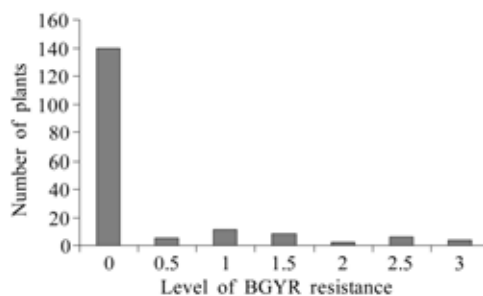


Figure 2. Distribution of DH lines with different BGYR infection levels.

QTL analysis

Two significant QTLs were identified for PM resistance (Figure 3). The major one is located at 1H and explains 56% of the genetic variation. The exact position of this QTL is similar to those previously reported for the series of *mLa* resistance genes (Wei *et al.*, 1999). In contrast, the other significant QTL, in 4H, only explains 4% of the genetic variation. Since there are not many markers in this region, it is possible that closer markers are missing, leading to less variation explained by the existing markers.

Three significant QTLs were identified for BGYR resistance: one on 7H and two on 5H (Figure 4). Among the three significant QTLs, the one at 7H explained 16.7% of the genetic variation, while the major one at 5H explained 11.1% of the genetic variation. The third one showed much less significance, only explaining 5.6% of the variation. As observed from the segregation of the resistance, only

two major genes existed for the resistance. It is possible that other minor genes were involved, leading to the different levels of susceptibility (0.5–3.0). This hypothesis was confirmed by the QTL analysis using two groups of DH lines, tolerant (0) and susceptible (0.5–3.0), with only two QTLs, BGYR-1 and BGYR-2, being significant.

In conclusion, at least one PM resistance gene existed in both Yerong and Franklin. Both showed medium tolerance. The combination of these two genes could lead to much greater tolerance to PM. In contrast, each parent had one resistant gene for BGYR but located on different chromosomes, one at 5H and the other one at 7H. The lines had either one or both genes tolerant to BGYR. There may possibly be another minor gene for BGYR resistance. When both major tolerance genes do not exist, the minor gene determines the level of BGYR infection.

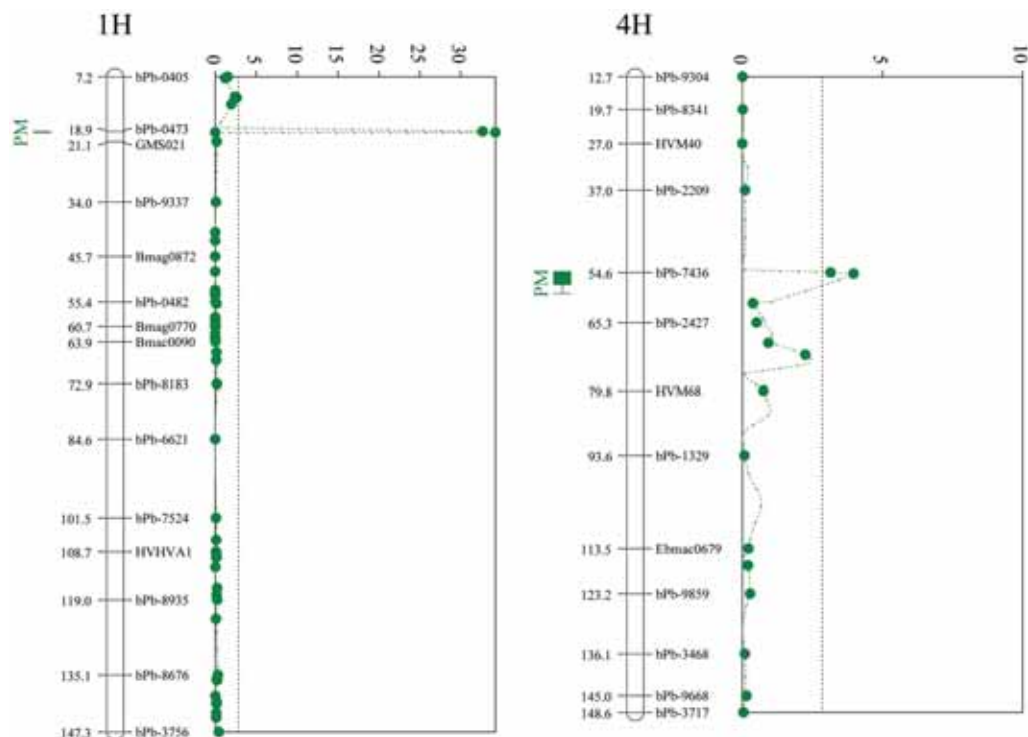


Figure 3. QTLs for PM resistance located on 1H and 4H.

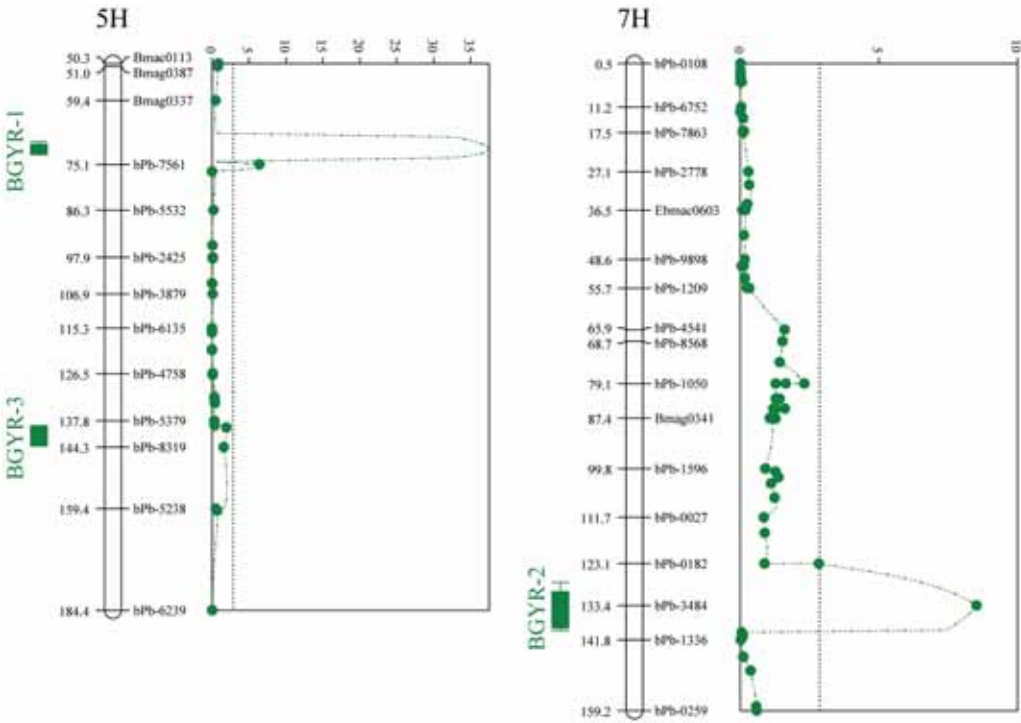


Figure 4. QTLs for BGYR resistance located on 5H and 7H.

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A barley homologue to an *Arabidopsis* susceptibility gene

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Abstract

Information available on completely sequenced genomes and expressed sequence tags (EST) from *Arabidopsis* and rice can be used to design primers to screen rapidly for potential candidate genes. PMR6, a pectate lyase-like gene, is required for powdery mildew susceptibility in *Arabidopsis thaliana*. As no genes for disease susceptibility have been recorded in barley (*Hordeum vulgare* L), we aimed to search for a PMR6 homologue in barley and rice. For this purpose, we blasted the coding sequence of PMR6 of the *Arabidopsis* against genomic rice sequences and barley ESTs. The results with the highest scores in barley and rice were TC148960 and Os04g0137100, respectively, both with predicted pectate lyase activity. We revealed the complete genomic sequence in barley by sequencing the PCR product from genomic barley DNA. With application of TILLING (Targeting Induced Local Lesions in Genomes) on PMR6 we found three mutations that were not confirmed with sequencing.

Introduction

The information available in genomic databases has become a powerful tool for biological research. The completion of genomic sequences for many organisms, and the identification of their genes, has led to an improved opportunity to identify homologues

between and within organisms. Powdery mildew is one of the most significant plant diseases, causing considerable yield losses. In barley, the disease is caused by the obligate biotrophic fungus *Blumeria* [syn *Erysiphe*] *graminis* f.sp. *hordei* (Jarosch *et al.*, 2003). The relationship between this pathogen and host plants seems to be controlled by genes that are required for pathogen growth, i.e. genes for susceptibility. Mutation in such host susceptibility genes, provided that the genes are not redundant or essential for host survival, can cause reduction in pathogen growth. Such mutants showed in the disease-resistant phenotype in *Arabidopsis* (Panstruga, 2003; Schulze-Lefert and Vogel, 2000). There is good knowledge of the plant genes associated with resistance, but only little is known about the plant genes required for susceptibility. Therefore, it is promising for future resistance sources to identify these genes. Using the model plant *Arabidopsis*, genes required for susceptibility have been identified and knocked out by mutational experiments. The isolated mutants have been reported to possess enhanced disease resistance to pathogens (Vogel and Somerville, 2000). PMR6, a pectate lyase-like gene is one of the genes required for powdery mildew susceptibility in *Arabidopsis*. Thus, *pmr6* resistance represents a novel form of disease resistance based on the loss of a gene required during a compatible interaction rather than the activation of known host

defence pathways (Vogel *et al.*, 2002). Since no genes for disease susceptibility have been recorded in barley, PMR6 identified in *Arabidopsis* controlling susceptibility was used to search for orthologous sequences in the barley EST database.

Materials and methods

For gene structures, gene sequences, ESTs and proteins, publicly available databases such as <http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi> were used. BLAST programs on TIGR barley (<http://tigrblast.tigr.org/tgi/>) and NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) were used for searching for orthologous sequences in barley EST databases and complete rice genome sequences. Primers development has been carried out using the Primer3 website (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi>). DNA was extracted according to the CTAB-protocol (Saghai-Marooof *et al.*, 1995). The genomic sequence in barley was revealed

by sequencing the PCR product. Alignment analysis between barley and rice sequences was done using Mega3 program software (Kumar *et al.*, 2004). Tilling was done based on Henikoff and Comai (2003), with minor modifications (Figure 1).

Results and discussion

Gene sequences identified in *Arabidopsis* controlling susceptibility were found with the aid of cross-database search and retrieval system ENTREZ. The coding sequence of this gene was used to search for orthologous sequences in barley EST databases. The EST with the highest score of probability identified by blast search was TC148960. The TC148960 EST in barley is predicted to code for an enzyme with pectate lyase activity. The sequence of the barley EST was again blasted against rice DNA sequences (BLASTn) as well as with the rice protein database (BLASTx). The highest score was found with Os04g0137100, a gene that is also

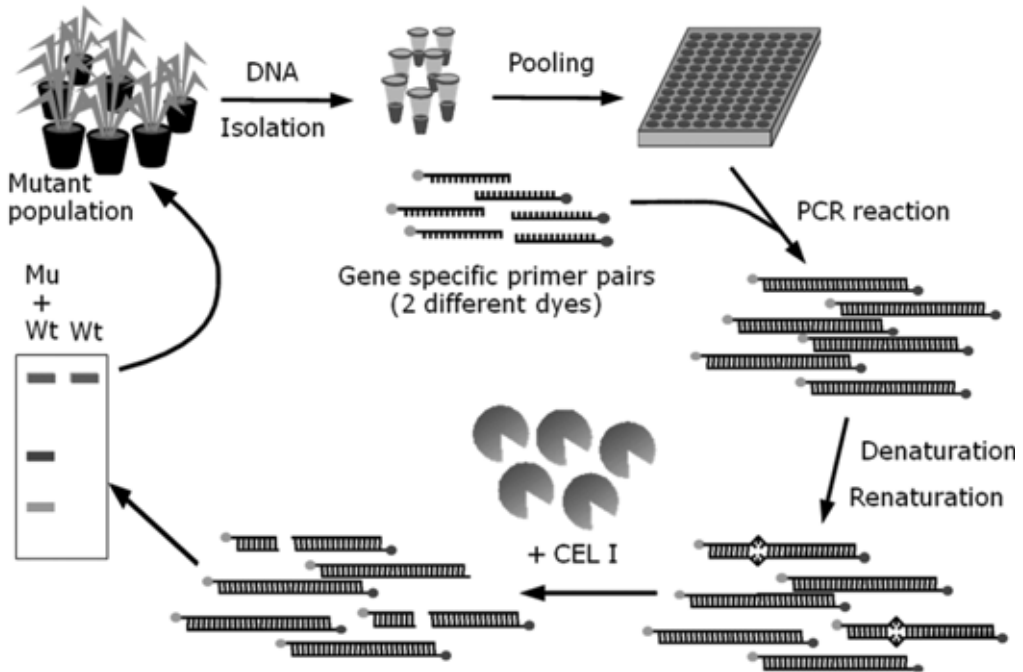


Figure 1. Steps in TILLING.

PMR6 structure

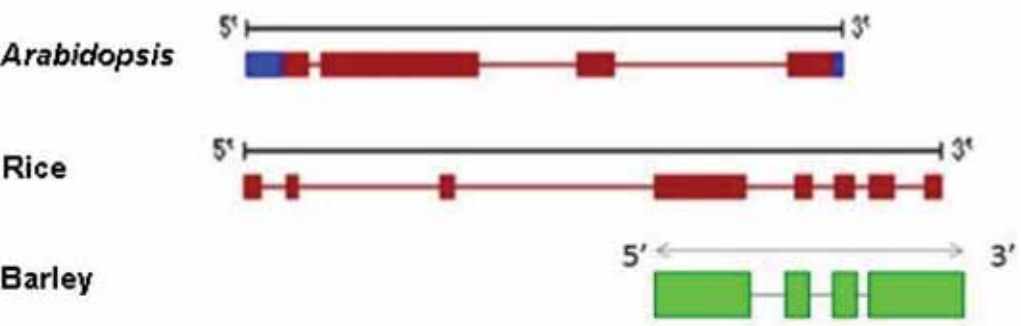


Figure 2. The *PMR6* gene structure in barley compared with *Arabidopsis* and rice plants.

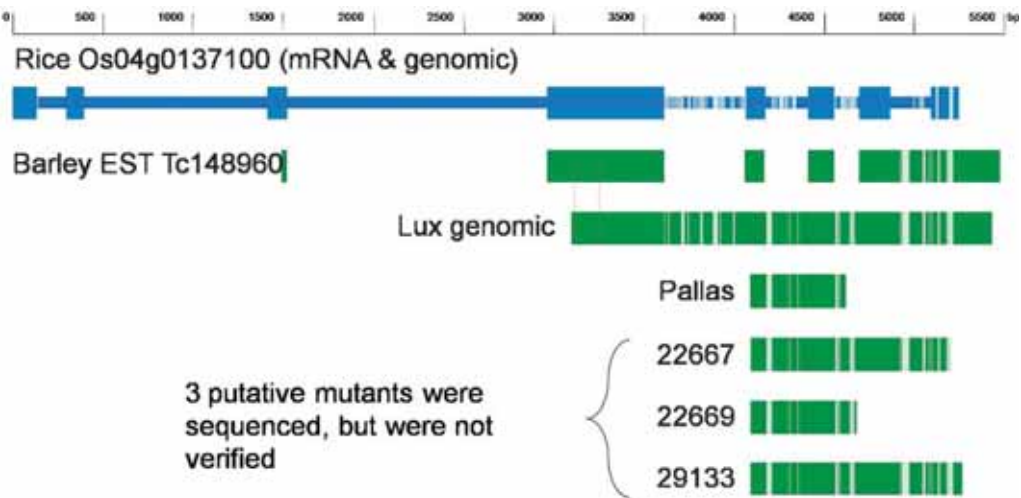


Figure 3. The *PMR6* gene structure in barley compared with *Arabidopsis* and rice plants.

predicted to code for pectate lyase activity in rice. The results showed that the BLAST searches can be used in identifying functional relationships between sequences (Figure 2).

Comparing the barley EST sequences and rice genomic sequences revealed the most likely positions of introns b and primers were designed to amplify the intron sequences. After sequencing of those PCR products, the position of intron and exon were identified in barley (Figure 3).

Our results confirm that ESTs sequences are valuable data for gene discovery, especially for plant species with large genomes that have not been fully sequenced.

The PCR from lines with detected mutations were sequenced and analyzed. Three mutations were detected in the pools. These three positive pools were confirmed by screening the ten individuals (Figure 4). Unfortunately, we were not been able to confirm those mutations with sequencing.

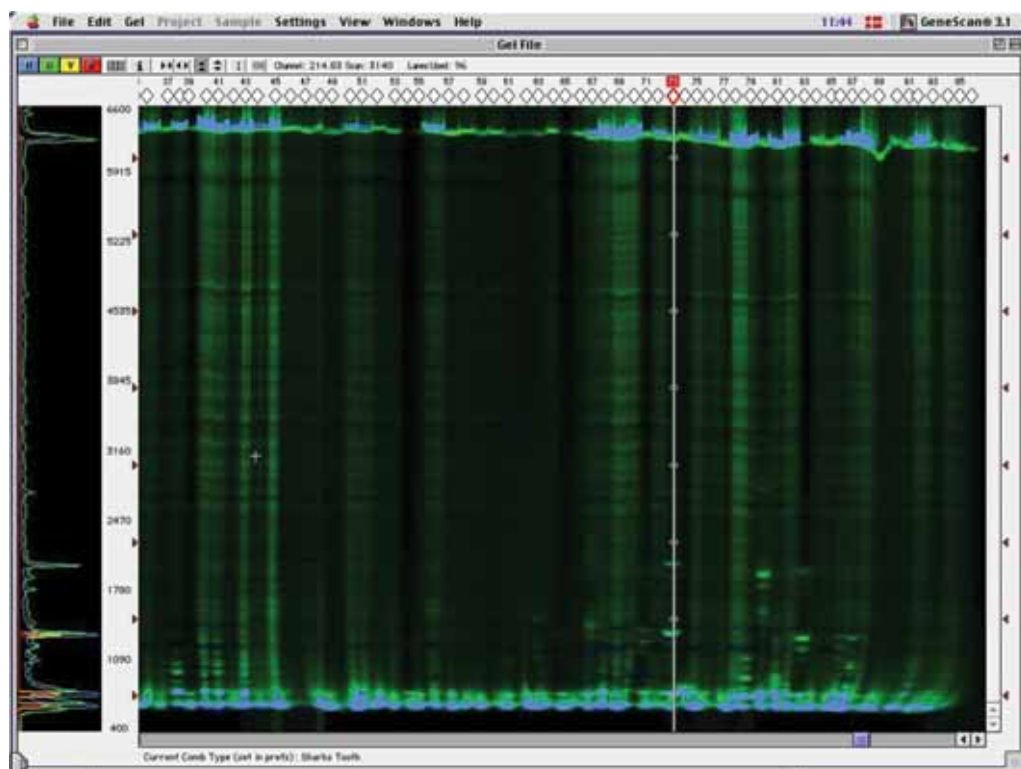


Figure 4. Gel image of one of the three detected mutations in the Lux mutagenized population.

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Screening for resistance donors to leaf rust and powdery mildew

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Abstract

Leaf rust (*Puccinia hordei*) and powdery mildew (*Blumeria graminis*) are among the most important barley diseases in the Czech Republic. The most effective method to protect barley from these diseases is to use resistant varieties. For the breeding of resistant varieties it is important to have sources of resistance available. There are many resistance genes in the gene stocks. The most expedient for practical use in breeding are *Rph7* and *rph16* for resistance to leaf rust, and *mlo11* for resistance to powdery mildew. This work aimed to find sources of these genes.

Introduction

Leaf rust (*Puccinia hordei*) and powdery mildew (*Blumeria* [syn *Erysiphe*] *graminis*) are among the most important diseases of spring barley in the Czech Republic. The intensity of their occurrence and impact on productivity differ in relation to the environmental conditions. The best and the cheapest way to avoid losses caused by these diseases is to use resistant varieties. For breeding success, it is important to have sources of resistance available. Within the genetic stocks there are many sources of genes with different levels of resistance to leaf rust and powdery mildew. Useful genes possessing full resistance to leaf rust in Europe are *Rph7* and *rph16*. For powdery mildew resistance, the gene *mlo11* is effective against all races of mildew. Our aim was to identify suitable donors carrying resistance

genes to powdery mildew and leaf rust in collections of genetic resources.

Methods

Plant material for testing was selected on the basis of glasshouse tests for resistance to leaf rust and powdery mildew. From individual plants, two pieces of leaf 3 cm long were collected and dried at 40°C. DNA was extracted from these segments using the CTAB method (Keb-Llanes *et al.*, 2002). DNA was analyzed by standard PCR with molecular markers MWG2133 (Ivandić *et al.*, 1998), cMWG691 (Graner *et al.*, 2000) and *Mlo* (Piffanelli *et al.*, 2004). PCR products were run on 1 or 2% agarose gels and visualized by ethidium bromide under UV light.

Results and discussion

We found 42 individuals with PCR products of marker cMWG691 specific for the *Rph7* gene. Greenhouse tests confirmed the resistance of these individuals to leaf rust. In the tested collection we detected several individuals with fragments unpublished in Graner *et al.* (2000). These fragments could indicate new alleles and will be an object of further study. We found no plant with PCR products of marker MWG2133 specific for the *rph16* gene. It is not surprising, because this gene was described in *Hordeum spontaneum* (Ivandić *et al.*, 1998), and our genetic resources were selected from *Hordeum vulgare*. We will continue searching for donors of this gene.

For gene *mlo11*, we found 141 individuals with PCR products of marker *Mlo* specific for this gene. Greenhouse infection tests verified resistance of these individuals to powdery mildew.

Conclusions

We found 141 donors of the *mlo11* gene, and 42 donors of the *Rph7* gene. It is possible to say that marker assisted selection is valuable in common breeding. The use of this method for identification of resistance donors is a worthwhile tool.

Acknowledgments

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Variation in virulence in Ethiopian and Jordanian barley scald (*Rhynchosporium secalis*) field populations

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Virulence variations in two Ethiopian (Haro-Wenchi and Negash, in central and northern Ethiopia, respectively) and one Jordanian (Al-Raba) populations of *Rhynchosporium secalis* were studied in a greenhouse test at ICARDA. A total of 58 isolates were sampled and tested for their virulence on a set of 17 differential cultivars. Kosman's index (KW), Shannon normalized index (Sh), Kosman's distance (KB) and Rogers' index (R) were used to estimate virulence diversity within and between populations.

Based on the reaction of the differentials, 54 phenotypes were identified with no phenotype shared among the populations. There were 17/17, 17/20 and 20/21 different phenotypes in the populations from Jordan, Haro-Wenchi and Negash, respectively. The populations also varied in the level of pathotype complexity (number of differentials to which an isolate is virulent) and distribution of virulence across the range of differentials. The pathotype complexity ranged from 3 to 15 in the Jordanian, 0 to 10 in the Haro-Wenchi and 1 to 8 in the Negash populations, with corresponding mean complexities of 10.9, 4.7 and 3.9, respectively. The frequency of virulent isolates on each of the differentials varied from 0 to 65%, 0 to 71% and 12 to 100% in the Haro-Wenchi, Negash and Jordanian populations, respectively, and there was more virulence frequency in the Jordanian than in the Ethiopian populations. All Jordanian isolates were virulent on differentials Kitchin,

La-Mesita and Forrajera, and three or more of the Jordanian isolates were virulent on the rest of the differentials. None of the Haro-Wenchi isolates were virulent on cultivars Osiris or Atlas-46, and no isolate from the Negash population was virulent on cultivars Osiris, Trebi, Abyssinian or Forrajera. There was relatively more phenotypic diversity in the population from Negash based on the Shannon normalized index ($Sh = 0.63$) than in the Jordan ($Sh = 0.60$) and Haro ($Sh = 0.57$) populations. But using the Kosman index (KW), the Jordan population was more diverse ($KW = 0.43$) than the Haro-Wenchi ($KW = 0.40$) and Negash ($KW = 0.33$) populations. According to the Rogers' distance index, the Jordanian and Ethiopian populations were dissimilar ($R = 1$). The dissimilarity between the two Ethiopian populations was also large ($R = 0.96$). The Kosman's distance index (KB) also showed similar results: KB values of 0.50 between Jordan and Haro-Wenchi populations, and 0.40 between the Jordan and Negash populations. The Kosmans' distance value between the two Ethiopian populations was 0.26.

The existence of such a high level of virulence in both Ethiopian and Jordanian populations indicates that the pathogen population is highly diverse, both in the centre of origin (Jordan) and in the centre of diversity of its host. The relatively larger within-population virulence diversity and relatively greater virulence frequency in the Jordanian population than in the Ethiopian

populations could partly be explained by the possible influence exerted by the pathogen population existing on the wild relatives of barley. In addition, observed differences in virulence diversity between the Ethiopian and Jordanian populations, as well as between the two Ethiopian populations, could possibly have resulted from regional differences in host populations differing in resistance that led to development of specific pattern of virulence-resistance associations. Thus different gene deployment strategies may have to be employed for each region. It would probably be essential to incorporate resistance genes similar to those of Igri,

Osiris and Atlas-46 for Haro-Wenchi and those of Abyssinian, Osiris, Forajjera and Trebi for Negash. The existence of larger virulence diversity, even within a single field, could indicate that it would be difficult to have successful control of barley scald using major gene resistance. For example, none of the differentials was resistant to all Jordanian isolates and it seems that none of the resistance gene(s) carried by the set of differentials was effective. Therefore the use of varietal mixtures with different resistance gene backgrounds relative to existing pathotypes of the fungus in each region would probably be a better option.

Session 6

Barley uses – Malt

Global warming impact – winter barley as reserve crop for the brewing industry in the traditional European countries declaring exclusive or dominant spring malting barley utilization

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Abstract

Middle and western Europe are among the producers of top malting quality spring barley for brewing purposes. Nevertheless, climatic changes and EU strategy are relevant issues governing sufficiency of supply for malting and the brewing industry. While in France the cultivation of winter barley has already for a long period been supported by the malting industry, in Germany and the Czech Republic the malting and brewing industry required exclusively spring barley. Nevertheless, when the shortage of preferred spring malting barley occurred, such as in 2000, 2006 and 2007, the malting industry also accepted winter barley of particular varieties (Tiffany in the Czech Republic and Esterel and Vanessa imported from France). It is obvious that six-row winter barley has many genetic and agronomic constraints. However, the yield stability due to its higher winter hardiness and the ability to escape high temperatures in summer can be decisive for its increased cultivation. In this paper we compare yield, economically important parameters and quality parameters of six-row and two-row winter malting barley and two-row spring malting barley. Also analyzed are the impacts of global warming resulting in increased virus epidemic challenge; economically

important parameters, including quality parameters; and the perspectives of marker assisted selection.

Introduction

Barley is a traditional crop in France, Germany and the Czech Republic. Germany and France were the largest producers of barley in Europe in 2007 (Table 1) with a production of 11.0 and 9.7 million tonne, respectively. Both in France and Germany, the total area of winter barley cultivation and production is greater than that of spring barley. In the Czech Republic, the area of winter barley cultivation is only one-third that of spring barley. In other countries of Europe, and

Table 1. Top ten barley producers in 2007 (from FAOSTAT)

Country	Production (×106 t)
Russia	15.6
Canada	11.8
Germany	11.0
France	9.5
Ukraine	6.0
Australia	5.9
United Kingdom	5.1
USA	4.6
Poland	4.1
China	3.8
World Total	140

other continents, particularly Australia, North and South America, and Asia, the production of spring barley dominates. Nevertheless, there are some countries in middle and western Europe that can take advantage of the cultivation of winter barley, which has a higher yield potential than spring barley. In particular, France and Great Britain, having traditionally mild winters, and due to global warming, other countries like Germany, the Czech Republic, Slovakia and Hungary. Due to shortages of malting barley from spring barley in 2006 and 2007, the maltsters and brewers in traditional countries like Germany and the Czech Republic directed their attention to winter malting barley varieties. According to Jochen Mautner GmbH Company, dealing with barley world markets, the prices of malting barley on the world market were expected exceed €250/t in 2008. The main cause is not unfavorable climatic conditions for cultivation of spring barley in many important regions of the world, but rather the demand for cereals for biofuel.

The main objective of this contribution is to evaluate advantages and disadvantages of winter barley cultivation, and especially to assess progress in genetics and breeding, in both agronomic and particularly malting quality parameters. Due to differences in the Czech Republic, Germany and France, the situation in individual countries will be analyzed independently; nevertheless the results and impacts will be summarized.

Material and methods

Despite the fact that demands on technical quality for malt used in the brewing industry are very similar in the Czech Republic (CZ), Germany (D) and France (F), the parameters of the malting quality differ. To compare malting quality of particular varieties of spring and winter (two-row and six-row) barley, the following basic parameters were selected: protein content % (PC); extract

content % (EC); relative extract % at 45°C – Hartong number (HN); Windisch-Kolbach units (°WK); final degree attenuation % (FDA); friability % (FRI); beta-glucan g/L of wort (BG); Diastatic power (DP); and malting quality index (1–10) (MQI).

Varieties of malting barley registered in more than one country were preferred where appropriate.

Results

Barley situation in France

Change in the area of cultivation

Starting from nearly 3×10^6 ha in the period between 1975 and 1980, there followed a great reduction of about 1×10^6 ha, the total area stabilizing at about 1.8×10^6 ha until 1992, with an almost constant production of 10×10^6 t.

From 1993, the reform of the Common Agricultural Policy (CAP) led to further reduction in the barley area (mainly the winter and feed types) so that in the last 10 years the area has varied from 1.5 to 1.6×10^6 ha, including 500 000 ha of spring barley (Figure 1), and 1.1×10^6 ha of six-row and two-row winter barley.

After the predominance of spring barley areas in the 1970s with the development of two-row varieties, the development of two-row winter barley areas in the 1980s, and then a period of roughly equal proportions of the three main types of barley in 1985, the current proportion, excluding the years 2001 and 2003 which were affected by extreme weather conditions, is approximately as follows:

- 35% spring barley;
- 45% six-row winter barley; and
- 20% two-row winter barley (a decrease that started with the 1998 harvest).

Yield changes

While the cultivated areas reduced and changes occurred in the proportion of the various types of barley, there was also a

significant improvement in productivity, especially of spring barley. In the last 30 years, there has been an average annual increase in the yields of spring barley of 0.12 t/ha for almost all varieties intended for brewing.

This progress can be attributed in equal proportions to genetic improvement in the form of new varieties, and improvements in agricultural practices, such as earlier sowing and the use of fungicides. As far as winter barley is concerned (six-row and two-row types combined), progress has been slightly slower (nearly 0.1 t/ha/year), but with a large final difference in productivity of more than 1 t/ha in favor of six-row varieties.

Madre (2004) reported a clear superiority in the yield of six-row winter barleys compared with two-row winter barleys. Two-row winter barley yield is rather higher than two-row spring barley. The relative productivity is: six-row winter barley > two-row winter barley > two-row spring barley. Together with the changes in productivity, other factors improving the reliability of yields have greatly improved, like resistance to fungal diseases, resistance to lodging,

and resistance to viruses (with generalized resistance to mosaic viruses in winter barley).

Change in the malting quality

Since the 1980s, with the urging of industrial maltsters and brewers, increasingly elaborate protocols for assessing the brewing quality of barleys have been gradually developed by official bodies of the Ministry of Agriculture (CTPS: Comité Technique Permanent de la Sélection) and malting and brewing professionals (CBMO: Comité Bière-Malt-Orge). These protocols include quality indices, the CTPS's list of barleys with brewing potential, functional indices, pilot tests, and lists of varieties preferred by French maltsters and brewers. Six-row winter barley recorded great progress in improved malting quality and the parameters are comparable with spring barley (Table 2). Despite that fact, it is not possible to generalize, as there are also dramatic differences among six-row winter barley varieties. Six-row winter barley variety Cartel provided comparable parameters to spring barley variety Cellar, preferred by the malting industry, in French pilot trials (Table 3). Cartel is a medium late

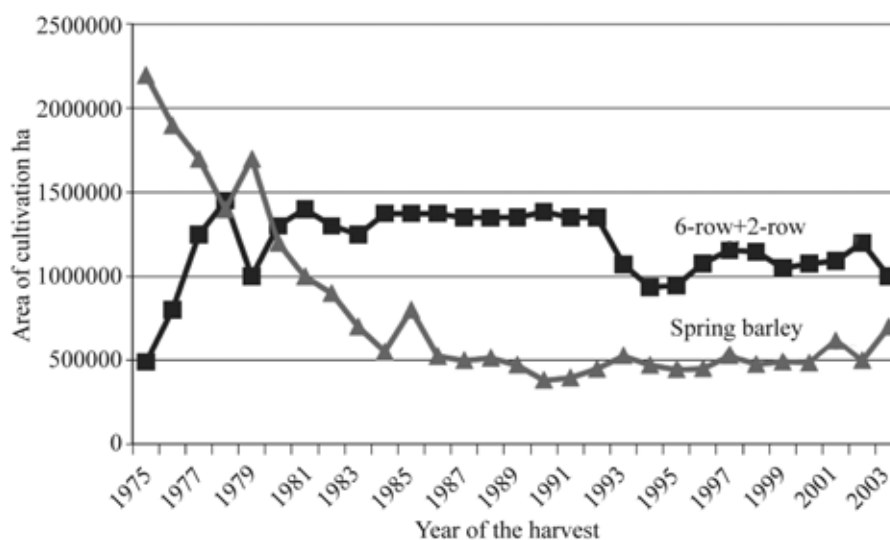


Figure 1. Development of barley cultivation in the period 1975–2003 in France.

variety with excellent resistance to lodging and diseases. Concerning quality, it is in the best class (A) in France thanks to a good extract, good diastatic power, low viscosity and good final attenuation. Especially valuable is the fact that Cartel provides higher and more stable yields in comparison with varieties Esterel and Vanessa (Table 4). Cartel has been intensively tested in many countries of Europe, with very promising results.

Due to the fact that two-row winter barley does not yield significantly more than spring barley, at least for the varieties in commercial evaluation for malting quality, only six-row winter barley varieties were considered for 2008 (Table 5). It is possible to expect that in addition to Cartel, other varieties such as Arturio, Azurel and Cervoise will be cultivated in some areas of France and Europe.

Barley in Germany

In some years, Germany is the largest producer of barley in Europe. For instance, in 2005, total production, at 11.7×10^6 t, was higher than in France. The total area of cultivation is $1.9\text{--}2.1 \times 10^6$ ha. As in France, in Germany the area of spring barley has strongly declined (Figure 2). Nevertheless, increased productivity of modern cultivars is often combined with a pronounced improvement in yield stability, due to widely improved disease and pest resistances. For example, barley cultivars released in Germany nowadays are characterized by considerably enhanced resistance against fungal pathogens like powdery mildew or rust. The yield of winter barley is about 15–25% higher than the yield of spring barley. In the event of more continental winter conditions, such as in 2003, the yield difference between winter and spring barley

Table 2. Change in malting quality - Global improvements in France. Data are average of criteria for varieties included in the CTPS approved malting barley list, 1989 to 2002 (Madre, 2004).

	No. of cvs. on CTPS list	PC (%)	EC (%)	KN (%)	DP (%)	FDA (%)
Spring barley	125	9.86	82.56	43.01	311	83.11
Two-row winter barley	65	10.34	81.29	40.18	347	82.54
Six-row winter barley	21	9.57	80.47	40.94	293	82.59

For key to column headings, see Materials and methods.

Table 3. Comparison of cvs. Cellar and Cartel in French pilot of the malting chain (3 t). Data from CBMO Harvest 2006.

Variety	PC (%)	EC (%)	HN (%)	KN (%)	DP (u. WK)	FDA (%)	FRI (%)	BG (ml/L wort)	pH
Cellar (spring barley)	10.2	82.6	39	34.0	270	80.0	87	292	5.92
Cartel (six-row winter barley)	10.8	80.0	46	42.9	497	82.5	92	86	5.78

For key to column headings, see Materials and methods.

Table 4. Comparison of cvs. Esterel, Vanessa and Cartel in French Official Technical Trials. Average of 6 micro-malting locations.

Variety	Category	Yield (%) of check)	PC (%)	EC (%)	(WK)	DP (%)	FDA (%)	FRI (%)	BG (%)	Color EBC phot.	MQI 2005
Esterel	six-row	76.1	9.1	80.6	42	330	82.0	77	202	2.6	
Vanessa	two-row	77.7	10.3	81.4	44	345	81.9	78	88	3.0	
Cartel	six-row	107.3	9.8	81.1	46	379	83.4	86	6.78	2.9	A

Table 5. Official list of preferred malting barley varieties in France (2008).

LIST OF PREFERRED VARIETIES BY MALTEURS DE FRANCE AND BRASSEURS DE FRANCE			
MALTING BARLEY – 2008 CROP			
ORGES DE PRINTEMPS - <i>SPRING BARLEY</i>		ORGES D'HIVER - <i>WINTER BARLEY</i>	
		2 rangs - 2 rows	6 rangs - 6 rows
VARIÉTÉS PRÉFÉRÉES - <i>PREFERRED VARIETIES</i>			
Polyvalentes - Multi-purpose	A usage spécifique - For specific use	VANESSA	ESTEREL
CELLAR / PEWTER / SCARLETT	HENLEY / NFC TIPPLE PRESTIGE / SEBASTIAN		
VARIÉTÉS EN OBSERVATION COMMERCIALE - <i>VARIETIES UNDER COMMERCIAL OBSERVATION</i>			
BEATRIX / BELLINI / PUBLICAN / QUENCH / SHAKIRA		ARTURIO / AZUREL CARTEL / CERVOISE	
VARIÉTÉS ADMISES EN VALIDATION TECHNOLOGIQUE - <i>VARIETIES UNDERGOING TECHNOLOGICAL VALIDATION</i>			
BERLIOZ / VIVALDI / JB MALTASIA / THORGALL			

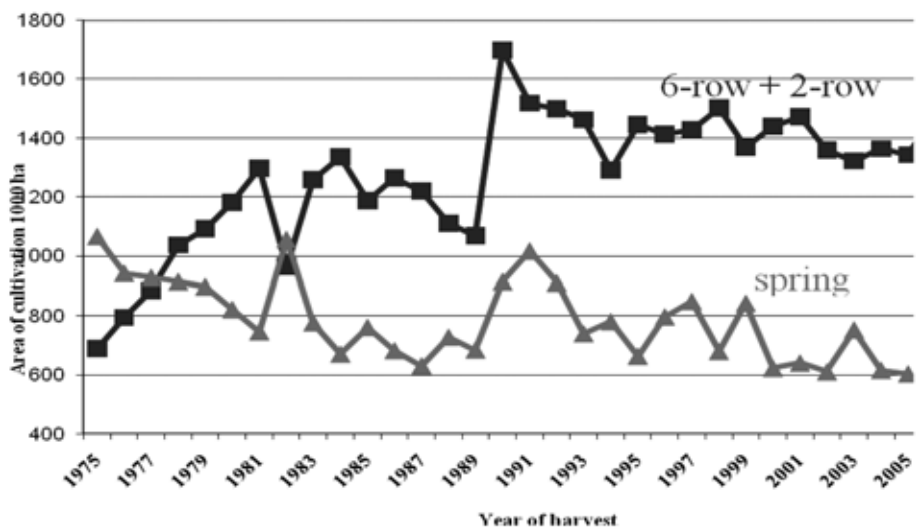


Figure 2. Development of barley cultivation in the period 1975–2006 in Germany.

is dramatically reduced (Figure 3). There are regions of Germany (e.g. Nordheim Vestfalia), where six-row barley is strongly superior to two-row winter barley in both yield and quality. In contrast, two-row barley is strongly competitive with six-row barley in Bavaria. The production of first class grain due to increased 1000-grain weight is highly appreciated. In the period 2006–2007, the dramatic increase in malting barley prices in Germany (Figure 4) opened possibilities for

the utilization of winter barley for malting purposes. Table 6 compares malting quality parameters of selected two-row, six-row winter barley and spring barleys. Despite the fact that six-row winter barley variety Dorothea does not compare well with two-row winter barley Wintmalt, and particularly to spring barley varieties Auriga and Marthe, the yield potential and yield stability are very attractive for both farmers and maltsters.

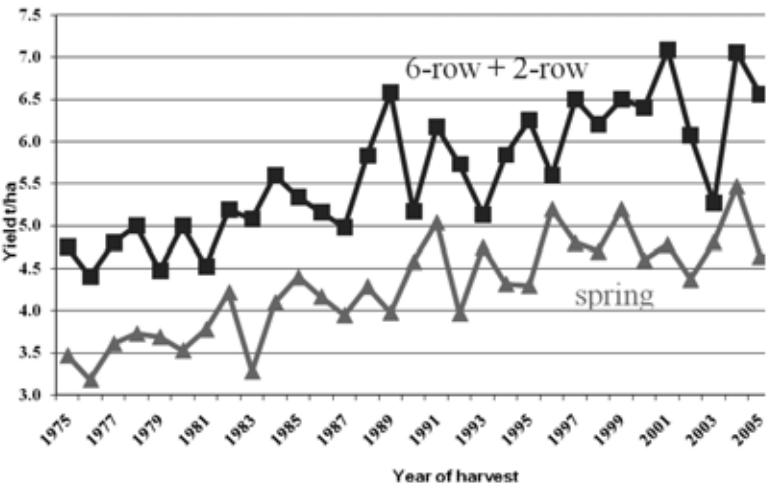


Figure 3. Global change in yields in the period 1975–2006 in Germany.

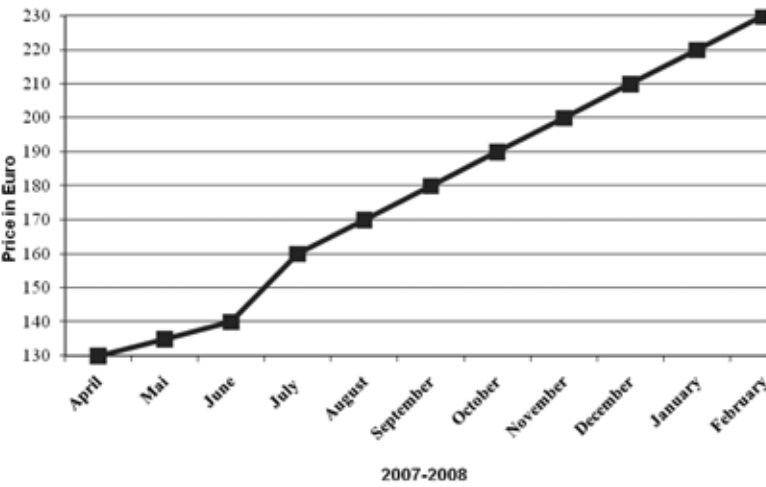


Figure 4. Development of malting barley price in the period April 2007–February 2008 in Germany.

Barley cultivation in the Czech Republic

In the Czech Republic, the cultivation, and particularly breeding, of top quality malting spring barley has a long tradition. Czech local varieties have been extensively used in the breeding programs of many countries in Europe and overseas since the beginning of the 20th century. Currently, spring barley is cultivated on about 400 000 ha and

represents 30% of the total area of cereal crops. Two-row barley with top malting quality strongly dominates among varieties. In contrast, six-row barley dominates among the winter barley varieties. The total area of winter barley is about 120 000 ha (Figure 5), with 90–95% of the winter barley being six-row varieties and 5–10% being two-row varieties. The comparison of acreage and yields for both spring and winter barley in

Table 6. Comparison of malting quality of selected winter and spring barleys in Germany. Results from 2005.

Variety	PC (kg/hL)	EC (%)	HN (%)	KN	FDA (%)	FRI (%)	COLOR EBC	pH	MQI 2005
Two-row winter barley ¹									
Tiffany	10.0	81.8	44.5	38.7	85.1	81.0	3.1	6.01	7.7
Vanessa	10.2	82.9	45.1	40.4	85.1	81.0	3.5	5.93	8.4
Carrero	10.4	81.8	41.5	39.2	84.8	75.7	3.2	5.97	7.5
Wintmalt	9.6	82.5	41.9	44.3	85.7	89.3	3.6	6.01	9.0
Malwinta	8.8	81.4	41.2	42.6	85.2	88.8	3.1	6.01	7.6
Six-row winter barley ²									
Ludmilla	10.7	77.4	42.5	36.3	84.2	63.2	3.2	6.03	4.1
Esterel	10.0	79.6	43.7	34.9	83.8	65.4	2.9	6.07	5.3
Dorothea	10.1	80.3	47.4	41.8	84.5	76.6	3.3	6.00	6.7
Spring barley ³									
Auriga	9.9	82.4	44.2	46.2	83.4	83.3	3.2	5.88	7.9
Marthe	9.6	83.8	45.8	46.2	83.2	90.8	3.8	5.92	9.1

For key to column headings, see Materials and methods.

Sources: ¹ Braugerste Jahrbuch 2007, IPZ 2b., AQU 4, Braueignungsprüfung 2005; ² Braugerste Jahrbuch 2007, IPZ 2b., AQU 4, Braueignungsprüfung 2005; ³ Braugerste Jahrbuch 2007, Bundessortenamt WPI (2003), WP II (2004), WP III (2005). Average of 22 trials.

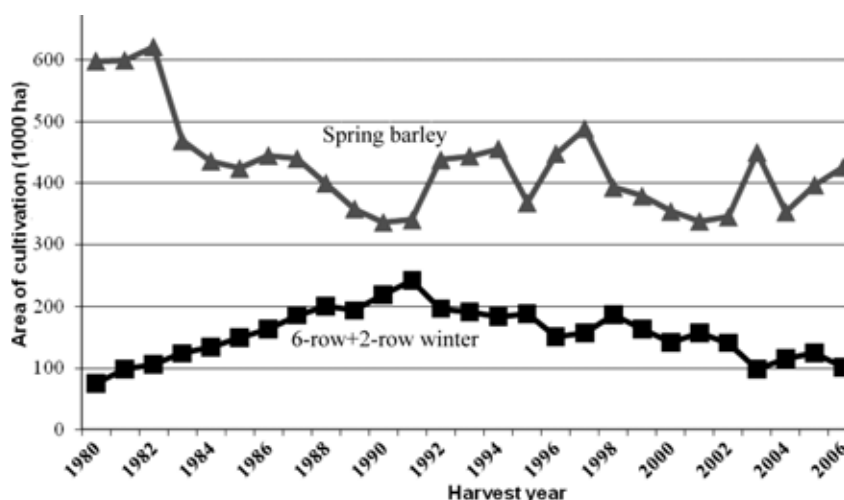


Figure 5. Development of barley cultivation in the period 1980–2007 in the Czech Republic.

the period 1974–2007 is presented in Table 7. The Czech Republic is situated on the border of maritime and continental climates, and barley is a crop that responds very sensitively to extremes in climate. The very continental spring and summer provide great advantage to winter barley, and winter barley was 25% higher yielding than spring barley in 2000. In contrast, the very continental winter in 2003

caused severe winterkill of winter barley, with a strong reduction both in the area harvested and in yields.

In 1999, the first two-row winter barley variety for malting purposes (cv. Tiffany, from Germany) was registered, in spite of the fact that the malting industry still strongly prefers spring barley. Nevertheless, the susceptibility of Tiffany to BYDV, the winterkill in 2003

Table 7. Area ($\times 10^3$ ha), yield (t/ha), and yield difference between winter and spring barley in selected years in the Czech Republic in the period 1974–2007.

Year	Winter barley		Spring barley		Difference	
	area	yield	area	yield	t/ha	%
1974	4	3.5	649	3.9	-0.36	-11
1984	123	5.1	469	4.5	0.53	13
1990	243	6.1	339	5.4	0.62	12
1994	185	4.2	495	3.7	0.47	13
1995	195	4.4	370	3.8	0.62	14
1998	187	4.1	393	3.8	0.31	8
2000	142	4.0	354	3.0	1.00	25
2001	157	4.4	338	3.7	0.70	18
2002	141	3.7	345	4.0	-0.30	-6
2003	98	3.1	450	3.9	-0.80	-22
2004	115	5.2	353	5.1	0.10	2
2005	125	4.8	397	4.2	0.60	14
2006	102	4.0	426	3.9	0.10	3
2007	129	4.8	369	3.7	1.10	23

and the overproduction of spring barley in 2004, led to the disappearance of Tiffany from the market. Due to the shortage of malting barley, winter barley was again utilized in the period 2006–2008, particularly the variety Wintmalt. There was a significant increase in malting quality parameters, particularly in extract content of both Czech (Malz, Respekt, Bojos) and foreign varieties (Jersey, Prestige, Sebastian, Marthe) registered in the period 2000–2007 (Table 8). Despite Wintmalt not achieving top malting quality among preferred malting barley varieties, the progress among two-row winter barley varieties is significant. The most famous Czech beer producer, Pilsen Urquell, was

going to contract about 20% of the barley demand as winter barley to reach higher stability in the supply of malting barley every year. As for two-row malting winter barley, winter hardiness is the most relevant problem, as the frequency of continental winters is much higher than in Germany or France. In contrast to Germany and France, six-row winter barley, although having better winter hardiness, is still not considered for malting purposes due to relatively low malting quality parameters. Some breweries in the Czech Republic, particularly Pilsen Urquell, consider the parameters of Hartong number and Kolbach number as too high for top malting quality. To achieve the particular

Table 8. Comparison of malting quality of selected winter and spring barleys in the Czech Republic based on results from 2005–2007 (Data from the Research Institute of Brewing and Malting, Brno).

Variety	Origin	PC of check)	EC (%)	HN (%)	KN u. WK	DP (%)	FDA (%)	FRI (%)	BG (ml/L wort)	MQI 2007
Two-row winter barley										
Babette	D	11.7	80.3	36.0	39.0	429	82.5	74	451	2.2
Caravan	D	11.3	80.6	34.9	41.5	409	81.9	75	425	2.3
Wintmalt	D	10.5	81.8	37.9	45.0	412	83.5	89	127	5.2
Spring barley										
Malz	CZ	11.2	83.2	39.4	42.9	339	81.1	84	238	6.4
Jersey	NL	10.9	82.1	42.6	44.3	383	82.3	88	174	6.2
Sebastian	DK	10.5	83.2	40.7	44.7	419	82.6	84	101	7.6
Marthe	D	11.0	82.8	48.0	47.8	412	84.0	89	131	8.3

For key to column headings, see Materials and methods.

taste of Czech lager beer due to the presence of residual extract, lower values for Hartong number and Kolbach number are required (Table 9). That is an opportunity for two-row winter malting barley having lower Hartong and Kolbach numbers, lower protein content but relatively high extract content.

Discussion

Friedt *et al.* (2000) and Madre (2004) presented great progress in the breeding of winter barley in Germany. The progress was evident in yield potential, resistances and malting quality, particularly for two-row winter barley.

Ahlenmeyear *et al.* (2006) showed that advances in plant breeding together with improvements in plant production led to an average annual increase of 77 kg/ha in winter barley in Germany over the last five decades. In order to specify the share of the genetic gain in this improvement, 64 six-row and 49 two-row winter barley varieties that were registered in Germany during the last 40 years and gained certain importance during that time were analyzed. The results showed that the improvements made in plant breeding accounted for about half of the gain in grain yield made on farmers' fields during the last decades. On the basis of molecular analysis by means of AFLP and SSR, it was concluded that for the

six-row material under investigation the genetic diversity remained constant, while in the two-row cultivars the genetic diversity has even increased. In combination with detailed phenotypic characterization of the cultivars, the genotypic data may facilitate the future detection of association between genetic markers and traits like yield, quality components or resistances.

Herz *et al.* (2007a, b) compared the malting quality of two-row winter barley and six-row barley. In spite of the progress, exclusively two-row winter barley Wintmalt is comparable to two-row spring varieties. Rath (2007) analyzed extensive data from EBC trials and agronomic parameters for both spring and winter malting barley varieties. He found that malting winter barley varieties yielded only 3% more than malting spring barley. No difference in resistance and yield stability were found. The advantage is only if summer hot periods occur in the period of spring barley maturation. Nevertheless, König (2007) considers the year 2008 as optimal to increase the area of spring and winter malting barley in Germany due to challenging prices.

According to Fouquin (unpublished data), only a few varieties of winter malting barley are available in France. In CTPS Registration 2001–2006, only 5% of A quality six-row winter barley varieties were tested. In two-

Table 9. Required parameters for malting barley in the Czech Republic, valid since 2003 (Psota and Kosar, 2002).

Quality parameter	Unit	Abbrev.	Minimal value 1 point	Optimal value 9 points	Czech beer Optimal value
Protein content	%	PC	9.5	10.2	
			11.7	11.0	
Extract content	%	EC	81.5	83.0	min. 81.5%
Hartong number	%	HN	35.0	40.0	max. 38%
			53.0	48.0	
Kolbach number	%	KN	40.0	42.0	39±1
			53.0	48.0	
Diastatic power	u.WK	DP	220	300	min. 220
Final degree attenuation	%	FDA	79.0	82.0	max. 80%
Friability	%	FRI	79.0	86.0	min. 75%
Beta-glucan content	mg/L wort	BG	250	100	max.250 mg/L

row spring and two-row winter barley, it was 50% and 41%, respectively. In spite of that fact, Fouquin is optimistic as to the future of six-row barley in France, Germany and central Europe.

Spunar *et al.* (2004) compared the yield potential and quality of spring and winter barley. It was concluded that two-row winter barley is competitive with spring barley in yield, particularly when winters are mild. Comparing the malting quality, only the variety Tiffany was able to reach the malting quality parameters of medium varieties. In the Czech Republic, the malt and beer industry strongly prefers spring barley (Spunar *et al.*, 2006). In spite of this, and because of the lack of spring malting barley, the winter barley variety Tiffany was accepted in the period 2000–2002. After, and particularly since 2003, winter barley was not accepted, but it was again accepted in the period 2006–2008. The breeding of a new variety takes 10–15 years. In the situation where the malt and beer industry decides which variety to accept for processing, farmers have to respect their requirements, and the breeders must intuitively modify their selection criteria to be competitive in the market. The prospect of barley as a crop for biofuel is under discussion in the EU. Hayes *et al.* (2006) evaluated winter barley as a reserve crop for malting in the USA due to serious problems caused by *Fusarium* head blight.

Conclusions

- Spring barley will in the future be the dominant crop for supplying the malt and beer industry. Malting quality parameters of newly registered varieties like Marthe registered in Germany and the Czech Republic further increase differences in malting quality between spring and winter barley.
- Winter barley offers higher acreage production but lower malting quality.
- Spring and winter malting barley have

strong competitors in highly stable crops like winter wheat, feed winter barley and triticale due to cultivation of these crops for biofuel.

- Spring and winter malting barley have a chance to be competitive if market prices increase from traditional levels of €120–140/t (as in 2005) to the €250–300/t of 2008 and the future.
- In France, more extensive cultivation of six-row winter barley variety Cartel is promising, which provides higher yields and better malting quality parameters than traditional varieties, both six-row varieties Plaisant and Esterel and two-row winter barley varieties like Vanessa.
- In Germany, six-row winter barleys Dorothea and Cartel are considered for use by malting industry in spite of the fact that two-row winter barley Wintmalt is preferred.
- In the Czech Republic, two-row winter barley varieties like Wintmalt, which possess comparable malting quality parameters to medium quality malting spring barley, have great prospects. Six-row winter barley is not considered at the present time.
- Further progress in winter barley breeding strongly depends on the response of the malting and brewing industry. The progress in genetics and breeding of malting winter barley depends on the amount of seed sales, to enable re-investing money in breeding and to limit the gap between malting quality of spring and winter barley.

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Pyramiding QTLs to improve malting quality in barley: genetic gains in phenotype and diversity

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Abstract

The ability of barley breeders to deliver germplasm that combines elite malt quality characteristics, disease resistances, and important agronomic traits has been greatly enhanced by the use of molecular marker technologies. These technologies facilitate the rapid transfer of desirable traits from diverse, elite, germplasm into locally adapted varieties. This present study sought to obtain an additive genetic effect by combining the malting quality of two elite donor parents (Harrington and Morex) such that the resultant progeny would possess quality superior to either parent. Quality data from micro-malting grains showed substantial improvements in the resultant progeny, compared with the recurrent parent. Malt extract levels were increased by 1.7–4.0 %, while diastase levels increased from approximately 306 WKE to between 400–465 WKE. Similarly, alpha-amylase levels were increased from 180 U⁻¹ kg to between 234 and 298 U⁻¹ kg, and wort viscosities were lowered from 2.11 cP to between 1.61 and 1.81 cP. Other quality improvements include increases in beta-glucanase levels from 386 to 492–537 units, and reductions in wort beta-glucan levels by 30–60%. Whilst the genetic

gains compared to the recurrent parent were impressive, quality of the derived lines were largely equivalent to the levels now available in the recently released varieties Buloke and Flagship. In a few cases, the MABC-derived lines also showed similarities to the original donors, Harrington and Morex, but in none of the cases did quality of these lines exceed those of either Harrington or Morex.

Introduction

A quantitative trait locus (QTL) target for molecular breeding, unique to barley (*Hordeum vulgare* L), is malting quality, as this trait attracts a substantial price premium (Koeber, 2004). Several QTL regions affecting barley malting quality have been reported in different population (Han *et al.*, 1997; Ullrich *et al.*, 1997; Mather *et al.*, 1997; Igartua *et al.*, 2000; Marquez-Cedillo *et al.*, 2000; Fox *et al.*, 2003; Emebiri *et al.*, 2004), making the parents the most likely donors of genes to improve malt quality. Here, we report an experiment initiated in 2000, which was designed to pyramid favorable alleles derived from two elite malting barleys (Harrington and Morex) into a genetic background that combined boron tolerance and disease resistance. The

key genomic regions influencing malting quality in these two varieties have previously been established by the North American Genome Mapping Project (Mather *et al.*, 1997; Marquez-Cedillo *et al.*, 2000) and the Australian National Barley Molecular Marker Program (AJAR Special, 2003). In our studies, molecular markers linked to the genomic regions were first validated (Michael *et al.*, 2003), before being used to monitor introgression of favorable alleles derived from Harrington and Morex in a nearly isogenic background. Although molecular markers were used to control the target genomic regions in *foreground* selection, no *background* selection was applied during the backcrossing process. Analysis of molecular diversity, based on whole-genome profiling with 700 DArT markers, showed clear separation of BC-derived lines from existing malting barley germplasm, indicating they represent a distinctly different source population for genetic improvement.

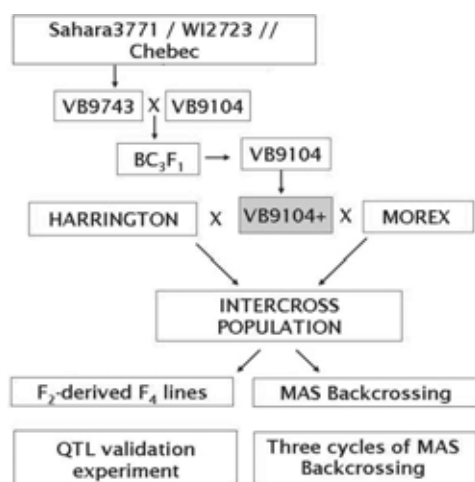


Figure 1. Flow chart showing the development of BC₆F₁-derived doubled haploid barley lines used in the study. During the backcrossing, molecular markers were used to control the introgression of genes influencing malting quality, boron tolerance and CCN resistance.

Materials and methods

A molecular breeding program was conducted at the Department of Primary Industries, Horsham Centre, Victoria, Australia, aimed at introgressing malting quality QTLs into an adapted breeding line (VB9104+) carrying boron tolerance and cereal cyst nematode resistance. A flow chart of the crossing scheme and parents contributing favorable alleles is shown in Figure 1. Molecular markers linked to genomic regions influencing malting quality were used to monitor introgression of segments derived from Harrington (chromosome 5H) and Morex (chromosome 7H) into the breeding line 96-121B*8-27 ("VB9104+"). We selected for presence of Harrington alleles at the distal end of chromosome 5HL (Bin 15), using the SSR marker GMS01, and for Morex alleles at a segment on chromosome 7H (Bins 4 and 6, respectively) using flanking SSR markers, EBmatc16 and Bmag914 (Ramsay *et al.*, 2000).

Three cycles of marker-assisted backcrossing (MABC) were conducted to VB9104+, and a final backcrossing to VB9104 was made to reintroduce top yield levels. After the final cross in 2003, 758 doubled-haploid (DH) lines were produced from the 19 resulting final backcross F₁ plants. The number of DHs created from each of the selected final backcross F₁ plants varied from 2 to 109.

Progressive culling with markers reduced the original population of 758 to 97, of which seeds of 81 were successfully multiplied to provide seed for field trials. All 81 lines possessed favorable alleles at malting quality QTLs, but it was not possible to select for CCN resistance using marker alleles due to a lack of polymorphism with the marker panel used between VB9104+ (CCN resistant) and VB9104 (CCN susceptible). A CCN-resistance bioassay was subsequently used to identify a subset of 40 DH lines from the population of 81, which possessed CCN resistance and were progressed to field assessment.

Field experiment

Field experiments were conducted in 2004 at two locations in southern Australia: Horsham in Victoria, and Roseworthy in South Australia. At both sites, 40 MABC-derived lines and 14 check varieties were grown in a randomized complete block design, with three replications. Plots measured 6 m in length at sowing, with six rows of plants spaced 15 cm apart, at a seeding rate of 40 g/plot or 50 kg/ha. Grain samples were passed through a 2.2 mm screen before micro-malting. The malting schedules were as described in Emebiri *et al.* (2004).

Statistical analysis

Data from each site were first tested for normality (Anderson-Darling Normality Test) before a combined analysis of variance (ANOVA) was performed using a linear model of the following format:

$$Y_{ijk} = \mu + S_i + R_{ij} + G_k + GS_{ik} + \varepsilon_{ijk}$$

where Y_{ijk} is observed phenotype, μ is the population mean, while S_i , R_{ij} , G_k , GS_{ik} and ε_{ijk} represent the effects of sites, replication within sites, genotype, genotype-site interactions and the experimental error, respectively. The analyses were carried out using MINITAB software, and considered S , R and G factors as fixed. Adjusted means were compared using the Dunnett Simultaneous Test, with parents specified as the control levels.

Molecular analysis

The 40 MABC-derived DH lines, along with 8 parents used in the complex crossing scheme, and 20 lines representing a range of malting barley varieties grown in Australia and advanced breeding lines originating from various breeding programs, were genotyped using 700 DArT markers (Wenzl *et al.*, 2004), covering approximately 96% of the barley genome (Wenzl *et al.*, 2006). The molecular data were used to generate precision graphical genotypes of each MABC-derived line,

using the Grafgen program (<http://fhospital.free.fr/fred/work/programs/grafgen/>) and to estimate the proportion of donor genome.

To examine genetic similarity to existing malting barley germplasm, the individual genotypic and phenotypic data were analyzed by the method of principal coordinate analysis, using Gen Stat v. 10. Further genetic characterization was conducted using STRUCTURE 2.2 (Pritchard *et al.*, 2000) to examine differentiation within the MABC-derived population. The analysis was performed with increasing levels of K (the number of inferred clusters), and the natural log probability of the data, which is proportional to the posterior probability of K (Falush *et al.*, 2003) peaked at $K = 5$. This was accepted as the smallest value that captured the major structure of the data.

Results

Introgression segments

Graphical genotyping confirmed successful introgression of the target QTL regions derived from Harrington and Morex into MABC-derived lines (Figure 2). For logistic reasons, no *background* selection was applied in this study during the backcrossing process. Genome composition was therefore assessed by whole-genome profiling of each of the MABC-derived DH lines, using multi-locus DArT markers. On average, each MABC-derived line carried 85% of the recurrent VB9104 genome, 3% of the target QTL segments and 12% of non-target chromosomal segments introgressed from donor parents. All lines carried the CCN-resistance gene, *Ha2*, located on chromosome 2H, but we could not determine the source of the favorable alleles, as both Sahara 3771 and Chebec carried similar allele-types at the region. In contrast, the introgression segment associated with boron tolerance on chromosome 4H was unequivocally traced to Sahara 3771 as the parent-of-origin.

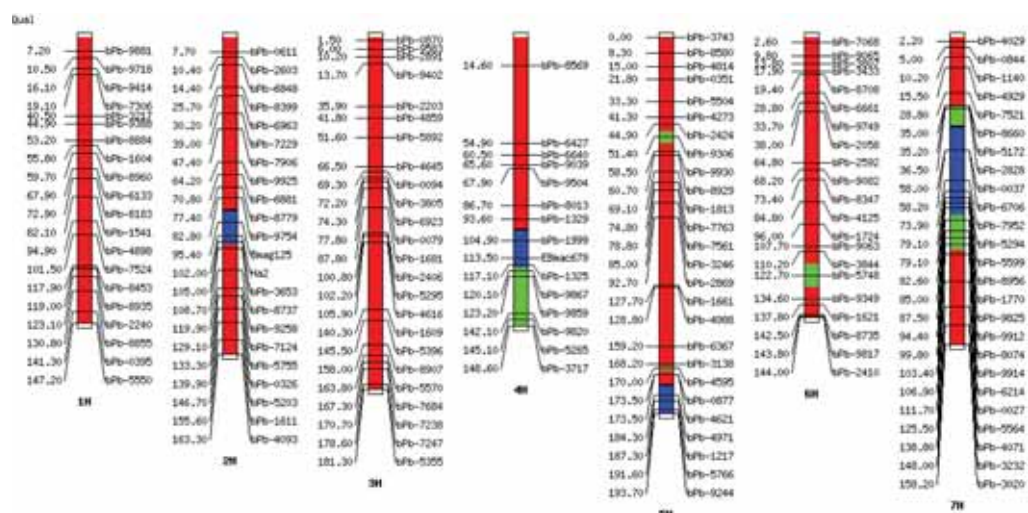


Figure 2. Precision graphical genotype of AB-36*17, one of the elite lines derived from the MABC program. Color scales reflect the probability (> 0.5) of being the recurrent parent (VB9104; red) genome, introgressed QTL (blue) or non-target introgression segment (green).

Gains in phenotype

Genetic gains for all measured quality traits in the MABC-derived population are described in Figure 3. A wide range of variation was observed for all measured traits, indicating substantial changes in individual quality parameters, which in some cases showed significant ($P < 0.05$) improvements over the recurrent. For instance, in the recurrent parent (VB9104), malt extract averaged 78.4%. The range in the MABC-derived lines was between 77 and 82%, with a median value of 80.1% (Figure 3). Statistical tests showed 5 of the MABC-derived lines were significantly ($P < 0.05$) higher than the recurrent parent by 2.0%, 10 were higher by 3.0%, while the most outstanding line (ABBD03-27*8) had 82.1% malt extract, a level similar to that of Harrington (82.4±0.47%).

Morex was chosen as a donor parent in this project due to the presence in this variety of a major QTL on chromosome 7H conferring high levels of beta-glucanase, hence low levels of both wort beta-glucan (WBG) and wort viscosity. Relative to the recurrent parents, all of the MABC-derived

DH lines showed improved (lower) levels of WBG and viscosity (Figure 3). The WBG levels were significantly ($P < 0.05$) reduced from an average of 1200 mg/L in the recurrent parent, to between 566 and 672 mg/L in the top quartile of the progeny, while viscosity levels were decreased from 2.1 cP to between 1.6 and 1.7 cP in the upper 25% of MABC-derived lines. This was accompanied by improvements in beta-glucanase levels, which was increased from 386 U/kg to between 508 and 536 U/kg in the top 25% of the MABC-derived lines (Figure 3).

Major genetic gains were also observed in other quality parameters. Diastase levels, for instance, increased from approximately 306 WKE in VB9104 to between 415 and 465 WKE in the top quartile of the progeny, levels superior to Harrington (413 WKE) and Morex (446 WKE) but lower than Franklin (474 WKE). Similarly, α -amylase in the recurrent parent was significantly increased from 180 U/kg to between 218 and 297 U/kg, levels slightly superior to those of Harrington (280 U/kg) and Morex (260 U/kg) (Figure 3).

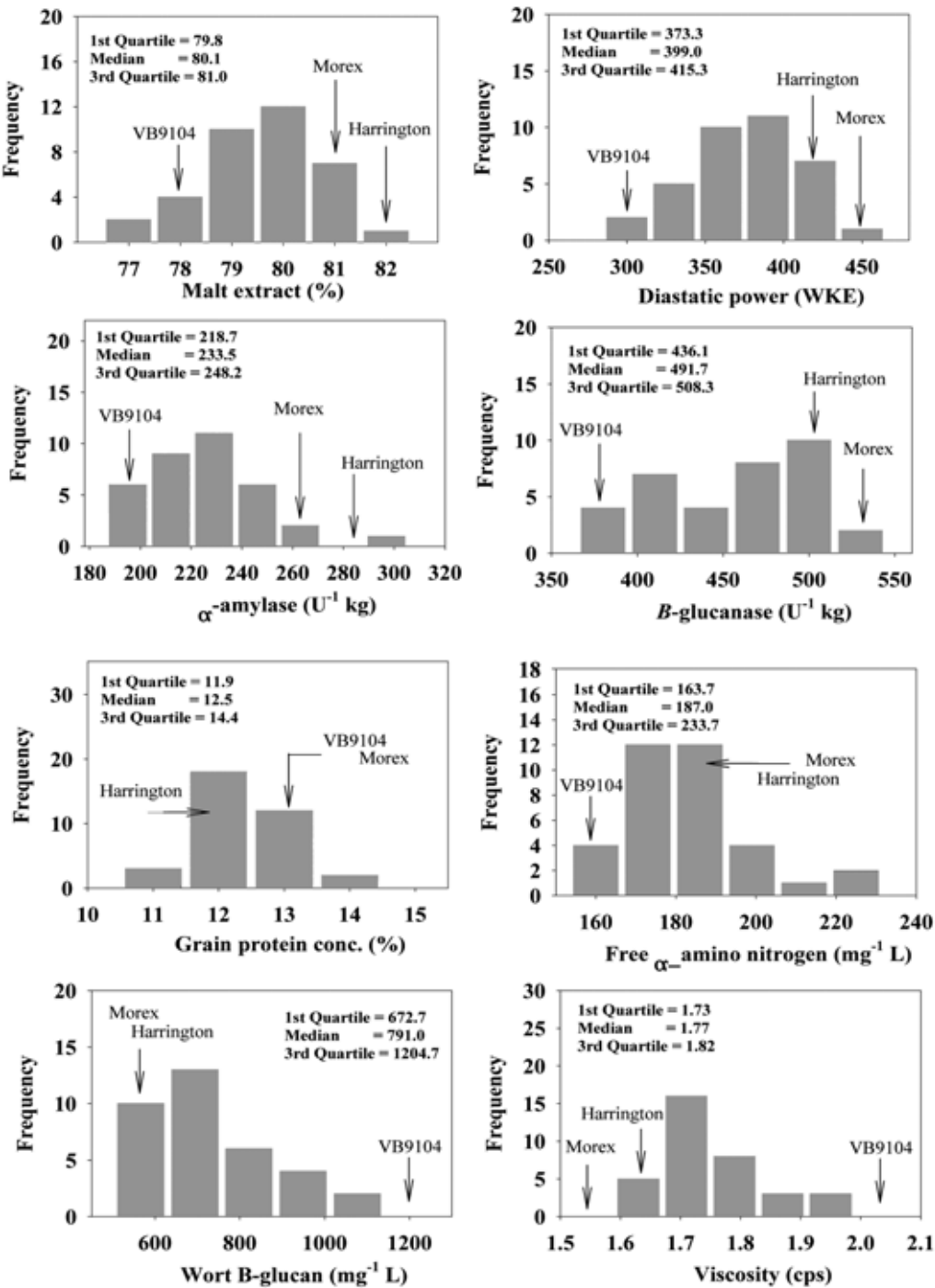


Figure 3. Descriptive histogram plots of phenotypes observed in a population of BC-derived doubled-haploid lines carrying independent malting quality QTLs derived from elite malting barley cultivars Harrington and Morex.

show the underlying divergence present within the MABC-derived population. STRUCTURE grouped the MABC-derived population into five genetically distinguishable groups of individuals with high levels of admixture (Figure 4). Such diversity confirmed the MABC-derived lines as a new source population for genetic improvement.

Multi-trait profile

Phenotypic diversity, when compared to existing germplasm, is shown in Figure 5. As expected, VB9104, which is known to be prone to high beta-glucan levels, was grouped with the commercial feed variety, Barque, while Gairdner and Gairdner+ also showed the expected high level of similarity. Gairdner+ was developed as a new version of Gairdner, incorporating CCN resistance and improved spot form of net blotch resistance, while maintaining malting quality profile.

The improvement in malting quality was clearly demonstrated in this plot as a shift away from feed varieties towards the cloud of commercial malting quality varieties in the centre of Figure 5. In most cases, quality improvements were similar or equivalent to the levels now available in commercial varieties, such as Sloop, Buloke and Flagship. In a few cases, the MABC-derived lines also showed similarities to the original donors, Harrington and Morex, but in none of the cases did the quality of the derived lines exceed those of either Harrington or Morex (Figure 5). The top 10% of the MABC-derived lines had malt extract levels greater than or equal to 81%, and had significantly ($P < 0.05$) elevated beta-glucanase levels compared with the recurrent parent, but wort beta-glucan levels and wort viscosities remained at the high end of the industry tolerance range.

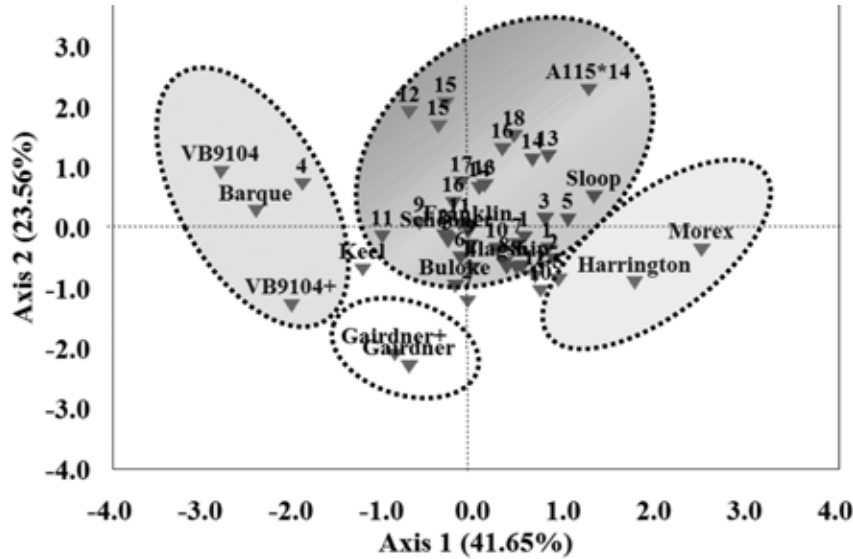


Figure 5. Patterns of similarity in malting quality profile of the BC-derived lines (coded in numbers) and commercial malting barley varieties. Percentage of variation represented by each axis is indicated. Analysis was based on average of malting quality parameters measured at two locations.

Discussion

In the present study, improvements achieved by pyramiding malt quality QTLs derived from elite donors were impressive, when compared with the recurrent parent (Figure 3). The quality of the top 10% of the lines was equivalent to that of the recently released varieties, Buloke and Flagship, and to the original donors Harrington and Morex (not shown). In this regard, our results are similar to the majority of reports published so far in the literature. Gur and Zamir (2004) evaluated the potential of breeding for increased tomato (*Solanum lycopersicum*) yield using genotypes carrying a pyramid of three independent yield-promoting genomic regions introduced from the drought-tolerant green-fruited wild species *Solanum pennellii*.

Yield of hybrids derived from the pyramided genotypes was more than 50% higher than that of a control market leader variety under both wet and dry field conditions that received 10% of the irrigation water. In maize, Ribaut and Ragot (2007) recently reviewed a study to improve drought adaptation by MABC introgression of favorable alleles at five target regions involved in the expression of yield components under water-limited conditions. Mean grain yield of MABC-derived hybrids was consistently higher than that of control hybrids (crosses from the recurrent parent to the same two testers as the MABC-derived families) under severe water stress conditions. Under those conditions, the best five MABC-derived hybrids yielded, on average, at least 50% more than control hybrids.

A recurrent criticism of QTL mapping is that the identified QTL in one population may not exert similar effects when introgressed into other genetic backgrounds. In this regard, our results suggest a very optimistic prospect for QTL pyramiding, and give support to theories advocating the use of genetic markers linked to a QTL to trace that QTL in a selection program. However, we

did not observe the expected recombinants possessing quality superior to either Harrington or Morex. Plausible reasons include epistasis and genetic background, but it should also be appreciated that single QTL introgressions do not necessarily work as simply as indicated by classical dominance-recessive relationships of genes. In addition to the introgressed QTL regions, the background genotype is expected to carry 3.1% of the donor's genomic DNA at the end of the backcross phase (Stam and Zeven, 1981; Young and Tanksley, 1989). Unintended or non-target introgressions can affect the phenotypic expression of a given gene. In a study to transfer grain yield QTLs derived from the high-yielding variety, Baronesse, to the North American two-row malting barley industry standard cv. Harrington, Schmierer *et al.* (2004) found that genome regions other than the major QTL targeted for selection influenced yield expression.

Conclusions

The present study has demonstrated substantial genetic gains from marker-assisted selection for malting quality in barley. A similar study by Kandemir *et al.* (2000a, b) examined three grain yield QTLs on chromosomes 2HS, 3H, and 1HL via MAS backcross breeding. While yield-related traits such as extended flowering time (2HS QTL) and reduced height, lodging, and head shattering (3H QTL) were successfully transferred (Kandemir *et al.*, 2000b), significant yield increases were not achieved (Kandemir *et al.*, 2000a). This highlights the need for basic research to better understand the behavior of QTLs when introgressed into different genetic backgrounds. The present study suffers from the fact that it was a variety development project, hence only individuals carrying the two introgressed regions were retained during backcrossing. It may well be that the 5HL region from Harrington was so dominant that it overshadowed the effect

of chromosome 7H (Morex) in a genetic background possessing both. One advantage of the Morex chromosome 7H allele(s) is that they have not been shown to be associated with pre-harvest sprouting, which is the case with the chromosome 5HL allele(s) from Harrington (Li *et al.*, 2004).

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Varietal and site interactions in the growing of malting barley for distilling and brewing

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Abstract

A range of spring and winter barley varieties (12–16) were grown on trial plots in Scotland and England (4 spring, 4 winter). These were grown and micro-malted by project partners as part of a HGCA/SAPPIO funded LINK Project, entitled Association Genetics of UK Elite Barley (AGOUEB), the aim of which is to identify genetic factors controlling economically important traits in malting barley for distilling and brewing. Predicted alcohol yield (PSY) data on samples from each site showed that although the site averages were different, the general trends on each site were similar, with current UK Recommended List varieties dominating on most sites. The results also confirmed that the malt total nitrogen contents were inversely related to PSY. These results are important since they show that important factors differentiating malting barley varieties are genetically determined and confirm that genetic mapping can be used to select for improvements in malting barley. The project data will be incorporated into the wider genetic studies in the AGOUEB Project and will help identify genes relating to specific traits, allowing barley breeders to select suitable parents to give offspring with improved qualities. This will provide benefits at all levels of the barley production chain, by giving guidance to plant breeders

and improved production for growers as well as industrial processors, such as maltsters, brewers and distillers.

Introduction

Barley is the 4th most important crop in the world, and is the second most important arable crop in the UK. Although UK production of barley has declined from about 11×10^6 t per year in 1984 to just over 5.5×10^6 t in 2005, the UK remains a major barley and barley malt producer, as it is associated with a reputation for high quality malting barley, which is an essential raw material for end-user applications such as distilling and brewing (AGOUEB, 2007). Currently UK maltsters are processing about 1.7×10^6 t of barley malt annually, based on figures for the 2006 harvest (Maltsters Association of Great Britain, 2007).

Scotch whisky is a unique and distinctive regional product of Scotland and is one of the most popular, and sought after, alcoholic beverages in the world. The export of Scotch whisky to 200 different countries generates about £ 2.5 billion (approx € 3.75 billion), making it one of the UK's top five export earners, accounting for more than 23% of UK food and drink exports (SWA, 2007). Scotch whisky distillers provide an important market for barley in the UK and are currently using around 442 000 t of barley per year (SWA,

2007). Distillers use about 37% of the malt produced in the UK (MAGB, 2007).

Over the last few years, poor harvest conditions coupled with substantial increases in barley prices have highlighted the vulnerability of distillers, maltsters and growers to seasonal variations. Additionally, as new global markets for Scotch whisky are opened up, particularly in India and China, there is increasing pressure for Scotch whisky distillers to increase production levels to satisfy future market requirements (Gray, 2007), as well as to replenish whisky stocks. This is expected to result in a further expansion in demand for distilling barley, increasing the importance of barley malt for distillers.

The Scotch Whisky Research Institute (SWRI) is a research organization directed by a consortium representing almost the whole Scotch Whisky Industry, and carries out research projects covering all aspects of Scotch whisky production, of which raw materials are a major component. The Institute has been an important essential component in the Scotch whisky distilling industry's strategy to maintain its long-term sustainability by supporting and sustaining links between the various components of the UK barley supply chain (plant breeders, barley growers, and end users such as maltsters, distillers and brewers). The Scotch whisky industry maintains an essential input in the selection of new malting barley varieties suited to growing in Scotland.

The UK barley selection process is operated by the Maltsters Association of Great Britain, since 2002, under the banner of the Malting Barley Committee of the Institute of Brewing and Distilling. The Recommendation system is designed to evaluate new barley varieties, to deliver material that provides advantages over established varieties, for the benefit of both growers and end users. However the current system for the development of new barley varieties is both time consuming and expensive, and there is a need to provide a

more efficient means of developing new barley varieties for the market. One approach that has been developed over the last decade or so is marker assisted selection, in which our knowledge of barley genetics can be used to identify genetic markers for barley traits that are important to end users.

Classical barley selection is both time consuming and expensive, not only for plant breeders, who wish to promote the barley varieties in which they have invested significantly, but also for the groups involved in the testing process, who have to support the existing system, both in terms of finance, but more importantly in terms of investing time, personnel and resources to ensure that that system is effective. More recently there has been a perception that the current techniques are now showing diminishing returns, and that it is becoming more difficult to differentiate between elite barley varieties (Bathgate, 1998; Steele, 2001; Thomas, 2003). There is thus a need to look beyond these parameters to achieve further major advances in distilling barley quality (Brosnan *et al.*, 2004).

The application of genetics to the selection of barley suited to particular end-user markets has been well established for many years, and during the last decade or so there have been major advances in our understanding of the barley genome. Now that the barley genome has been mapped extensively, some quantitative trait loci (QTLs) have been identified for economically important quality traits, such as hot water extract, malt fermentability and predicted spirit yield (PSY), which are important for Scotch whisky distillers (Swanston *et al.*, 1999, 2000; Meyer *et al.*, 2000, 2001; Thomas, 2003; Powell *et al.*, 2004). Most importantly, a linked genetic marker was developed for epiheterodendrin (EPH), a precursor of ethyl carbamate, which is an undesirable contaminant that can be found at trace levels in whisky (Cook *et al.*, 1990), and which is subject to regulation in several markets for

Scotch whisky (such as Canada, USA and the Czech Republic). The marker for EPH has now been refined to a candidate gene, and is now actively used in the selection of non-EPH-producing distilling barley in the official UK barley selection process.

Many of these advances were further highlighted at the 2004 International Barley Genetics Symposium in Brno (Spunar and Janikova, 2004), and work in this area continues to improve our knowledge and understanding, leading to the development of more advanced precision tools, such as association mapping (Hayes and Szücs, 2006; Rostoks *et al.*, 2006) which can be used more efficiently to locate the genetic factors identified with the major quality traits of barley that are of interest to end users, such as distillers.

The Association Genetics of UK Elite Barley (AGOUEB) project is a £ 1.9 million collaborative barley research project. The principal aim of the project is to use association genetics to identify genetic markers controlling economically important barley traits in both current and 'traditional' elite malting barley varieties. This information will be used to develop a database based on both genetic and analytical parameters, which can be used by plant breeders to inform the selection process for new barley varieties. This new technology will soon enable researchers to develop new 'designer' barley varieties to suit end user requirements. This will accelerate 'classical' (non-GMO) breeding programs to provide both improved agronomic yield for growers and enhanced barley quality, not only for distillers, but also for other end users of barley, such as maltsters and brewers.

The principal role of the SWRI in the AGOUEB Project is to provide analytical data on a range of parameters that are fundamentally important to distillers (including maltsters and brewers) in defining the distilling quality of barley. The main quality selection parameters of interest to

distillers are hot water extract, fermentability and predicted spirit yield (PSY) of the malt, which are usually best expressed under lower grain nitrogen levels. These are established as standard quality parameters for the commercial trading of malted barley, and determine the amount of alcohol that can be produced from a given batch of malt. Bathgate *et al.* (1978), Buckee (1985), Dolan *et al.*, (1981), Dolan (1983), Bringham *et al.*, (1996) and Dolan (2000) summarize the development of standard analytical methods for these parameters and demonstrate how these analyses can be used to assess the performance of distilling barley malt aimed at Scotch whisky production.

This paper will show how some of the analytical data for distilling quality parameters can provide useful information that can be used by geneticists to provide suitable tools to develop barley varieties that will deliver improvements in distillery performance.

Production of Scotch whisky

The generic identity of Scotch whisky is tightly controlled and protected by UK and EU regulations defining Scotch whisky production (The Scotch Whisky Act, 1988; The Scotch Whisky Order, 1990; European Commission Regulation (EEC) No1576/89). These specify that Scotch whisky must be made in Scotland from water, malted barley, unmalted cereals and fermented by yeast. With the exception of spirit caramel and water, no other substances, process aids or other additives, are permitted. Whisky must be matured in oak casks for at least 3 years, although typically malt whisky is generally matured for at least 8 years. For high quality malt brands, spirits can be matured for much longer (>15–21 years).

There are three types of Scotch whisky: malt whisky, grain whisky, and blended whisky. Malt whisky is produced exclusively

from malted barley, and is produced in a relatively small batch-production process, using pot stills. On average, a typical malt distillery will produce around 2.5 ML of pure alcohol (LPA) per year, but capacity can vary substantially from 0.9 ML up to 10 ML LPA per year (Gray, 2007). In contrast, grain whisky is produced on a much larger scale, and uses unmalted cereals, primarily wheat, although maize is still sometimes used. In comparison to the 92 or so malt distilleries, there is a very small number of grain distilleries (7), but individually, these have a much larger production capacity, on average about 45 ML LPA per year (ranging from 12 to 70 ML LPA per year). Blended Scotch whisky is a mix of malt and grain whisky and is the most important in terms of market volume.

The technical aspects of the production of Scotch whisky have been described comprehensively in the literature. Simpson (1968), Bathgate (1998, 2003), Dolan (2003), Nicol (2003) and Lyons (2003) describe various aspects of the production of Scotch malt whisky, while Pyke (1965), Bringham *et al.* (2003) and Campbell (2003) summarize the processes involved.

Materials and methods

Samples

Grain samples (96) were from 16 spring and 9 winter barley varieties grown at four sites for harvest 2006 went for micro-malting by commercial maltsters, under the direction of the Maltsters Association of Great Britain (MAGB), who provided Total Nitrogen (TN) and a measure of Hot Water Extract (HWE).

The varieties grown on each site included current UK Recommended List distilling and brewing varieties, such as Optic, Oxbridge, Cocktail, Cellar and Westminster, as well as some older varieties, such as Triumph, Chariot and Blenheim. Some non-UK malting varieties, such as Scarlett and Astoria, were also included to provide a representative

selection of the development of spring and winter malting barley over the past 25 years.

Methods

Micromalt samples were sent to SWRI for analysis of moisture, another measure of HWE (Miag 7 grind), fermentability and PSY, using the approved standard methods for these analyses as specified by the Institute of Brewing and Distilling (IOB, 1997a, b, c). The data is reported on a dry weight basis.

Results and discussion

The data that is considered in this paper primarily represents one of the main quality traits required for high quality distilling barley, namely is 'high alcohol yield'. The main analysis used to estimate this parameter was the standard IOB Fermentability method (IOB Method 2.16 (unboiled worts)), which together with the IOB method for HWE (IOB, 1997b) provides data on malt fermentability, fermentable extract and PSY, which can be used to predict alcohol yield performance (Dolan, 2003). These parameters are important components of currently accepted benchmarks for the commercial trading of distilling malt in the UK. Distillers generally express HWE as a percentage, rather than as litre degrees per kilogram (L°/kg), the measure more commonly used by UK brewers and maltsters, since the percentage figure is used directly in the calculation of the fermentable extract and PSY (Dolan *et al.*, 1981; Bringham *et al.*, 1996).

Samples were assessed over a range of nitrogen levels on 4 spring and 4 winter sites and the data for PSY correlated well with malt total nitrogen (TN) levels over the range of samples studied. Overall, the results show that the range of material that has been studied in the context of the AGOUEB project represents a wide range of genotype quality, grown on a number of sites under different nitrogen conditions. These were subjected to a wide range of environmental conditions

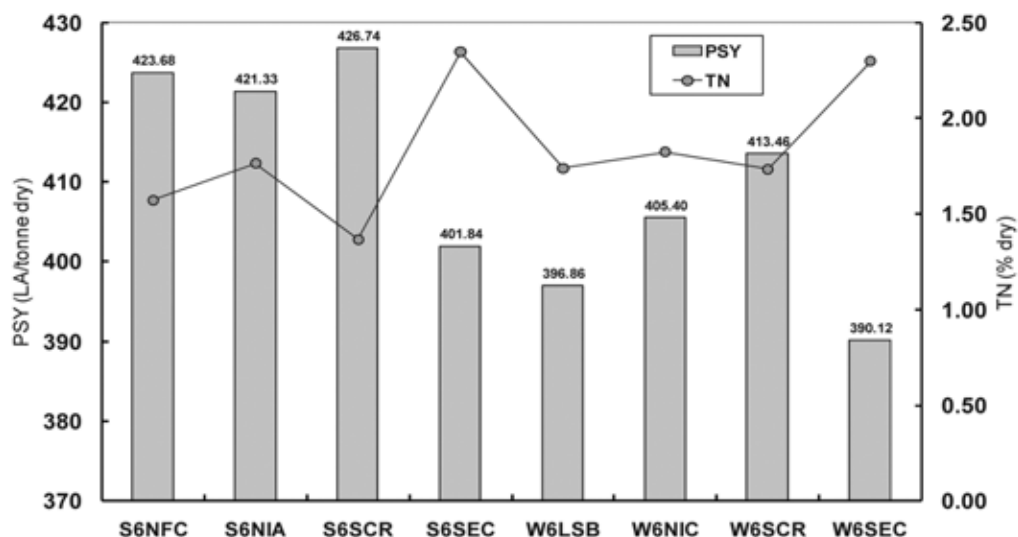


Figure 1. Average alcohol yield (PSY), and malt total nitrogen (TN) for individual trial sites for spring and winter barleys.

during growing, and this is reflected in the site averages, which are shown in Figure 1 and indicate a wide range in performance between sites.

In general, the correlation between alcohol yield and total nitrogen over the whole data set was good for spring barley ($r = -0.8765$; $R^2 = 0.7682$), but less so for the winter varieties ($r = -0.5468$; $R^2 = 0.299$), and this is reflected in the site averages in Figure 1. Importantly the range of site trends that were seen contributes to the robustness of the data, since the performance of individual varieties on each site was largely dominated by that of the elite Recommended List varieties.

Genetic diversity of spring and winter barley varieties

One of the reasons for selecting the particular samples chosen was to provide a range of 'distilling quality' amongst accepted malting barley varieties, reflecting not only the 'normal' quality range for distilling malt, but also the more extreme ends of the quality spectrum. This would make it easier to identify more clearly the contrasts between the factors representing 'poor' as

well as 'good' quality, which would be useful in guiding the genetic studies within the AGOUEB Project.

Figure 2 shows the distribution of PSY data for spring and winter barley, and shows the proportion of samples in the different quality ranges. The bulk of the samples (80%) covered the 'normal' spectrum of malt quality (401–440 litres of alcohol per tonne (LA/t); dwb), this includes both poor and good quality malts. Although, in reality, deliveries to distilleries are generally in the upper reaches of this range (> 430 LA/t (dwb)), much poorer batches than this can still be occasionally encountered in practice. As expected, most spring barley varieties performed better than the winter ones, with a higher proportion of these performing at the 'high' quality end of the range. The relatively poorer performance of winter barley reflects distillers' classification of these as poor distilling malts.

The range of data shown confirms that the samples that were selected represented a reasonably broad spectrum of quality and illustrates the fact that not all malting varieties are suitable for distilling.

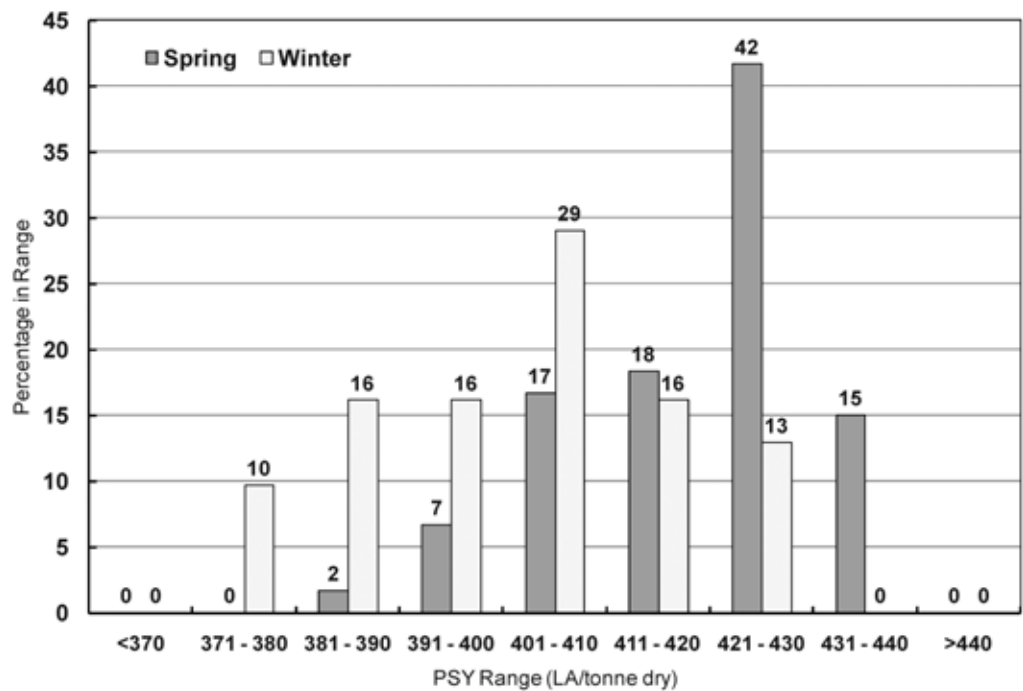


Figure 2. PSY range distribution for micromalts prepared from spring and winter barley varieties.

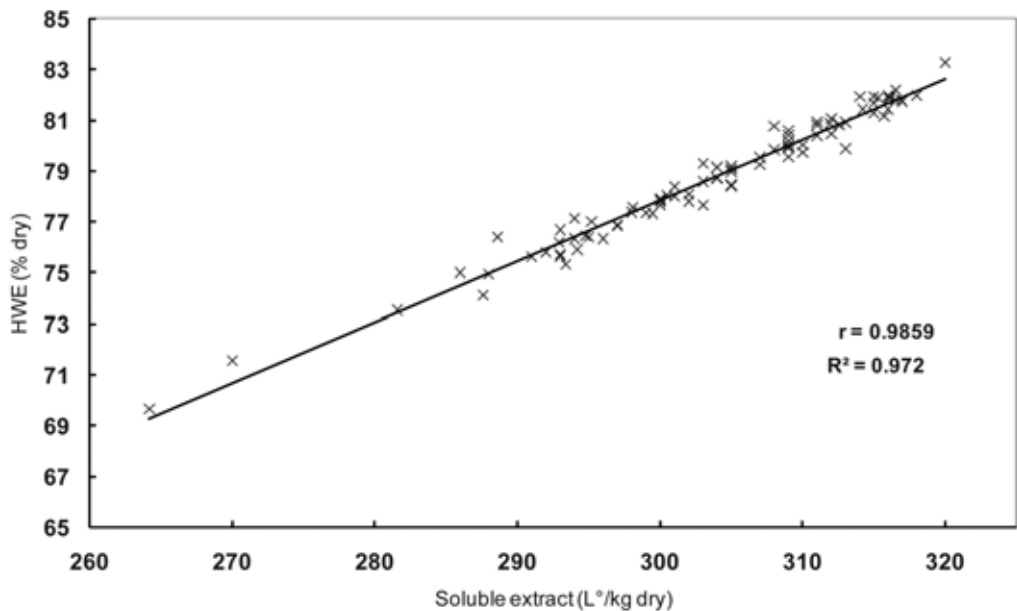


Figure 3. Correlation between HWE data supplied by SWRI (%) and maltsters (L°/kg) (Full sample set: spring and winter barley).

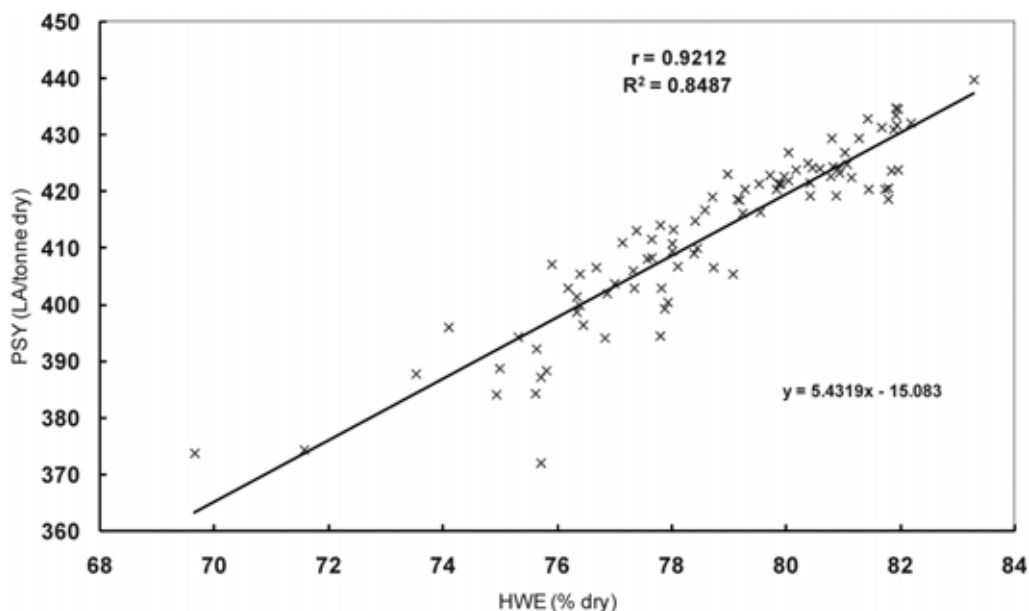


Figure 4. Relationship between HWE and PSY of micromalt samples over the full data set (spring and winter barley varieties).

Relationship between HWE and PSY

Comparison of the SWRI soluble extract data (% HWE) with soluble extract (L°/kg) data supplied by project partners, showed that these had a very high coefficient of correlation ($r = 0.98$; $R^2 = 0.9679$) (Figure 3) indicating that the methods used by project partners were comparable over the full range of samples, supporting the robustness of the methods that were used at different laboratories.

The relationship between HWE and PSY was also very significant (Figure 4), with a high correlation between these parameters ($r = 0.92$; $R^2 = 0.84$) over the whole dataset of spring and winter varieties. In general the data points were well distributed about the correlation lines, indicating that the relationship was maintained over the full range of samples that were studied. This illustrates the fundamental dependence of the potential for alcohol yield on the soluble HWE value, and indicates that the analyses

were performed consistently. These strong correlations indicate that the soluble extract data supplied by SWRI and project partners were strongly related to each other as well as to the SWRI PSY results, and helps to strengthen the data produced.

Spring barley

Spring barley varieties were grown on each of the trial sites, and the potential alcohol yield of the resulting micromalts was reported as predicted spirit yield (PSY) (litres of alcohol per tonne (LA/tonne)). The results are summarized as the average of 4 sites and as shown in Figure 5. The charts also show the malt total nitrogen levels, and show clearly how high nitrogen content relates to low PSY values (and vice versa).

The results show that overall the best performing spring barley varieties were recent varieties, such as Westminster, Cocktail, Oxbridge, Troon and Appaloosa, which are all current UK Recommended Varieties.

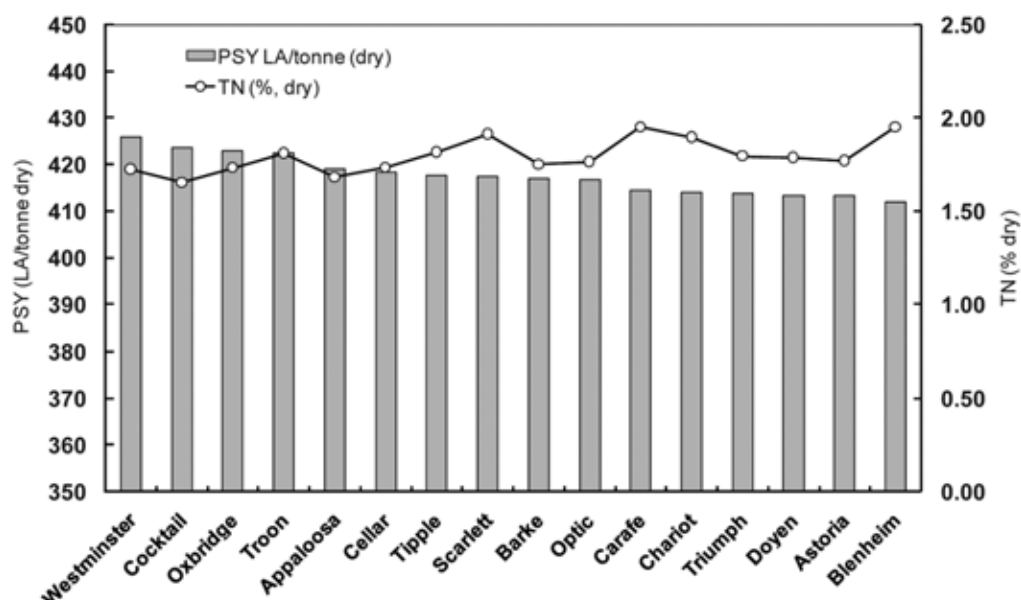


Figure 5. Alcohol yield (PSY) ranking of spring varieties together with malt Total Nitrogen (TN) levels (Average of 4 sites).

Also interesting is that when considering the averaged data, the nitrogen trend reflects the accepted view that alcohol yield is inversely proportional to nitrogen levels. This is confirmed by a strong correlation between these parameters for spring barley ($r = -0.88$; $R^2 = 0.77$).

The alcohol yield trends on individual sites largely reflected those shown in Figure 5, and it is not surprising that the top ranking spring barleys, on 3 out of the 4 spring sites, largely represented recent varieties on the UK Recommended Lists varieties for distilling and brewing. Westminster, although the best performer overall, does not have Approval for use as a distilling variety, since it is an EPH producer.

There were some exceptions to the trends described above, such as Scarlett and Astoria, which performed very well on individual sites but not on others. Appaloosa gave a general level of performance similar to Optic on all sites. Interestingly, some of the older varieties, such as Triumph and Chariot, performed reasonably well on some sites.

These results lend further support to the robustness of the current selection process for barley grown in the UK. Interestingly these trends were consistent on both high- and low-nitrogen sites.

Winter barley

A smaller number of winter barley varieties were grown on the winter trial sites. These were ranked in a similar way (Figure 6).

As with the spring varieties, the ranking was mainly dominated by Recommended List brewing varieties, such as Flagon and Fanfare. Pearl performed consistently well on 3 sites. Maris Otter, which is not currently a UK malting variety, also performed well on 3 out of the 4 sites.

Unsurprisingly, the malts deriving from winter barley did not give as good a PSY as the spring ones, these also gave poor filtration rates and high levels of β -glucans (data not shown) and emphasizes that, in general, winter barley is considered unsuitable by distillers. However, the wider aims of

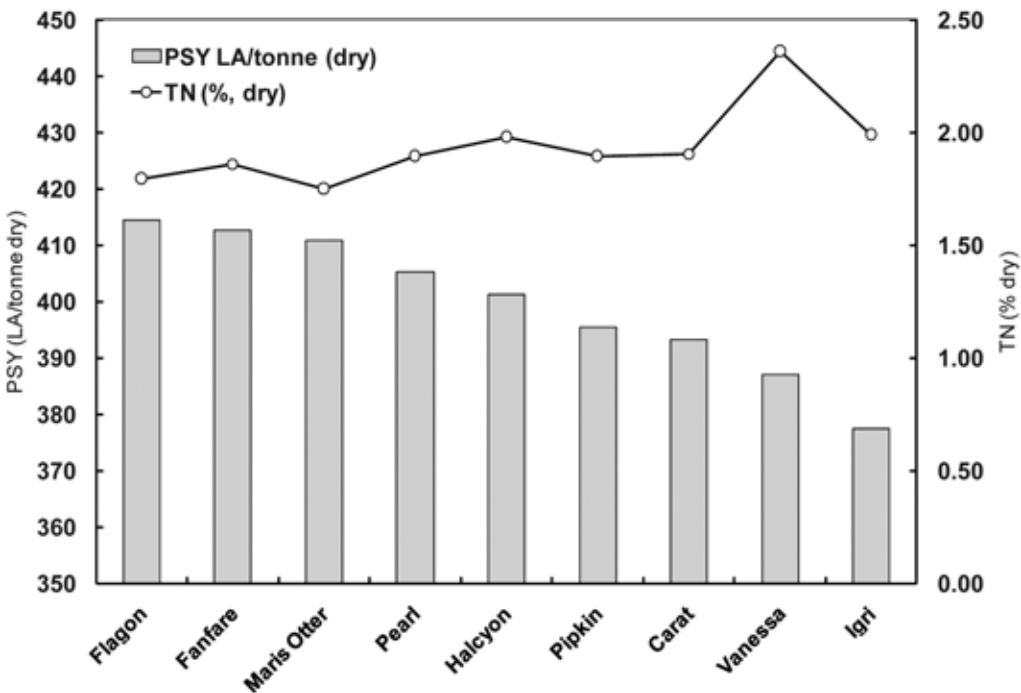


Figure 6. Alcohol yield (PSY) ranking of winter varieties together with malt Total Nitrogen (TN) levels (Average of 4 sites).

the AGOUEB project include maltsters and brewers as well as distillers, and the inclusion of this material will contribute to the robustness of the data.

Currently the main selection criteria for new barley varieties is that in order to progress through the system, they must be shown to be as good as, or better than the best currently established control varieties. In practice, the assessment is carried out against a range of parameters, relating to agronomic, malting, brewing and distilling performance, of which HWE and PSY are major indicators.

That the system has been effective can be shown by Figure 7, in which mean phenotypic performance of varieties is regressed against the year of its first recommendation. This shows a general, sustained, increase in the performance of new malting and distilling barley varieties over the years, both in terms of agronomic yield and distilling performance.

Both spring and winter barley varieties have progressed in terms of both agronomic yield and hot water extract. From the perspective of Scotch whisky distillers, the alcohol yield performance of spring barley has increased substantially, on average by around 10 litres of alcohol per tonne.

Conclusions

The work reported in this paper illustrates how genetic factors are able to overcome environmental challenges, to provide barley germplasm with performance traits that are suited to particular end users, in this case Scotch whisky distillers. Clearly the impact of genotype is of fundamental importance, since the current UK MAGB/IBD Recommended List varieties (such as Optic, Cocktail, Cellar, Oxbridge, Troon, Westminster and Appaloosa) were dominant on most sites, and were reasonably comparable with

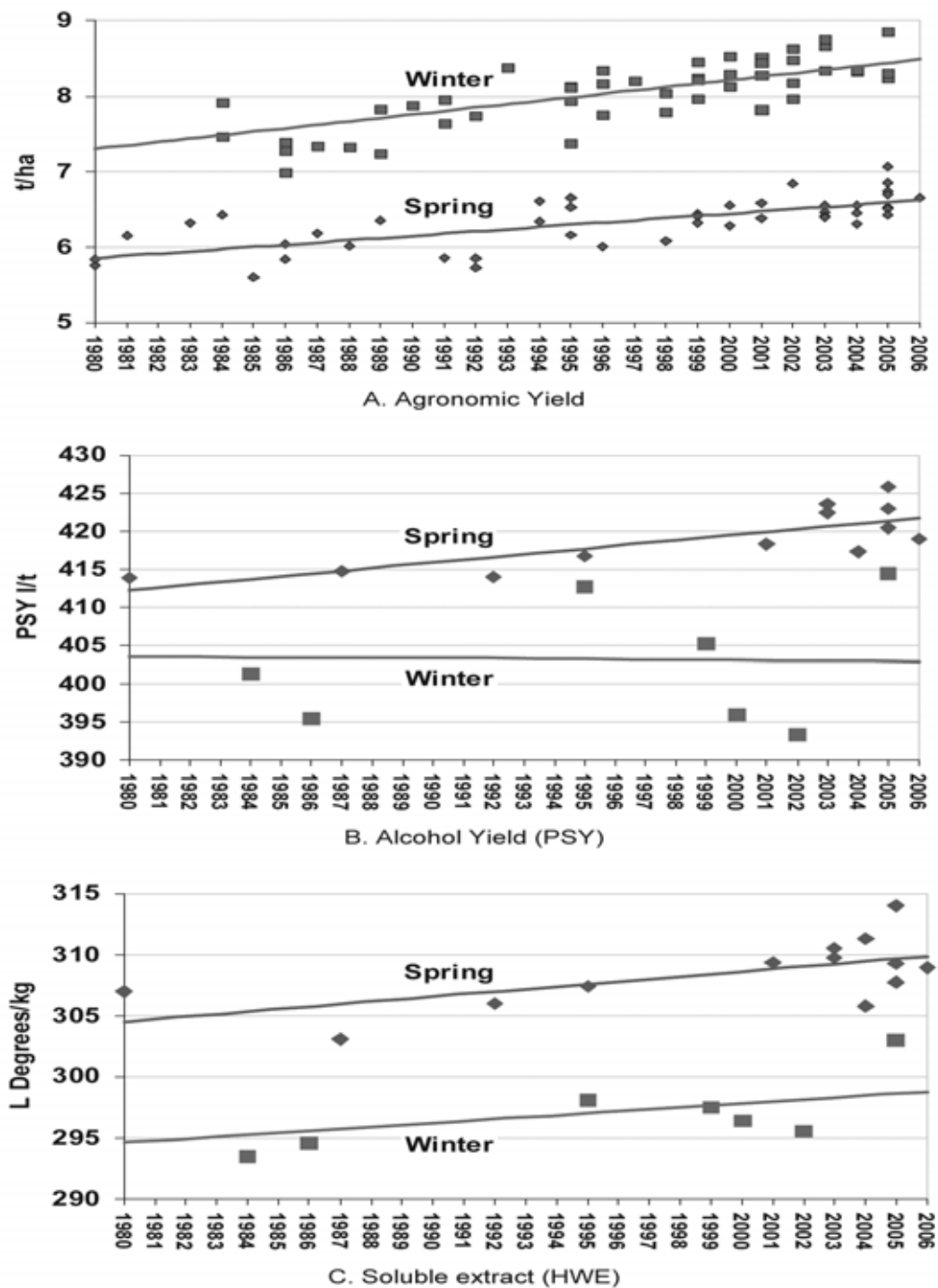


Figure 7. Mean Varietal phenotypes for (A) Agronomic yield (tonne/ha); (B) Alcohol Yield (PSY) (Litres of alcohol per tonne); and (C) Extract (HWE) (L°/kg) performance of barley malt plotted against year of first recommendation.

each other. Hence the data lends support to the robustness of the current MAGB/IBD systems for assessing and approving new barley varieties. Westminster, one of the best performing varieties, is not suited to distilling, since it is an EPH producing variety. Oxbridge, Troon and Appaloosa are all EPH non-producers.

However, the importance of these results lies not in the ranking of individual varieties that we have seen on these sites, but in the consistency in the trends that were observed, which indicates that both varietal and site \times environmental factors (as measured by malt nitrogen levels) were important in maintaining performance. Previous workers have shown that genotypic and environmental effects both have a significant influence on barley malting quality (Molina-Cano *et al.*, 2004), and it is considered important to select genotypes carefully to give optimal performance in different locations. This helps to explain why barley varieties suited for growing in the UK may not necessarily match up with those that perform better in other geographical regions.

From the work that has been described, it is clear that in the UK there is a robust system for assessing new distilling (malting and brewing) barley varieties. The unique dataset that has been generated from this system over many years will be very useful in facilitating further genetic studies. The use of association genetics using elite barley information will provide a useful strategy for improving barley varieties aimed at particular end-user requirements, such as those of Scotch whisky distillers.

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Proteome analysis of wort, boiled wort, and beer proteins

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Abstract

We previously reported on identification of major proteins in beer using two-dimensional gel electrophoresis (2DE) and mass spectrometry analysis (Iimure *et al.*, 2007). In the present study, in order to discuss changes in protein composition and modification during beer production, we analyzed and identified major proteins in wort. Wort, boiled wort, and beer samples were prepared from the barley malt of cultivar Haruna Nijo. The wort proteins were separated using 2DE, and 202 protein spots were analyzed by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS), with 72 protein spots identified and categorized into 32 protein species. Among the 32 proteins, 14 proteins were newly identified in wort, which were retrieved only in the unpublished barley EST and cDNA databases constructed by Okayama University. Comparison of the 2DE patterns of wort, boiled wort, and beer proteins suggested that the degrees of protein precipitation depended on the protein species. Additionally, we found that pI of lipid transfer protein 1 (LTP-1) in wort was different from that in beer, indicating that the conformation of LTP-1 changes during wort boiling and/or fermentation processes.

Introduction

Proteins play important roles in each step of the brewing process. In malt and wort, protein-related indexes, such as total nitrogen, soluble

nitrogen and Kolbach index, are key factors determining the brewing quality. However, there is little information on the relationship between individual protein species and beer quality except for a few findings such as beer foam stability (Evans *et al.*, 2002) and haze formation (Leiper *et al.*, 2003).

Recently, proteome analysis has been advanced in many research fields. In brewing science, proteins in barley grain, malt and beer proteins have been investigated comprehensively using two-dimensional gel electrophoresis (2DE) and mass spectrometry analysis. Østergaard *et al.* (2004) separated the proteins in barley grain by 2DE and identified major protein spots on 2DE gels. Iimure *et al.* (2007) and Perrocheau *et al.* (2005) identified proteins in beer by 2DE and mass spectrometry analysis.

The purpose of this study is to construct a novel proteome database of wort proteins. We separated wort proteins using 2DE and then identified major protein spots using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS) followed by peptide mass fingerprinting (PMF) analysis. To identify protein species, we used both disclosed databases, such as NCBI-nr and HarvEST unigene, and novel databases containing barley cDNA and EST sequences constructed by Okayama University. Additionally, we compared the 2DE patterns of wort, boiled wort and beer proteins to investigate changes in peptide profiles during wort boiling and fermentation processes.

Materials and methods

Wort, boiled wort and beer sample

Barley grain of cv. Haruna Nijo was harvested in Japan in 2002. Wort was prepared according to the standard methods of the European Brewery Convention (EBC, 1987), with 30 mL of the wort boiled at 100°C for 120 min in a 100 mL flask, with reflux. The wort and boiled wort were used in proteome analysis. For malting, 75 kg of barley grain (>2.5 mm screen) was malted according to our previous report (Ogushi *et al.*, 2002). A beer sample was brewed from the barley malt of cv. Haruna Nijo according to the standard method of the Product and Technology Development Center of Sapporo Breweries.

Two-dimensional gel electrophoresis (2DE)

After the wort, the boiled wort and the beer samples were desalted using PD-10 column (GE Healthcare Biosciences, Japan), and the proteins were lyophilized. The lyophilized proteins were completely dissolved in the dissolving buffer (8 M urea (Wako, Japan) + 2% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) (Dojindo Laboratories, Japan) solution containing 0.28% dithiothreitol (Wako, Japan)), then applied to two-dimensional gel electrophoresis. First dimensional isoelectric focusing was carried out using IPG dry strips pI 3-10 or 4-7 or 6-9 (GE Healthcare Biosciences, Japan). Second-dimensional SDS-PAGE was performed using a precast XL 12–14% gradient gel (GE Healthcare Biosciences, Japan). First and second dimensional gel electrophoreses were carried out using Multiphor II system (GE Healthcare Biosciences, Japan). The gel was stained with a Silver Staining Kit Protein (GE Healthcare Biosciences, Japan). For the mass spectrometry analysis, protein spots were stained by Silver Stain MS Kit (Wako, Japan).

Mass spectrometry analysis

After 2DE and silver staining, the protein spots were cut from the gels, then each protein spot was collected and preserved in stock solution (5% (v/v) acetic acid and 50% (v/v) methanol) at 4°C until mass spectrometry analysis was carried out. After digestion of the protein sample by trypsin, the solution was desalted using Zip-Tip (Nihon Millipore Ltd., Japan), and eluted by matrix solution. This eluent was then analyzed using MALDI TOF-MS carried out using a Voyager-DE STR (Applied Biosystems, USA). Protein species were identified by peptide mass fingerprinting (PMF) using the National Center for Biotechnology Information non-redundant database (NCBI-nr). Database searching was performed using the MSCOT search engine. If a protein could not be identified, re-searching was carried out under the same conditions using the Institute for Genome Research (TIGR) barley gene index (<http://compbio.dfci.harvard.edu/tgi/>), HarvEST unigene #31 (<http://harvest.ucr.edu/>), and the mass spectrometry protein sequence database (MSDB) (<http://csc-fserve.hh.med.ic.ac.uk/msdb.html>). If a protein was still unidentified, re-searching was performed under the same conditions using in-house nucleotide databases constructed by Okayama University, Japan, comprising barley ESTs and cDNA sequences.

Results and discussion

Identification of major proteins in wort

The wort sample prepared from the barley malt of cv. Haruna Nijo germinated at 37% of ex-steep moisture was desalted and then applied to 2DE (pI 4-7, 6-9) (Figure 1). The subsequently obtained major protein spots were analyzed by MALDI TOF-MS. In order to identify proteins, a database search was carried out using disclosed databases (NCBI-nr database, etc.) and the in-house databases constructed by Okayama University. As

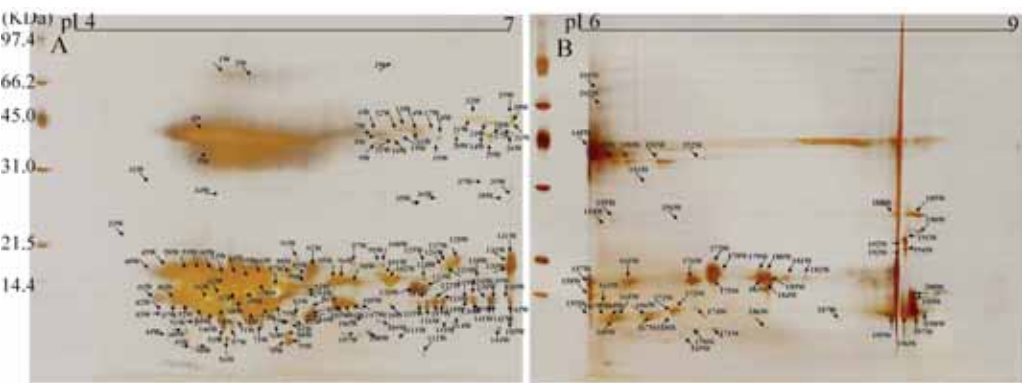


Figure 1. The two-dimensional gel electrophoresis (2DE) patterns of the proteins in the wort prepared from the malt of cv. Haruna Nijo (A: 2DE pattern of pI 47-; B: 2DE pattern of pI 69-. Arrows indicate spot numbers).

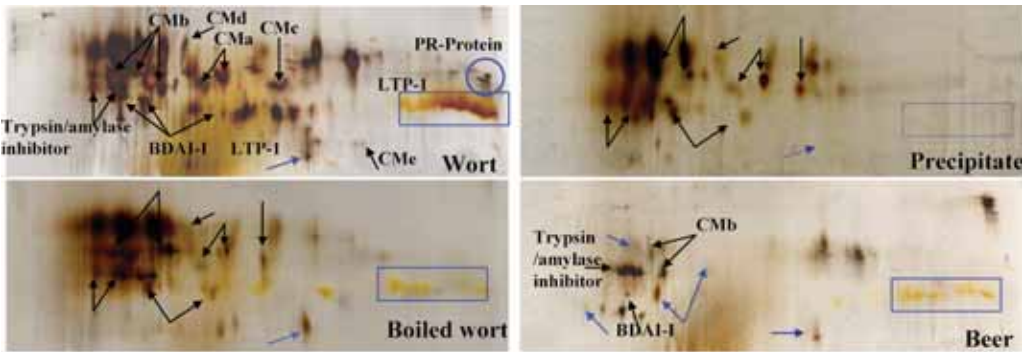


Figure 2. Comparison of low molecular weight region of two-dimensional gel electrophoresis patterns of the wort, the boiled wort, the precipitant by wort boiling and the beer. Blue arrows and the blue square indicate LTP 1.

a result, 72 out of 202 protein spots were identified and categorized into 32 protein species. Among the 32 proteins, 14 protein species were newly identified proteins in wort, found only in the unpublished data set of the EST database and cDNA database of Okayama University. The information on the spots identified in this study may contribute to both brewing and barley breeding. The functions of the newly identified proteins are not known at present. However, our future aim is to determine the relationships between these proteins and certain qualities of beer and wort.

Changes of 2DE patterns from wort to beer

Proteins in wort are precipitated during wort boiling. The wort, the boiled wort and their precipitant, and the beer samples prepared from the barley malt of cv. Haruna Nijo were compared with each other in terms of the 2DE patterns of proteins. Figure 2 shows enlarged images of the 2DE patterns of the low molecular weight region.

Five species of CM (chloroform-methanol soluble) protein (CMa, b, c, d and e) were identified in the wort sample, whereas only CMb was observed in the beer

sample. Distinct spots of CMa and CMc were observed in the precipitant after wort boiling. However, relatively faint spots of these proteins were observed in the boiled wort sample. In contrast, the spots of CMb and CMd in boiled wort were distinct. These results suggest that CMa and CMc are relatively heat-susceptible proteins in the CM protein species. Pathogen-related proteins (PR-protein) such as thaumatin-like protein TLP-7 were identified in the wort sample. However these spots disappeared in the boiled wort, the precipitant and the beer sample. The distinct spots of LTP-1 were observed in the wort, the boiled wort and the beer samples; however, these spots were not detected in the precipitant sample (Figure 2). These results suggest that LTP-1 is relatively resistant to boiling. By comparison of 2DE patterns of the wort and the beer sample, it was seen that the pI of the LTP-1 spots dramatically changed. The pIs of the LTP-1 spots in the wort sample were relatively basic, whereas the pIs of LTP-1 spots in the beer sample were shifted to the acidic side. Moreover, the apparent molecular weight of the LTP-1 protein in the beer changed to higher values compared with the apparent molecular weight of the wort LTP-1 protein. These results suggest that the conformation of LTP-1 changed during wort boiling or fermentation processes, or both.

Conclusion

In this study, we constructed a novel proteome database of wort proteins using an in-house data set of the barley EST and cDNA sequences constructed by Okayama University. Using this proteome database, we investigated the changes in the 2DE patterns during the wort boiling and fermentation processes. As a result, it was suggested that the degrees of heat resistance during wort

boiling depends on protein species, and that changes in conformation of protein occur in these processes. In future work, we will be using this database to examine the relationship between beer qualities and proteins.

Acknowledgements

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Improvement of two-row malt barley through two- × six-row hybridization in India

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Abstract

Barley cultivation in India is now becoming more and more oriented towards industrial utilization, with a changing scenario in southeast Asia with respect to beer and other malt products consumption. In addition to the quantity, the quality of grain is becoming a constraint for the domestic malting industry. Nearly 20–25% of barley production is used by the malting industry and this malt is further utilized in brewing (about 50%), malt whiskies (20–25%), energy drinks and baby foods (20–25%), and medicinal syrups and vinegar (5%). Traditionally, six-row barley is cultivated in the country, but recently production of two-row malt barleys in the states of Punjab, Haryana and Rajasthan started with newly released cultivars. As barley was largely considered a crop for marginal and problematic soils under rainfed condition, the quality of the barley produced from these areas did not meet industrial requirements. The barley varieties available for the malting industries possessed more husk, a higher proportion of thin grains, poor grain filling, and lower wort filtration or higher wort viscosity, resulting in poor malting quality. Therefore, introductions of two-row malt barley varieties were made in early 1990s. Two such introductions, Alfa 93 (introduced from Argentina) and BCU 73 (an ICARDA line introduced into Australia and released under the name of Yagan) were released for commercial cultivation in the northwestern plains (NWPZ) of India in 1994 and 1997, respectively. However, these varieties did not match the yield levels of the

improved six-row types and were not well accepted by farmers. Therefore, there was the need for a separate breeding program for malting barley in India. In order to evaluate and classify the barley variety as malt or non-malt type, the minimum standards for various grain and malt traits were finalized. Though there are various grain and malt parameters important in barley, the hot water extract (HWE) is paramount, and the price of malt is mainly dependent on its HWE values, along with diastatic power, friability, homogeneity, etc. Our regression studies indicated that measurement of grain traits like hectolitre weight, hull content and malt friability can predict HWE, with hectolitre weight the single largest contributor. Therefore hectolitre weight can be effectively used in initial screening of breeding lines in early generations (without actual malting), and the remaining traits can be assessed in elite material and advanced breeding lines. Following a two- × six-row hybridization program for grain yield improvement in two-row barleys, significant yield gain (37.5 to 45.1 q/ha) has been made in newly bred varieties like DWRUB52, DWR28, RD2668 compared with earlier introductions in NWPZ under limited irrigation, almost equivalent to six-row types. In addition to grain yield improvement, incorporation of resistance to yellow rust and leaf blight has been another significant achievement. These developments have now opened up the possibilities of cultivating two-row barleys in India, which were shunned by farmers because of their lower yield, despite possessing good malting quality.

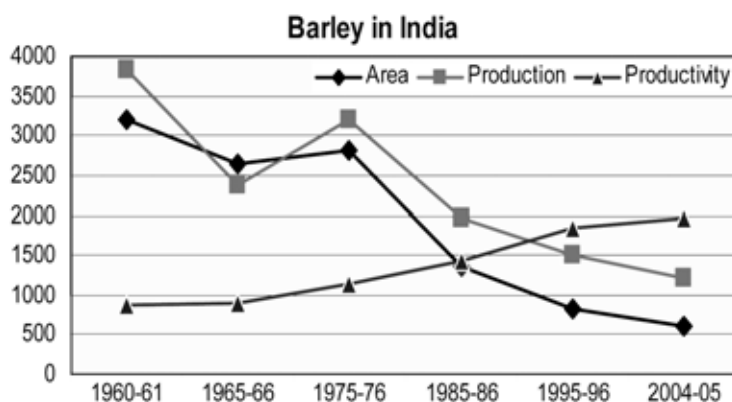


Figure 1. Trends in barley area ('000 ha), production ('000 t) and productivity (kg/ha) in India.

Introduction

Barley has been traditionally considered as poor man's crop in India because of its low input requirement and better adaptability to harsh environments, like drought, salinity and alkalinity, and marginal lands. It has been utilized as food, cattle feed and industrial raw material in the country. Currently, its utilization as food crop (mainly huskless types) is restricted to the tribal areas of hills and plains in India. Barley products like *Sattu* (consumed in summer because of its cooling effect on the human body) and *missi roti* (for its better nutritional quality) have been traditionally used in India. Under semi-arid conditions it is also used as dual crop for green fodder and grain from the re-growth crop. The barley area is concentrated in the states of Uttar Pradesh, Punjab, Rajasthan, Haryana, Madhya Pradesh and Bihar in plains and Himachal Pradesh, Uttarakhand and Jammu and Kashmir in the hills. Barley occupies nearly 620 000 ha, producing nearly 1.21 million tonne of grain, with a yield of 19.58 q/ha (Verma *et al.*, 2005). Despite productivity gains through research efforts on varietal development and production technology, the area under barley has reduced in the country over the last four decades,

basically because of a shift towards wheat (in irrigated areas) and mustard (in rainfed areas) (Figure 1).

Traditionally, six-row barley has been cultivated in the country, and since barley was largely considered a crop for marginal and problematic soils under rainfed condition, the quality of the product was not aligned to industrial requirements for malting. Currently available barley for the malting industries possessed more husk, a higher proportion of thin grains, poor grain filling and lower wort filtration or higher wort viscosity, resulting in poor malting quality. Barley cultivation in India is now becoming more and more oriented towards industrial utilization, with a changing scenario in southeastern Asia with respect to consumption of beer, malt whiskies and other malt products. The increased domestic demand for barley for industrial utilization, as well as the establishment of several malting and brewing units, including several multinational malting and brewing companies, necessitated the initiation of a malt barley breeding program. In addition to quantity, the quality of available barley grain had now become a constraint for malting industry. The brewing industry now prefers to use two-row barley because of its better performance for brewing traits like higher

extract, better wort filtration rate and low viscosity.

Nearly 20–25% of the production (250–300 000 t) is consumed by the malting industry and this malt is further utilized in brewing (about 60%), malt whiskies (8–10%), energy drinks and baby foods (25%), and medicinal syrups and vinegar (7%) (Source: M/s Barmalt India (Pvt.) Ltd., Gurgaon – the largest malt company in India, in absence of official figures) (Figure 2). Only recently has production of two-row malt barley in the states of Punjab, Haryana and Rajasthan been initiated with newly released cultivars under contract farming with the malting and brewing industry.

Coordinated barley improvement program

The Barley Network Program of ICAR, under the All India Coordinated Wheat and Barley Improvement Project (AICW&BIP), involves seven funded centers located in major barley growing states, and a few voluntary research centers, including one R&D unit of M/s United Breweries Ltd., Bangalore. Each center has its local as well as zonal or regional objectives, depending on the agro-climatic conditions as well as the prevalent biotic challenge situation. In the case of barley improvement for difficult areas and as a low-input crop, the program made significant progress. The research and development efforts on barley as a low-input crop at various centers were strengthened and

the incorporation of resistance to different diseases and pests was made mandatory.

More emphasis is now being laid on irrigated barley for industrial utilization, with the intention of shifting towards two-row barley for this purpose. The barley network refined its research priorities for barley improvement in view of the changing scenario of the last 15 years and updated its objectives to address the needs of malt, feed and fodder types of barley in the country. These priorities are:

- Development of high yielding varieties with superior malting qualities.
- Development of cultivars for problematic environments such as rainfed, saline or sodic soils, brackish water and *diara* lands (lands with annual recurrence of floods).
- Development of dual-purpose varieties for green fodder and grain as cattle feed.
- Incorporation of resistance to various biotic stresses like rusts, leaf blights, aphids and cereal cyst nematode.
- Development of suitable crop protection and production technologies.

After re-prioritization of the research objectives, the barley network program has been able to make improvement in the desired direction. A number of varieties suitable for different cultural and agroclimatic conditions have been developed. In addition, varieties with specific attributes like cold tolerance in the Northern Hills and cereal cyst nematode (*Heterodera avenae*) resistance for sick soils of Rajasthan and Haryana were also made available to the farmers. Systematic research efforts resulted in gradual improvement in productivity of the varieties under different production conditions in various areas. The genetic gain in yield potential under different production conditions is quite evident through new varieties, where a gradual increase in grain yield of the new cultivars has been achieved with the continuous breeding efforts of the barley research centers in the country. In addition to grain yield, the barley breeders

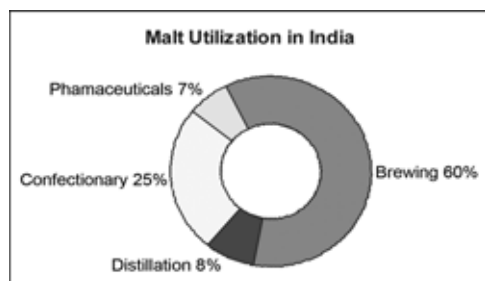


Figure 2. Proportions of barley malt utilization for different purposes in India.

have successfully incorporated resistance to major biotic and abiotic stresses in the barley genotypes.

Barley improvement for malting purposes

Research on barley improvement dates back to the 1920s and 1930s in India, when, by pure line selections, a number of improved barley varieties, including C 251, Type 4, Type 5, were developed (ICAR, 1961; Vasudeva *et al.*, 1979). Though these varieties were not developed for malting purposes, they were used for malting because of their good grain type. Amongst these, C 251 was exported to England for industrial utilization (Roberts and Singh, 1951). Research efforts on two-row malting barley started at the Indian Agricultural Research Institute (IARI), New Delhi, in the late 1960s after the inception of the ICAR AICW&BIP, simultaneously with the initiation of research into semi-dwarf six-row barley for irrigated cultivation.

Several introductions, including Peatland and Pedigree (USA), Manchuria (Germany) and Odessa (Russia), were evaluated at IARI, New Delhi, along with indigenous varieties like Type 4, C 251 and NP 113 in the late 1960s. These exotic lines were found to be inferior for traits like 1000-kernel weight (tkw) and high N content (Anon., 1968), and differential requirements for brewer's and distiller's malt were clear to the then researchers. Another set of introductions—Clipper (two-row, semi-winter type malt barley, introduction from Australia), Prior, Triga and AQ 769—were evaluated in a separate coordinated multi-location trial for two-row barley. Clipper was yielding about 22% less (22.0 q/ha vs. 29.3 q/ha) than the six-row zonal check (Anon., 1971). In the following year, Union, Ciro, Clipper, Alsha, Firecheck and a few others were tested in this trial and again it was observed that the two-row types yielded between 20 and 45% less than the six-row checks (Anon., 1972).

In order to meet industrial demand for better quality in the northern plains of India, Clipper was released for commercial cultivation in 1972. However, Clipper was not popular with farmers due to poor grain yield, late maturity and poor price support from industry as compensation for less yield but good grain quality. During 1974–75, three more introductions (Golden Promise, Universal and Midas) from UK were evaluated under the AICBIP in two-row barley trial at four locations, but they also could not match the yield of six-row barley check Jyoti (Anon., 1975). Separate trials for two-row barley were also organized in 1976 in the northern plains. These trials were extended to the southern hills (Nilgiri Hills and nearby areas in Tamilnadu) to explore the possibilities of growing two-row barley with relatively longer growth period in the relatively cooler climate there. However, the results did not change and the trials were inconclusive in the area.

Though breeding efforts were made on a limited scale for introgression of the desirable features of two-row barley (grain plumpness, low husk and low N content) into six-row barley, not much success was obtained. During this phase, more emphasis was given to the development of huskless barley for human consumption and breeding hulled barley for low input, rainfed and marginal soils, which resulted in development of tall type cultivars with less input requirement, and whenever these were irrigated and fertilized everything lodged badly. Based on these experiences, as well as because of poor industrial growth and irregular requirement for the raw material, the evaluation program for two-row barley came to a halt for a long period.

Resurgence of malt barley demand

Though barley has been utilized in malting in the country for a long time, it was only

in the late 1980s that demand picked up because of the opening up of the economy and liberalization policies of the government towards issuing licences to new breweries, after a long hiatus. By this time, the barley area and production in the country was considerably reduced and shortage of good quality raw material was felt. During this period, the malting and brewing companies even sought permission to import barley grain for malting purposes. The annual requirement for barley for malting purposes since then has risen. Estimates based on the installed capacity and production of major malting units in India indicate that total quantity of barley needed annually for malting purposes is between 240 000 and 250 000 t. An expected growth rate of 10% per year in barley requirement for industrial purposes is predicted, with several new malting and brewing units entering the field. At present, the quantity of barley available for malting is not a major problem, but its quality is a major concern as it does not meet the minimum grain standards for malting. The barley currently available for malting has more husk, a greater proportion of thin grains, and less carbohydrate in the endosperm, resulting in poor malting quality.

In order to meet the demand for better malt-type barley indigenously, it was planned to first screen and evaluate the available six-row cultivars for their acceptability as malting barley by the industry. This preliminary evaluation of released varieties for malting quality indicated that varieties RD 37, RD 57, RS 6, Bilara 2 and C 138 could be utilized for malting. However, all of them were old, tall barleys for rainfed cultivation, and could not tolerate the improved management needed for high yield. All other improved high yielding cultivars were not found suitable for malting, because of various undesirable grain characters (higher husk, higher proportion of thin grains and less carbohydrate in endosperm). Some of the good six-row barley cultivars released recently, such as

RD 2503 (NWPZ), K 551 (NEPZ) and DL 88 (Peninsular Zone) for cultivation under irrigated, timely sown conditions were also recommended for use as malting barley (Table 2). All these cultivars were better than the other six-row barleys, but nowhere near to the quality of two-row types.

Identification of suitable environments for malt barley cultivation

Several grain and malt parameters influence the malting quality of barley and these are in turn affected by the location or the area of cultivation. Environmental effects on malting quality have been studied and locations with better malting potential were also identified (Verma and Nagarajan, 1996). In the relatively drier belt of the NWPZ with lighter soils in the states of Punjab, Haryana, Western Uttar Pradesh and Northern Rajasthan, with mild winters, where barley is grown under 2–3 irrigations, malting barley of good quality can be grown. The high humidity and high temperature conditions of eastern India promotes a higher incidence of diseases like leaf blights, which adversely affect the appearance of grain, malt quality and yield, making it an unfavorable area for malting barley cultivation.

Understanding the industrial requirement

In order to evaluate and classify a barley variety as malt or non-malt type, the exact requirements of the industry for various grain and malt traits were not conclusive. At the same time there was no input from the industry in the R&D efforts of the national program. However, the continuous shortage of good quality raw material forced the industry to seek cooperation with the barley breeders to address the issue, and for the first time several industry delegates joined the annual conference of the AICBIP in 1992.

The barley network program interacted with the representatives of the malting and brewing industries and a National Core Group on Malt Barley Development (NCGMBD) was established, involving representatives of the malting, brewing and confectionary industries together with barley researchers from ICAR and universities, to understand the specific grain quality requirements for malting and brewing.

The minimum standards of barley grain and malt quality traits (Table 1), as a guideline to barley breeders and all other agencies concerned, were finalized in 1995 by the core group, taking into consideration the European Brewery Convention (EBC), American Malt Barley Association (AMBA) and ISI guidelines (Verma *et al.*, 2005). These standards now guide the program when selecting or promoting any barley variety as a malting barley in the network evaluation trials and for commercial release.

Establishment of a malt quality evaluation laboratory

Consequent to the visit of the first author to Carlsberg during the 6th IBGS in 1991, the concept of micro-malting analysis was conceived and implemented in the Indian barley program. A well furnished laboratory (the only of its kind in the public sector in the country) was established at DWR, Karnal, as a central facility for the Barley Network to evaluate and analyze the breeding material for malting traits and to help the varietal improvement program in addition to the regular evaluation of the test entries of coordinated yield trials. The laboratory facilities now include an automatic micro-malting system (Phoenix), protein auto analyzer, malt friability meter, Sortimat, malt mashing bath and viscometer, etc., most of which are EBC-approved instruments. The

Table 1. Analytical standards for malting barley in India¹

		Type of barley	
No.	Parameter	2-row	6-row
BARLEY GRAIN			
1.	Moisture (%)	<12.0	<12.0
2.	Kernel Shape	Elliptical	Elliptical
3.	Kernel Size	Uniform plump	Uniform Plump
	Bold (on 2.5 mm)	90%	80%
	Thin (through 2.2 mm)	<3%	<3%
4.	Skinned/broken grains	<10%	<10%
5.	1000-kernel weight (g)	>45	>42
6.	Husk Content	<11.0%	<11%
7.	Protein Content (DM basis) ²	9.0–11.0%	9.0–11.5%
8.	Germination Capacity	>96%	>96%
9.	Germination Energy (72 hrs)	>96%	>96%
10.	Beta-glucans	3.7–5.0%	3.7–5.0%
BARLEY MALT			
1.	Malt Modification	Satisfactory modification with four-day germination cycle	
	Malt Homogeneity	>90%	>90%
	Malt Friability	>60%	>60%
2.	Kolbach Index (S/T protein ratio)	40–44%	40–44%
3.	Malt Extract (minimum)	80%	78%
4.	Wort Viscosity	<1.5	<1.5
5.	Wort turbidity	Clear	Clear
6.	Diastatic Power (L.V.)	80–120	90–130

¹ Finalized in the first meeting of the NCGMBD at DWR, Karnal, on 12 December 1995. ² Revised during 43rd Annual Meeting of the AICW&BIP, at IARI, New Delhi (27–30 August 2004).

regular analysis of the material through multi-location grain samples of the coordinated yield trials has resulted in classifying several new varieties as malt barley, with prescribed standards.

Recent efforts for malt barley improvement

Evaluation of two-row malt barley introductions

Germplasm introductions, mostly two-row types from ICARDA, Australia, Denmark and Argentina, were evaluated in multi-location yield trials and nurseries during 1989–1993 and, as a stop-gap arrangement, two-row introductions Alfa 93 (introduced from Argentina as Quilmes Alfa) and BCU 73 (Yagan, described earlier) were released for commercial cultivation under irrigated, timely sown conditions in NWPZ, India, in 1994 and 1997, respectively (Table 2). However, these two-row varieties did not match the yield levels (being some 20% less) of the improved six-row types and were not well accepted by the farmers. Late maturity, weak straw, poor grain filling under heat stress and low grain yield remained the points of concern for farmers and researchers.

Significant reduction in the performance of the physical grain quality traits was observed for most of these accessions when grown under Indian conditions, compared with the original seed lots from the respective sources. Introductions like Clipper, Quilmes

Pampa, Quilmes 27-1, Caruso, Canut, Marnie, Henley, Xanadu, Carafe and Prestige, coming from different continents, behaved in the same manner. The thin straw and profuse tillering gives a grassy look and the late maturity severely reduces both yield and quality in the late heat experienced at the grain development stage in late March and early April. The sudden rise in temperature coupled with hot winds leads to forced drying and only a period of 30–35 days is available to the crop. The application of additional irrigation at this stage does not help and results in severe lodging, which again reduces the grain quality.

Two- x six-row hybridization for two-row malt barley improvement

The experience with two-row introductions in the late 1960s and recent renewed efforts led to a separate breeding program for two-row malt barley in India. The idea was to introgress the local adaptability and higher yield of six-row indigenous types into two-row exotics. Although grain yield remained the primary objective in this program, quality was made an integral part. A two stage strategy was implemented in the breeding program. In stage one, priority was given to increase adaptability and grain yield with changes in plant type of the two-row barley. In stage two, more emphasis is given to utilize/involve the advanced two-row breeding lines with improved plant

Table 2. Barley varieties released for malting purposes in India.

Variety	Year of release	Production Condition	Area of Adaptation	Developed at
Alfa 93*	1994\$	Irrigated (TS)	NWPZ	DWR Karnal
BCU 73*	1997\$	Irrigated (TS)	NWP, PZ	DWR Karnal
RD 2503	1997	Irrigated (TS)	NWPZ	ARS (RAU) Durgapura
K 551	1997	Irrigated (TS)	NWPZ	CSAUA&T, Kanpur
DL 88	1997	Irrigated (TS)	PZ	IARI, New Delhi
DWR 28*	2002	Irrigated (TS)	NWPZ	DWR Karnal
DWRUB52*	2006	Irrigated (TS)	NWPZ	DWR Karnal
RD 2668*	2006	Irrigated (TS)	NWPZ	ARS (RAU) Durgapura

\$ = Introductions. * Two-row type barley. NWPZ = Northwestern plains zone. PZ = Peninsular zone.

type developed under the program using the new exotics in a systematic hybridization program to increase the frequency of the desirable quality traits. The germplasm screening program for malting quality taken up separately under AP Cess fund project has helped to evaluate more than 5500 accessions (exotics and indigenous) available in our genebank and to identify new sources with excellent malting quality, in both two-row and six-row backgrounds (Verma, 2008). As a consequence of these efforts, two-row genotypes having thick stems with broad leaves and erect spikes (preferred by farmers for vegetative appearance) were developed in place of grassy-looking thin-leaved plants with drooping spikes. The choice of six-row parents in the hybridization program was made for bold seeded, thin husked, disease resistant and high yielding types to achieve such improved two-row types.

The comprehensive breeding and multi-location coordinated evaluation to improve barley for malting resulted in development of the first two-row malt barley variety DWR 28, which was released in 2002 for commercial cultivation in NWPZ (comprising the states of Northern Rajasthan, Haryana, Western Uttar Pradesh and Punjab). The variety is a result of hybridization between two-row malt barley BCU73 (Yagan) and a popular six-row barley variety of Punjab (PL172), and has improved yield and adaptability with better malting quality. In NWPZ, DWR28 is comparable in grain yield to the six-row good malting type varieties like K 551 and RD 2503, though a yield gap of about 10% between two-row malt barley and high

yielding feed barley varieties still remained in NWPZ. This gap needed to be reduced further to encourage their economic and sustainable cultivation by the farmers.

Recently, as a result of the first private-public collaborative research partnership in malt barley improvement in the country between M/s United Breweries Ltd., Bangalore, and DWR, Karnal, another two-row variety, DWRUB52, with good malting and brewing quality, was developed (Verma *et al.*, 2007). This variety is a result of hybridization between two-row barley, DWR17 (22nd IBON-25 from ICARDA-CIMMYT) and a popular six-row malt barley variety (K551). It was observed that in the NWPZ, the two-row barley DWRUB52 is even comparable in grain yield to the six-row high yielding feed barley (Table 3). The yield gap between malt barley and feed barley, which was still about 10–15% in NWPZ, has been reduced further, thus supporting the cultivation of malting varieties by the farmers without yield loss.

The grain samples from most of the locations of the yield evaluation trials for each variety have been analyzed as a regular practice in all the three years of coordinated testing under AICW&BIP, and the mean zonal performance for individual traits can be seen in Table 4. There has been an improvement in the grain and malt quality traits of the new cultivars, and yield has not been obtained at the cost of grain quality.

It can be concluded that by following a two- × six-row hybridization program for grain yield improvement in two-row barley, significant genetic gain (from 36.8 q/ha of

Table 3. Grain yield (q/ha) of DWRUB52 under irrigated, timely sown conditions in NWPZ.

Year	No. of trials/locations	DWRUB52	RD2668	Two-row DWR28	Six-row K551	C.D.
2003–04	6	41.2	37.3	37.5	41.0	1.6
2004–05	8	45.7	44.4	41.8	45.8	1.4
2005–06	10	47.0	44.2	43.2	43.2	0.9
MEAN	24	45.1	42.5	41.3	43.5	—
Frequency in the top group (over 3 years)		11/24	9/24	3/24		

CD = Critical difference

Table 4. Mean performance of new varieties for some important malting quality traits over three years involving 24 locations.

Variety	Grain Traits				Malt Traits					Score
	BD (kg/hL)	BG (%)	Husk (%)	GP (%)	Frib. (%)	HWE (%db)	DP (°L)	KI	Viscosity (mPa s)	
TDWRUB52	64.0	83.3	10.5	11.4	57.7	78.2	105	0.44	1.597	18/27
RD2668	60.0	64.0	10.5	11.8	46.0	77.7	122	0.49	1.550	13/27
DWR28(C)	61.0	92.0	12.0	12.0	45.0	76.7	121	0.42	1.707	11/27
K551 (6-R)	56.9	73.5	13.2	10.7	38.0	76.0	106	0.43	1.744	11/27

BD = bulk density (kg/hL); BG = % bold grains; Husk = % husk content; GP = % grain protein; Frib. = malt friability (%); HWE = hot water extract (% fgdb); DP = malt diastatic power in degree Linter; KI = Kolbach index; Score = weighted score for desirable range out of 9 traits.

Alfa 93 to 45.1 q/ha in latest variety DWRUB 52) has been made in newly bred varieties over direct introductions in NWPZ under limited irrigation (Figure 3). In addition to grain yield improvement, incorporation of resistance to yellow rust and leaf blights has been another significant achievement.

These developments have now opened up the possibilities of cultivation of two-row barley in the zones. The initiation of contract farming by industry, with some premium for better quality, in the states of Punjab and Haryana, with involvement of government agencies, has mitigated the hesitation of farmers concerning two-row types. The help of extension agencies through the *front line demonstration* scheme has been phenomenal, where complete packages of cultural practices, including the maximum inputs, were provided and the potential of new

varieties was demonstrated in farmer fields. The misconceptions of the farmers were also clarified by educating them that the yield loss in two-row barley because of only two rows of grains in the spikes is compensated for by better tillering and higher 1000-kernel weight. The involvement of breeders in this group gave them the opportunity to obtain useful feedback from the farmers about their requirements and preferences for a good barley variety.

Now that we have attained almost equivalent yield levels in two-row and six-row types under optimum management conditions, the utilization of the new sources of quality is targeted to bring further improvement in specific quality traits like malt friability and lower viscosity to meet international specifications.

Association studies and selection criteria in malting barley breeding

Malting quality is a combination of several grain and malt traits, many of which are negatively correlated. It is near impossible to get maximum value for each trait in a variety in addition to grain yield and resistance to various biotic and abiotic stresses. The analysis of these traits before and after the malting process is a tedious job and involves a high level of technical expertise, in addition to significant cost. The amount of grain sample needed for analysis and the stage of

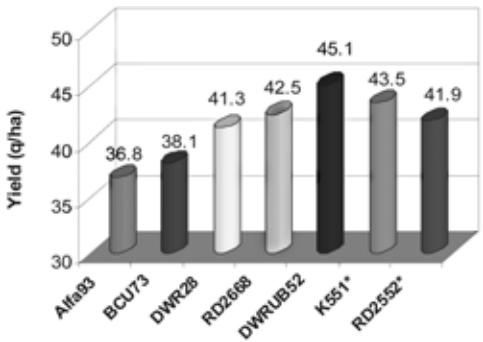


Figure 3. Yield gain in two-row barley through 2- × 6-row hybridization in NWPZ of India (* = 6-row barley).

evaluation in a breeding program are two important components. Theoretically, little variation is expected for selection at advanced stages of breeding material. Thus in order to practice selection for malting quality in early generations under Indian conditions, one must have certain traits to look for, which can help in the ultimate recovery of genotypes with good malting quality. Although there are various grain and malt parameters important in barley, hot water extract (HWE) is paramount and the price of malt is mainly dependent on its HWE value, along with diastatic power, friability and homogeneity, etc. Knowledge of the relationship among the different grain and malt parameters could help in indirect selection in breeding programs. The studies of material available in the coordinated program revealed that grain beta-glucan content, malt friability and wort viscosity are the parameters that need an urgent improvement in addition to HWE.

It has been observed that grain beta-glucan content is significantly negatively correlated with wort viscosity and wort filtration rate (Sewa Ram and Verma, 2002) under Indian conditions. The hordein profile study indicated positive association of the D-subunit with HWE (Sewa Ram and Verma, 1998). Another set of correlation studies indicated that HWE is correlated with a number of grain (hectolitre weight, plump%, thin%, protein%, 1000-kernel weight and husk%) and malt (friability, homogeneity, wort viscosity, filtration rate and Kolbach Index) traits, either positively or negatively (Verma *et al.*, 2008). Multiple regression analysis has indicated that measurement of grain traits like hectolitre weight, hull content and malt friability can predict HWE, with hectolitre weight as the single largest contributor. Therefore, hectolitre weight can be effectively used in initial screening of breeding lines in early generations (without actual malting) or for screening of germplasm accessions. The remaining traits (1000-kernel weight, husk content and malt

friability) can be used to predict HWE in short-listed material and advanced lines of a breeding program (Verma *et al.*, 2008).

Genotypes like DWR 37 (low husk and low beta-glucan content), DWR 38 (superior hectolitre weight), DWR 39 (low beta-glucan and wort viscosity) and DWR 51 (good overall malting quality) have been identified as sources of specific malting quality traits and were registered by the germplasm registration committee at NBPGR, New Delhi. In another AP Cess Fund study, the entire set of accessions (>5500) has been evaluated for all important grain quality parameters, and the promising ones were short-listed for detailed micro-malting analysis. A number of accessions, both indigenous and exotic, have been observed with very good overall malting quality and may be utilized in the malt barley breeding program in the country (Verma, 2008). The availability of new sources for malting quality and suitable selection criteria at appropriate stages of the breeding program will now help in further improvement in malt barley development in India.

Future research priorities

Barley cultivation in India is now becoming more and more oriented towards industrial utilization. Although at present the proportion in malt and brewing utilization is low, it is expected to increase sharply in the near future with the changing scenario in southeastern Asia with respect to beer and other malt product consumption. In addition to the quantity of barley, the quality of grain to be utilized for these purposes (which is currently meeting international grading requirements) is going to be a constraint on the indigenous malting industry being able to compete with malt being produced abroad. The Barley Network plans to take up the research program on the following specific issues to meet future requirements.

- Improvement of two-row malt barley genotypes having early maturity, better

adaptability across environments and improvement of six-row genotypes for grain uniformity, plumpness, low husk content and better malt extract recovery.

- Incorporating resistance to leaf blights (which are becoming more important under irrigated conditions), in addition to stripe and leaf rusts.
- Supplementing varietal improvement efforts with “Pilot-Scale Tests” in association with industry for the malt barley varieties prior to their commercial release.
- Use of exotic sources for improvement in specific traits such as high enzymatic activity for malting quality, and novel genes for resistance to diseases and pests.
- The use of marker assisted selection for efficient and fast evaluation and screening for malting quality traits and resistance to aphids. Work on identification of molecular markers for low beta-glucan content and aphid-resistance traits has been initiated at DWR, Karnal, to strengthen the varietal development efforts.

These new research options look very promising to convert barley to an industrial cereal, from a poor man's crop for food, cattle feed and fodder. However, considerable effort needs to be made in this direction, and effective implementation will depend upon cooperation between R&D units of public and private systems for both research and extension purposes. The initiation of contract farming by some industrial houses has opened up new possibilities for two-row malt barley cultivation, as well as providing a stimulus for the varietal development program in the country, which has long been advocated and only now is being realized by industry.

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Improvement of candidate malting barley cultivars for diverse agro-ecologies of Turkey

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Abstract

The aim of this project was to test yield and malting quality of 36 winter barley lines and cultivars under 6 different agro-ecological conditions of Turkey, and to develop new candidate cultivars for each zone. For this purpose, 26 yield trials, each consisting of 30 advanced lines and 6 checks, were conducted during the 2006–07 season. Due to the severe drought, the majority of these trials were negatively affected and six failed. Average yield was 20–90% lower than the long-term national average. The highest mean yield was 5.6 t/ha in Edirne (Trace region), while the lowest mean yield was 0.69 t/ha in Amasya (Northern Transitional region). Mean protein content was in an acceptable range for high malting quality in Edirne (11.5%), but higher than the acceptable limit in many locations, including Amasya (17.28%) due to severe drought. Generally, there was a strong relationship between yield levels of high yielding lines and cultivars and their selection regions or almost similar agro-ecologies. Some lines and cultivars out-yielded others at the sites where they were selected, such as Line 28, Sladoran and Zeynelaga cultivars in Trace and Northern Transitional Zone; Line 11 and Zeynelaga and Sladoran cultivars in Western Transitional Zone; Line 10 and Line 8 and cultivars Aydanhanım, Cumra and Catalhoyuk in Central Anatolia region; and Line 25 and Zeynelaga and Aydanhanım in Eastern Transitional Zone. The first year results indicated that yield and quality of

malting type Zeynelaga used as common check, was also very promising across almost all agro-ecologies. Micro-malt analyses of these genotypes by location are in progress.

Introduction

Turkey is one of the important gene centers for barley (*Hordeum vulgare* L.) and for barley production globally, with 9 million tonne grain production. Barley is mainly produced under dryland conditions in the Turkish highlands, and 90% of the production is used as animal feed. However, demand for malting barley both inside and outside the country has been gradually increasing during the last ten years. In order to cover all aspects of malting barley, including breeding for every agro-ecology, good malting production zones, better agronomic practices for dryland and irrigated conditions, disease resistance and integration of anther culture methods for shortening the breeding cycles, a countrywide malting barley breeding project was started in 2006, involving all breeding institutes and private companies. In the first year, we conducted national yield trials in order to test performance of 30 genotypes in different zones of the Turkish highlands.

Materials and methods

The Central Research Institute for Field Crops (CRIFC) prepared and distributed 26 yield trials, each consisting of 30 advanced lines and 6 checks, jointly provided by 5 government

and 1 private breeding institutes. They went to all institutes for the 2006 planting season. All the trials were winter planted, in 7 agro-ecological zones of the country, during the 2006–07 season using a triple lattice design (Figure 1). Analysis of variation for yield was performed for each sub-agro-ecology using the MSTAT software package. Differences among genotypes were calculated by using the LSD test. Additionally, mean and coefficient of variation were calculated. Standard cultivation practices were applied to all trials and morphological and disease data were collected during vegetative growth. After harvest, physical and chemical malting analyses were conducted (Yazicioglu *et al.*, 1976).

Results and discussion

In 2007, Turkey experienced a very dry season, especially in Central Anatolia and the Transitional Zones, so that 9 out of 26 trials failed and yield in Amasya (690 kg/ha) was 90% lower than the long-term average yield. These unusual weather conditions, especially 60% less and very erratic rainfall and a temperature 2–3°C higher than the long-term average during anthesis and grain

filling negatively affected the physical and chemical pre-malting quality parameters in every zone of Turkey in which the trials were conducted (Tables 1 to 4). Genotypes were selected and tabulated according to their yield performance and pre-malting qualities among the 36 checks and advanced lines.

Yield and pre-malting quality parameters collected from all trials indicated that growing season effects on yield and grain quality are higher than those of location and genotype, a finding paralleling Ewertson's (1977) findings that these can be the result of very unusual seasonal effects on barley germplasm. There was great variation for yield and pre-malting quality parameters in 4 of the 7 agro-ecological zones in which the genotypes were tested in 2007. The highest yield level was 4727 kg/ha in Trace, while it was 2606 kg/ha in South Eastern Anatolia (Tables 1 to 4). In addition to yield performance, protein content and physical pre-malting parameters were also collected in Trace (Table 1) and it was followed by Western Transitional Zones (Table 2). The other two zones (Central Anatolia and South), were more severely affected by drought and protein and sieve analysis scores did not meet basic malting criteria. Like previous studies



Figure 1. Experimental sites of the malting barley project in 2007.

Table 1. Yield and quality performance of some genotypes in Trace in 2007.

Genotypes	Yield* (kg/ha)	HW (kg/hL)	1000-KW (g)	Protein content (%)	Sieve Analysis (>2.5 mm)
Line 28	6457a	72.7	33.7	11.5	74.2
Sladoran	6381a	70.0	32.5	12.3	79.6
Zeynelaga	6255ab	72.6	36.4	11.8	70.4
Line 11	5859bc	66.2	30.3	11.6	39.7
Line 25	5820cd	70.9	33.8	11.9	77.5
Line 27	5623cde	70.7	37.2	11.7	75.5
Line 29	5537cde	68.9	35.2	12.7	74.5
Line 4	5447cde	65.3	27.9	10.9	18.7
Line 9	5421de	69.1	33.2	12.1	41.6
Line 10	5310e	69.7	32.7	11.0	64.5
Mean	4727	67.4	33.6	11.8	49.7
CV (%)	9.1	3.7	7.9	5.4	39.9

HW = hectolitre weight; 1000-KW = thousand kernel weight; CV = Coefficient of Variation. * = means followed by the same letter(s) are not significantly different.

Table 2. Yield and quality performance of some genotypes in Western Transitional Zone in 2007.

Genotypes	Yield* (kg/ha)	HW (kg/hL)	1000-KW (g)	Protein content (%)	Sieve Analysis (>2.5 mm)
Line 11	4796a	65.9	32.2	12.9	60.4
Line10	4397ab	69.3	35.8	14.1	61.8
Line 9	4396ab	66.6	31.7	12.8	47.2
Line 14	4280ab	66.8	32.4	12.3	39.6
Zeynelaga	4204b	64.2	41.0	14.1	69.6
Line 32	4136b	69.8	42.7	12.3	82.2
Line 2	4113b	71.4	33.0	13.0	80.2
Line 35	4094b	69.4	40.8	13.5	77.8
Line 4	4089b	67.7	35.1	14.7	59.3
Line 25	4081b	66.6	34.1	15.3	65.8
Mean	3824	68.4	37.1	13.3	68.4
CV (%)	11.9	2.3	9.0	7.5	17.8

HW = hectolitre weight; 1000-KW = thousand kernel weight; CV = Coefficient of Variation. * = means followed by the same letter(s) are not significantly different.

Table 3. Yield and quality performance of some genotypes in Central Anatolia in 2007.

Genotypes	Yield* (kg/ha)	HW (kg/hL)	1000-KW (g)	Protein content (%)	Sieve Analysis (>2.5 mm)
Line10	4046a	71.0	30.2	12.8	38.3
Line 35	3828ab	70.4	44.8	17.1	85.4
Line 32	3771ab	71.6	46.3	15.7	90.4
Line 19	3746ab	71.7	38.3	16.4	62.1
Aydanhanim	3714ab	71.0	43.1	15.6	83.2
Line 17	3647ab	69.2	39.8	15.6	59.4
Line 31	3644ab	71.8	43.8	15.1	84.3
Line 9	3537ab	68.9	26.0	12.5	15.0
Line 20	3520ab	70.9	39.7	16.6	72.4
Line 22	3483ab	72.8	39.1	15.9	79.6
Mean	3286	70.9	39.1	15.3	67.0
CV (%)	20.8	2.3	11.1	8.5	29.0

HW = hectolitre weight; 1000-KW = thousand kernel weight; CV = Coefficient of Variation. * = means followed by the same letter(s) are not significantly different

Table 4. Yield and quality performance of some genotypes in South Eastern Anatolia in 2007.

Genotypes	Yield* (kg/ha)	HW (kg/hL)	1000-KW (g)	Protein content (%)	Sieve Analysis (>2.5 mm)
Line 25	3235a	71.5	43.2	17.2	60.6
Line 27	3139ab	70.3	46.6	16.2	71.3
Line 11	2955ab	68.5	42.4	14.8	65.5
Line 7	2939ab	71.2	47.3	16.2	73.5
Zeynelaga	2888ab	69.8	42.6	15.7	68.3
Line 13	2838ab	70.4	51.4	15.3	77.9
Line 1	2831ab	71.4	41.9	15.8	74.3
Line 28	2823ab	70.4	41.8	16.4	57.2
Line 17	2801b	70.4	47.8	15.8	70.3
Line 21	2797b	72.2	45.2	16.4	80.5
Mean	2606	70.5	45.5	16.5	70.2
CV (%)	16.2	1.8	11.3	3.9	20.7

HW = hectolitre weight; 1000-KW = thousand kernel weight; CV = Coefficient of Variation. * = means followed by the same letter(s) are not significantly different

conducted by Yazicioglu *et al.* (1976) and Ewertson (1977), malting barley evaluated in diverse agro-ecological zones results in different yield and quality levels. Yield and pre-malting quality of one of the checks, Zeynelaga, across the agro-ecological zones was highly striking (Tables 1 to 4), as it was the most productive common check among the top ten genotypes in every zone. It was followed by Sladoran in Trace and by Aydanhanım in Central Anatolia. However, these checks were out-yielded by advanced lines in terms of yield and pre-malting quality parameters (Tables 1 to 4). Variation among the cultivars and advanced lines for malting quality were documented by Akkaya and Aktan (1990) and Basgul and Engin (1992).

Conclusion

According to the first year of data collected from the different agro-ecological zones of Turkey, the weather was the most important factor affecting yield and pre-malting quality parameters, and it was followed by locations and genotypes. Striking yield and pre-malting

quality of some advanced lines in diverse agro-ecological zones compared to the check cv. Zeynelaga is highly promising for development of new malting barley cultivars and use of them as parents for further winter malting barley breeding studies in the Turkish highlands.

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Agronomic practices affecting winter-type malting barley in dryland agriculture

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Abstract

In Turkey, the supply of malt extract has not been meeting the increasing local demand. Our objective is to produce malting barley on the dryland plateau to fill the gap between demand and supply. Four barley cultivars (Aydanhanım, Avci, Zeynelaga and Tarm-92) were grown in 2- and 4-year crop rotation systems, under macro- (N, P, S) and micro- (Zn, B) nutrient conditions. The first two cultivars were also tested for different seed rates, N rates and N rate \times application dates. Because of severe drought prevailing throughout the Central Plateau, only the plots of barley cultivars following winter lentil, winter vetch and fallow provided grain yields in 2-year (with 9 rotations) and 4-year (with 5 rotations) cropping systems. The barley succeeding spring legumes and oil crops and continuous cereal failed in the early stages of developments. Crop yields ranged from 0.8 to 3.0 t/ha among trials. Crop rotations did not affect the quality parameters. Zeynelaga grown in rotation trials was the best cultivar with respect to malting quality parameters, especially protein content and grain yield. Zeynelaga and sole P application in fallow/wheat system was the promising cultivar \times agronomy practice combination for malt production in dry seasons. Zn and B application to soil did not affect quality parameters and yields. No significant responses to varying seed and N rates were observed affecting quality parameters and grain yield. For good quality malting barley, N fertilizer should be applied late for low

protein and high yield. Research showed that it is possible to grow malting barley in dryland areas with special techniques.

Introduction

Barley, sown annually on 3.5 million hectare and yielding 8 million tonne production at 2.2 t/ha is a key crop for Turkish agriculture. Most of the production is utilized by factories producing animal feed and concentrates (Anon., 2002). Most malting barley is currently imported.

The malting industry is driven by grain quality, probably more than any other cereal-based industry. Two key quality factors are kernel size and grain protein. Maltsters like grain of uniform size with at least 70% plump kernels (retained on a 6/64 inch (2.38 mm) screen) and less than 5% thin kernels (passing through a 5/64 inch (1.98 mm) screen). Grain that does not meet the plumpness standard is rejected and substantially reduces the value to the grower (Oscarsson, 1997).

The other important factor that determines malting barley quality is grain protein. Excessively high or low protein levels are unacceptable to the malting industry. Six-row malting barley should have from 11.5 to 13.5% protein. Too high rates of N fertilization often lead to excessively high grain protein levels.

Common drought occurrences in Turkey and in the Northern Hemisphere result in quality problems in malting barley, and push up market prices. From US\$ 200/t in 2006, it went up to US\$ 310/t in 2007. In addition to

price increases, the quality criteria for malting barley have changed. Protein levels of 13–13.5% were adopted by the industry due to insufficient supply of barley in markets. The same trend can be seen in Turkey, and 20% of plump kernels passing through a 5/64 inch (1.98 mm) screen has been accepted by the industry (Engin, pers. comm.). Zn deficiency and B toxicity are the nutrient problems in the Central Plateau (Avci and Akar, 2005; Çakmak *et al.*, 1996).

In Turkey, the demand for malt extracts has been increasing, but supply cannot meet this increasing demand. The objective of this research was to investigate a number of agronomic practices that could help to produce good quality malting barley on the dryland plateau, to fill the gap between demand and supply for malt extract products, by using newly developed barley cultivars.

Material and methods

Seven dryland field experiments were conducted. They were 4-year and 2-year crop rotations, with various seed rates, N rates and N rate \times time of application, and macro- (N, P, and S) and micro- (Zn, B and S) nutrient application.

Three barley varieties were grown in 9 different 2-year and 5 types of 4-year crop rotations in 3 replicated experiments. The varieties were Aydanhanım, Zeynel and Tarm. The first two are two-row malting types, while Tarm is two-row feed barley. Fallow/barley, sunflower/barley, winter lentil/barley, spring lentil/barley, barley/barley, safflower/barley, chickpea/barley, winter vetch/barley and vetch + barley mixture/barley were the crop rotations used. The first 5 were also used in 4-year crop rotations in addition to the 2-year crop rotations.

Five seed rates (175, 275, 375, 475, 575 seed/m²) and five N rates (0, 40, 80, 120, 160 kg/ha N) were used in 3 replicated trials, with cvs. Aydanhanım and Avci. Avci is a dual-purpose malting barley for irrigated areas.

In another trial, N rates of 40 and 80 kg/ha \times N application dates (all at planting; all at tillering; all at heading; $\frac{1}{2}$ N at planting + $\frac{1}{2}$ at tillering (common practice); $\frac{1}{2}$ N at planting + $\frac{1}{2}$ at heading; $\frac{1}{2}$ N at tillering + $\frac{1}{2}$ at heading; $\frac{1}{2}$ at planting + $\frac{1}{4}$ at tillering + $\frac{1}{4}$ at heading; and 0 N application) were tested using cvs. Avci and Aydanhanım.

In other group of experiments, cvs. Tarm, Aydanhanım, Avci and Zeynelaga were grown in N, P and NP and no fertilizer (0); with and without S; with and without Zn; and with and without B; all with 3 replicates. This experimental field had been maintained in the same place since 1998 so that the same treatments were applied to the same plots.

The 2006/2007 cropping season was extremely dry in the Turkish plateau. Total precipitation during the growing season was 200 mm, which was 150 mm less than the long-term average, with temperature 2°C more than the long-term average.

Results and discussion

Crop rotations

In 2- and 4-year crop rotations, only crop rotations of fallow/barley, winter legumes (lentil and vetch)/barley provided seed yield, due to severe drought, while the other crop rotations failed. However, barley following fallow in the other series of 4-year crop rotations can produce economic seed yields.

None of the 2-year crop rotations differed in terms of yield and quality parameters. Bulk weight of cv. Tarm was statistically higher than the others (Table 1).

Of the sources of variation, only the cultivar made a statistical difference for all parameters in the series of fallow/barley/alternative crops/barley of 4-year rotation systems. Cv. Zeynelaga predominated with respect to quality parameters, especially protein content and grain yield (Table 1).

In the other series of 4-year rotations (fallow/barley/alternative crops/barley), none of the quality parameters were statistically

Table 1. Effects of crop rotation systems on malt quality and the grain yields of barley in dryland areas, 2007.

Crop rotation	Protein (%)	Starch (%)	Bulk weight (kg/hL)	Yield (t/ha)
2-year rotations				
Winter lentil/barley	15.8	59.6	62.8	1.37
Winter vetch/barley	16.0	59.5	63.8	1.16
Fallow/barley	15.9	59.7	62.0	1.43
Crop rotations	ns	ns	ns	ns
Cultivars	ns	ns	*	ns
4-year crop rotation				
Fallow/barley/winter lentil/barley	16.0	59.2	63.8	1.13
Fallow/barley/fallow/barley	16.0	59.2	63.3	1.42
Crop rotations	ns	ns	ns	ns
Cultivars	*	*	*	*
4-year crop rotations				
Fallow/barley/winter lentil/barley	16.2	59.3	63.1	1.50
Fallow/barley/fallow/barley	16.0	59.2	62.1	1.40
Fallow/barley/sunflower/barley	16.3	58.9	61.2	1.19
Fallow/barley/barley/barley	16.1	58.9	61.1	1.29
Fallow/barley/spring lentil/barley	16.4	59.1	62.4	1.51
Crop rotations	ns	ns	ns	** (Interaction)
Cultivars	*	**	**	*

*, ** = statistically significant at $P < 0.05$ and $P < 0.01$, respectively. ns = not significant.

Table 2. Effects of applied macronutrients on malting quality of barley grown in a dryland fallow/barley rotation, 2007.

Nutrients applied	Protein (%)	Starch (%)	Bulk wt. (kg/hL)	Yield (t/ha)
N only	16.3	59.1	62.7	1.71
N+P	15.6	59.7	64.1	1.83
Zero N (control)	13.9	60.5	62.0	1.62
P only	12.5	61.6	63.6	1.77
Macronutrients	*	*	ns	ns
Cultivars	ns	ns	ns	**
With S	14.7	60.1	64.4	1.83
Without S	14.8	60.4	64.3	1.75
Macronutrients	ns	ns	ns	ns
Cultivars	ns	ns	ns	**

*, ** = statistically significant at $P < 0.05$ and $P < 0.01$, respectively. ns = not significant.

significant due to rotation type. However, the cultivars were statistically different in terms of quality parameters. Zeynelaga was superior to Aydanhanım because of its lower protein and higher bulk weight. Rotation and cultivar interaction for grain yield was also significant (Table 1). Zeynelaga was the second-highest-yielding cultivar in rotations comprising wheat, spring and winter lentil crops, followed by cv. Tarm. Consequently, Zeynelaga is likely to be a suitable malting cultivar in 4-year crop rotation systems, even in a dry year.

Macronutrients

Among the macronutrients, phosphorous application positively and significantly affected the malting quality parameters. N application increased significantly the protein content of the barley cultivars. It is interesting to note that P application lowered the protein content more than zero N application. Sulphur application did not affect quality parameters and yield. Cvs. Zeynelaga and Tarm were the best with respect to grain yield levels (Table 2). Therefore, Zeynelaga and only P application in fallow/wheat system is

the promising cultivar \times agronomic practices combination to produce malting barley, even in such a dry season.

Micronutrients

Zn and B application to soil did not affect quality parameters and yields of barley cultivars. We only observed a genotypic difference between grain yields (Table 3). In the B and Zn experiments, cvs. Tarm and Zeynelaga yielded much more than cvs. Aydanhanım and Avci.

Nitrogen rates

Nitrogen rates did not affect quality parameters and yield (Table 4). The data suggest that no N fertilizer application tended to provide the highest yields for both of the

cultivars. This result indicates relatively high residual nitrogen in the soil and low response to the N application due to severe drought.

Seed rates

No significant responses to varying seed rates were observed for quality parameters and grain yield (Table 5). However, non-linear grain yield responses are evident for both of the cultivars. The maximum yield was from around 375 and 475 seed/m² for Aydanhanım and Avci, respectively.

N rate and time of application

There were no differences between the N doses of 40 and 80 kg/ha in terms of all quality parameters and grain yields for both of the cultivars. Timing of N application affected

Table 3. Effects of applied micronutrients on malting quality of barley crops grown in a dryland fallow/barley rotation, 2007.

Nutrients applied	Protein (%)	Starch (%)	Bulk wt. (kg/hL)	Yield (t/ha)
With B	15.0	60.3	65.0	2.35
Without B	15.2	60.1	64.8	2.22
Micronutrients	ns	ns	ns	ns
Cultivars	ns	ns	ns	**
With Zn	14.5	60.2	63.4	1.75
Without Zn	15.1	59.9	63.8	1.65
Micronutrients	ns	ns	ns	ns
Cultivars	ns	ns	ns	**

*, ** = statistically significant at $P < 0.05$ and $P < 0.01$, respectively. ns = not significant.

Table 4. Effects of some applied nitrogen rates on malting quality of barley grown in a dryland fallow/barley rotation, 2007.

N rate (kg/ha)	Protein (%)	Starch (%)	Bulk wt (kg/hL)	Yield (t/ha)
cv. Aydanhanım				
0	15.5	59.8	61.2	1.31
40	15.7	59.5	61.7	1.11
80	16.9	59.1	60.9	1.39
120	17.4	58.5	59.9	1.04
160	16.4	59.1	60.4	1.11
	ns	ns	ns	ns
cv. Avci				
0	16.3	58.7	59.9	1.04
40	15.6	58.8	59.4	0.83
80	16.8	58.0	58.5	0.85
120	16.4	58.8	61.8	0.83
160	17.9	57.3	59.9	0.91
	ns	ns	ns	ns

ns = not significant.

significantly all parameters of Avci except bulk weight (Table 6). The data suggest that early season applications (at planting and tillering) tended to give more protein than late season application. However, the late season application of N fertilizer increased grain yields more than early season application. Thus, for good quality malting barley N

fertilizer should be applied late to obtain low protein and high yield.

The results implied that it seems possible to grow malting barley with acceptable quality under dryland and dry conditions by employing appropriate techniques such as fallow, P fertilizing, late N application and appropriate cultivar.

Table 5. Effects of seeding rates on malting quality of barley grown in a dryland fallow/barley rotation, 2007.

Seed rate (seed/m ²)	Protein (%)	Starch (%)	Bulk wt. (kg/hL)	Yield (t/ha)
cv. Aydanhanım				
175	16.4	59.1	60.0	1.07
275	16.0	59.4	60.9	1.24
375	16.0	59.5	61.6	1.57
475	17.5	58.6	60.6	1.59
575	16.0	59.5	61.1	1.59
	ns	ns	ns	ns
cv. Avci				
175	16.9	58.7	61.2	1.44
275	15.3	59.1	59.8	1.52
375	16.2	58.4	59.5	1.78
475	17.5	57.6	59.3	1.95
575	17.2	57.8	59.6	1.9
	ns	ns	ns	ns

ns = not significant.

Table 6. Effects of nitrogen timing on malting quality of barley grown in dryland fallow/barley, 2007.

N timing	Protein (%)	Starch (%)	Bulk wt. (kg/hL)	Yield (t/ha)
cv. Aydanhanım				
All at planting	17.3	58.7	63.3	1.19
½ of N at planting + ½ at tillering (common practice)	17.6	58.2	61.5	1.03
½ of N at planting + ½ at heading	17.0	58.8	61.8	1.28
½ at planting + ¼ at tillering + ¼ at heading	16.8	58.9	62.0	1.13
All at tillering	16.5	59.0	62.1	1.39
½ of N at tillering + ½ at heading	16.4	59.2	62.1	1.34
All at heading	16.9	58.9	62.7	1.51
No N	16.3	59.3	61.7	1.33
N rate	ns	ns	ns	ns
Timing	ns	ns	ns	ns
cv. Avci				
N rate	ns	ns	ns	ns
All at planting	17.2	57.7	58.7	0.67
½ of N at planting + ½ at tillering (common practice)	16.6	58.4	60.5	0.83
½ of N at planting + ½ at heading	16.7	58.4	60.0	0.88
½ at planting + ¼ at tillering + ¼ at heading	16.6	58.4	60.2	0.86
All at tillering	16.1	58.7	59.9	1.02
½ of N at tillering + ½ at heading	16.0	58.9	61.0	1.27
All at heading	16.2	58.5	59.7	1.05
No N	15.7	59.0	60.2	1.29
N rate	ns	ns	ns	ns
Timing	**	**	ns	**

** = statistically significant at $P < 0.01$. ns = not significant.



Figure 1. A view from seed rate trial.



Figure 2. Effect of drought in a two-year crop rotation.

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Variability of limit-dextrinase activity

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Abstract

Limit-dextrinase is the only endogenous barley enzyme that hydrolyses alpha 1-6 bonds of starch, releasing fermentable sugars and small dextrans. Limit-dextrinase activity has been shown to significantly affect wort fermentability, despite difficulties in quantifying the enzyme due to inhibitors. Activity is generally classified as total or free, depending on extraction conditions. Variability of these activities, as affected by genetics and processing, is poorly understood. Cv. Harrington has shown higher limit-dextrinase activity than cv. AC Metcalfe, which could explain similar fermentation properties of these varieties despite higher diastatic power in AC Metcalfe. The consistency of this advantage was examined under varying malting conditions: supplementation with gibberellic acid (GA); anaerobic germination; and varying kilning temperatures. Barley, grown in Manitoba during 2006, was malted in Phoenix Micromaltings and analyzed using standard methods. Total and free limit-dextrinase activities were significantly higher in Harrington despite higher diastatic power in AC Metcalfe. GA supplementation significantly increased free limit-dextrinase activity in both varieties but had no effect on total limit-dextrinase activity. Anaerobic germination decreased total limit-dextrinase activity but had no effect on the free form. Higher kilning temperatures reduced total activity, but only in Harrington. The importance of limit-dextrinase was supported by a significant positive relationship of total limit-dextrinase activity and fermentability. In summary, processing conditions affected

limit-dextrinase activity significantly, but Harrington maintained higher levels of both free and total limit-dextrinase than AC Metcalfe. The research supported potential breeding for limit-dextrinase, which could result in varieties with more predictable fermentability.

Introduction

Fermentability is the key process in the brewery, whereby fermentable sugars are converted to ethanol by yeast. Fermentability must be predictable to produce specific beer types and to produce them economically. Traditional methods of determining malt quality are not always pertinent to prediction of fermentability.

Limit-dextrinase is one member of a family of barley enzymes that degrade starch to fermentable sugars during the mashing step of brewing. The other enzymes are alpha-amylase, beta-amylase and alpha-glucosidase. Limit-dextrinase is the only one that hydrolyses alpha 1-6 bonds in starch and it often limits fermentability (Evans *et al.*, 2005). Measurement of limit-dextrinase is complicated by a limit-dextrinase inhibitor that results in two forms of the enzyme: free and inhibited. Activity is routinely reported as total, the combination of free and inhibited limit-dextrinase, or free activity, depending on presence or absence, respectively, of a reducing agent during extraction. The amount of each type of activity varies considerably in final malts, with effects from both genetics and malt processing.

Levels of both total and free limit-dextrinase activity are known to vary among barley varieties. Lee and Pyler (1984), using

a short (2.5 hour) extraction without reducing agent, found significant differences in free limit-dextrinase activity among varieties. Using longer extraction times and extracting with the reducing agent, diathiothreitol (DTT), levels of total limit-dextrinase also varied significantly among varieties (Schroeder and MacGregor, 1998). Ross *et al.* (2003) found the rate at which free limit-dextrinase developed during germination was also dependent on variety. They concluded that differences were due to variable levels of inhibitor.

Malt processing can affect levels of limit-dextrinase. Limit-dextrinase activity is present in unprocessed barley (Lee and Pyler, 1984; Schroeder and MacGregor, 1998) but mostly in the bound form (Kristensen *et al.*, 1993). Total limit-dextrinase continues to increase as barley germinates (Schroeder and MacGregor, 1998) but the major change, especially at later stages of germination, is the release of free limit-dextrinase (Kristensen *et al.*, 1993). Supplementation with gibberellic acid accelerated the formation of total limit-dextrinase during germination and resulted in higher plateaus of total limit-dextrinase activity at 4 days of germination (Schroeder and MacGregor, 1998). In a separate study, gibberellic acid had a greater effect on release of the free form of limit-dextrinase, although the effect was most apparent after 5 days of germination (Lee and Pyler, 1984). Anaerobic growing conditions, achieved with wet germination, produced increased levels of total limit-dextrinase during the first few days of germination, but free enzyme increased rapidly starting at day 5 of germination, with almost all activity in the free form by day 9 (Longstaff and Bryce, 1993).

Kilning can significantly reduce limit-dextrinase activity. Lee and Pyler (1984) found that activity, essentially free limit-dextrinase, was stable at temperatures of 49°C, but declined dramatically at temperatures of 82°C. Kristensen *et al.* (1993) found 50% of free limit-dextrinase activity was lost at

75°C, but bound limit-dextrinase was stable up to end point kilning temperatures of 85°C.

Several studies have found a significant relationship between limit-dextrinase activity and fermentability, despite differences in assay methods. Lee and Pyler (1984) found a significant effect of free limit-dextrinase activity on fermentability and ethanol. Stenholm and Home (1999) also found fermentability was highly dependent on free limit-dextrinase and not on total limit-dextrinase activity. In contrast, Evans *et al.* (2005) found both forms correlated with fermentability, with each explaining similar degrees of variability. Each lab used extensively different extraction methods for measuring both total and free limit-dextrinase, which probably affected results.

The present study investigated differences in effects of barley variety and malt processing on levels of total and free limit-dextrinase activity in malt. Cvs. Harrington and AC Metcalfe were selected because of significant differences in limit-dextrinase potential (Langrell and Edney, 2006). Processing treatments were selected on the basis of their potential practicality in a malt plant. Supplementation with gibberellic acid simulated more intensive modification of barley. An anaerobic treatment could have practical implications for adjusting aeration during germination. Kilning is always carefully monitored to manipulate enzyme levels in final malts.

Materials and methods

Variability in development of limit-dextrinase activity during malting was investigated with a complete block design involving two barley varieties, addition of gibberellic acid, anaerobic versus aerobic germination and a low temperature versus regular kilning regime. The resulting 16 treatments were duplicated for a total of 32 malts.

Harrington and AC Metcalfe, Canadian malting barley varieties (two-row), were grown in Hamiota, Manitoba, Canada,

in 2006 and malted with a Phoenix Micromalting System using standard 2006 micro-malting conditions (Steeping: 10 h wet, 18 h dry, 8 h wet, 12 h dry @ 13°C; Germination: 96 h @ 15°C; Regular kilning: 12 h @ 55°C, 6 h @ 65°C, 2 h @ 75°C, 4 h @ 85°C or Low-temperature kilning: 12 h @ 55°C, 6 h @ 65°C, 6 h @ 75°C). Gibberellic acid was mixed by hand with barley (2 ppm) at steepout. Control samples were mixed with similar quantities of water (0 ppm gibberellic acid). Anaerobic conditions were produced by limiting roller drive time, during germination, to 20% compared with standard, aerobic conditions with roller drive on 100% of the time.

Total and free limit-dextrinase activities were determined by extracting malts for 5 hours at 40°C with and without diathiothreitol (DTT), respectively. Extracts were assayed for limit-dextrinase activity according to Megazyme kit instructions (Megazyme International Ireland Ltd.). Diastatic power and alpha-amylase activity were determined using standard American Society of Brewing Chemists methods (ASBC, 2004). Apparent Attenuation Limits (AAL) were determined using a small-scale version (Logue *et al.*, 1997) of an official AAL method (Analytica-EBC, 1998).

Results and discussion

Harrington consistently produced more limit-dextrinase activity than AC Metcalfe (Table 1). Harrington's superiority had been previously reported (Schroeder and MacGregor, 1998; Langrell and Edney,

2006). Total and free limit-dextrinase activities were both significantly higher in all Harrington samples regardless of treatment. Two treatments, gibberellic acid supplementation and anaerobic conditions (Table 2), did significantly affect free and total limit-dextrinase, respectively. Kilning had no significant effect on limit-dextrinase activity (Tables 2, 3 and 4), but there was an interaction with variety. Higher kilning temperatures significantly reduced total limit-dextrinase activity in Harrington but not in AC Metcalfe. Harrington still maintained an advantage (Figure 1). Overall the results support the assumption that barley varieties can have a genetic potential to produce more limit-dextrinase activity.

Malt processing had several different effects on limit-dextrinase activity. Enhanced germination, as promoted by gibberellic acid supplementation, significantly increased free but not total limit-dextrinase activity (Table 2). Limit-dextrinase was probably activated during germination through destruction of inhibitors. Interestingly, anaerobic conditions, which should have intensified germination, as suggested by significantly higher alpha-amylase activity (Table 2), actually lowered total limit-dextrinase significantly with no effect on free limit-dextrinase. This is in contrast to the literature (Longstaff and Bryce, 1993), where even more severe anaerobic conditions, as produced with excess water, actually increased limit-dextrinase activity, especially the free form. The results support further investigations on development of total limit-dextrinase activity. As well, the high-

Table 1. Effect of variety on enzyme activities and fermentability.

Variety	Barley protein (% DM)	Total Limit-dextrinase (U/kg)	Free Limit-dextrinase (U/kg)	α -Amylase (DU)	Diastatic Power (°Lintner)	AAL (%)
Harrington	12.4	557 \pm 68.2a	271 \pm 50.6a	92.4 \pm 13.6	130 \pm 8.2b	87.3 \pm 0.65a
AC Metcalfe	12.7	457 \pm 30.4b	191 \pm 45.9b	88.7 \pm 13.3	140 \pm 5.8a	86.7 \pm 0.74b

Means followed by different letters differ significantly ($P < 0.01$) based on Duncan's Multiple Range Test; DM = dry matter; U/kg = Megazyme units per kg malt; DU = dextrinizing units; AAL = Apparent Attenuation Limit. Values are average \pm standard deviation.

Table 2. Effect of malt processing treatments on enzyme activities and fermentability.

Treatment	Total Limit-dextrinase (U/kg)	Free Limit-dextrinase (U/kg)	α -Amylase (DU)	Diastatic Power ($^{\circ}$ Lintner)	AAL (%)
Added Gibberellic acid	502 \pm 76.8	272 \pm 48.1a	98.2 \pm 10.5a	138 \pm 9.0a	87.0 \pm 0.80
No supplementation	513 \pm 70.2	190 \pm 46.3b	82.9 \pm 11.4b	133 \pm 6.8b	87.1 \pm 0.58
Anaerobic (20% roller)	486 \pm 65.0b	237 \pm 58.5	95.4 \pm 12.4a	133 \pm 8.1	86.8 \pm 0.63a
Aerobic (100% roller)	529 \pm 75.3a	226 \pm 67.8	85.7 \pm 12.8b	137 \pm 8.1	87.3 \pm 0.78b
Low temperature kiln	523 \pm 89.1	232 \pm 64.9	89.8 \pm 13.9	136 \pm 7.4	87.0 \pm 0.9
High temperature kiln	492 \pm 49.2	230 \pm 62.2	91.3 \pm 13.1	135 \pm 9.3	87.0 \pm 0.6

Means followed by different letters differ significantly ($P < 0.01$) based on Duncan’s Multiple Range Test; DM = dry matter; U/kg = Megazyme units per kg malt; DU = dextrinizing units; AAL = Apparent Attenuation Limit. Values are average \pm standard deviation.

Table 3. Statistical analysis (ANOVA) of free limit-dextrinase activity as affected by treatment.

Source	Mean Square	F Value	Pr > F
Gibberellic acid addition	54 285.1	94.95***	<0.0001
Variety	51 200.0	89.56***	<0.0001
Anaerobic	1 012.5	1.77	0.20
Kiln	36.1	0.06	0.80

*** = Significantly different from zero at the 0.001 level of probability.

Table 4. Statistical analysis (ANOVA) of total limit-dextrinase activity as affected by treatment.

Source	Mean Square	F Value	Pr > F
Variety	79 600.5	40.82***	<0.0001
Anaerobic	14 878.1	7.63**	0.01
Variety*Kiln	9 660.5	4.95*	0.04
Kiln	8 001.1	4.10	0.06
Gibberellic acid addition	968.0	0.50	0.49

*, **, *** Significantly different from zero at the $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels of probability, respectively.

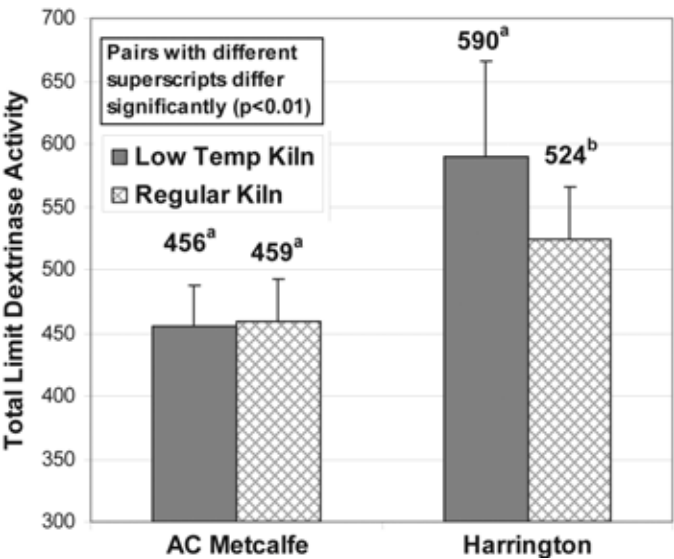


Figure 1. Interaction of kiln and variety on total limit-dextrinase activity (Megazyme units).

temperature kilning significantly lowered total limit-dextrinase in Harrington samples, but had no effect on free limit-dextrinase, which is in contrast to the literature, where the free form was more heat labile (Kristensen *et al.*, 1993). The greater heat stability of total limit-dextrinase activity in AC Metcalfe supported a genetic component to heat stability, as was previously reported (Evans *et al.*, 2003).

AC Metcalfe averaged significantly higher levels of diastatic power than Harrington (Table 1). The AC Metcalfe sample had a slightly higher level of grain protein (Table 1) but higher diastatic power was more likely to be genetic (Legge *et al.*, 2003). Gibberellic acid significantly increased diastatic power in both varieties, probably through activation of beta-amylase as the majority of diastatic power is formed during grain development. AC Metcalfe maintained higher levels of diastatic power even with the gibberellic acid treatment. Other treatments had no significant effect on diastatic power (Table 2). Alpha-Amylase was not significantly affected by variety, but gibberellic acid and anaerobic conditions significantly increased alpha-amylase. Enhanced germination would have led to increased synthesis and secretion of alpha-amylase (Schroeder and MacGregor, 1998).

Total limit-dextrinase activity was the only activity that correlated significantly with fermentability (Figure 2). All other enzyme activities, free limit-dextrinase, alpha-amylase and diastatic power showed no relationship with fermentability (Figures 3, 4 and 5). Harrington had significantly better fermentability than AC Metcalfe, probably due to higher total limit-dextrinase and despite significantly less diastatic power (Table 1). The importance of total limit-dextrinase activity was reinforced by comparing individual treatments. Gibberellic acid had no significant effect on fermentability despite significantly higher levels of alpha-amylase, diastatic

power and free limit-dextrinase (Table 2). In contrast, the anaerobic treated samples, with significantly less total limit-dextrinase, had significantly poorer fermentability despite superior alpha-amylase levels. Results emphasized the importance of total limit-dextrinase, supporting some reports in the literature (Evans *et al.*, 2005) but not others, where free limit-dextrinase was of greater importance (Lee and Pyler, 1984; Stenholm and Home, 1999). The results also indicated that diastatic power and alpha-amylase, which were at typical levels for Canadian malt, were not limiting to fermentability in the samples tested.

The insignificant effect of free limit-dextrinase on fermentability was not expected, as free limit-dextrinase should have been more active at producing fermentable sugars. However, the assays used for both total and free limit-dextrinase did not involve a mashing-type step, relying on a simple extraction method. It is likely that inactive limit-dextrinase, measured as total with the assay, was activated during the mashing step. None of the assays reported in the literature, nor the standard Megazyme method, use a mashing-like step for extraction of limit-dextrinase. Relationships between the results from these methods and the actual activity mashing need to be examined.

Conclusions

Harrington had significantly higher levels of both total and free limit-dextrinase activities than AC Metcalfe, regardless of the treatment, supporting the breeding for controlled levels of activity. Results suggested that breeding could target the synthesis of the limit-dextrinase, as indicated by the effect on total limit-dextrinase, as well as target reduction of limit-dextrinase inhibitor, as suggested by the effect on free activity. The greater susceptibility to heat inactivation, specifically with Harrington, suggested heat stability could also be a breeding target.

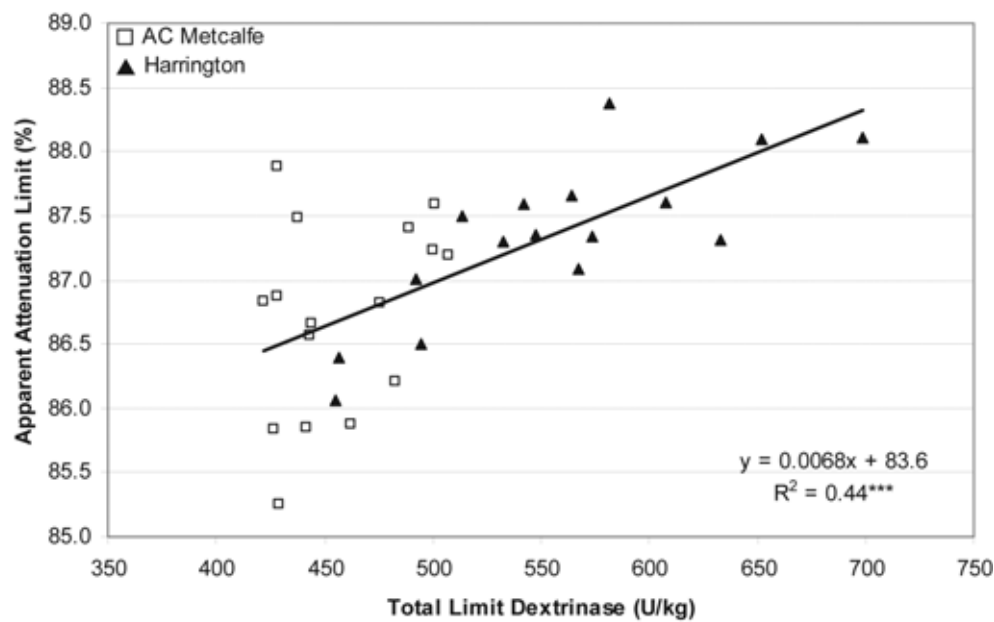


Figure 2. Relationships between total limit-dextrinase activity and fermentability for all samples including both varieties.

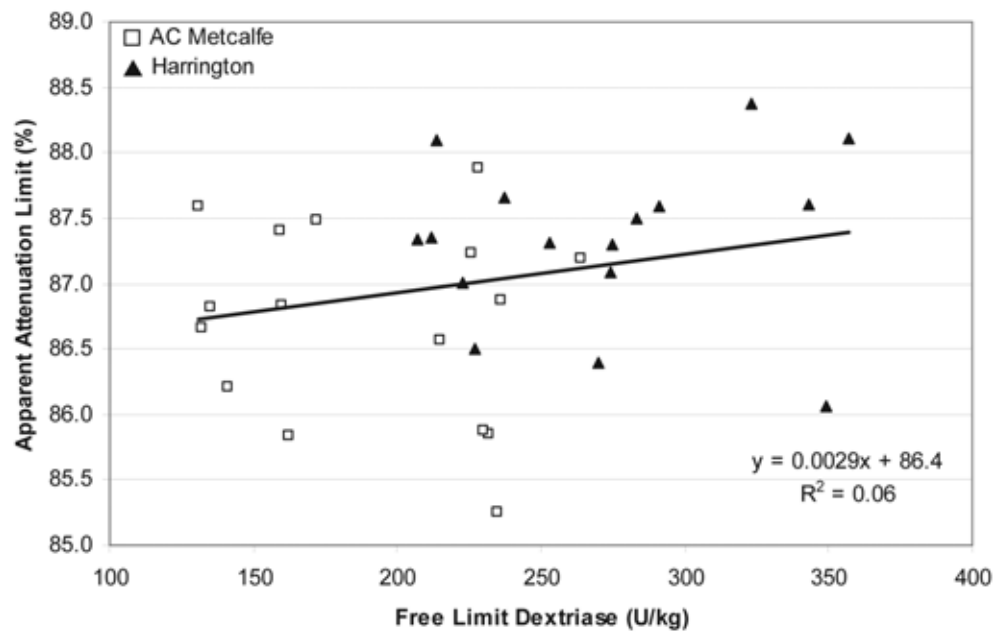


Figure 3. Relationships between free limit-dextrinase activity and fermentability for all samples including both varieties.

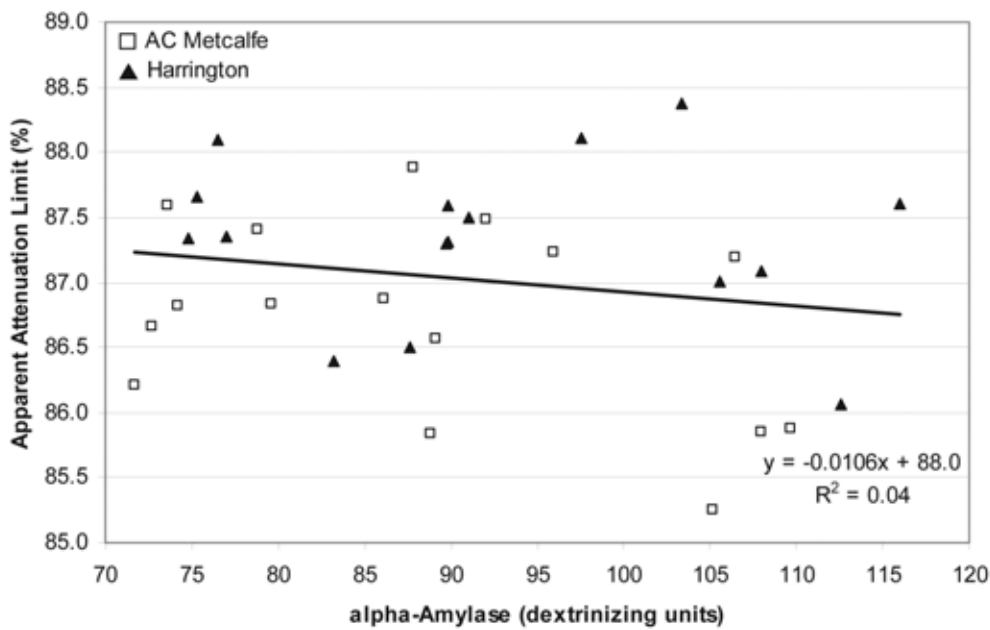


Figure 4. Relationships between alpha-amylase activity and fermentability for all samples including both varieties.

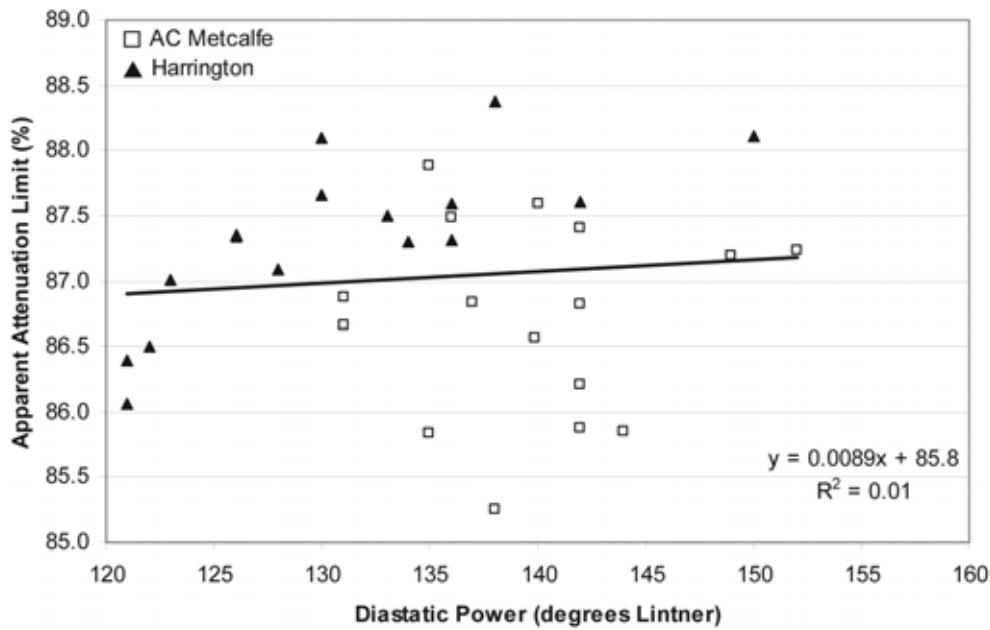


Figure 5. Relationships between diastatic power and fermentability for all samples including both varieties

Limit-dextrinase was the most important starch-degrading enzyme for determining fermentability. However, total limit-dextrinase was the form affecting fermentability, not free activity, supporting the need for research on measurement of limit-dextrinase active during mashing. Factors affecting total limit-dextrinase also require further investigation.

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Detection of functionally pleiotropic QTLs linked to malt extract in two barley populations

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Abstract

Malt extract, the principal indicator of malting quality in barley (*Hordeum vulgare* L), is considered a ‘mega-trait’ because it is influenced by a number of component traits. However, in the vast majority of previous QTL studies involving malting quality parameters, each measure has been analyzed independently. Single-trait analysis has the advantage of computational simplicity, but does not capitalize on the multivariate structure of the data. Several researchers have shown that multi-trait QTL analysis is statistically more appropriate, and may assist in testing several biologically important hypotheses, such as the distinction between linkage and pleiotropy as mechanisms of genetic correlations, or the problem of QTL \times environment interactions. When a large number of traits are considered simultaneously, however, the value of multi-trait QTL analysis is decreased due to the increased number of parameters to be estimated. The study reported here used path analysis as a tool for choosing functionally related traits in two-trait pairings. An advantage of the approach is that it allows a reduction of the multi-trait complex to a two-trait set, which can then be easily analyzed by a combined multi-trait (MT), multi-interval (MIM), and multi-environment (ME)

approach without building complex models. Micro-malting data obtained from two previously published barley populations—the VB9524 \times ND11231*12 and Arapiles \times Franklin populations—were used to illustrate the approach. In the two populations studied, increased malt extract was driven by two different parameters: grain protein in VB9524 \times ND11231*12 and wort β -glucan in the Arapiles \times Franklin population. Furthermore, the re-analyses revealed more QTLs than previously reported for the same populations, and provided a valuable insight into the cause of QTL \times environment interactions.

Introduction

Malt extract, the principal indicator of malting quality in barley (*Hordeum vulgare* L), is considered a ‘mega-trait’ (Ullrich and Han, 1997) because it is influenced by a number of component traits. Consequently, many traits are often tested in gene mapping studies with the expectation that this can identify both quantitative trait loci (QTLs) that are specific for each trait and genetic pathways that are shared between correlated traits. However, in the vast majority of previous QTL studies involving malting quality parameters, each measure has been analyzed independently (Ullrich and Han, 1997; Mather *et al.*, 1997; Marquez-Cedillo *et al.*, 2000; Emebiri *et*

et al., 2004; Okada *et al.*, 2006). Single-trait analysis has the advantage of computational simplicity, but does not capitalize on the multivariate structure of the data (Ferreira *et al.*, 2006), and there are unresolved issues regarding how best to adjust for the multiple testing of correlated measures, for which Bonferroni corrections are over-conservative (Marlow *et al.*, 2003). In response to these shortcomings, several groups (Calinski *et al.*, 2000; Korol *et al.*, 2001; Hackett *et al.*, 2001; Marlow *et al.*, 2003) proposed a multivariate approach to QTL analyses. Multivariate analysis, besides being statistically more appropriate, may assist in testing several biologically important hypotheses, such as to distinguish between linkage and pleiotropy as mechanisms of genetic correlations, or to address the problem of QTL \times environment interactions (Jiang and Zeng, 1995). When a large number of traits are measured in different environments, however, the increased number of parameters to be estimated can complicate the multivariate approach. In multivariate analysis, a set of parameters is estimated for each trait. Thus when the number of traits in the model increases, so does the number of parameters to be estimated, and as a result the power and precision of multivariate analysis decreases (Stearns *et al.*, 2005). To overcome the increase in parameters, Weller *et al.* (1996) proposed an approach based on a principal component analysis (PCA) of the phenotypic data. The principal purpose is to reduce dimensionality of the dataset by identifying uncorrelated linear combinations of traits, the principal components. Disadvantages of PCA in QTL analyses include unresolved issues regarding the number of principal components that should be retained for use in subsequent analysis and interpretation of QTL effects relative to the original traits (Andersson-Eklund *et al.*, 2000; Musani *et al.*, 2006). Korol *et al.* (2001) suggested a two-trait approach, but arbitrary two-trait pairing can create statistical difficulties due

to the large number of trait pairs and issues regarding how best to adjust for the multiple testing.

The study reported here explored the potential of path analysis as a tool for choosing functionally related traits in two-trait pairing. Path analysis is a statistical tool for testing hypotheses of cause-effect relationships between variables in a complex system (Guillen-Portal *et al.*, 2006). It partitions correlation coefficients into direct and indirect effects, and thus allows the separation of the direct influence of each component trait from the indirect effects caused by interdependence of independent variables. It has been applied in QTL analyses of multiple quantitative traits with the aim of identifying causal networks (Stein *et al.*, 2003; Steege *et al.*, 2005; Li *et al.*, 2006; Vargas *et al.*, 2007; Dhungana *et al.*, 2007). While this approach was intended for identification of functionally pleiotropic QTLs underlying the complexity of malt extract in barley, it can be applied to multi-trait QTL analysis of any other plant or animal species. An advantage of the approach is that it allows a reduction of the multi-trait complex to a two-trait set, which can then be easily analyzed by a combined multi-trait (MT), multi-interval (MIM) and multi-environment (ME) approach without building difficult models. Data from two previously published barley populations were used to illustrate and compare results.

Materials and methods

Source of data

Data analyzed were obtained from two QTL mapping populations, the VB9524 \times ND11231*12 and the Arapiles \times Franklin populations, constructed as part of the Australian National Barley Molecular Marker Program. The Arapiles \times Franklin population was developed with the aim of detecting malting quality QTLs (Panozzo *et al.*, 2007), while the VB9524 \times ND11231*12 population was specifically constructed to

specifically identify regions of the barley genome that influence variations in grain protein concentration (Emebiri *et al.*, 2004). Arapiles, which was released in 1993, is a slow modifying variety with moderate levels of malt extract. Franklin, in contrast, has high levels of malt extract and low levels of wort β -glucan (Panozzo *et al.*, 2007).

The two populations are somewhat related; VB9524 is an advanced selection from a cross of Arapiles with Franklin, but rejected for release due to a blue aleurone characteristic observed during malting. ND11231*12 is a two-row barley line from the breeding program at North Dakota State University (NDSU). The line is early maturing with good extract, moderate diastatic power but low in α -amylase activity (Franckowiak, pers. comm.).

The Arapiles \times Franklin population consists of 225 doubled-haploid (DH) lines for which 240 molecular markers were scored. VB9524 \times ND11231*12 comprised 180 DH lines, and is well characterized (Emebiri *et al.*, 2003, 2004). Malt quality data for the VB9425 \times ND11231*12 population were derived from 2 trial sites, Horsham, Victoria, and Yanco, New South Wales, conducted in 2000. Data for the Arapiles \times Franklin population were selected from the 2 trial sites with the most complete data-points, namely those at Horsham, Victoria, and Esperance, Western Australia, conducted in 2000 and in 2001, respectively. Grain samples from the trials were assessed for protein content, using Near Infrared Spectroscopy, while other malting quality parameters were measured by the micro-malting procedures described in Emebiri *et al.* (2004).

Genetic correlations and path analyses

Data were analyzed for genetic correlations using GENSTAT v10 (Lawes Agricultural Trust) codes described by Williams and Matheson (1994), in which the quality traits were paired in all possible combinations and

values of each pair were summed to estimate the covariance, according to the formula:

$$\text{COV}_{xy} = \frac{\sigma^2_{x+y} - \sigma^2_x - \sigma^2_y}{2}$$

and genetic correlation as:

$$r_g = \frac{\text{COV}_{xy}}{\sqrt{(\sigma^2_x)(\sigma^2_y)}}$$

Genetic correlations were tested for significance, using a Student's *t*-value with (*n*-2) degrees of freedom, where *n* is the number of experimental units:

$$t = \frac{r_g}{\sqrt{[(1 - r_g^2)/(n - 2)]}}$$

The genetic correlations were then used as inputs for path coefficient analysis (Dewey and Lu, 1959). The structural model considered malt extract (ME) as the only endogenous (dependent) variable, and others (Diastatic power (DP), alpha-amylase (AA), Grain protein (GPC), Soluble protein (SP), Viscosity (VISC), and wort beta-glucan (WBG)) as exogenous. The direct and indirect effects were calculated by setting up the simultaneous equations in a matrix format:

$$\begin{bmatrix} r_1 \\ r_2 \\ \vdots \\ r_N \end{bmatrix} = \begin{bmatrix} 1 & r_{12} & \dots & r_{1N} \\ r_{12} & 1 & \dots & r_{2N} \\ \vdots & \vdots & \ddots & \vdots \\ r_{1N} & r_{2N} & \dots & 1 \end{bmatrix} \times \begin{bmatrix} P_1 \\ P_2 \\ \vdots \\ P_N \end{bmatrix}$$

where r_i is the genetic correlation coefficient between malt extract and the independent variables, r^{ij} is the correlations amongst the independent variables, and P_i is the path coefficient. If *A* is defined as the column vector for the r_i , *B* as the correlation matrix, and *C* as the column vector of path coefficient, then, $A = B \times C$. The path coefficients (*C* column vector) were calculated by solving this equation as $C =$

$B^{-1}A$ (Singh and Chaudhary, 1977), and can also be modeled graphically using a path diagram (Figure 1). In the graphical model, a single uni-directional arrow indicates a direct causal effect (path coefficient) of a parameter on malt extract (Y), while indirect effects are indicated by alternative paths from a variable (i) through another variable (j) to the endogenous variable (y). A single indirect effect is quantitatively equal to the product of path coefficients along a given path:

$$\text{Indirect Effect} = (r_{ij}) \times (P_{jy})$$

The total indirect effect (I) is the sum of individual one-directional indirect effects, and the effect coefficient (C) is the sum of direct and total indirect effects.

The computations can be performed with freely available software packages, such as AGRICOLAE, written in R language by Mendiburu (2007), and also with statistical software packages such as MINITAB (Minitab Inc.), while graphical modeling can be conducted using AMOS 4.0 (Arbuckle and Wothke, 1999).

Multi-Environment-Multi-Interval QTL analyses

Molecular and phenotypic data for the two populations, generated as part of the Australian National Barley Mapping Program (Emebiri *et al.*, 2003; Panozzo *et al.*, 2007), were used for this paper, although the marker data for both populations were updated by addition of EST-derived markers in the course of this work. The genetic linkage maps were also constructed de novo, using the evolutionary strategy algorithm implemented in the Multipoint mapping software (Mester *et al.*, 2003).

QTL analysis was conducted using the multiple trait algorithm implemented in the MultiQTL package (www.multiqtl.com) to apply a combined multiple interval (MT), multi-trait (MTA), multiple environment (ME) model (Korol *et al.*, 1998). Hypotheses of interest were compared by bootstrap analysis with 1000 iterations.

Results and discussion

Path analysis

Genetic correlation coefficients were fairly consistent in the two barley populations, as indicated by their sign (direction) in Figure 1. DP, for instance, showed a positive association with AA and BG activities, and also with free alpha-amino nitrogen (FAN), but was negatively correlated with VISC. These correlations agreed well with the findings of Mather *et al.*, (1997), and were expected since DP reflects the level of enzymatic activity needed to break down starch in the kernel into sugars that in turn are converted into alcohol by yeast.

Path analysis explained 45-50% of the phenotypic variation in ME in the two populations, and showed a clear partitioning of the correlation coefficients into direct and indirect effects (Figure 1). DP, a trait customarily associated with increased malt extract, had negligible direct effects on ME in both populations (Figure 1). Indeed, the correlation of ME with DP varied across populations, being positive in the Arapiles \times Franklin population but negative in the VB9524 \times ND11231*12 population (Figure 1). In contrast, the direct effect of DP on ME was consistently negative in the two populations, and this represents a clear advantage of path analysis over multivariate analysis, which is based on phenotypic covariance or correlation matrix. DP is a general measure of amylolytic enzymes levels in the malt, with AA the major enzyme involved in starch conversion.

In the Arapiles \times Franklin population, WBG was identified as the main determinant of malt extract, that is, the variable having the largest direct effect (P -value = -0.69). Since path coefficients are standardized partial regression coefficients, the P -value indicates that a unit change in WBG would result in a change of 0.7 standard deviation unit in ME levels. VISC also had a large direct effect on ME (P -value = 0.22), but this was largely

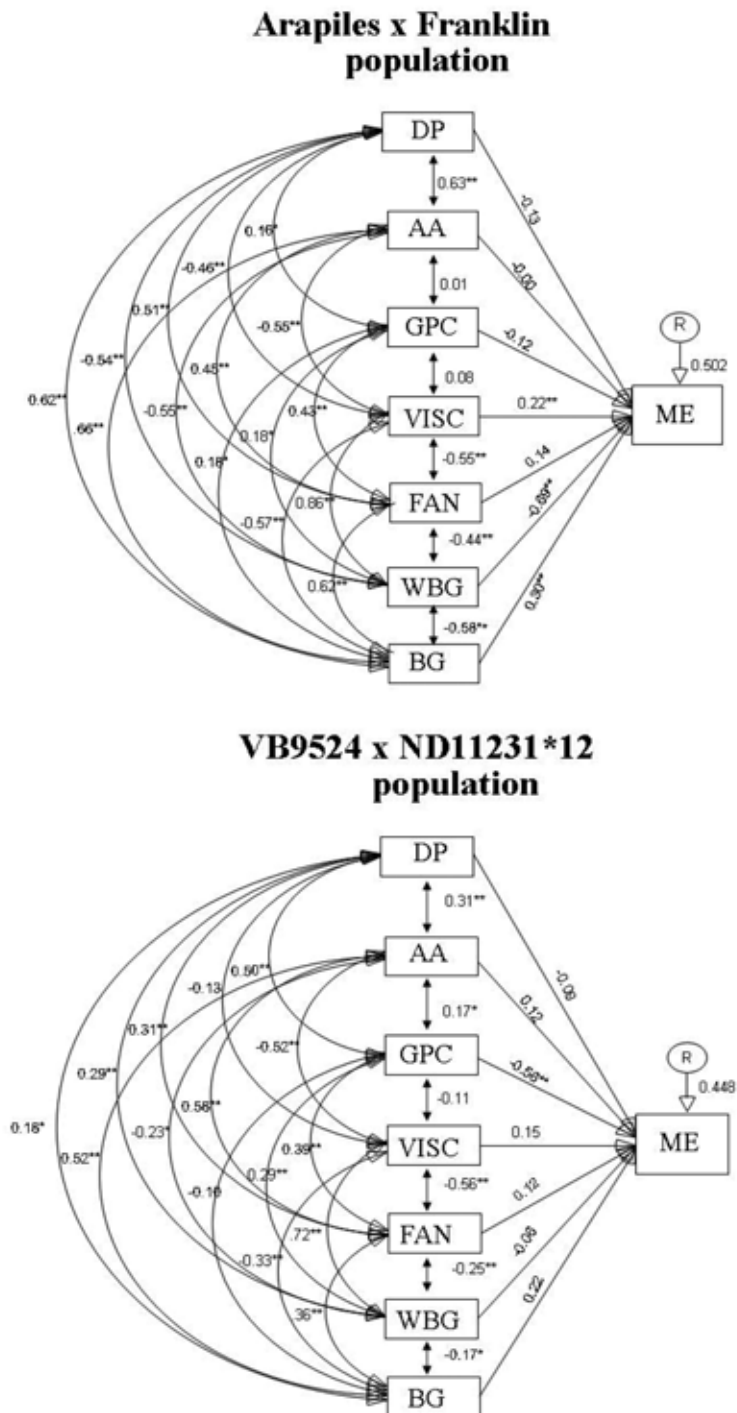


Figure 1. Path diagram of malting quality traits measured in two barley populations. One-directional arrows indicate direct effects while double arrows are correlation coefficients.

through WBG (Figure 1). Even though AA had a highly significant positive correlation with ME ($r = 0.42$; $P = 0.001$), its direct effect was close to zero (Figure 1). This is good information because it showed that, at least in the Arapiles \times Franklin population, malt extract can be increased without a concomitant increase in the levels of AA activity. There are industry concerns that increased levels of AA enzymatic activity in new barley varieties might be associated with sprouting problems under wet harvest conditions.

Grain protein content (GPC) was the major determinant of ME in the VB9524 \times ND11231*12 population, as indicated by the large direct effect (P -value = -0.56) (Figure 1). VISC showed close to zero correlation with ME ($r = -0.03$), but its direct effect was positive, although largely through the activities of BG (P -value = 0.22). A similar situation was observed for AA, but in the case of WBG, the bivariate relationship, quantified by correlation coefficient, was significant. The total indirect effect, however, was three times larger than the direct effect (Figure 1). Furthermore nearly all of the indirect effect was through GPC, and this was negative. In contrast, the observed correlation between GPC and WBG was positive ($r = 0.29$; $P = 0.001$), and consistent with reports in the literature. These results suggest that, in this population, the well known association of WBG with ME results largely from the fact that the trait varies along with GPC, which, in actuality, is the true predictor of ME.

Multi-trait QTL analysis

The main determinants of ME in the two populations (WBG and GPC) were used in a two-trait QTL mapping to re-analyze previously published data by a combined multi-trait (MT), multi-interval (MIM) and multi-environment (ME) approach. The accuracy of this approach is illustrated in Figure 2, which shows the profile of the QTL scan of chromosome 1H in the Arapiles \times

Franklin population. The chromosome 1H region is associated with multiple quality traits considered to be indicators of modification, a process in which the cell walls and protein matrix of the barley endosperm are degraded through enzymatic action during malting (Wentz *et al.*, 2004). With single-trait analysis (no effect of WBG), results showed poor QTL resolution, with QTL peaks spread along the chromosome (Figure 2), and a decrease in mapping precision (standard deviation of QTL location = 39.1 cM). With the current approach, the maximum LOD score was 49.3, and mapping accuracy was 4 cM. The estimated chromosomal position of the QTL (close to Bmag345) is consistent with Panozzo *et al.* (2007), but the current approach allowed greater power of QTL detection, as evident in the maximum LOD score.

In the Arapiles \times Franklin population, Panozzo *et al.* (2007) applied a factor analytic model in QTL analysis to account for spatial variation in the field and in each phase of the malting quality analysis in the laboratory. The authors identified four QTLs for ME, located on chromosomes 1H, 3H, 5H and 7H. In the current study, two additional QTL regions, not previously identified by Panozzo *et al.* (2007), were detected on chromosomes 2H and 6H. The 6H locus was independent, and detected regardless of the effect of WBG (results not shown). In contrast, the ME QTL on chromosome 2H was functionally related to WBG (Figure 3), as explained by Li *et al.* (2006). There was little evidence of a QTL for ME on chromosome 2H when data were analyzed with a single-trait model (sub-model with no effect of WBG across sites). With a two-trait model (ME, WBG), however, a large and highly significant change in LOD score (Δ LOD = 18.3; $P < 0.001$) was observed close to the SSR marker, *AWBMS93*, indicating a QTL for ME causally related to WBG. The location of the QTL, close to the *Ha2* gene, is similar to those identified in several barley

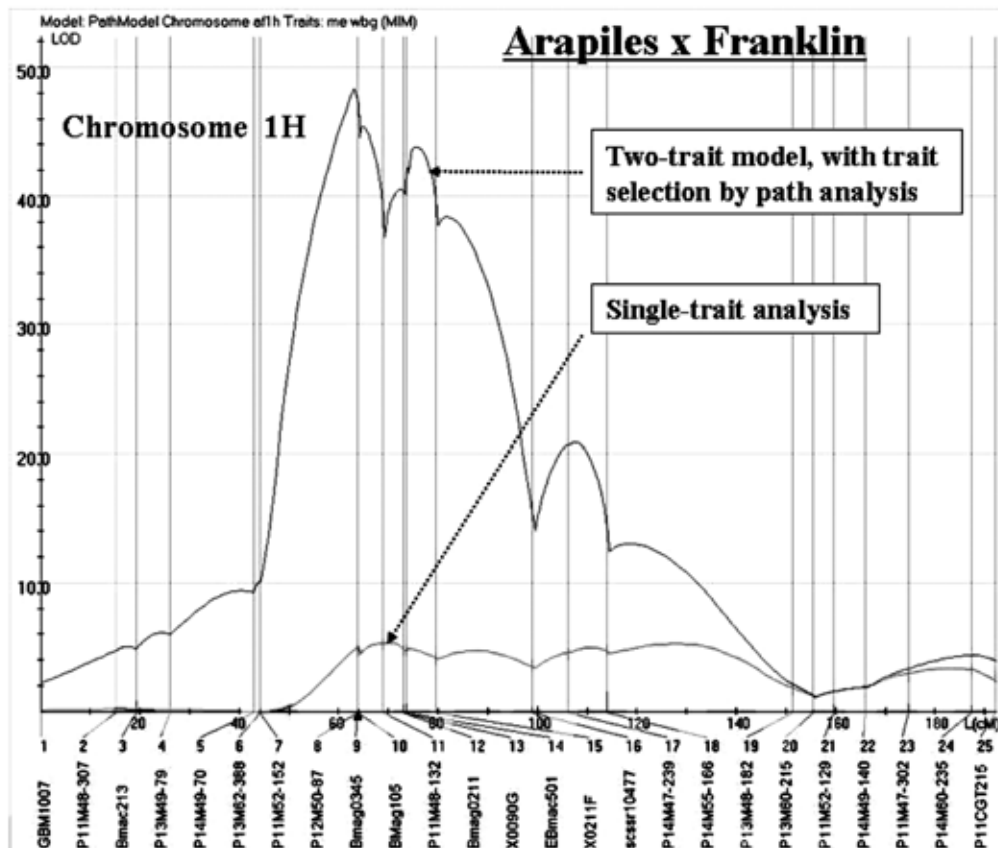


Figure 2. The profile of QTL scan of chromosome 1H in the Arapiles \times Franklin population, based on single-trait analysis and after fitting a two-trait model (ME, WBG), with traits selected by path analysis.

populations (see Collins *et al.*, 2003), and is consistent with the views of Barr *et al.* (2001) that the 2H locus for malt extract is common in Australian germplasm.

In the VB9524 \times ND11231*12 population, Emebiri *et al.*, (2004) used a single-trait approach and identified QTLs on chromosomes 2H and 7H. In the present study, two additional QTLs were found to be segregating in the population, located on chromosomes 4H and 5H. The 5H region comprised two QTLs, one at the centromeric region (identified by Emebiri *et al.* (2003) and Moralejo *et al.* (2004) to harbor a major

locus for GPC) and the other on the long arm of 5H. The long arm of chromosome 5H is known to harbor QTLs for ME in different barley populations (see Collins *et al.*, 2003; Fox *et al.*, 2003; Karakousis *et al.*, 2003; Okada *et al.*, 2006; Panozzo *et al.*, 2007). The chromosome 4H region, however, is less common, but has been reported in other barley populations (see Fox *et al.*, 2003). LOD score profiles of chromosome 4H are shown in Figure 4, where comparison with the sub-models showed a large change in the LOD scores, indicating a 'functionally pleiotropic' relationship with GPC.

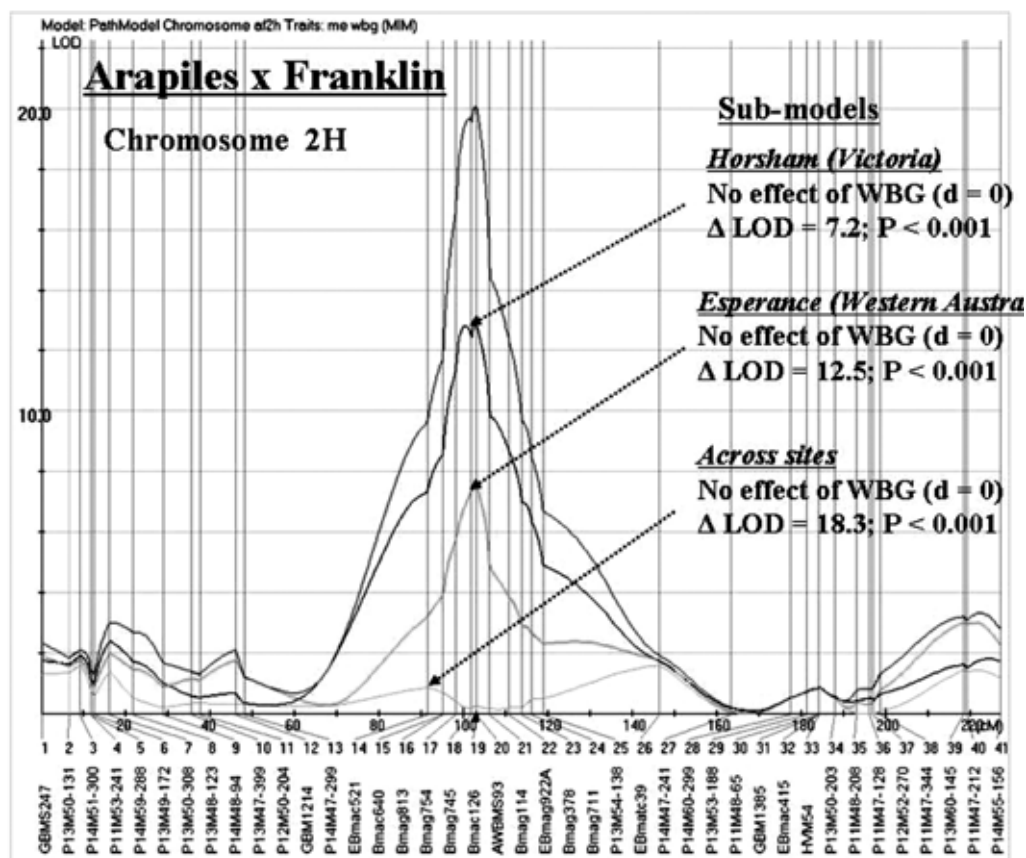


Figure 3. The LOD score profile of chromosome 2H in the Arapiles \times Franklin population, after fitting a two-trait model (ME, WBG), with traits selected by path analysis. Included in the display are profiles of QTL scans obtained with sub-models in which QTL detection was conditional on the effect of WBG across sites, or at specific sites.

QTL \times environment interactions

In addition to gains in power to detect QTLs, the current approach allows testing the hypothesis of QTL \times environment (QTL \times E) interactions by fitting environment-specific models (Figures 3 and 4). QTL \times E interactions can arise from two possible sources: (i) the difference in responses of the same set of genes to different environments; and (ii) the expression of different sets of genes in different environments (Falconer, 1952; Robertson, 1959; Cockerham, 1963). A direct consequence is a change in genetic

correlations from one environment to the next. In the present approach, a significant change in the LOD score profiles resulting from QTL scan with environment-specific models indicates a significant QTL \times E interaction.

The magnitude of the change in LOD scores (ΔLOD) can also provide insights on the choice of environments for QTL detection. In the current study, for instance, fitting a sub-model with no effect of GPC or WBG at Horsham had less impact on QTL detection than the alternative model.

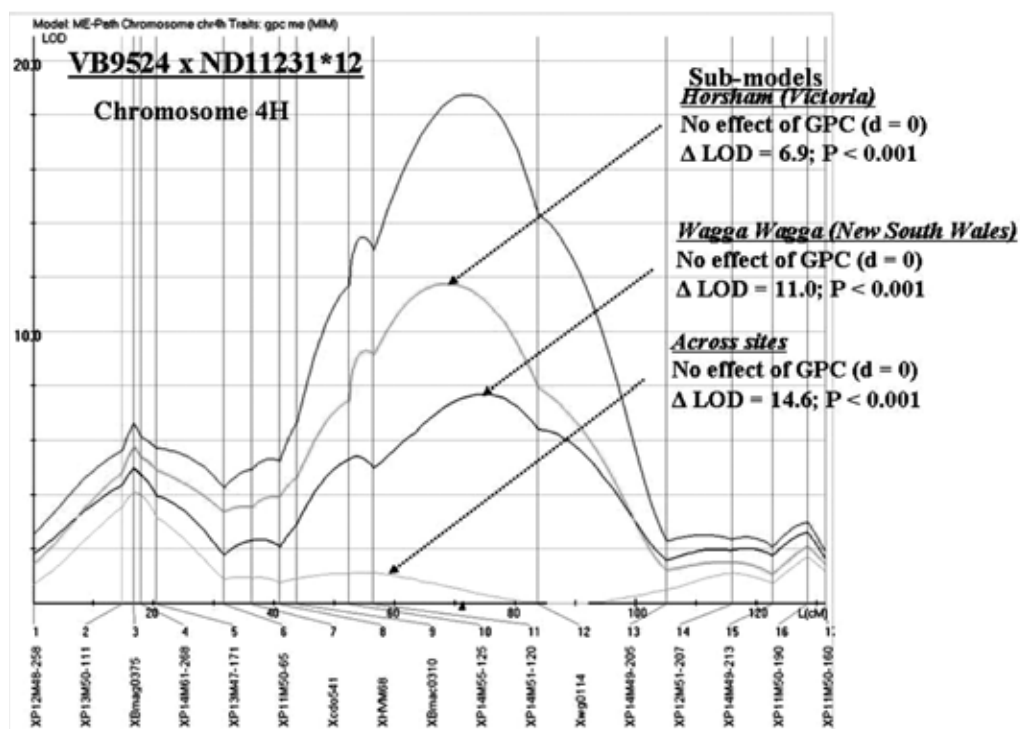


Figure 4. The LOD score profile of chromosome 4H in the VB9524 \times ND11231*12 population, after fitting a two-trait model (ME, GPC), with traits selected by path analysis. Included in the display are profiles of QTL scans obtained with sub-models in which QTL detection was conditional on the effect of GPC across sites, or at specific sites.

Conclusion

Merits of the analytical approach proposed in this paper lies with the fact that it allowed for the detection of ‘functionally pleiotropic’ QTLs, and in the process revealed more QTLs than previously reported for the same populations. An additional and major merit is the improved interpretation of results, which could be extremely important for designing a successful marker-assisted breeding strategy. In the two populations analyzed, increased malt extract was apparently driven by two different parameters: grain protein, and wort beta-glucan. This information is a definite advantage in QTL analyses, and furthermore, the results showed good agreement with general expectations. In the last 50 years, the malt extract of barley varieties has steadily

increased from around 75% to roughly 80%, and we are now close to reaching the upper limit, estimated to be about 85% (Wright, 2000). There are only a few ways that extract could increase beyond this limit. One is through the use of hullless barley, but this might require changing the brewing process, as barley hulls are an important component of the wort-filtering process and contribute essential flavor to the beer. Since the brewing industry, as a whole, is very conservative, and changes slowly, a more direct, cost-effective approach is by reducing grain protein content (Wright, 2000). Less protein is related to more starch in the grain, and high starch content increases the availability of fermentable sugars, which yeasts convert into alcohol and CO_2 . The large direct effect of grain protein on ME in the VB9524 \times

ND11231*12 population was therefore as expected.

In the Arapiles \times Franklin population, the large direct effect of WBG on ME is new, useful information. The QTL on chromosome 2H mapped to the proximate location of the cloned gene, *CsIF*, which mediates the synthesis of cell wall (1,3;1,4)-beta-D-glucans (Burton *et al.*, 2006). Numerous reports from the literature suggest that beta-glucan levels play an important role in determining the level of extract yield (Agu, 2007).

Acknowledgement

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Validation of the improvement of barley malting quality by analyzing NILs containing four different alleles of the gene encoding beta-amylase 1

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Abstract

A set of NILs was generated by introgressing four different alleles of the beta-amylase 1 gene into two contrasting malting barley varieties by a marker assisted backcrossing approach. Published sequences were used to develop a pyrosequencing assay for the specific selection of all four alleles at the *bmy1* locus. As a measure for the activity of the enzyme beta-amylase diastatic power (DP), a parameter summarizing the activity of the amylolytic enzymes alpha- and beta-amylase, was chosen. The total set of 107 NILs was analyzed for diastatic power under different temperature conditions to investigate the thermostability of the enzyme. In addition, 12 general malting quality parameters were assessed by a standardized micro malting analysis. The results show the influence of the different *bmy1* alleles in contrasting genetic backgrounds and indicate that the improvement of malting quality by introgression of thermostable beta-amylase depends on the genetic background of the recipient. The use of single favorable alleles for breeding and improvement of malting quality is discussed.

Introduction

As a globally grown cereal, barley shows a high tolerance for different climatic and geographical conditions. Successful varieties are adapted to give maximum yield in

contrasting environments. Although yield is the focus of every breeding program because it is the economic basis of agriculture, and since the most important use of spring barley is malting and brewing, the improvement of malting quality of barley varieties is crucial. Single parameters may be of different importance in different countries or for different applications, but the key role of particular enzymes is confirmed by most users and researchers.

The introgression of particular enzymes or enzyme types with improved performance appears to be an approach that both gives breeders a tool to accelerate their breeding programs and gives the processing industry an instrument for the quality assessment of the raw material. To obtain detailed information about a single character or gene, nearly-isogenic lines (NILs) are the most suitable kind of plant material. They represent a set of lines which possess the same genetic background but differ only in a particular gene or parameter. NILs are a common tool in plant breeding to assess particular traits of interest in different genetic backgrounds and to introgress important traits into agronomically adapted germplasm.

The importance of the starch degrading enzyme beta-amylase was documented early because its activity delivers the small units of reducible sugar that are required for the attenuation of the beer (Narziss, 1999). The gene has been located on the long arm

of barley chromosome 4H (Kleinhofs *et al.*, 1993) and its sequence is publicly available. Early studies have revealed the existence of different forms of this enzyme, characterized by different stability when exposed to high temperature (Kaneko *et al.*, 2000; Logue *et al.*, 1999). The effect of the different types of enzymes on malting and beer quality was demonstrated by several studies comparing the quality of different barley varieties (Kihara *et al.*, 1999; Zhang *et al.*, 2004). The effect on diastatic power was also shown in segregating populations (Edney *et al.*, 2007).

Using the sequence information, genetic markers have been developed to identify the different alleles of the underlying genes (Herz *et al.*, 2004; Kaneko *et al.*, 2000; Ovesna *et al.*, 2006). The distribution of the alleles in accessions from Australia, Germany, Europe and the Czech Republic (Chiapparino *et al.*, 2006; Herz *et al.*, 2004; Malysheva *et al.*, 2004; Ovesna *et al.*, 2006) has been documented by the use of these markers. A clear correlation was found between the thermostability of beta-amylase and the final attenuation of the malt (Zhang *et al.*, 2004) in a wide range of barley varieties. The final attenuation parameter plays an important role in the characterization of a variety, and many superior varieties already have a very high level for this trait.

The objective of the present study was to find out if the introgression of the different thermostable beta-amylase alleles into varieties with a contrasting genetic background would also improve the level of the overall malting quality of these varieties. A comparison of all four alleles in the same genetic background was considered to be essential to obtain a comprehensive data set.

Materials and methods

Plant material

The varieties Hendrix and Auriga were chosen as recurrent parents for the development of NILs. The varieties were registered in the

German variety list in 2002. Both varieties provide good malting quality but they differ significantly in their final attenuation (Figure 1). Hendrix possesses a rather low level of this trait while Auriga shows a good performance regarding final attenuation.

Auriga as been grown widely in Germany and still is a variety of common importance there (Figure 1). Table 1 summarizes the donors of the single alleles at the *bmy1* locus used for the generation of the NILs.

The offspring of each generation following the initial cross was backcrossed to the respective recurrent parent until the BC₃ generation. In order to obtain genetic homozygosity, the selected lines were self fertilized twice. Following each step of backcrossing and each selfing generation, a marker analysis of the progeny was performed and the carriers of the desired allele were selected for further crossing or selfing respectively. Seeds of the homogenous lines were increased in South America in the off season. In 2006, a replicated field trial with 5 m² plots was grown in Freising, Germany, to harvest a sufficient amount of grains for micro-malting.

Table 1. Origin of the beta-amylase alleles used for the introgression into the recurrent parents.

<i>bmy1</i> Allele	Accession
Sd1	Hendrix, Auriga, Kenia
Sd2H	Haruna Nijo, Ryota, PI296897, Delta
Sd2L	Golf, Emir, Aramir
Sd3	<i>H. spontaneum</i> 77146-33

Marker analysis

DNA for the marker analysis was prepared as previously described by Herz *et al.* (2004). PCR primers were designed using Vector NTI Suite 8.0. Sequencing primers were identified using the internet Pyrosequencing application (www.pyrosequencing.com). All primer sequences are shown in Table 2. The further SNP analyses of the *bmy1* locus were performed using the pyrosequencing method as described by Kaneko *et al.* (2000).

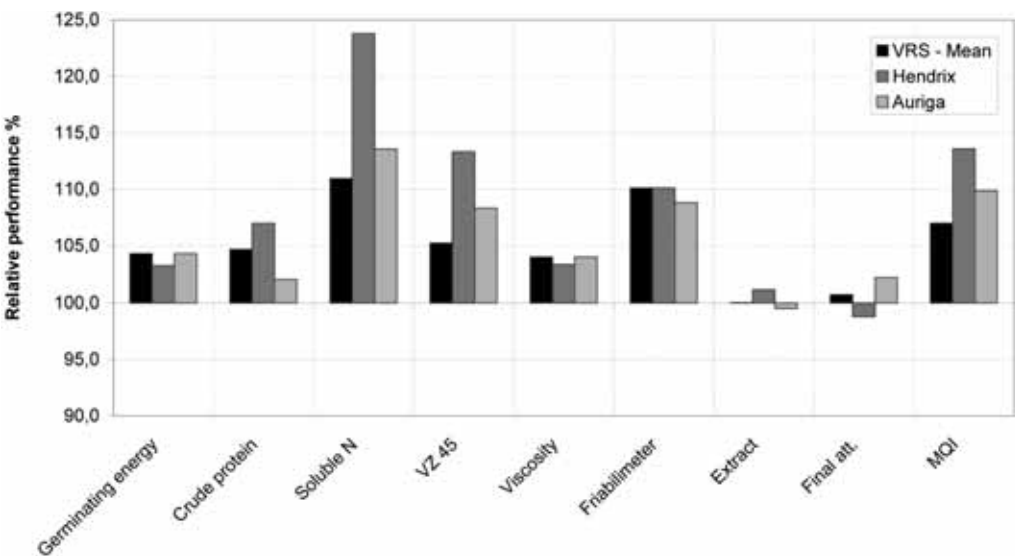


Figure 1. Relative performance of malting quality characters of the recurrent parents Auriga and Hendrix. 100% represents the basic value for good malting quality. (Germinating energy: 93%; Crude protein: 10%; Soluble N and Hartong number (VZ 45°C): 42%; Viscosity: 1.50 mPa*s; Friabilimeter: 85%; Extract and Final attenuation: 82.5%; MQI: 8.1). SOURCE: German registration trials 1999–2001, mean from 21 trials.

Table 2. Sequences of PCR and sequencing primers for identification of the β -amylase alleles by pyrosequencing.

SNP 1	
Primer	5'-Sequence-3'
	Biotin-
PCR Primer F	GCTGTGACAGATGTATGCCGAT
PCR Primer R	GGTACCTCATCTCTCCAGCTGG
Sequencing Primer	CACCAGCATCCAAGAA
SNP 2	
Primer	5'-Sequence-3'
	Biotin-
PCR Primer F	GCACGATAATATATACCATTGCC
PCR Primer R	TTGTTGGAGTACCATGCAAGGAA
Sequencing Primer	GTGTCATTGTACTGTCCG

Analysis of malt quality

From each sample harvested from the field trials, 300 g of the 2.5 mm grading fraction were micro-malted in an automatic micro-malting machine (Phoenix, Australia). Micro-

malting conditions followed the standard procedure described by MEBAK. Analysis of malting quality parameters was performed according to the MEBAK protocol.

Analysis of enzyme activity

The diastatic power (DP) of all lines was analyzed in order to determine the thermostability of the enzyme types coded by the different alleles. The DP of malted barley was measured according to the following protocol: from the ground malt sample, 10 g were solved in 200 ml 0.5% NaCl. The sample was incubated for 2 h at 20°C and stirred frequently. The DP was measured according to MEBAK after incubating the samples for 10 min under different temperature conditions. To generate a profile of the DP relative to different temperatures, the samples were exposed to 20°C, 50°C, 57°C and 65°C.

Results

A total of 107 BC₃S₂ lines resulted from the marker assisted backcrossing program, with each line harboring one of the four alleles. The proportion of lines with different alleles is shown in Table 3. The number of lines containing the respective alleles varies because of the different segregation of the alleles in the particular crosses.

The values of the parameters representing the malting quality of the lines were in the range generally observed in Bavaria in the 2006 harvest. Due to the unusual hot and dry weather conditions during ripening, the general enzyme activity remains at a relatively low level, as does the gelatinization temperature, which shows higher values than usual and is expressed by a low VZ 45°C (Hartong number) according to Keßler *et al.* (2005).

Table 3. Allocation of the *bmy1* alleles in the population of NILs (including standards).

Recipient	Allele	No.
Hendrix	Sd1	5
Auriga		18
Hendrix	Sd3	12
Auriga		25
Hendrix	Sd2H	26
Auriga		3
Hendrix	Sd2L	19
Auriga		9

The overall results illustrate the basic quality differences between cvs. Hendrix and Auriga (Table 5). Most obviously they differ in the final attenuation character, but there are differences between the two recipients also in Kolbach index, VZ 45°C and extract. The malt quality index, which summarizes the most important quality traits according to their meaning for the processing industry, shows the highest value for Hendrix. The lines possessing the allele *Sd2H* show the second-best malt quality index *Sd* because, in general, the most important parameters are on a high level. The lines with the *Sd3* allele have a lower mean quality, which might be the result of a small part of unadapted germplasm remaining in the lines despite selected backcrossing.

The largest effect of the single alleles that could be measured was the influence on final attenuation.

As shown in Table 4, the thermostability of beta-amylase is also significantly correlated to extract yield and final attenuation. There are also close correlations with crude protein content and malt extract. Both characters show a general correlation to each other because protein content and N solution affect malt extract. As this trait differs strongly between the recipients, it becomes evident that the main influence from the thermostable alleles affects final attenuation.

Table 4. Correlation matrix of diastatic power measured under different temperature conditions and parameters for malting quality in 118 barley lines.

Parameter	DP 20°C	DP 50°C	DP 57°C	DP 65°C
Crude Protein	0.53	0.54	0.55	0.14
Soluble N	0.15	0.13	0.07	0.17
Kolbach index	-0.15	-0.17	-0.23	0.08
VZ 45°C	0.34	0.33	0.29	0.38
Extract	-0.57	-0.59	-0.63	-0.14
Final attenuation	0.55	0.55	0.62	0.44
Viscosity	-0.29	-0.27	-0.22	-0.57
Brabender	0.22	0.25	0.26	-0.27
Friabilimeter	-0.09	-0.13	-0.17	0.39
Ggls	-0.02	0.01	0.00	-0.17
pH Value	0.07	0.08	0.07	0.07

*** = Significantly different from zero at the 0.001 level of probability.

More interesting results become clear when having a look in detail at the changes in the parameters regarding the respective recipients (Table 5).

The allele *Sd2L* reduces the final attenuation in any genetic background. The level of final attenuation regarding *Sd1* mirrors the level of the original varieties. Significant differences in the final attenuation between the recipients Auriga and Hendrix can be observed for the two alleles considered as thermostable. The final attenuation of Hendrix, which has been identified as less than Auriga, could be considerably increased to a level comparable to Auriga, while the high level of Auriga was not affected significantly by the introgression of thermostable alleles.

As shown for the overall means of all parameters analyzed, the differences in other important traits correlated significantly to neither the presence of the different alleles nor to the DP at 57°C. The friabilimeter of the lines with the donor Hendrix is in all lines higher than in the lines with the recipient Auriga. Total extract values are also higher for the Hendrix-derived NILs, independent of the

introgressed allele. Figure 2 illustrates the relative performance of the single quality parameters and DP of the NILs compared with the recipient lines.

The behavior of the introgression lines gives good evidence for the successful transfer not only of the genetic basis for the respective enzymes but also their effect on DP under exposure to different temperatures. The graph (Figure 3) displays clearly that the enzymatic activity increases to a temperature

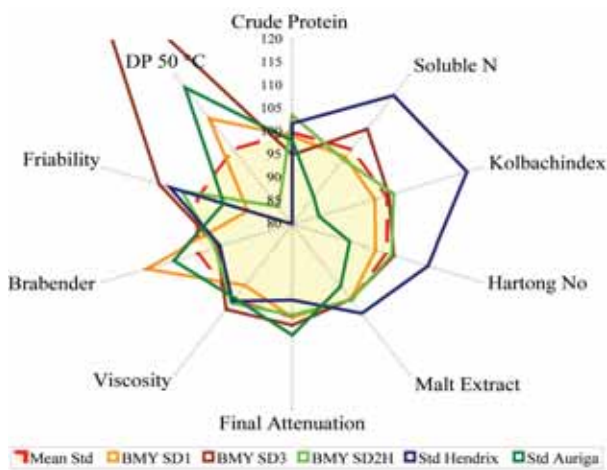


Figure 2. Graphical illustration of the relative performance of NILs containing different alleles of the *bmy1* alleles. (Relative quality performance basis, as explained in Figure 1).

Table 5. Effects on malting quality parameters and DP of the different alleles at the *bmy1* locus in the two recipients.

Recipient	Allele	Extract (%)	Final Att. (%)	Friabili-meter (%)	Kolbach Index (%)	MQI	DP 57°C WK
Auriga	Sd1	81.3	83.0	75.6	37.6	6.6	84
Hendrix		84.0	80.2	83.9	44.2	7.9	42
Auriga	Sd3	80.2	84.3	72.9	36.8	6.2	141
Hendrix		81.5	84.3	77.3	43.6	7.0	104
Auriga	Sd2H	80.4	83.9	59.1	33.3	5.4	101
Hendrix		83.2	83.0	79.8	41.8	7.6	57
Auriga	Sd2L	81.0	82.9	74.4	36.5	6.4	87
Hendrix		83.4	79.6	80.8	42.7	7.3	38
Auriga	mean	80.7	83.6	73.3	36.9	6.3	91
Hendrix		83.0	82.0	79.9	42.6	7.4	42
Mean Total		81.9	82.8	76.8	39.9	6.9	83

MQI = Malt Quality Index; DP 57°C WK = Diastatic Power in Windisch-Kolbach units

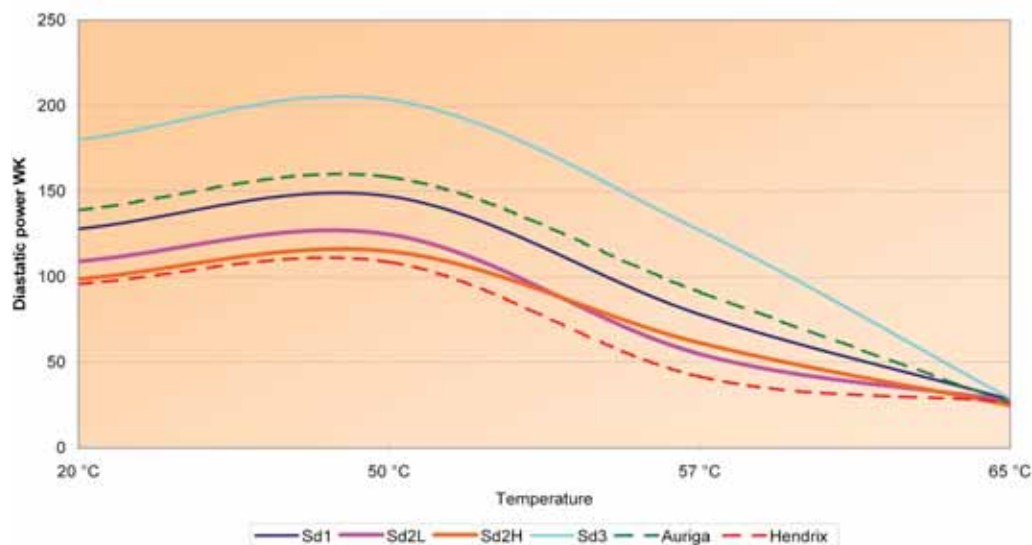


Figure 3. Performance of DP in NILs carrying the different alleles under varying temperature conditions.

optimum of 50°C. A further increase of temperature leads to a significant reduction in enzyme activity, until at 65°C the beta-amylase breaks totally down. The remaining level of DP represents the activity of alpha-amylases, which are synthesized after induction by malting and are not affected by high temperature. The temperature profile of DP shows the different reaction of the carriers of the single alleles under increasing temperature conditions and also the general differences in DP (Figure 3).

In order to confirm that not only have the genetic markers been transferred but also that the transferred genetic segments improve the thermostability of the enzyme, the entire population was analyzed for DP under a temperature gradient (Figure 3). The graph shows the differences of the recurrent parents in the parameter DP. The contrast in DP in the lines with different *bmy1* alleles is also evident. The lines with the allele *Sd2L* show in general a lower DP than all other lines, while the lines with the allele *Sd3* have the highest DP among the analyzed material.

All lines had reduced DP at a temperature above 57°C. The lines carrying *Sd2L* show the steepest reduction in the curve at a temperature of 57°C. The decrease of the DP at higher temperatures is lower in the lines containing the alleles with an expected improved thermostability (*Sd2H*, *Sd3*) than in the material with the alleles *Sd1* and *Sd2L*.

Plot yield, grading fraction >2.5 mm and thousand-grain weight (TGW) of the NILs did not exceed the values of the recurrent parents Auriga and Hendrix (Table 6). From the NIL population the lines carrying *Sd1* show the highest mean plot yield but also the lines with *Sd2L* yield more than the mean of the entire population. Grading fraction >2.5 mm is lowest in the subpopulation of the *Sd3* carriers. The mean TGW of the NILs enclosing *Sd2H* is in the range of the varieties Auriga and Hendrix, which have a higher TGW than the mean of their offspring. The exotic origin of the allele *Sd3* appears to have an effect also on the yield parameters, most likely due to the inheritance of additional genomic regions with a depressing effect.

Table 6. Yield parameters of the NILs and the standard varieties.

	Plot yield (g/3.75 m ²)	Grading fraction >2.5 mm	TGW (g)
Mean Sd1	1562.3	91.4	45.5
Mean Sd3	1377.3	88.1	45.8
Mean Sd2H	1347.4	91.5	47.9
Mean Sd2L	1504.9	91.9	47.0
Mean All	1437.6	90.6	46.6
Mean Auriga	1667.3	93.1	45.3
Mean Hendrix	1617.8	94.4	47.5
Mean of standard cvs.	1644.8	93.7	46.3

TGW = thousand-grain weight.

Conclusions

The effect of the introgression of the thermostable type of the beta-amylase enzyme has been demonstrated for a barley variety with a low level of final attenuation. Without considerable influence on the other quality parameters, the level of final attenuation could be strongly increased.

Nevertheless, a positive effect of the introgression of thermostable beta-amylase cannot be assumed generally, at least not for the barley accessions prevalent in the European elite germplasm pool. Malting quality, and in particular final attenuation, of this intensively selected material is already at a level upon which it is almost impossible to improve. This means that an introgression of the favorable alleles with the aim to improve final attenuation will only be successful when the target germplasm offers good overall malting quality with the exception of final attenuation.

It is not just the breeders take can take advantage of this efficient method. The improved thermostability might have advantages for brewing in some special cases. In 2006, a general reduced activity of amylolytic enzymes was observed in combination with an increased gelatinization temperature (Kreis, 2006). Under these circumstances a higher temperature for mashing might be required, which has in general a negative effect on the activity of beta-amylase. Such problems could be reduced with varieties containing the thermostable isoform.

Based on these results, the small proportion of varieties in Europe that carry the thermostable beta-amylase alleles can be explained. A long period of selection, also with respect to the German purity law, for good extract and final attenuation in Europe resulted in superior genotypes that did not require any improvement regarding diastatic power or enzyme activity. In contrast, in countries where the enzymatic activity of the malt plays an important role because of the use of other starch-containing grains in brewing, the selection for thermostable beta-amylases resulted in a greater number of registered cultivars with this trait.

The identification of additional genetic factors influencing final attenuation will be a challenge for breeding research in the future.

The directed introgression of favorable alleles of a gene has been successfully demonstrated. Although the parameters final attenuation and malt extract in general have so far been considered as the sum of the effects of a number of genes, the results show that only a single gene has major influence on these traits. The marker-based introgression of the alleles from the exotic parents could minimize the decrease in yield. Nevertheless, the reduction of yield and grain quality by introgression of the thermostable alleles has to be confirmed by further field trials. Obviously the genetic background of a variety has to be considered by the breeder when planning a crossing program.

The results have shown that a breeding program using molecular markers during the

breeding process allows a very rapid adaption of the breeding material to current demands. In particular, the introgression of particular genes that encode improved types of enzymes offer the chance to improve malting quality parameters. Thermostable beta-amylase represents a unique example, where the combined knowledge of gene sequence, enzyme activity and malting quality can be utilized for efficient improvement of malting barley. With a growing number of traits selectable by markers, the combination of favorable quality parameters with a high yield potential will be the most efficient method to bring superior varieties to the market.

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QTL analysis for malting characteristics based on the high-resolution mapping populations from a cross between Japanese malting barley cultivar Haruna Nijo and wild barley (*Hordeum vulgare* subsp. *spontaneum*) H602

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Abstract

A high density linkage map with EST markers was constructed using doubled-haploid populations from a cross between Japanese malting barley cultivar Haruna Nijo and wild barley (*Hordeum vulgare* subsp. *spontaneum*) accession H602. In the present study, we evaluated malting-related characteristics (test weight per litre, 1000-kernel weight, kernel plumpness, and grain protein) of the population to identify quantitative trait loci (QTLs) controlling each characteristic. The analyses indicated that the malting-related QTLs were located on all chromosomes except chromosome 7H. One QTL for crude protein, which was detected on the long arm of chromosome 5H, was previously reported in another population from a cross between Japanese malting barley cultivar Mikamo Golden and standard North American malting barley cv. Harrington. This result suggests that the QTL located on chromosome 5H has a common effect of controlling the amount of grain protein in Japanese malting barley germplasm.

Introduction

In the past ten years, genetic analyses with a molecular marker have been widely attempted in barley, and various quantitative trait loci (QTLs) have been observed for agronomic and industrially important characteristics. The previous results of genetic analysis indicate some QTL regions for the malting quality in various barley varieties (Hayes *et al.*, 1993; Han *et al.*, 1995; See *et al.*, 2002). In addition, many researchers have reported QTLs for enzyme activity related to the malting quality (Han *et al.*, 1997; Mather *et al.*, 1997; Ullrich *et al.*, 1997; Kihara *et al.*, 2006).

Map-based cloning is a powerful tool to identify genes responsible for useful traits in barley. QTL cloning requires additional resources to ensure that the QTL really is localized within the target marker interval. This necessitates high-resolution mapping with closely linked markers and a segregating population of several thousands of individuals.

In a previous study, 93 doubled-haploid lines (DHLs) from a cross between the Japanese malting barley cv. Haruna Nijo and wild barley (*Hordeum vulgare* subsp. *spontaneum*) accession H602, which was used to construct a linkage map encompassing 1106 loci covering 1362.7 cM (Sato *et al.*, 2004), were analyzed for the QTL of several agronomic characteristics (Hori *et al.*, 2005). In the present study, the DHLs were evaluated for malting-related characteristics (test weight per litre, 1000-kernel weight, kernel plumpness, and grain protein) to identify the QTLs controlling each characteristic.

Materials and methods

Plant materials

A set of 93 DHLs derived from Haruna Nijo and H602 and their parents were grown in a field at Kurashiki, Japan, in the 2006–2007 barley growing season.

Phenotype evaluation

Four malting-related characteristics (test weight per litre, 1000-kernel weight, kernel plumpness, and grain protein) were evaluated in 93 DHLs and their parents. Kernel plumpness was measured by the percentage of grain weight per total grain weight retained after sieving on a 2.5 mm screen. Grain protein was analyzed with the Kjeldahl method in Analytica-EBC.

QTL analysis

PCR primers developed from the 3' EST sequences generated at the Research Institute for Bioresources in Okayama University were used as EST markers (Figure 1), and QTL analysis was performed using MapQTL5 (Kyazma).

Results and discussion

Four characteristics were examined in the parental lines. Test weight per litre, 1000-kernel weight and kernel plumpness showed a difference between parents, with

those in Haruna Nijo higher than those in H602 (Table 1). In contrast, grain protein in H602 was higher than that in Haruna Nijo.

These characteristics varied among the population of 93 DHLs, and their means \pm standard deviation were: test weight per litre 626 ± 74 g/L (min. 456; max. 949); 1000-kernel weight 34.6 ± 4.7 g (min. 25.0; max. 43.8); kernel plumpness $33.9 \pm 27.2\%$ (min. 1.4; max. 87.0); and grain protein $11.1 \pm 1.5\%$ (min. 8.1; max. 15.1) (Table 1). There were positive correlations between pairs among test weight per litre, 1000 kernel weight and kernel plumpness (Table 2).

QTL analysis was conducted for these characteristics (Figure 1). One QTL was detected on chromosome 1H for test weight, and several QTLs were identified on chromosomes 2H, 3H and 6H for 1000-kernel weight. QTLs for kernel plumpness were coincident with those for 1000-kernel weight.

The level of grain protein is one of the most important characteristics in malting barley, because nitrogen in the grain influences the various malting quality characteristics. Three grain protein QTLs were identified in two regions on chromosome 4H and one region on chromosome 5H. In the QTL on 5H, the LOD score and variance explained were 6.36 and 27.0%, respectively. In the same region, a QTL was also detected in the cross between the Japanese malting barley cv. Mikamo Golden and the North American malting barley cv. Harrington (Okada *et al.*, 2006). These facts suggest that this QTL contributes to the accumulation of grain protein in Japanese malting barley.

In the present study, we confirmed that the cultivated vs. wild barley mapping population is useful to detect malting-related characteristics as well as agronomic characteristics. In the near future, the markers closely linked with these QTLs could contribute to efficient marker assisted selection for these characteristics in malting barley breeding, and promote the cloning of these QTLs.

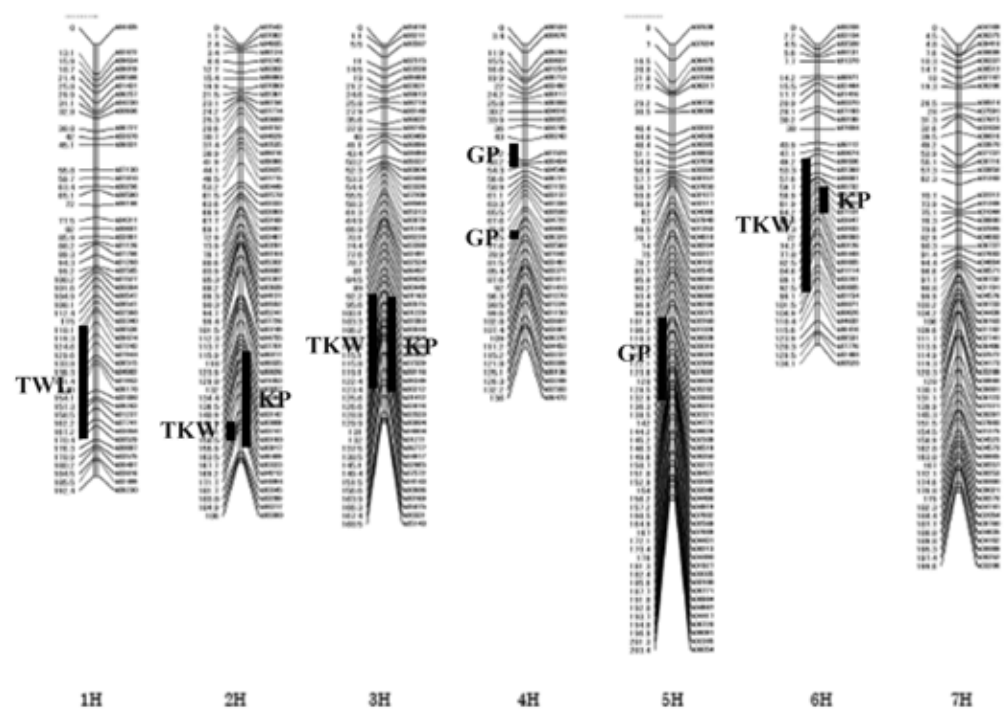


Figure 1. QTL positions of four malting-related characteristics on the map developed by 93 DHLs derived from the cross between Haruna Nijo × H602. QTLs are indicated by black bars. Abbreviations are: TWL = test weight per litre; TKW = 1000-kernel weight; KP = kernel plumpness; and GP = grain protein. All the QTLs detected in this study have positive values by the loci from Haruna Nijo.

Table 1. Malting-related characteristics in the parent strains

Parameter	Test weight per liter (g/L)	1000-kernel weight (g)	Kernel plumpness (%)	Grain protein (% dry basis)
Haruna Nijo	719	46.1	92.3	12.0
H602	575	31.1	2.4	15.0

Table 2. Correlation between the four characteristics examined.

	Test weight per liter	1000-kernel weight	Kernel plumpness	Grain protein
Test weight per litre	1.00	—	—	—
1000-kernel weight	0.21*	1.00	—	—
Kernel plumpness	0.39**	0.65**	1.00	—
Grain protein	-0.01	-0.08	0.09	1.00

* = Significant at the 5% level; ** = significant at the 1% level.

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Characteristics of malting barley in southeast Europe. I. Spring barley

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Abstract

High temperatures and water deficit during the growing season have a negative effect on yield and quality of spring malting barley (SMB). The aim of this work was to evaluate agronomic and quality traits of SMB in Novi Sad (45°20' N, 15°51' E, 86 masl) in southeast Europe. Eight SMB varieties were tested in small-plot trials for seven years. Variations in yield, thousand-grain weight (TGW), grain hectolitre weight (GHW), grain protein concentration (GPC), fine extract content (FEC) and viscosity, and the relationships of these traits, are discussed in this paper. All the traits varied more across years than across varieties. The average yield, TGW and GHW were 6.23 t/ha, 43.3 g and 73 kg/hL, respectively, while GPC varied from 11.5% to 14.6%. The average extract content across years and varieties was 78.3% and viscosity 1.498. The analysis of components of variance showed that the highest variation for all traits was due to year and genotype \times year (G \times Y) interaction. The heritability ranged from 35.9% for yield to 76.3% for FEC. Yield was positively correlated with temperature during the growing season and precipitation during grain filling period (GFP). FEC was positively correlated with precipitation during the growing season. Genetic variability of SMB enables selection of varieties possessing appropriate agronomic and quality traits for the given conditions. To forestall negative weather effects on SMB production, it is suggested to grow at least two varieties per production region.

Introduction

Malt quality depends first and foremost on the biochemical properties of the mature grain, which in turn are determined by genotype, environment and their interactions. Malting quality may vary on a regional basis due to differences in growing conditions, and from one year to another. Barley produced in regions with appropriate temperature and favorable water supply, such as most of the countries in central and western Europe, will give good malt. Weather conditions in southeast Europe are more variable, particularly with respect to the amount and distribution of precipitation.

High temperature and drought are two environmental phenomena frequently occurring during grain filling of spring barley in southeast Europe (Dragović and Maksimović, 1995). The effects of moderately high temperature are difficult to compare with effects of very high temperature since the responses to each of these thermal regimes may be substantially different (Wardlaw and Wrigley, 1994). Heat stress in the post-anthesis period can significantly decrease grain yield and quality in barley (Savin *et al.*, 1996; Wallwork *et al.*, 1998).

Timing of the stress relative to the stage of grain growth has a different effect on yield and quality. Grain weight of wheat is most sensitive to an early exposure, about 10 days after anthesis, to moderately high temperature and short heat stress, and it becomes progressively less sensitive at later stages of grain filling (Tashiro and Wardlaw,

1990; Randall and Moss, 1990). Savin and Nicolas (1999) found also greater reduction in grain weight, test weight and yield when heat and drought stress occurred in an early phase of grain filling. The effect of drought on grain weight was smaller than that of heat stress. Starch content is more severely affected by heat stress and drought than grain weight (Savin and Nicolas, 1999). The amount of nitrogen per grain was reduced when grains were exposed to either heat stress or drought. Since the variation in grain nitrogen content paralleled that in total grain weight, grain nitrogen concentration varied little among treatments.

The present study was undertaken to evaluate the range of variation in some agronomic and quality traits in eight spring barley varieties, and to detect relationships between agronomic and quality traits. The study aimed at determining genotype variation in agronomic and quality traits in barley to indicate the feasibility of using such variation in barley breeding and growing. The analysis of variance components provided information about possibilities of increasing yield of grain and malt quality.

Material and methods

The 8 two-row spring malting barley varieties listed in Table 1, released by the Institute of Field and Vegetable Crops in the 1980–2000 period, were studied over a seven-year period (1998–2004) in Novi Sad. A complete randomized block design with three replicates was used. Plots 5 m × 1 m in

size, with 10 cm between rows, were sown at the rate of 400 germinable grains per m². The sowing dates were 16 February 1998, 1 March 1999, 15 February 2000, 13 February 2001, 13 February 2002, 13 March 2003, and 12 March 2004. The preceding crop in all seven years was soybean. The cultural practices applied were those regularly used for large-scale malting barley production. Anthesis occurred when the total spike of the main culm had emerged from the flag leaf sheath. The duration of grain filling period was defined as the time between anthesis and the loss of green color from the spike.

Analysis of grain and malt from each plot was conducted four months after harvesting, using the EBC-Analytica methodology. Two-way analysis of variance and estimates of the components of variance due to variety, year, and variety × year interaction were made. A mixed model was used, with variety considered as fixed and years as random effects (Zar, 1996). The analysis of variance and estimates of the components of variance due to variety (σ^2_G), variety by year interaction (σ^2_{GY}), and error were calculated according to Comstock and Moll (1963). The significance of mean squares for the variety was tested using mean squares of G×Y interaction, and the significance of G×Y interaction was tested against the pooled error. The percentage contribution of each variance component was estimated by summing the appropriate terms to give an estimate of total variance and dividing the specific variance component by the total variance (Brandle and McVetty, 1988). The

Table 1. Pedigrees of the varieties and lines, year of release, and relative maturity class.

Variety	Abbr.	Pedigree	Release year	Relative maturity
Novosadski 294	NS 294	NS. 39/Emir//Union	1980	ME
Viktor	Viktor	Akka/2*NS96//2*NS185/3/Spart/4/NS297	1994	ME
Novosadski 450	NS 450	Safir/2*Fj. 6850-85//Bonus	2001	E
Novosadski 454	NS 454	L.142-83/Menuet	2001	E
Novosadski 456	NS 456	Osk.5.241-1-83/Menuet// Viktor	2001	E
Novosadski 460	NS 460	L. 153-83/Severa// NS 316	2002	E
Novosadski 462	NS 462	Fj. 8168-85/Menuet	2002	ME
NS 466	NS 466	NS 300/Menuet//Koral/3/NS 292	2000	ME

E = early; ME = medium early.

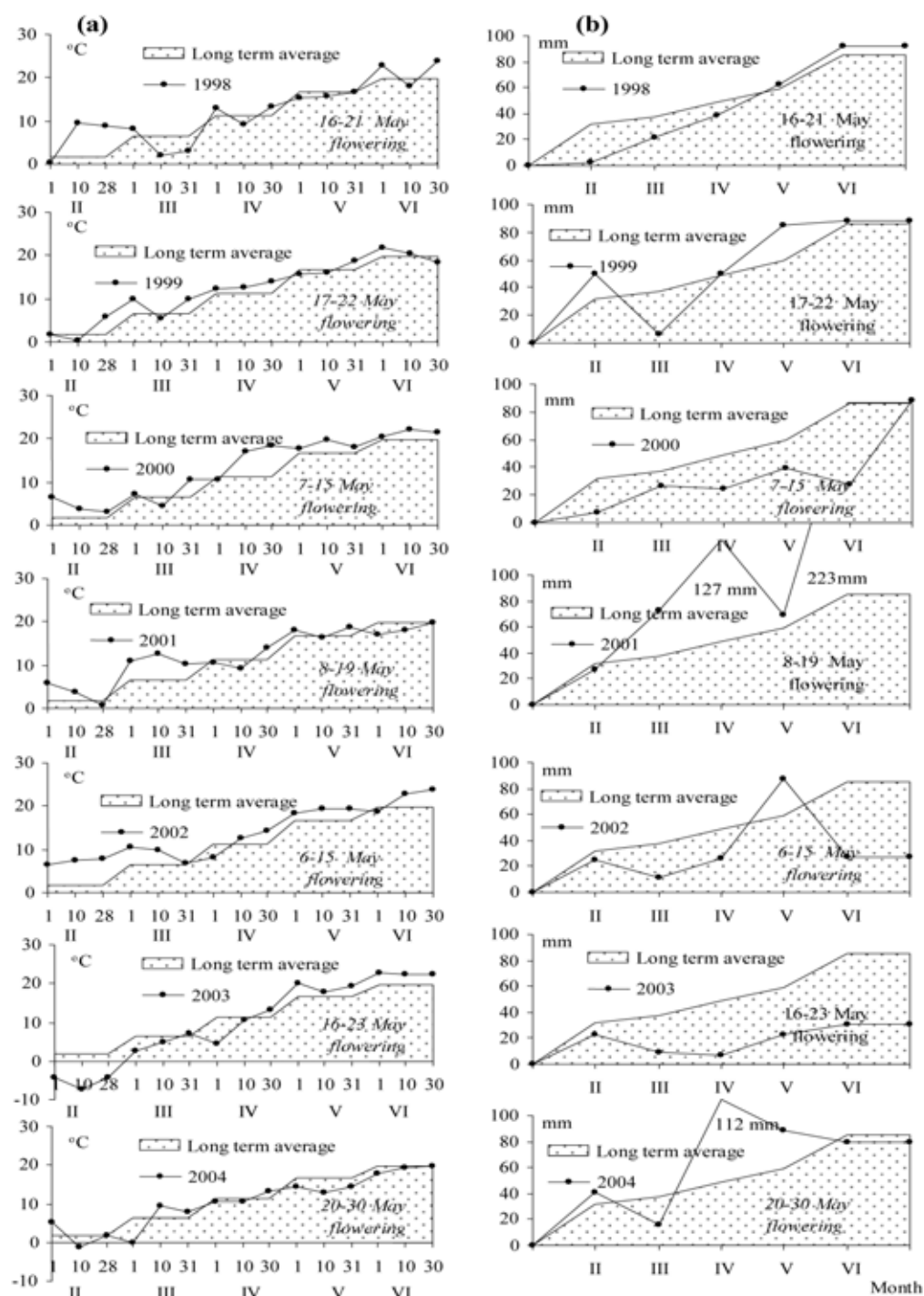


Figure 1. Long term average (1964-2004 period) and 10 days average (1998-2004 period) of (a) temperatures and (b) precipitation, from sowing to harvest of spring barley.

Pearson coefficient of correlation was used to calculate simple correlations between the characters, based on their yearly mean performance. Accumulated growing degree-days (GDD) were calculated by summing up daily degree-days. Daily degree-days were calculated as $T_n = (T_7 + T_{14} + 2T_{21})$ where T_7 , T_{14} , and T_{21} are temperatures at 07:00, 14:00 and 21:00, respectively.

The heritability of the genotype mean over years was estimated by $(M_g - M_{gy})/M_g$, where M_g is the mean square of the genotype and M_{gy} the mean square of G×Y interaction (DePauw and McCaig, 1991).

Weather

The 1998 barley growing season was characterized by high temperatures and water deficit during tillering, and moderate temperatures and precipitation during grain filling (Figure 1). Temperatures above and precipitation below long-term average were the main characteristics of weather in the period from emergence to stem elongation in 1999. Temperatures and precipitation during the grain filling period were similar to the long-term averages. The 2000 growing season was considerably warm, but not hot, with precipitation below the long-term average. Warm and wet weather persisted

during the entire 2001 growing season. In 2002, temperatures during tillering were much higher than the average, while they were close to the long-term average during grain filling, except for May, when spring barley passed through the stage of flowering, the precipitation in all other months was below the long-term average, but it could not be considered as dry. A cold period during tillering and a very warm period during grain filling were the main thermal features of 2003. That year was extremely dry. Moderate temperatures and precipitation during tillering and cold and very wet weather during grain filling characterized the 2004 growing season.

Results

Components of variance

The period from emergence to flowering, i.e. the vegetative period (VP), was influenced mainly by year and variety, and to a lesser extent by G×Y interaction (Table 2). The highest percentage of variance, 69.5%, was due to the year. The ranges of genotype and annual mean values show almost twofold variation due to year compared with variety (Table 3).

Low values for the G×Y interaction indicated a similar ranking of the varieties

Table 2. Mean squares, variance components (%) and heritability of yield, 1000-grain weight (TGW), grain hectolitre weight (GHW), grain protein concentration (GPC), fine extract content (FEC), and viscosity in 8 spring barley varieties in 7 years (1998–2004).

Parameter	DF.	VP (GDD, °C)	GFP (GDD, °C)	Yield (t/ha)	GHW (kg/hL)	TGW (g)	Grain protein (% DM)	Fine extract (% DM)	VIS (mPa s, 8.6%)
Variety (G)	7	36 216.97**	36 216.97**	3.81	30.37*	139.20**	8.17**	33.011**	0.056*
Year (Y)	6	136 233.59**	16 578.18**	37.03**	144.00**	462.70**	29.34**	76.757**	0.393**
G × Y	42	2 024.57**	2 024.57**	1.25**	7.73**	20.18**	1.40**	3.015**	0.017**
Pooled error	110	228.09	228.09	0.10	0.41	5.72	0.03	0.113	0.001
% of variance									
		20.2	53.2	5.8	11.2	16.4	2.0	25.6	7.8
		69.5	19.8	71.1	59.1	53.2	16.4	55.1	65.7
		7.4	19.6	18.3	25.4	13.9	59.0	17.3	22.4
		2.8	7.5	4.9	4.3	16.5	23.1	2.0	4.2
		66.8	94.4	35.9	56.5	66.4	65.8	76.3	44.9

* = Significant at the 0.05 probability level. ** = Significant at the 0.01 probability level. VP = vegetative period to anthesis. GFP = grain filling period. GDD = growing degree-days. GHW = hectolitre weight. TGW = 1000-grain weight.

Table 3. Ranges of variety and year mean values for the characteristics tested of 8 spring barley varieties grown in 1998–2004 growing seasons

Parameter	Genotype (n=8)			Year (n=7)			Mean
	Min.	Max.	Diff.	Min.	Max.	Diff.	
VP (GDD)	707	825	118	635	860	225	777
GFP (GDD)	734	852	118	750	821	71	782
Yield (t/ha)	5.39	6.73	1.34	3.68	7.18	3.5	6.23
GHW (kg/hL)	70.5	74.3	3.8	68.8	76.6	7.8	73.1
TGW (g)	39.4	46.7	7.3	37.7	49.5	11.9	43.4
GPC (%)	12.1	14.1	2.0	11.5	14.6	3.1	13.1
FEC (% DM)	76.3	80.3	4.0	76.1	80.9	4.8	78.3
Viscosity (mPa s 8.6%)	1.439	1.603	0.164	1.315	1.692	0.377	1.498

VP = vegetative period, GFP = grain filling period, GDD = growing degree-days. GPC = grain protein content; FEC = fine extract content; DM = dry matter.

in the 8 test years (data not shown). The longest VP of 860 GDD across varieties was recorded in 2001, which was characterized by warm and wet weather during the tillering period. Dry and cold weather during spring barley tillering in 2003 shortened the VP, the value of which across years was 225 GDD less than in 2001. Contrary to VP, the period from flowering to physiological maturity, i.e. the grain filling period (GFP), was mostly determined by the genotype, where more than half of the variation was due to the variety and one fifth to the interaction $G \times Y$ (Table 2). Year determined about one-fifth of the GFP variation. Very high heritability for GFP and relatively high heritability for VP may be expected (Table 2).

ANOVA showed that the grain yield in the tested varieties was affected by the year and by the $G \times Y$ interactions (Table 2). The absence of significant genotype effects on yield could be partially explained by the close genetic base of the tested varieties and by a very strong influence of the year. Yield had the lowest heritability of all tested traits. Indeed, genetic determination of yield in this trial was expressed through the interaction $G \times Y$, i.e. the specific behavior of the varieties in the years tested. Yield varied more among the years (3.68–7.18) than among the varieties (5.39–6.73) (Table 3). Plump, well-filled grain is an important requirement for malting barley, which is determined through organogenesis, flowering

and grain filling. During this long period of grain development, the influence of non-genetic factors could be significant, and a high percentage of variation due to the year and interaction $G \times Y$ can be expected (Table 2). One quarter of the genetic variation was due to the genetics of the tested varieties. The year determined the highest variation of GHW and TGW (Table 2). Genetic effects on GHW variation were less prominent and significant only at the $P=0.05$ probability level (Table 2). Percentage components of variance are consistent with the ranges of variety and year mean values, which show greater difference between maximum and minimum values due to the year than due to the variety (Table 3). Broad sense heritability for GHW and THW was 56.6 and 66.4, respectively.

Protein concentration in barley grain was mainly determined by specific behavior of the tested varieties in the 7 years, and 59% of the variation was due to the $G \times Y$ interaction (Table 2). Keeping in mind the high value of the $G \times Y$ interaction, it is not so unexpected that the percentage of the genetic component of variance was very low. The high value of error could be due partly to sampling method and differences in the performance of the chemicals used. The range of variation in grain protein concentration was more influenced by the year than by the variety (Table 3). Among the traits studied related to barley grain properties, fine extract content (FEC) depended mostly on the genotype,

where one quarter of the variation was due to the variety (Table 2). More than one half of the variation of fine extract content was caused by the year. Viscosity depended mostly on the year and G×Y interaction (Tables 2 and 3). Heritability for fine extract content was 76.3.

Interaction G×Y

Since the ranking of the varieties tested changed according to the year for all traits, data are discussed separately for each year. Significant differences ($P < 0.01$) in the VP duration were observed among varieties, years and G×Y interaction (data not shown for VP duration). Across the years, the variety NS 460 had the shortest and the variety NS 294 the longest VP, with values of 707 and 825 GDD, respectively. Across the varieties, the shortest VP was in 2003, 635 GDD, and the longest in 2001, 860 GDD. All varieties, without exception, had the shortest VP in 2003 and 6 of the 8 varieties had their longest VP in 2001.

GFP differed significantly ($P < 0.01$) among the varieties across years, among the years across varieties and among the years for specific variety by G×Y interaction (data not shown for GFP duration). Time of planting, weather conditions during pre-anthesis period and during GFP, as well as the genotype of the varieties tested determined the duration

of VP and GFP. Contrary to the duration of VP, varieties with short VP had long GFP and vice versa. This rule is not confirmed for years across varieties. Due to extremely unfavorable weather conditions in 2003 and generally a very short total barley life cycle, this year recorded the shortest GFP when compared across varieties. The longest GFP across varieties was recorded in 1998. The shortest GFP was recorded for one variety in the year 2000, for 2 varieties in 1999 and 2003, and for 3 varieties in 2004. The longest GFP was recorded for one variety in 2000, for 2 varieties in 2001, and for 5 varieties in 1998.

There was no significant difference in yield among the varieties tested across years (Tables 2 and 4). The 2003 season was very unfavorable for spring barley growing and the average yield across varieties was only 3.68 t/ha. Across varieties, highest yields were seen in 2000 and 1999, 7.18 and 7.02 t/ha, respectively. Significant differences ($P < 0.01$) existed for each variety in the 7 experiment years. It is clear that all varieties had the lowest yield in 2003. Three varieties (NS 454, NS 456 and NS 466) had the highest yield in 1999, three (NS 294, NS 450 and NS 460) in 2000, and 1 in each 2001 (NS 462) and 2002 (Viktor).

Significant differences ($P < 0.01$) were found among varieties and years for GHW

Table 4. Yields (t/ha) of 8 spring barley varieties across 7 years (1998–2004)

Variety (A)	Year (B)							Average
	1998	1999	2000	2001	2002	2003	2004	
NS 294	5.66	5.73	7.03	4.67	6.28	3.67	4.69	5.39
Viktor	5.07	7.89	7.60	7.93	8.57	3.93	6.12	6.73
NS 450	6.49	5.97	7.03	6.10	6.61	3.59	5.50	5.90
NS 454	6.66	7.71	6.82	7.31	7.04	3.18	5.95	6.38
NS 456	6.61	7.89	7.22	7.13	6.95	4.06	6.50	6.62
NS 460	6.35	6.71	7.45	7.35	5.80	3.84	5.85	6.19
NS 462	7.81	6.75	7.11	7.89	6.80	3.64	4.65	6.38
NS 466	6.75	7.49	7.18	6.93	6.40	3.52	5.63	6.27
Average	6.42	7.02	7.18	6.91	6.81	3.68	5.61	6.23
	LSD @ 0.05		A	B		AB		
	LSD @ 0.01		NS	0.18		0.52		
			NS	0.24		0.68		

NS = not significant

Table 5. Protein concentration (% DM) in 8 spring barley varieties over 7 years (1998–2004).

Variety (A)	Year (B)							Average
	1998	1999	2000	2001	2002	2003	2004	
NS 294	13.6	14.5	15.1	12.9	12.0	13.1	13.6	13.6
Viktor	13.3	13.0	12.8	10.6	10.3	12.3	12.4	12.1
NS 450	15.3	12.8	15.3	13.1	14.6	13.2	14.3	14.1
NS 454	13.5	14.8	15.3	12.8	11.4	13.3	13.3	13.5
NS 456	13.4	13.8	14.3	11.2	10.1	12.4	12.5	12.5
NS 460	13.8	14.8	14.6	11.6	11.2	12.7	13.0	13.1
NS 462	12.8	14.8	14.6	11.4	10.7	13.2	12.7	12.9
NS 466	13.7	12.7	15.0	11.7	11.7	13.3	12.9	13.0
Average	13.7	13.9	14.6	11.9	11.5	12.9	13.1	13.1
LSD @ 0.05		A		B		AB		
LSD @ 0.01		0.11		0.09		0.28		
		0.14		0.13		0.37		

Table 6. FEC (% DM) in 8 spring barley varieties over 7 years (1998–2004).

Variety (A)	Year (B)							Average
	1998	1999	2000	2001	2002	2003	2004	
NNS 294	76.2	76.9	76.4	79.6	81.4	77.3	77.8	77.9
Viktor	75.7	79.3	78.9	81.6	81.3	80.3	80.4	79.6
NS 450	74.6	78.6	75.5	79.3	80.7	76.4	78.6	77.7
NS 454	73.7	75.5	73.8	77.1	78.7	74.6	80.6	76.3
NS 456	78.0	80.1	78.4	82.3	83.1	79.7	80.5	80.3
NS 460	75.6	77.5	76.2	79.0	80.7	77.9	76.5	77.6
NS 462	76.7	77.4	76.6	79.8	80.7	75.5	79.8	78.1
NS 466	78.2	78.2	76.0	79.9	81.0	78.2	79.5	78.1
Average	76.1	77.9	76.5	79.8	80.9	77.5	79.2	78.3
LSD @ 0.05		A		B		AB		
LSD @ 0.01		0.2		0.2		0.5		
		0.3		0.3		0.7		

(data not shown). The variety NS 454 had the highest hectolitre weight across years (74 kg/hL), the variety NS 294 the lowest (70 kg/hL). Across varieties, the lowest value was recorded in 1998 and the highest in 2000. Generally, 1998 was very unfavorable for grain filling and grain development. In that year, 6 varieties had the lowest values of GHW. One variety had the lowest GHW in 1999 and one in 2004. All varieties had the best GHW in 2000, with values ranging from 75 to 79 kg/hL.

Across years, the varieties significantly differed ($P < 0.01$) in TGW (data not shown). Cv. Viktor had the greatest TGW (46.1 g) and the variety NS 450 the least (39.4 g). Similarly to GHW, the greatest grain weight across varieties was observed in 2000 and the lowest in 1998. Six varieties had their greatest TGW in 2000, and one each in 2001

and 2002. The low average TGW in 1998 was due to 5 varieties that had the smallest grain in that year. For TGW, 1999 was the worst for three varieties, NS 454, NS 456 and NS 462.

Grain protein concentration is an important requirement for malting quality of barley. Generally, low protein means better quality of barley for malting. Although the variance component due to the genotype was very small (Table 2), a high proportion of G×Y variation identified genotypes with favorable protein concentration in certain years. Across years, the lowest protein concentration was found in the variety Viktor (Table 5). The 2002 growing season was very favorable for malting barley production, when the lowest grain protein concentration across varieties was recorded, 11.5% DM. Except for the variety NS 450, all other varieties had their lowest protein concentrations in

2002. In contrast, according to GPC, 2000 produced the poorest malting barley. Indeed, 5 of the 8 varieties had their highest protein concentrations (more than 13% DM) in that year. Two varieties had their highest GPCs in 1999 and one in 1998.

FEC is the most important economic trait of malting barley. After 7 years of testing, the varieties NS 456 and Viktor had the highest FECs, 80.3 and 79.6, respectively (Table 6). Obviously, 2002 was the most favorable year in Vojvodina Province for malting barley production, since the average fine extract content across varieties was 80.9. In 2001 and 2004, fine extract content was also acceptable, with averages of 79.8 and 79.2, respectively. In 1998, 2000 and 2003, fine extract content was very low, 76.1, 76.5 and 77.5 respectively. The G×Y interaction was significant for all varieties. The varieties NS 294, Viktor, NS 450 and NS 454 had the lowest fine extract contents in 1998 and the highest in 2002, 2001, 2002 and 2004, respectively. Two varieties, NS 456 and NS 460, had their best results in 2002 and worst in 1998.

Viscosity is the measure of cytolytic degree of modification during malting of barley grain. There were significant differences ($P < 0.01$) in viscosity across years, across varieties and among varieties in different years (data not shown for viscosity). Across years, the variety Viktor had the lowest and the variety NS 454 the highest viscosity. The best modification of malt across varieties was in 2001 and the worst in 2003. All varieties

tested had their lowest viscosity value in 2001. Six of the eight varieties had their highest viscosity in 2003 and two in 2000.

Correlations

Yield of spring barley was significantly and positively ($P < 0.01$) correlated with temperature accumulated during the period up to anthesis, while there was no significant relationship with rainfall for that period. These relations were expected due to two facts: (i) tillering of spring barley in southeast Europe takes place during the spring months when generally there is enough water from winter reserves and current rainfall, and (ii) spring months are rather cold and each increase in temperature increases the rate of growth, i.e. increases the tillering capacity. Faster development enables the accumulation of increased quantities of nitrogen needed for translocation during grain filling and earlier completion of the life cycle, before very high temperatures occur in the second half of June (Pržulj and Momčilović, 2001a, b).

Grain protein concentration is significantly and negatively correlated with grain weight. Consequently, all malting barley breeding programs focused on the selection of genotypes with increased TGW. Although there is no significant statistical evidence of increased protein concentration in grain due to drought (Table 7), it is well known that hot weather increases the protein concentration and decreases the fine extract content in barley grain. It is confirmed by our results, with a significant negative correlation of

Table 7. Simple correlation coefficient between the means of 8 spring malting barley varieties in 1998–2004 (n=56).

Trait	VP		GFP		GHW	TGW	Yield	GPC	FEC
	GDD	RF	GDD	RF					
Yield	0.62**	0.26	0.32*	0.41**	0.28*	0.18	—	—	—
GPC	-0.07	-0.18	-0.13	-0.36**	0.01	-0.35**	-0.06	—	—
FEC	0.31**	0.28*	-0.10	0.31*	0.12	0.27*	0.16	-0.72**	—
Viscosity	-0.56**	-0.59**	-0.12	-0.72**	0.15	-0.15	-0.46**	0.40**	-0.50**

* = Significant at the 0.05 probability level, ** = Significant at the 0.01 probability level, VP = period till anthesis, GFP = grain filling period, GDD = growing degree-days, RF = rainfall, GHW = hectolitre weight, TGW = thousand-grain weight; FEC = fine extract content.

-0.35**. The significant negative correlation between viscosity and the amount of precipitation during barley development confirmed that barley achieves good quality and low viscosity in conditions of available water.

Discussion

One of the main tasks of barley breeders is to breed varieties whose pattern of development fits the prevailing growing conditions. In addition to identifying barley varieties with adequate developmental responses for certain environments, it is important to define the relationship between the durations of VP and GFP, with the aim of maximizing yield and quality. For a certain genetic constitution, a long GFP has a positive effect on yield and quality, but within certain limits. Generally, it could be expected that moderate temperatures and high precipitation are positively correlated with long GFP. Depending on plant habit, soil properties, biotic stresses and weather conditions, a prolonged GFP could even decrease the yield. In our trials, GFP was longest in 1998, at 821 GDD, but the yield was 11% lower in relation to 2000, when GFP was 786 GDD and the yield across varieties was 7.18 t/ha. If GFP coincides with high precipitation, losses can be expected from disease attack and lodging. The relationship between GFP and yield is described by a second-order polynomial, where duration of GF that fits maximum yield can be easily determined (Schelling *et al.*, 2003).

High yield potential correlates with long pre-anthesis period (Dofing, 1995). Our results also showed a significant positive correlation ($P < 0.01$) between grain yield and duration of pre-anthesis period (Table 7). Two out of three yield components are determined during pre-anthesis period, namely potential number of spikes per unit area and number of kernels per spike. Since conditions that increase density are unfavorable for yield, it can be concluded that a negative ecological

correlation exists between number of spikes per unit area and yield.

Temperature is supposed to be the main factor determining GFP and grain filling rate (Chowdhury and Wardlaw, 1978; Wheeler *et al.*, 1996; Pržulj 2001). Water supply and drought stress are other important factors influencing grain filling (Long *et al.*, 1998). When GFP is shortened as a consequence of high temperature, drought stress, or both, the yield of barley is decreased (Savin and Nicolas, 1996; Savin *et al.*, 1997). In 2003, temperatures and precipitation were extremely unfavorable for barley development. During the entire life cycle (March-June), total precipitation was three times lower than average while temperatures were lower than average from emergence till anthesis and very high from anthesis till maturity, i.e. during GFP. In that year, the yield across varieties was 3.68 t/ha, or only 41% of the 7-year average yield. Chowdhury and Wardlaw (1978) estimated that optimum mean daily temperature during grain filling of cereals is between 15 and 18°C. Wardlaw *et al.* (1989) found that every 1°C of increase above a mean daily temperature of 15°C reduces the grain yield of wheat by 3–4%. Grain filling rate (GFR) is positively associated with temperature, and if temperature is not so high, increased GFR can buffer the negative effect of decreased GFP (Pržulj, 2001). It is obvious that negative effects of high temperature coupled with water deficit could not be prevented by increased GFR.

In addition to grain yield reduction, unfavorable weather conditions during grain filling, i.e. heat stress, drought and insufficient water, also decrease grain weight and quality in barley. In the very dry and hot 2003, TGW was 5.4% lower than the 7-year average, indicating that the grain yield reduction of 41% in that year resulted mainly from reduction in spike number per

unit area. This reduction was expected in the light of the very unfavorable weather conditions during the tillering period. The third component of yield, number of grains per spike, was affected by heat stress and drought to a limited extent (data not shown).

Adequate precipitation during GFP seems to exert a stronger effect on grain quality than high temperature and drought. The main effect of low rainfall during grain filling is the smaller grain. Thus TGW in 1998 and 1999 was 37.7 and 39.2 g, respectively, which is much lower than the 41.1 g of 2003. In addition to decreasing the average grain size, unfavorable conditions tend to increase the screening percentage, the proportion of grains <2.5 mm increasing by 15–20% (data not shown). Grading percentages higher than 90% in German conditions is achieved in years with temperatures during grain filling below 16°C (Schelling *et al.*, 2003). Grain weight is most sensitive to high temperature in the early phase of grain filling, about 10–15 days after anthesis, and it becomes progressively less sensitive at later stages of grain filling (Tashiro and Wardlaw, 1990).

Reduction in the final grain weight is linearly related to the reduction in starch content (Savin *et al.*, 1997). It means that the main effect of heat stress on grain mass is due to changes in starch accumulation and formation of smaller grains. Accordingly, the starch content in barley grains across varieties was reduced in 2000 and 2003 due to high temperatures and drought. MacLeod and Duffus (1988) found the enzymes involved in starch synthesis to be highly susceptible to high temperature, which leads to decreased starch synthesis. Since enzymes involved in protein synthesis are less affected than those involved in starch synthesis, the amount of proteins in grain is not changed, but the amount of proteins appears to be increased in relation to the decreased starch (Jenner, 1994). Schelling *et al.*, (2003) found that, in German conditions, a favorable protein concentration below 10.5% required

less than 17°C, at least 116 mm of rainfall and a minimum air relative humidity of 75%. High nitrogen percentage forms a protein matrix which could limit the activity of beta-glucanases on the endosperm cell walls and generate lower beta-glucan degradation (Savin *et al.*, 1997).

Conclusion

Unfavorable weather conditions, i.e. either high temperature and drought, or cold temperature and water excess, reduce grain yield and quality of barley. The scope of the present study is limited to a single location and to varieties with a rather narrow genetic base. To be more general, the investigation should be extended over a broader range of environmental conditions and larger genetic variability. Still, this study provided information that a sufficiently large genetic variability allows selection of varieties with good agronomic and quality performances in the conditions of southeast Europe.

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Characteristics of malting barley in southeast Europe. II. Winter barley

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Abstract

In southeast Europe, where water deficit and high temperature stress occur frequently in the spring, winter malting barley (WMB) is grown on a larger area than spring malting barley (SMB). The aim of this work was to study agronomic and quality traits of eight WMB varieties in Novi Sad (45°20' N, 15°51' E, 86 masl), in southeast Europe. Across years, the vegetative period (VP) from emergence to anthesis varied from 985 to 1296 GDD, and the grain filling period (GFP) from 839 to 972 GDD. Average yield varied much more across years (4.77–11.45 t/ha) than across varieties (8.26–9.31 t/ha). Thousand-grain weight and test weight were fairly stable across years, with variations from 40.4 to 46.7 g and from 70.7 to 76.4 kg/hL, respectively. Across years, fine extract content and viscosity were 77.4 and 1.583, respectively. Most of the variation for all traits was due to the interaction Genotype \times Year (G \times Y) and years (Y). Heritability was rather low for all traits and ranged from 12.2% for grain yield to 64.4% for protein concentration. Yield was positively correlated with precipitation and GDD during VP, and negatively correlated with GDD during GFP. Fine extract was positively correlated with temperature during VP. Although the year has a strong influence on agronomic and quality traits, the results of individual varieties indicated that it is possible to produce good WMB in Southeast Europe.

Introduction

The major breeding objectives in malting barley breeding programs are high yield

and good malting quality. Malting quality is a complex character determined by both the growing environment and the variety. The quality of malt depends on physical characteristics of mature grain and the enzymes synthesized during germination. Significant grain quality characters include grain size, i.e. grain weight and grain plumpness, and grain protein content. Significant malt quality characters include malt extract and diastatic power (Henry, 1990).

Malting barley must have a number of well-defined characteristics to provide good material for malt production (Narziss, 1976). Regions with moderate temperature and sufficient precipitation are the most suitable for the production of good quality malting barley. However, malting barley is also produced in arid and semi-arid regions in which climatic conditions are considerably worse. A moisture deficit occurring in the spring in such regions does not allow the production of good spring malting barley each year, so it is important to have some acreages of winter barley in the growing structure as well (Fuchs *et al.*, 1979; Schildbach, 1994). Long-term droughts also occur in the humid regions of western Europe, significantly decreasing the quality of spring malting barley. In such years, winter malting barley has better malt quality and grain characters (Baumer *et al.*, 1994; Pržulj *et al.*, 1998). Even long-term studies conducted in these regions have shown that winter malting barley has better malt than spring barley (Beer *et al.*, 1994). This suggests an advantage of growing winter malting barley not only in the steppes but also in humid regions. In some

countries, the winter malting barley acreage amounts to 20% of the spring malting barley acreage (Baumer *et al.*, 1994; Pržulj and Momčilović, 1995).

The aim of this study was to investigate the genotype (G), year (Y) and genotype \times year (G \times Y) interaction effects on yield and some grain and malt characteristics of winter malting barley in the conditions of southeast Europe, i.e. the Pannonian Plain.

Material and methods

Eight spring malting barley varieties (Table 1), released by the Institute of Field and Vegetable Crops, were studied during a 7-year period (1998–2004) in Novi Sad. A complete randomized block design with three replicates was used. Plots 5 m \times 1 m in size with 10 cm between rows were sown at the rate of 350 viable grains/m². The sowing dates were 1 October 1997, 1 October 1998, 2 October 1999, 1 October 2000, 3 October 2001, 12 October 2002 and 13 October 2003. The preceding crop in all seven years was soybean. The cultural practices applied were those regularly used for large-scale winter malting barley production. Anthesis was recorded as the date when the spike of the main culm had fully emerged from the flag leaf sheath. The time between anthesis and green color loss of the spike was considered as the duration of grain filling period (GFP).

Analysis of grain and malt from each plot was conducted four months after harvesting,

using EBC-Analytica methodology. Two-way analysis of variance and estimates of the components of variance due to variety, year, and G \times Y interaction were made (using MSTAT-C software, 1990).

A mixed model was used, with variety considered as fixed and years as random effects (Zar, 1996). The analysis of variance and estimates of the components of variance due to variety (σ^2_G), G \times V interaction (σ^2_{GY}), and error were calculated according to Comstock and Moll (1963). The significance of means squares for the variety was tested using mean squares of G \times Y interaction and significance of G \times Y interaction by pooled error. The percentage contribution of each variance component was estimated by summing the appropriate terms to give an estimate of total variance and dividing the specific variance component by the total variance (Brandle and McVetty, 1988). Pearson's coefficient of correlation was used to calculate the simple correlation between the characters based on their yearly mean performance. Accumulated growing degree-days (GDD) were calculated by summing daily degree-days with base temperature of 0°C. Daily degree-days were calculated as $T_n = (T_7 + T_{14} + 2T_{21})$ where T_7 , T_{14} , and T_{21} are temperatures at 07:00, 14:00 and 21:00, respectively. The heritability of the genotype mean over years was estimated by $(M_g - M_{gy})/M_g$, where M_g is the mean square of the genotype and M_{gy} the mean square of genotype \times year interaction (DePauw and McCaig, 1991).

Table 1. Pedigrees of the varieties and lines tested, year of registration and relative maturity.

Variety	Abbrev.	Pedigree	Year registered	Relative maturity
Novosadski 293	NS 293	Fr33/NS185-2	1982	ME
Novosadski 183	NS 183	Ager/Emir	1977	E
Novosadski 519	NS 519	Rodnik/Corona	1998	E
Novosadski 525	NS 525	Ranij-1/NS293//NS327/3/Slad	1999	E
Novosadski 529	NS 529	OJK 8-82/NS 293//Slad	1999	E
Novosadski 535	NS 535	L07-87/Sladoran	2000	E
NS 543	NS 543	L122-87/Rodnik//NS327	1997	ME
NS 545	NS 545	NS333/Alpha	1997	ME

E = early and ME = medium early.

Weather

Temperatures close to the long-term yearly average and available water characterized the weather during emergence and tillering of winter barley in autumn 1997 (Figure 1). End of tillering and beginning of shooting in spring 1998 were coupled with high temperatures and water deficit, while moderate temperatures and precipitation were registered during grain filling. The autumn of 1998 was favorable for emergence and tillering, as well as spring 1999 for stem elongation and grain filling. The growing season 1999/2000 was characterized as very favorable for winter barley development, i.e. moderate temperatures and precipitation in autumn provided good conditions for tillering, and abundant winter precipitation provided high reserves of winter moisture. Increased temperatures during end of tillering and during shooting increased the rate of barley development in spring 2000. Winter water reserves combined with current precipitation and elevated temperatures during grain filling were suitable for grain development. Increased temperatures and water deficit were not good enough conditions for winter barley development during autumn 2000. Warm and wet weather was registered during shooting and grain filling in 2001. In the 2001/02 growing season of winter barley, the autumn weather was unfavorable, with high temperatures and drought during germination and seedling growth and cold and wet weather at the beginning of tillering. During tillering and shooting in the spring, as well as during grain filling, temperatures and precipitation were close to the long-term averages. Sufficient water during October and very warm weather during November did not provide good acclimatization and winter barley suffered frost damage in the first half of December, while density was drastically reduced. Drought and high temperatures during spring months of 2003 were unfavorable for shooting and grain development. In terms of temperature and

precipitation, the 2003/04 winter barley growing season was very close to long-term average values.

Results

Components of variance

The main task of all breeding programs is creating varieties with high yield and good quality. Phenology of the selected varieties must fit ecological conditions of the growing area. The genetic base and climatic conditions determine length of pre-anthesis period (vegetative period – VP) and post-anthesis period, i.e. grain filling period (GFP) and their relationship. Varieties originating from same breeding center usually have a similar relationship between these periods, and this could be an explanation for the very low and non-significant participation of the genotype in total variation of VP and GFP (Table 2). Indeed, the lengths of VP and GFP were mainly determined by the year and G×Y interaction. Accordingly, the heritability for these traits was very low (Table 2). The low variability due to the variety was confirmed by the ranges of variety and year mean values, which showed that the range of VP variation was 8.9 times less among the varieties than across the years, and for GFP it was 4 times lower among the varieties than among the years (Table 2).

The year defined 96% of grain yield variation (Table 2). Heritability for yield was rather low at 12.5%. Grain quality traits, grain hectolitre weight (GHW) and 1000-grain weight (TGW), were mainly determined by the year and specific behavior of varieties in the year. The broad sense heritabilities for GHM and TGW were 74.3 and 65.4, respectively.

The highest partition of variation for grain protein concentration in grain belonged to the non-genetic component, i.e. the year. Significant contribution ($P < 0.01$) of the variety to the variation was expressed mainly through the specific behavior of the varieties tested in the different years.

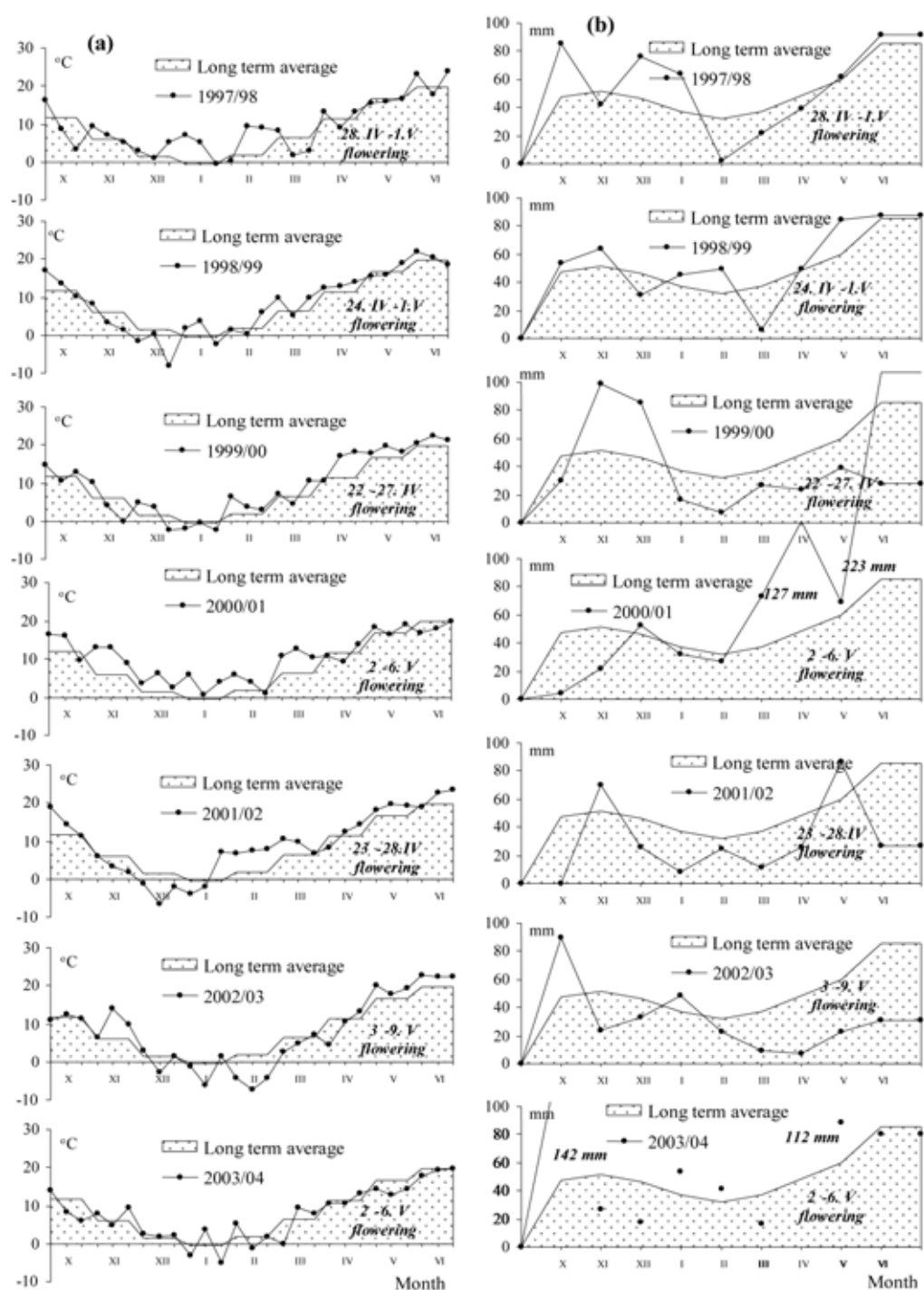




Figure 1. Long-term average (1964–2004, ) and 10-day average (for the period 1997/98–2003/04, ) for (a) temperatures and (b) precipitation from sowing to harvest of winter barley.

Table 2. Mean squares from the analysis of variance, percentage of variance components and heritability of pre-anthesis period duration (VP), grain filling period duration (GFP), grain yield, grain hectolitre weight (GHW), 1000-grain weight (TGW), grain protein concentration (GPC), fine extract content (FEC), and viscosity (VIS) in 8 winter barley varieties over a 7-year period (1998–2004).

Parameter	DF	VP (GDD °C)	GFP (GDD °C)	Yield (t/ha)	GHW (kg/hL)	TGW (g)	Grain protein (% DM)	Fine extract (% DM)	VIS (mPa s 8.6%)
Variety (G)	7	2643.26	2643.26	4.28	34.33*	20.61	2.88*	1.231	0.031
Year (Y)	6	346232.26**	70333.24**	102.93**	78.2**	100.30**	9.17**	6.074**	0.189**
G × Y	42	1331.41**	1331.41**	2.518**	7.96**	11.60**	0.88**	4.826**	0.010**
Pooled error	110	142.80	142.80	0.21	0.39	0.66	0.02	0.178	0.001
% of variance components									
σ_G^2		0.4	1.8	0.4	17.7	5.1	12.7	0.0	8.0
σ_Y^2		96.0	82.7	96.0	41.3	43.8	46.2	2.9	59.9
σ_{GY}^2		2.7	11.4	2.7	35.6	43.2	37.9	87.1	24.1
σ_e^2		0.9	4.1	1.0	5.5	7.9	3.2	10.0	8.0
σ_b^2		2.9	13.0	12.5	74.3	43.6	65.4	0.0	47.5

* = Significant at the 0.05 probability level. ** = Significant at the 0.01 probability level.

Heritability for grain protein concentration was relatively high, which means that the genotype variation within the total variation was rather high. Although the variation for fine extract content due to the year was significant, it could be concluded on the basis of ANOVA results that the performance of this trait depended on the specific behavior of the variety under the specific conditions of each year. Year and G×Y interaction were responsible for variation in viscosity.

G×Y interaction

The life of barley begins with seed germination in the seedbed and ends with physiological

maturity of the spike. More discrete phenological stages of plant development can be defined easily between these two points. Heading or anthesis is usually used to divide the life cycle in two phases: vegetative period (VP) and grain filling period (GFP). In our study, VP across the years ranged from 1144 GDD for the variety NS 519 to 1178 GDD for the variety NS 545 (data not shown for VP duration). Across varieties, the shortest duration of VP, 985 GDD, was in 2003, and the longest, 1296 GDD, in 1998.

A considerably higher variation of VP among years in relation to varieties can be seen in Table 3. The small portion of G×Y interaction in the total variation was

Table 3. Ranges of variety and year mean values for the traits tested in the 8 winter barley varieties grown in the 1998–2004 growing seasons.

Parameter	Genotype (n=8)			Year (n=7)			Mean
	Min.	Max.	Diff.	Min.	Max.	Diff.	
VP (GDD)	1144	1178	35	985	1296	311	1169
GFP (GDD)	897	932	35	833	972	138	907
Yield (t/ha)	8.26	9.31	1.05	4.77	11.45	6.68	8.92
GHW (kg/hL)	72.6	75.7	3.2	70.7	76.4	5.7	73.8
TGW (g)	42.6	45.2	2.6	40.3	46.7	6.3	43.8
GPC (%)	11.8	12.8	1.0	11.2	13.2	2.0	12.3
FEC (% DM)	77.0	77.6	0.6	76.4	78.0	1.5	77.4
Viscosity (mPa s, 8.6%)	1.533	1.653	0.120	1.446	1.708	0.262	1.583

VP = vegetative period, GFP = grain filling period, GDD = growing degree-days, GPC = grain protein content; FEC = fine extract content; DM = dry matter.

confirmed by the distribution of maximum and minimum VP values. Indeed, seven out of the eight varieties had the shortest VP duration in 2003 and all varieties had the longest GFP in 1998.

The year defined 96% of grain yield variation (Table 2). Heritability for yield was rather low, 12.5%. Grain quality traits, GHW and TGW, were mainly determined by the year and specific behavior of varieties in the year. The broad sense heritabilities for GHM and TGW were 74.3 and 65.4, respectively.

The highest partition of variation for grain protein concentration in grain belonged to the non-genetic component, i.e., the year. Significant contribution ($P < 0.01$) of the variety to the variation was expressed mainly through the specific behavior of the tested varieties in the different years. Heritability for grain protein concentration was relatively high, which means that the genotype variation within the total variation was rather high. Although the variation for fine extract content due to the year was significant, it could be concluded on the basis of ANOVA results that the performance of this trait depended of specific behavior of the variety under specific conditions of each year. Year and G×Y interaction were responsible for variation in viscosity.

The duration of grain filling (GF) differed significantly ($P < 0.01$) across years, varieties and for each variety among the years (data not

shown for GFP). The variety NS 545 had the shortest and the variety NS 519 the longest GFPs, 897 and 908 GDD, respectively. In 2000 and 2004, GFP was shortest, 833 and 839 GDD, respectively. In 2000 and 2004, five and three varieties, respectively, had their shortest periods of grain filling. Across varieties, the longest GFP was in 1999, at 972 GDD. In both 1999 and 2003, four varieties had their longest GFPs.

GHW was among the most stable traits. The difference between maximum and minimum value across years was 3.2 kg, and across varieties 5.7 kg (Table 3). The varieties NS 525, NS 529 and NS 535 had the lowest (72 kg/hL) and the variety NS 545 the highest GHW (76 kg/hL) (data not shown for GHW). The lowest GHW across varieties was in 1999, 71 kg/hL, and the highest in 2000, 76 kg/hL. Four varieties had their lowest GHW values in 1999 and seven their highest values in 2000.

Barley grain size could also be expected to be fairly uniform across years, which implies a stability of this trait. There is a relationship between grain size on the one hand, and grain number per spike and growing conditions on the other. The variety NS 453 had the smallest (42.6 g) and the varieties NS 293 and NS 525 the largest TGW (45.2 and 45.1 g, respectively). In 1998, the average TGW for the 8 varieties was the lowest (40.4 g) and in 2003 the highest (46.7 g). Most of the

Table 4. Yield (t/ha) of the 8 winter varieties in 7 years (1998–2004).

Variety (A)	Year (B)							Average
	1998	1999	2000	2001	2002	2003	2004	
NS 293	9.34	9.30	10.75	8.47	8.56	2.69	8.79	8.27
NS 183	9.54	8.76	10.27	6.88	8.65	6.21	7.51	8.26
NS 519	10.03	10.36	11.67	8.35	9.58	4.62	9.73	9.19
NS 525	8.87	10.40	12.51	8.39	8.79	4.94	9.90	9.11
NS 529	11.07	9.57	11.61	9.53	9.47	3.88	10.01	9.31
NS 535	9.17	9.76	11.45	8.51	9.55	2.13	10.16	8.67
NS 543	10.68	9.69	11.29	8.24	8.65	7.67	8.84	9.30
NS 545	10.91	9.76	12.03	8.41	8.66	6.01	8.97	9.25
Average	9.95	9.70	11.45	8.35	8.99	4.77	9.24	8.92
				B		AB		
LSD @ 0.05				0.26		0.74		
LSD @ 0.01				0.34		0.98		

varieties had their smallest grain size in 1998 and largest in 2003.

Although the differences in grain yield were high among the varieties, they were not significant (Tables 2 and 4). Variation across years was 6.4 times higher than variation across varieties (Table 3). The average yield across varieties ranged from 4.77 t/ha in a very unfavorable year (2003) to 11.45 t/ha in 2000. Significant differences ($P < 0.01$) were found for all varieties among the years. All varieties had their maximum yield in 2000 and their minimum yield in 2003.

Across years, NS 519 and NS 525 had the lowest GPC, 11.8 and 11.9%, respectively (Table 5). According to GPC in this study, the oldest varieties—NS 183 and NS 293—can no longer be considered as good raw material

for malting. The lowest GPC was in 2001, the highest in 2002, 11.3 and 13.3%, respectively. All varieties had their lowest GPC in 2001. Half of the varieties had their highest GPC in 2002 and the other half in 2003 (Table 5).

There were no significant differences in FEC in malt among the varieties across years (Table 6). The variation across varieties for this trait was 1.5%; the maximum value was in 1999 and the minimum in 2004. Five varieties had their highest extract in 1999 and one in each of 1998, 2000 and 2003. In 2004, four varieties had lowest extracts and one in each 1998, 1999, 2001, and 2003. Diverse conditions in the years suited different varieties, which resulted in a very high $G \times Y$ component of variance (Table 2).

Table 5. GPC (% DM) in the 8 winter barley varieties over 7 years (1998–2004).

Variety (A)	Year (B)							Average
	1998	1999	2000	2001	2002	2003	2004	
NS 293	12.0	12.8	13.1	11.6	13.2	13.5	12.7	12.7
NS 183	12.9	12.8	12.7	12.1	13.9	12.6	12.9	12.8
NS 519	11.6	11.7	12.1	10.9	12.2	12.4	11.8	11.8
NS 525	11.8	11.9	11.7	10.8	12.2	12.6	12.1	11.9
NS 529	11.8	12.1	11.9	10.7	14.5	13.9	11.8	12.4
NS 535	12.0	11.3	12.0	11.0	12.5	13.6	12.4	12.1
NS 543	11.7	11.8	13.1	11.3	13.6	11.5	12.1	12.2
NS 545	12.2	12.4	12.5	11.7	13.9	11.8	12.8	12.5
Average	12.0	12.1	12.4	11.3	13.3	12.7	12.3	12.3
LSD @ 0.05		A		B		AB		
LSD @ 0.01		0.09		0.09		0.25		
		0.13		0.12		0.33		

Table 6. FEC (% DM) in the 8 winter barley varieties in 7 years (1998–2004)

Variety (A)	Year (B)							Average
	1998	1999	2000	2001	2002	2003	2004	
NS 293	76.5	79.6	77.4	77.5	78.1	76.7	74.7	77.2
NS 183	77.4	79.0	77.1	77.7	77.8	77.8	76.6	77.6
NS 519	76.2	79.2	77.8	77.1	76.8	78.1	77.9	77.6
NS 525	77.4	78.7	77.3	77.6	77.6	76.7	77.8	77.6
NS 529	77.5	78.7	78.0	75.3	77.3	75.5	77.4	77.1
NS 535	77.6	78.5	78.9	76.7	78.6	77.2	75.5	77.6
NS 543	79.5	74.5	77.8	77.1	75.5	79.8	76.9	77.3
NS 545	79.3	75.3	77.6	77.2	76.6	78.1	75.1	77.0
Average	77.7	78.0	77.7	77.0	77.3	77.5	76.5	77.4
LSD @ 0.05				B		AB		
LSD @ 0.01				0.24		0.68		
				0.32		0.90		

Correlations

Grain yield was positively correlated with duration of VP and precipitation up to anthesis, and negatively correlated with GFP duration (Table 7). A negative correlation was found between yield and grain protein concentration. Due to the negative correlation between grain yield and grain size, genotypes with a moderate grain size are preferred in selecting for high yielding varieties. Reduced grain protein concentration was correlated with increased rainfall during VP and GFP and decreased GHW. A positive correlation was found between fine extract content and GFD.

Discussion

Grain filling (GF) is the result of photosynthate translocation from the source to the grains. Grain filling rate (GFR) represents the rate of dry matter accumulation per grain during GFP. Physiological maturity represents the point at the end of the GFP beyond which there is no significant increase in grain dry matter. GF duration is in strong negative correlation with temperature (van Sanford, 1985; Stapper and Fischer, 1990). Genetic factors determine the GFR to a large extent, while environmental factors, first of all temperature, primarily determine the GF duration (Wardlaw *et al.*, 1980; Triboi 1990; Hunt *et al.*, 1991).

In this study, out of the 7 seasons, the 1999/00 growing season was the most favorable for all varieties, with an average yield across varieties of 11.45 t/ha. Indeed, in this season all varieties had the highest

yield. What were the main features of the 1999/00 growing season in which the highest yield across varieties was obtained? The trial was sown on an optimum date, 2 October. An optimum available amount of water and optimum temperature in October and the first half of November increased the rate of development during seedling growth and tillering so that the plants entered the winter in a robust condition. Good acclimatization and deep snow provided good winter survival. Main characteristics of the winter period were very high precipitation and temperatures close to the long-term average (Figure 1). In a 12-year experiment, we found that winter precipitation is the main factor in high and stable yield (Mladenov and Pržulj, 1999). Moderate precipitation and the temperature 2°C higher than the long-term average were the climatic conditions dominating the period of stem elongation and GFP. Chowdhury and Wardlaw (1978) estimated that the optimum mean daily temperature during GF of cereals is between 15 and 18°C. In our study, the temperature during grain filling was between 17.7 and 19.7°C, without heat stress. It is obvious that the increased temperature during GF increased GFR, which resulted in high yield. At the same time, temperatures were not high enough to limit the GF duration, which was 1243 GDD across varieties or only 53 GDD less than 1998, which had the longest GF duration. That means that the 2000 growing conditions enabled a high GFR and long GF duration. In previous work, it was found that for southeast Europe, genotypes with

Table 7. Simple correlation coefficient between the means of 8 spring malting barley varieties in 1998–2004 (n=56).

Trait	VP		GFP		GHW	TGW	Yield	GPC
	GDD	RF	GDD	RF				
Yield	0.61**	0.57**	-0.44**	0.10	-0.03	-0.42**	—	—
GPC	-0.06	-0.59**	-0.10	-0.54**	0.40**	0.23	-0.30*	—
FEC	0.11	0.00	0.34*	-0.15	-0.14	-0.07	0.17	-0.14

* = Significant at the 0.05 probability level; ** = Significant at the 0.01 probability level; VP = vegetative period to anthesis; GFP = grain filling period; GDD = growing degree-days; RF = rainfall; GHW = grain hectolitre weight; TGW = 1000-grain weight. GPC = grain protein concentration; FEC = fine extract content.

a high GFR and moderate GF duration are convenient for growing (Pržulj, 2001).

A temperature increase up to a certain point does not negatively affect yield, since the intensification of physiological processes can compensate for the reduced GFP. Longer duration of high temperatures reduces the GFP to such an extent that a faster GFR cannot prevent yield losses (Wardlaw *et al.*, 1980). When GF duration is severely limited by temperature, the final grain weight is proportional to the GFR (Wiegand and Cuellar, 1981). What were the main features of the 2002/03 growing season in which the lowest yield across varieties was obtained? The sowing was performed on 12 October 2002, and high temperature and high precipitation enabled good emergence and tillering, but without adequate acclimatization. Extensive frost damage in the first part of December reduced crop density in some varieties. Temperatures in the spring, at the time of tillering, were exceedingly high and a severe drought occurred. During stem elongation and grain filling, plants received only 16 mm of rain and average temperatures were about 2–3°C higher than the long-term average. Severe drought was the main reason for reduced yield in this growing season. Savin and Nicolas (1999) also reported that drought has a stronger negative effect on yield than does elevated temperature. Due to unfavorable conditions, the VP in the growing season 2002/03 was the shortest among the 7 years. Earlier anthesis enabled longer GF duration and it was on the level of 1999, in which GFP was the longest. In 2002/03, reduced density and reduced number of grains per spike caused a serious yield reduction. In this growing season, the grain had more-or-less good quality, it had the largest size of all years and the GHW was high.

Numerous authors have investigated the effect of temperature and drought during GF on barley yield and quality (Fathi *et al.*, 1997; Roxana *et al.*, 1997; Roxana and Nicolas 1999). Very few authors discussed the effect

of environment before anthesis on barley quality properties, especially winter barley (Beer *et al.*, 1994; Pržulj *et al.*, 1998). Pre-anthesis environments have an indirect effect on grain quality through mineralization of plant residues from the preceding crop, crop density, nitrogen uptake, etc. In southeastern Europe, the environmental conditions during seedling growth and tillering generally fit plant growth requirements and enable good plant development, formation of appropriate density and grain set per spike. In a normal growing season, even higher soil nitrogen (N) content from the preceding crop does not have a negative effect on grain quality. Indeed, in favorable pre-anthesis conditions, plants use most of the soil N before anthesis, so soil N is no longer available to plants during GF (Malešević and Starčević, 1992). Extremely unfavorable conditions during the pre-anthesis period, as in 2003, did not have negative effects on grain quality, because plants were not able to access N due to drought (Pržulj and Momčilović, 2001a).

High temperature and drought are environmental factors that frequently occur during grain filling of barley. In southeastern Europe, moderately high temperature and moderate drought occur more frequently than very high maximum temperatures and severe droughts. The effects of moderately high temperature and drought on crop quality are difficult to estimate (Wardlaw and Wrigley, 1994). It is especially difficult to estimate for winter cereals, where winter water reserves are of crucial importance in yield and quality determination (Mladenov and Pržulj, 1999). If the analysis were confined only to the spring months of 2000, when high temperatures and water deficit occurred, an erroneous conclusion could have been reached, i.e. that the spring of 2000 was unfavorable for malting barley. In contrast, the maximum yields were obtained in that year, grain properties were excellent, protein content was slightly high and fine extract content was close to that obtained in years with the highest extract content.

Decreased grain quality can be a consequence of increased temperatures and drought during winter and early spring, as in 2002. In such conditions, significant amounts of N remain in the soil after stem elongation and it is potentially available for plants in the GFP. High precipitation and elevated temperatures during GF enable N uptake during GF, which results in increased grain protein concentration and decreased fine extract content. In favorable years, when water is not limited for barley development and large vegetative biomass is developed, high amounts of N can be translocated from vegetative parts to grain. Pržulj and Momčilović (2001b) found that in favorable growing seasons translocated N is 2.3 times greater than in poor growing seasons. The same authors found that the contribution of translocated N to grain N varies from 42 to 85%. This data suggests that adequate agronomic practices must be applied in malting barley production. Consequences of high temperatures and drought during GF are much more prominent in decreasing yield than in decreasing quality. In 2003, when grain yield was 2.4 times lower than in 2000, protein content increased but fine extract content was on the level of quality of the best years. The negative effects of high temperature and drought during GF can be partially buffered by applying adequate agronomic practices.

Conclusion

The environments in southeastern Europe during the pre-anthesis period are not restrictive for high yield and acceptable quality of winter malting barley. Coupled with production technology, the environment during grain filling determines the agronomic and quality properties of winter malting barley. Selection of varieties adapted to the environments of southeastern Europe, and application of appropriate technology, make it possible to have grain yields as high as 7–8 t/ha, and a fine extract content of 78–80%.

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Characterizing Vlamingh, a new malting barley cultivar from Western Australia

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Abstract

The Western Australian barley industry has recently made available to growers a new, two-row spring malting barley (*Hordeum vulgare* L.) named Vlamingh (tested as WABAR2175). The cultivar was bred by the Government of Western Australia's Department of Agriculture and Food. The accreditation of the cultivar was overseen by Barley Australia, and its release in Western Australia by the Western Region Barley Council. Agronomic information on the adaptation of Vlamingh was compiled from yield performance and agronomic management research trials and is presented in this paper. Vlamingh is seen as a competitor to the sowing of Baudin and Gairdner, which currently occupy nearly 45% of the barley acreage.

Vlamingh is suited to the medium and high rainfall regions of Western Australia, and has a slight grain yield advantage over Gairdner and Baudin in state-wide cultivar evaluation trials. It has the same maturity as Gairdner and Baudin but has wider regional adaptation. This is because of a rounder grain shape and higher probability of meeting malting receival specifications for grain plumpness. It has a similar disease resistance profile to Gairdner and advantages over Baudin for scald, net type net blotch and barley leaf rust. Lodging may occur in very high yielding situations, but that is a risk similar to Gairdner, but a higher risk relative to Baudin.

Vlamingh could displace all acreage currently sown to Gairdner and Baudin because of its superior agronomic performance. It may even be grown more widely and be sown in medium rainfall areas where Hamelin and Stirling are currently sown. An economic analysis of 263 research trials conducted over all rainfall zones from 2002 to 2007 found that Vlamingh had a AUS\$ 13/ha advantage over Baudin, AUS\$ 23/ha over Hamelin, AUS\$ 39/ha over Stirling and AUS\$ 44/ha over Gairdner. This was largely in part because Vlamingh met malt barley receival standards for screenings, grain protein and grain brightness in 41% of trials compared to 32% for Baudin and 20% for Gairdner. Hamelin and Stirling met malt barley receival standards in 44% of trials, whilst Stirling met *shochu* (a Japanese distilled white spirit) standards in 19% of trials.

Micro-malting results indicate that the malting features of Vlamingh differ from those of Gairdner and Baudin. It is expected that Vlamingh will suit international markets, where lower levels of starch adjuncts are used in the brewing process. Pearling tests show that even though Vlamingh is much softer than Stirling, it is performing about equal to Stirling for broken pearl and sound pearl ratio. As a softer-grained cultivar, Vlamingh could also have a role in the *shochu* market in Japan as an alternative to Schooner, subject to its performance in distilling tests.

The level of substitution for Gairdner and Baudin will be determined by international market acceptance for it as both a malting and a *shochu* cultivar, the potential upgrade of another cultivar in commercial malt accreditation trials with Barley Australia, and the acceptance by growers of five new feed barley cultivars released in 2006 and 2007.

Introduction

Western Australian growers have traditionally sown barley cultivars that can be received into the malting segregation. Since 1983, around 80% of the barley acreage has been sown to cultivars that can be received as malting at the time (Table 1). This is because very few feed barley cultivars have yielded enough to make up the difference in price paid for malting barley relative to feed barley. At a feed price of AUS\$ 200/t and a malt premium of AUS\$ 40/t, feed barley needs to yield 1.20 times or 20% more than a malting barley for the

same gross return. This assumes 100% of the malting barley meets malting specifications and they have the same input costs. At a 50% probability of meeting malting specifications, feed barley needs to yield only 10% more than malting barley for the same gross return. As a consequence, Western Australia has traditionally produced significant tonnages of malting barley.

Western Australia is an important supplier of malting barley traded to the international malting and brewing market. Over the last five years, Western Australia has supplied nearly 20% of the malting barley required by the international market, primarily to Japan, China, South America and Korea. Since the release of Gairdner in 1997, growers in WA have had access to cultivars including Hamelin and Baudin, that better suit the adjunct brewing styles of our customers in Japan and China. Western Australia has consolidated its position as the major barley producing state in Australia, accounting for

Table 1. Percentage (%) of the Western Australian barley area sown to different cultivars averaged over a 3-year period since 1983 (Source: Cooperative Bulk Handling Pty Ltd, Perth, Western Australia).

Cultivar	1983–1985	1986–1988	1989–1991	1992–1994	1995–1997	1998–2000	2001–2003	2004–2006
Cultivars currently segregated as malting								
Baudin						0.0	0.6	19.1
Gairdner					0.0	8.9	24.2	23.8
Hamelin						0.0	0.3	7.1
Schooner	0.2	1.0	0.1	0.0	0.1	5.9	6.1	2.5
Stirling	35.9	75.4	83.0	76.5	69.5	65.7	54.7	26.6
Vlamingh								0.1
Cultivars previously segregated as malting								
Clipper	20.0	2.5	0.9	0.5	0.0			
Dampier	8.2	0.4	0.0					
Franklin			0.0	2.1	6.9	1.6	0.0	
Harrington				0.0	1.2	2.1	1.2	0.4
Unicorn					0.0	1.0	1.4	0.2
Cultivars never segregated as malting								
Beecher	11.9	4.8	2.2	0.9	0.2	0.2	0.2	0.1
Forrest	22.0	5.2	2.0	0.4				
Mundah					0.8	2.6	5.4	11.2
O'Connor	1.8	10.4	7.4	4.1	2.3	1.2	0.4	0.3
Onslow			1.2	8.1	9.2	2.8	0.5	0.2
Skiff		0.0	0.6	3.9	6.9	4.2	1.1	0.6
Yagan		0.0	1.0	1.7	1.9	1.0	2.2	4.5
Other feed	0.0	0.4	1.5	1.9	1.2	2.7	1.8	3.3

44% of the national crop in 2006/07 (www.abareconomics.com/interactive/cr_dec07/). The value of Western Australia's barley exports was AUS\$ 334 million in 2006/07. The value of barley exports were expected to climb to over AUS\$ 600 million at the 2007/08 harvest due to recent increases in the world barley price.

Barley as a commodity has continued to climb in importance in Western Australia as an alternative to wheat in the cereal rotation over the last 20 years. Much of this is because barley offers agronomic advantages to growers in terms of sowing time, harvest management, business management and weed management, as well as economic advantages due to an improvement in the price of barley relative to wheat. Over the last twenty years, the area sown to barley has doubled from just under 0.5 million ha to just over 1 million ha today. Average grain yield has risen from 1.4 t/ha in the 1980s to 1.7 t/ha in the 1990s to 2 t/ha in the 2000s. Annual production now averages over 2 million tonne per annum.

The increase in grain yield and annual production has been associated with a swing to cultivars with a semi-dwarf habit, such as Gairdner and Baudin, and the availability of higher yielding feed barley cultivars. The proportion of the barley area sown to cultivars with a semi-dwarf habit has increased from less than 1% (~1000 ha) in the mid-1980s to around 20% (~205 000 ha) in the mid-1990s to just under 50% (~467 000 ha) in the mid-2000s.

The dominance of Stirling as the most widely sown malting cultivar is declining as the adoption of the new malting cultivars Baudin, Gairdner and Hamelin expands due to their grain yields being equivalent to the best feed cultivars available at the time. Market signals have also seen a swing to Hamelin as an alternative to Stirling in lower-rainfall environments.

The release of the tall medium spring cultivar Vlamingh could change the swing

to cultivars with a semi-dwarf habit and significantly modify the area sown to different cultivars.

The objective of this paper is to describe the process to release Vlamingh, its malting and *shochu* (a Japanese distilled white spirit) potential, its agronomic characteristics and where it could be adopted in Western Australia. It is expected that Vlamingh will directly compete with Baudin and Gairdner for a place in a grower's cropping program. It therefore focuses on the performance of Vlamingh relative to Baudin and Gairdner, but refers to Hamelin, Stirling and Schooner where relevant.

Commercial accreditation process

Since the release of Baudin and Hamelin in 2002, the protocol adopted by the malting and brewing industry to classify the suitability of barley cultivars for brewing has changed. The accreditation of barley cultivars as being suitable for use in the brewing industry is now managed and coordinated nationally by Barley Australia (www.barleyaustralia.com.au). This has happened to allow more national uniformity in how cultivars are classified, greater transparency in the decision-making process and to better enable the presentation of individual cultivars to the marketplace. In the past, the accreditation process was managed by the regional barley advisory councils, such as the Western Region Barley Council (Paynter *et al.*, 2004).

For each new cultivar of malting barley bred in Australia, there are a rigorous series of evaluations and minimum standards the cultivar must pass in order to gain accreditation as a nationally recognized malting barley cultivar.

After new barley cultivars have been routinely screened for agronomic and processing performance by the breeding institutions, they are subjected to further micro- and commercial-scale testing, using

methodology for both sugar adjunct brewing and starch adjunct brewing. Standardized methods based on commercial practice are adhered to in order to replicate the conditions the cultivars will meet in the commercial environment. Sugar adjunct brewing mimics the brewing process used in Australian breweries, whereas starch adjunct brewing mimics the brewing process in Chinese and Japanese breweries. The quality parameters of barley required for each brewing market differs, hence the use of the two brewing protocols to assess the potential end-use of a new cultivar.

The final accreditation section of the evaluation process takes place over two seasons to ensure a commercial examination of the cultivar. Full accreditation is achieved after the second season's evaluation results have been examined and approved by the Australian barley industry's technical evaluation panel - the Malting and Brewing Industry Barley Technical Committee (MBIBTC). MBIBTC is the national industry body, comprising a panel of malting and brewing experts, who assess each cultivar to establish if it will meet international and/or domestic market performance requirements. Once the cultivar has been accredited by MBIBTC it is posted on the Barley Australia website and can be classified as a malting barley.

It should be noted that national accreditation by Barley Australia does not mean that the cultivar will be received as malting in Western Australia. The decision to commercially accept (or purchase) a cultivar as malting in Western Australia is made by export grain licence holders (i.e. Grain Pool) and domestic malt manufacturers (i.e. Kirin Australia and Joe White Maltings). These companies are members of the Western Region Barley Council (WRBC). The WRBC, which includes grower representatives, meets twice yearly to provide:

- a forum for information exchange for the whole barley industry in Western Australia;
- geographical, end use and agronomic feedback for barley cultivars—both old and new—from a Western Australian perspective; and
- production recommendations to barley growers on an annual basis on the suitability of each cultivar by agro-ecological region, taking into consideration the end use products evaluation from Barley Australia.

Breeding and release of Vlamingh

In February 2006, the Government of Western Australia's Department of Agriculture and Food released the barley breeding line WABAR2175. WABAR2175 was named Vlamingh after the Dutch explorer Willem de Vlamingh and continues the tradition of naming cultivars after people of significance to Western Australia. Willem de Vlamingh was one of the first explorers to chart the west coast of Australia. On 29 December 1696, he landed on an island 19 km off the coast of Western Australia, near Fremantle. He saw numerous quokkas there and thinking they were large rats, he named the island "rats' nest" or Rottnest because of them. On 10 January 1697 he made his way up the river near Fremantle and named it the Swan River after the large numbers of Black Swans that he observed there.

Vlamingh is a two-row spring cultivar bred by the F_2 bulk progeny method at the Department of Agriculture and Food, Western Australia, in 1992. Vlamingh is the result of a controlled pollination cross between the seed parent WABAR570 (synonym 76T110/409) and the pollen parent TR118 (Anon., 2007). The breeding of Vlamingh is another example of where Australian breeders have benefited from international germplasm exchange programs as it involves a cross between the local crossbred WABAR0570 and international crossbred TR118, which was bred by the University of Saskatchewan, Canada.

Malt quality analysis

In 2006, Vlamingh completed commercial malting and brewing trials under the management and co-ordination of Barley Australia and was accredited as a malting cultivar. It was deemed to be suitable for both export (starch adjunct) and domestic (sugar adjunct) brewing markets. On 19 October 2006, the WRBC announced that Vlamingh barley had been granted general malting status for Western Australia. This meant that grain samples meeting malt barley receival standards would now be paid the malting premium on receival at selected sites in Western Australia for the 2007–08 harvest onwards.

European Brewing Convention (EBC) micro-malting data collected from 14 trials

conducted from 2003 to 2006 show that Vlamingh has improved malting quality compared with Stirling (Figure 1; Table 2). It has a higher malt extract level, similar to that of Gairdner but lower than Baudin. Diastase activity is similar to that of Stirling but Vlamingh has higher alpha-amylase and beta-glucanase activity. Diastase activity is lower than in Baudin with lower alpha-amylase and limit-dextrinase activity. Vlamingh malt produces wort with a lower beta-glucan level than Stirling and similar to Baudin. Wort viscosity is lower than that found in wort made from Stirling barley. Apparent fermentability is similar to that from Gairdner malt. Protein modification is slightly higher than for Stirling, as measured by the Kolbach Index and FAN levels.

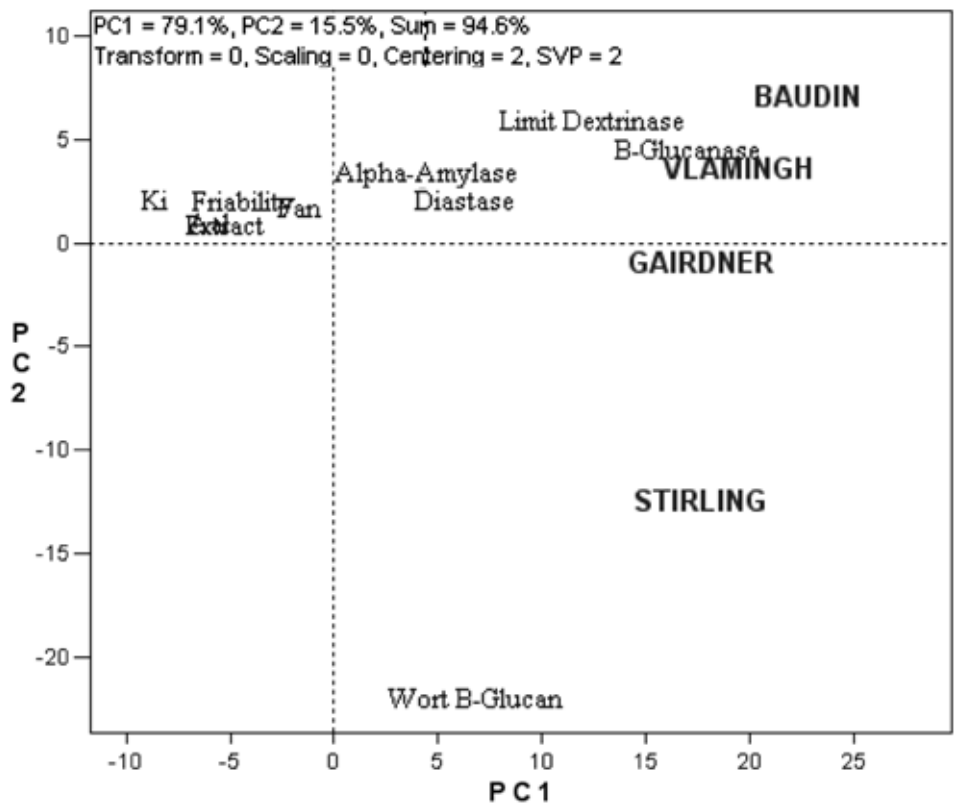


Figure 1. Bi-plot (principal component analysis) of micro-malting quality data of four barley cultivars, Baudin, Gairdner, Stirling and Vlamingh, based on data presented in Table 2.

Table 2. Micro-malting data of Vlamingh in comparison with Baudin, Gairdner and Stirling.

Parameter	Baudin	Gairdner	Stirling	Vlamingh
Malt yield (%)	91	91	91	90
Extract Fine Grind (%)	80.8	80.0	78.8	80.2
Extract F-C difference (%)	0.5	1.0	1.7	0.9
Diastase (WK)	340	210	240	250
Alpha-amylase (U/g)	245	160	160	200
Limit-dextrinase (U/kg)	550	185	305	300
Wort Viscosity (cps)	1.46	1.49	1.67	1.47
Malt protein (% db)	10.1	10.2	10.9	10.3
Malt soluble N (% db)	0.65	0.5	0.56	0.59
Kolbach Index	40	31	33	36
Wort soluble N (% db)	730	560	635	665
FAN (mg/l)	160	110	125	135
Wort beta-glucan (mg/L)	160	230	540	155
Beta-glucanase activity (U/kg)	490	470	330	480
Wort pH	6.0	6.0	6.0	6.1
Wort color	3.5	3.0	3.7	3.4
AAL (%)	84	80	79	82
Friability (%)	89	85	71	89

SOURCE: Allen Tarr and Stefan Harasymow, DAFWA Grain Products Laboratory. Data from 14 research trials from 2003–2006 using 250/500 g EBC tests.

Overall, the micro-malting results indicate the malting features of Vlamingh are different from that of Gairdner and Baudin (Figure 1). It is expected that Vlamingh will suit international markets where lower levels of starch adjuncts are used in the brewing process. The first shipments of Vlamingh grain would be received by international customers in the 2008 season.

Hardness and pearling characteristics

Stirling and Schooner barley are the two main barley cultivars exported from Australia to Japan for use in the production

of the distilled white spirit *shochu*. Stirling is a relatively hard-grained cultivar and Schooner is a soft-grained cultivar (Figure 2; Tables 3 and 4). The *shochu* market size for barley grain is around 0.2 million tonne per annum, of which Australia dominates supply. Grain meeting *shochu* receipt specification is paid a premium relative to grain delivered as malting in Western Australia.

As new malting and feed barley cultivars are being released the area planted with Stirling and Schooner is decreasing due to declining popularity (Table 1). None of the new malting cultivars, namely Baudin, Flagship, Gairdner and Hamelin, have yet been approved for

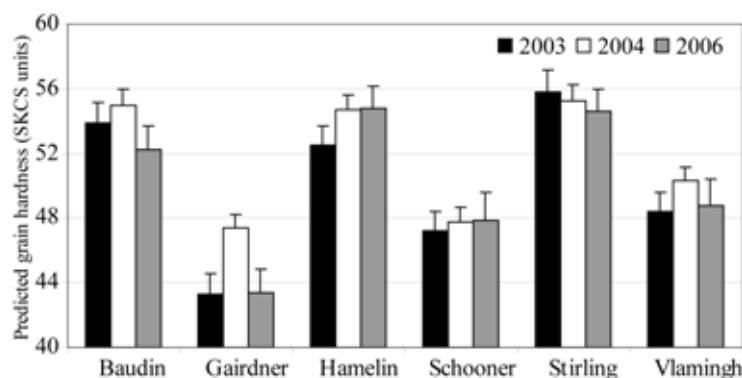


Figure 2. NIRS predicted grain hardness (SKCS units \pm SE) for six cultivars from 59 observations in 2003, 54 observations in 2004 and 33 observations in 2006.

Table 3. Average SKCS grain hardness (SKCS units), average grain weight (mg) and grain diameter (mm), pearling yield (%), pearling time to ~65% yield (min), sound pearl % and broken pearl ratio (%) for five cultivars from 38 observations from 2003 and 2004.

Cultivar	SKCS hardness (SKCS \pm SD)	SKCS av. grain weight (mg \pm SD)	SKCS grain diameter (mm \pm SD)	Pearl yield (% \pm SE)	Pearl time to ~65% yield (min \pm SE)	Sound pearl (% \pm SE)	Broken pearl ratio (% \pm SE)
Baudin	55.3 \pm 12.1	41.1 \pm 6.0	2.35 \pm 0.29	65.5 \pm 0.1	4.31 \pm 0.15	59.6 \pm 3.1	37.0 \pm 3.2
Hamelin	53.7 \pm 12.1	41.7 \pm 6.1	2.41 \pm 0.31	65.6 \pm 0.1	3.84 \pm 0.12	64.6 \pm 2.5	31.6 \pm 2.8
Schooner	47.2 \pm 11.5	43.1 \pm 5.5	2.37 \pm 0.27	65.3 \pm 0.1	3.89 \pm 0.13	56.3 \pm 3.1	39.7 \pm 3.3
Stirling	53.9 \pm 11.7	42.1 \pm 5.7	2.43 \pm 0.29	65.5 \pm 0.1	3.93 \pm 0.08	59.8 \pm 1.9	37.3 \pm 1.9
Vlamingh	45.5 \pm 12.0	41.1 \pm 5.8	2.42 \pm 0.29	65.4 \pm 0.1	3.55 \pm 0.12	62.1 \pm 2.8	34.1 \pm 3.0

supply to Japan for use in the manufacture of *shochu*. The question is what is the suitability of these new cultivars received in Western Australia for the manufacture of *shochu*? This is a difficult question to answer as there is a lack of facilities and experience with *shochu* manufacture in Australia, so we can only evaluate the grain hardness and pearling attributes of new cultivars.

Using both single kernel characterization (SKCS) analysis (Perten SKCS 4100) and near infrared (NIRS) prediction (Foss NIRS6500), the hardness and pearling attributes of Vlamingh were determined in relation to Stirling and Schooner. As the *shochu* market is like the malt market, a cultivar-segregated market, the hardness and pearling attributes were compared against the three other cultivars currently received as malting in Western Australia, namely Baudin, Gairdner and Hamelin.

The hardness of Vlamingh grains was found to be similar to Schooner grains and 6 to 8 SKCS units softer than Stirling grains (Figure 2; Table 3). This suggests that Vlamingh is a soft-grained cultivar (Table 4). Relative to the other malting cultivars received in Western Australia Vlamingh is slightly harder than Gairdner, but softer than Baudin and Hamelin (Figure 2). Baudin and Hamelin are both hard-grained cultivars with a hardness similar to Stirling (Table 3). Variation in the grain hardness of individual grains, as measured by standard deviation, was similar across cultivars.

The SKCS analysis also showed that Vlamingh had a similar grain diameter to Stirling and Hamelin and was slightly rounder than Baudin and Schooner (Table 3). This reflects field observations for screenings (Tables 9, 10, 11 and 12). Grain diameter variation was similar.

When the grain samples were pearled to approximately 65% yield, using a Satake Test Mill with a revolving speed of 1130 rpm and a grit wheel graded 36P, the pearling tests showed that Vlamingh had superior pearling quality to Schooner. Vlamingh produced a pearled product with less broken kernels (Table 3). Vlamingh grain took on average 20 seconds less time to pearl to approximately 65% yield, produced 6% higher sound pearl and had a lower broken pearl ratio.

Relative to the harder grained Stirling, Vlamingh grain took 23 seconds less time to pearl to approximately 65% yield, produced slightly more sound pearl and had a slightly lower broken pearl ratio. The pearling quality of Baudin was similar to Stirling but it took 23 seconds longer to approximately 65% yield. Vlamingh was not as good for pearling as Hamelin whose grains were the least susceptible to breakage. Hamelin produced a higher proportion of sound kernels than Baudin, Schooner, Stirling and Vlamingh. The hardness and pearling tests suggest that Vlamingh could have a role in the *shochu* market in Japan as a soft-grained alternative to Schooner, subject to its performance in distilling tests.

Table 4. Comparison of Stirling, Hamelin, Gairdner, Baudin and Vlamingh for a range of phenological, physical and disease resistance traits.

Trait	Baudin	Gairdner	Hamelin	Stirling	Vlamingh
Spring maturity group	Medium	Maturity Medium	Early	Early	Medium
Basic vegetative period	Short	Medium-Long	Short	Short	Short-
Medium					
Daylength sensitivity	Very high	Medium	High	High	Medium
Physical traits					
Coleoptile length (mm) ¹	68	67	68	70	70
Early growth habit	Prostrate	Prostrate	Erect	Erect	Erect
Boron leaf symptoms	Medium	Medium	High	High	High
Physiological leaf spotting	Medium	Low	High	Low	Low
Plant height at maturity	Short	Medium	Tall	Tall	Tall
Straw strength	Very good	Fair-Good	Fair	Fair	Good
Head retention	Very good	Mod. good	Fair	Mod. poor	Good
Grain plumpness	Mod. good	Fair	Good	Very good	Good
Grain protein potential	Low	Low	Moderate	Moderate	Low
Pre-harvest sprouting risk	Low	Medium	High	Low	Low
Grain hardness rating	Hard	Soft	Hard	Hard	Soft
Pearling efficiency rating	High	Low	High	High	High
Disease resistance ²					
Scald	I	I	S	S	MR
Net-type net blotch	S	I	S	S	I
Spot-type net blotch	S	S	MS	MS	S
Powdery mildew	S	MS	S	S	S
BYDV	MR	R	ES	I	MS
Barley leaf rust	S	S	S	S	MS
Cereal cyst nematode	S	S	S	S	S

¹ Data from Paynter *et al.* 2007. ² Leaf disease resistance ratings: ES = extremely susceptible, S = susceptible, MS = moderately susceptible, I = intermediate, MR = moderately resistant and R = resistant (SOURCE: Sanjiv Gupta and Jason Bradley, DAFWA Plant Pathology).

Table 5. Flowering times of Vlamingh (days earlier (-) or later (+) than) relative to Baudin, Gairdner, Hamelin and Stirling for April through to July sowing based on awn emergence data collected from hill plot trials.

Parameter	Sowing date			
	April	May	June	July
Baudin	+2	-2	0	+3
Gairdner	+3	-6	-2	-7
Hamelin	+17	+11	+8	+8
Stirling	+15	+9	+7	+7

Agronomic characteristics

Maturity

Vlamingh is a barley with a medium spring maturity. Its development pattern is based on a short-medium basic vegetative period and a medium level of daylength sensitivity. This pattern is different from that of Baudin, Gairdner, Hamelin and Stirling (Table 4).

Vlamingh takes around 100 to 110 days to reach awn emergence when sown in May and June in Western Australia. For May to July sowing, Vlamingh flowers one to two weeks later than Stirling and Hamelin and a little over two weeks later when sown in April (Table 5). Vlamingh is generally earlier flowering than Gairdner for May to June sowing and similar to Baudin. When sown in

June it is later flowering than Baudin, but still earlier flowering than Gairdner.

Appearance

Vlamingh has an erect early-growth habit (Table 4). Other malting cultivars of a similar maturity, Baudin and Gairdner, have a prostrate early-growth habit. Stem elongation occurs 9 to 11 weeks after sowing. Its plants and heads lack the red anthocyanin pigmentation found in Baudin, Gairdner, Hamelin and Stirling plants and heads (Figure 3). Vlamingh plants have an erect leaf architecture not unlike that observed in Dash, a cultivar carrying the semi-dwarf gene *ari-e*. *GP* from Golden Promise. Its leaves are less floppy than observed in Baudin, Gairdner, Hamelin and Stirling. As a consequence its canopy is more open before stem elongation (Paynter and Hills, 2007, 2009). Vlamingh plants are therefore distinct from those of the other cultivars currently received as malting in Western Australia.

Leaves of Vlamingh show symptoms of boron toxicity when grown on alkaline soils where sub-soil pH (CaCl_2) is >6.5 . These appear as small black spots appearing on the leaf tip and leaf margins, which occasionally have yellow margins. The level of expression is similar to that found in Stirling and

Hamelin and greater than in Baudin and Gairdner (Table 4).

Vlamingh leaves do not express symptoms of physiological leaf spotting to the same degree as Hamelin and Baudin (Figure 4; Table 4). In Hamelin and Baudin, physiological leaf spotting may occur over the entire leaf and appear as small brown spots that do not have a yellow margin. The brown spots may even have a fingerprint-like pattern or concentric circle pattern. In some cases up to 50% of the leaf area can be affected. In Vlamingh the spots are more defined, smaller and without a fingerprint pattern. Environmental stresses such as nutrient deficiencies, drought and high temperatures can intensify the spotting.

Rates of leaf emergence in Vlamingh are similar to that found in other malting cultivars at around 70 to 80 GDD/leaf (Table 6). Rates of grain filling during the linear phase for Vlamingh are, however, slightly slower than that found in Baudin, Gairdner, Hamelin and Stirling. The number of grains produced per ear is more than Stirling and Baudin, less than Gairdner and similar to Hamelin (Table 6; Paynter and Hills 2007, 2009).

At maturity, the straw of Vlamingh is similar in length to Stirling and Hamelin (Tables 4 and 6; Paynter and Hills 2009). Its lodging resistance is higher than Hamelin



Figure 3. From left to right: Vlamingh plants with erect leaf architecture; auricles lacking anthocyanin pigmentation; and head auricles lacking anthocyanin pigmentation.

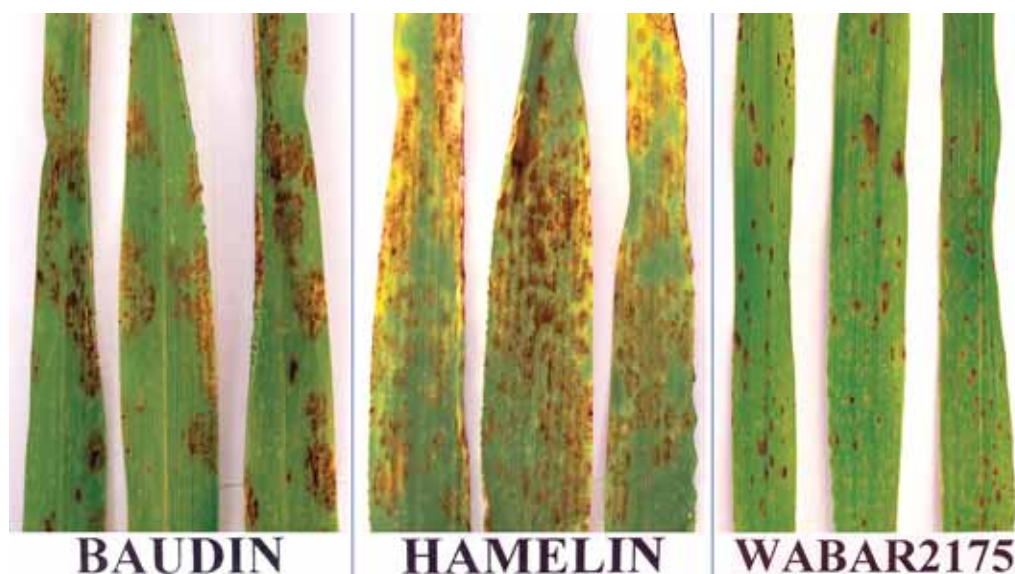


Figure 4. From left to right: symptoms of physiological leaf spotting observed on leaves of Baudin, Hamelin and Vlamingh (WABAR2175).

and Stirling, similar to Gairdner, but not as good as in Baudin (Table 4).

Vlamingh's resistance to head loss is higher than Hamelin and Stirling and is between that of Baudin and Gairdner (Tables 4 and 6). In southern high-rainfall areas, where swathing is practised to minimize head loss and manage harvest moisture to allow earlier harvesting, it is expected that Vlamingh would directly replace Gairdner and be managed with similar farming practices. Baudin has an advantage over

both Gairdner and Vlamingh in that it does not require swathing to minimize the risk of lodging and head loss.

Disease resistance and management

Disease control is now a major cost in growing barley in Western Australia. The two diseases to which Vlamingh is susceptible are spot-type net blotch (*Pyrenophora teres* f. *maculata*) and powdery mildew (*Blumeria* [syn *Erysiphe*] *graminis* f. sp. *hordei*) (Table

Table 6. Rates of leaf emergence, rates of grain filling and grain number per ear measured at York from an 11 June 2007 sowing, and head loss measured at Esperance from an 8 May 2006 sowing.

Cultivar	York, 2007				Esperance, 2006
	Phyllochron (GDD/leaf)	Linear rate of grain fill (mg/GDD)	Grain number per ear	Plant height to base of ear at maturity (cm)	Head loss (heads/m ²)
Baudin	76 ± 1	0.112 ± 0.005	23.0 ± 0.6	58 ± 2	3 ± 3
Gairdner	78 ± 1	0.106 ± 0.007	29.7 ± 0.6	67 ± 2	39 ± 33
Hamelin	75 ± 1	0.105 ± 0.009	25.3 ± 0.8	82 ± 3	114 ± 18
Stirling	73 ± 1	0.115 ± 0.004	21.7 ± 0.4	82 ± 2	104 ± 53
Vlamingh	74 ± 1	0.092 ± 0.004	26.1 ± 0.1	82 ± 3	18 ± 4

4). Overall, it has a similar disease resistance profile to Gairdner, and advantages over Baudin, Hamelin and Stirling for scald (*Rhynchosporium secalis*), net type net blotch (*Pyrenophora teres* f. *teres*) and barley leaf rust (*Puccinia hordei*).

To minimize the development of smut and reduce early infection of powdery mildew, Vlamingh seed should be uniformly dressed with a full-spectrum seed dressing. As foliar fungicides have a much bigger impact on reducing disease levels than any decrease in the supply of nitrogen to the crop or the application of potassium, it is important that Vlamingh crops are setup to match the season's potential. Fungicides and rotation can then be used to manage disease levels. Timely fungicide applications are an important tool to manage the risk of high screenings in Vlamingh and reduce the likelihood of downgrading to feed.

Foliar fungicides are applied to delay disease development and to maintain green leaf area. They need to be applied in response to the disease threat and at timings appropriate to protect upper canopy leaves, which are most important for yield production. Fungicides can be used to manage powdery mildew and spot-type net blotch in Vlamingh.

In high-risk powdery mildew environments, growers should look to use an in-furrow fungicide such as flutriafol to reduce the risk of infection during tillering. Powdery mildew infection during tillering can reduce grain yield by up to 25%. Once powdery mildew is established in the canopy it is very difficult to control. In many situations the use of an in-furrow fungicide can delay the need for a powdery mildew spray until mid-stem-elongation. This may avoid a later spray during ear emergence.

If an in-furrow fungicide is not used, then growers should look to spray for powdery mildew as soon as they see it during tillering. This may be between mid-tillering and stem elongation. A follow up spray will often be required before flag leaf emergence to protect the flag-1 leaf. Fungicide options for

powdery mildew control include flutriafol, propiconazole, tebuconazole and triadimefon.

Potassium-deficient barley is more susceptible to powdery mildew infection (Jayasena and Brennan, 2007). The application of a potassium fertilizer to Vlamingh growing on potassium-deficient soil (Colwell K < 50 ppm) can reduce powdery mildew infection and increase grain yield. Muriate of potash is more effective at reducing powdery mildew infection than sulphate of potash.

In the management of spot-type net blotch, avoiding barley stubble will significantly reduce the likelihood of infection. Vlamingh should be grown on paddocks that have had at least a two-year break from barley, and not sown next to paddocks which had infected barley stubble. Should infection occur, a single spray between stem elongation and flag leaf emergence may often be enough to control the disease. In high-disease-risk scenarios it may be necessary to apply two sprays, such as at stem elongation, with a follow up spray 3 to 4 weeks later at flag leaf emergence. Fungicide options for spot-type net blotch control are azoxystrobin plus cyproconazole and propiconazole. In-furrow fungicides offer no protection against a stubble-borne disease like net blotch.

Growers in high leaf rust risk environments will need to look at a fungicide program for controlling barley leaf rust. Where an early outbreak occurs, an initial spray should be applied at the onset of the disease and a second application is often needed 3 to 4 weeks later. Fungicide options for barley leaf rust are azoxystrobin plus cyproconazole, propiconazole and propiconazole plus cyproconazole. In all other areas, the moderately susceptible rating of Vlamingh should be enough to hold off the development of leaf rust in the canopy.

Grain yield

Vlamingh is a high yielding cultivar in medium- and high-rainfall environments

Table 7. Grain yield of Vlamingh compared with Baudin, Gairdner, Hamelin and Stirling (as a percentage of Stirling) for different seasons, dates of seeding, Agzones and soils types. Analysis is a direct analysis with all five cultivars sown in all 263 trials.

Agzone ¹	Season	Baudin	Gairdner	Hamelin	Vlamingh	Stirling	Stirling (t/ha)	No. of observations
	2002	116%	114%	108%	115%	100%	1.56	34
	2003	109%	108%	105%	114%	100%	3.16	59
	2004	111%	105%	100%	110%	100%	2.30	62
	2005	111%	108%	103%	114%	100%	2.60	26
	2006	97%	92%	102%	100%	100%	2.05	56
	2007	105%	102%	103%	105%	100%	2.57	26
	Average	108%	105%	103%	110%	100%	2.40	263
	late May	112%	110%	104%	113%	100%	2.82	83
	early June	108%	106%	102%	109%	100%	2.56	85
	late June	103%	98%	104%	107%	100%	2.04	73
	early July	100%	84%	105%	102%	100%	1.41	22
Agzone 1	May	111%	116%	104%	123%	100%	2.94	7
	June	115%	106%	107%	116%	100%	2.76	6
Agzone 2	May	117%	115%	105%	118%	100%	2.94	26
	June	106%	100%	102%	108%	100%	2.23	58
Agzone 3	May	112%	114%	105%	110%	100%	3.11	7
	June	103%	103%	102%	105%	100%	2.88	23
Agzone 4	May	105%	104%	102%	103%	100%	2.40	10
	June	96%	90%	103%	98%	100%	1.77	13
Agzone 5	May	109%	106%	102%	106%	100%	2.31	19
	June	107%	107%	105%	110%	100%	2.01	38
Agzone 6	May	110%	105%	104%	112%	100%	3.36	14
	June	107%	107%	102%	112%	100%	2.76	20
Soil type								
	non-alkaline duplex soils	108%	104%	104%	109%	100%	2.45	106
	alkaline duplex soils	107%	106%	102%	108%	100%	2.43	54
	gravels	107%	104%	103%	111%	100%	2.51	56
	earths and sands	109%	105%	105%	110%	100%	2.12	47

¹ Agzones represent different regions for which cultivar performance is evaluated and recommendation made, see Figure 5. 2. Soil type was assessed according to Schoknecht (2002).

SOURCE: DAFWA agronomy and some CVT data 2002–2007.

(Tables 7 and 8). Its yield advantage over Stirling was influenced by season, sowing date and agricultural zone (Agzone), but was stable across soil types. The yield advantage is smallest in Agzone 4 (the low-rainfall regions of the eastern and northern wheat belt) (Tables 7 and 8) and there was no advantage with June sowing (Table 7). The yield advantage relative to Stirling also decreases with delays in seeding after late May, especially in Agzones 1, 2 and 3. The yield advantage of Vlamingh was lowest in the 2006, a season of low in-crop rainfall.

Across all seasons, Agzones, sowing dates and soil types, the grain yield of Vlamingh is

similar to Baudin and greater than Gairdner (Tables 7 and 8; Figure 5). Any advantage Vlamingh did have was the highest in Agzones 1, 4 and 5, and the least in Agzones 2, 3 and 6 (Table 8). This suggests that Vlamingh has advantages in environments which are more marginal for Baudin and Gairdner, or shorter-season environments. Vlamingh appears to have an advantage over Baudin in Agzone 1 (the high- and medium-rainfall zone of the northern wheatbelt), especially with May sowing (Table 7). Overall the yield of Vlamingh is similar to Gairdner when sown in May, but as seeding is delayed Baudin and Vlamingh become progressively higher

Table 8. Grain yield of other barley cultivars compared to Vlamingh (as a percentage of Vlamingh) grown in Western Australia for each of the six Agzones in Western Australia. Data only presented where there are 5 or more observations in an Agzone. Analysis is an indirect analysis with not all cultivars sown in all 335 trials.

Cultivar	Agzone 1	Agzone 2	Agzone 3	Agzone 4	Agzone 5	Agzone 6
Cultivars currently received as malting						
Baudin	93	101	97	98	98	97
Gairdner	96	100	98	96	96	99
Hamelin	88	97	91	96	92	90
Schooner	88	95	89	96	90	89
Stirling	87	95	88	96	92	87
Cultivars currently received as feed						
Barque	94	102	98	106	102	100
Buloke	101	105	103	107	104	105
Dash	–	103	101	107	107	109
Doolup	97	103	95	101	99	97
Fleet	–	–	104	–	109	109
Hannan	99	105	98	112	102	101
Hindmarsh	–	–	102	–	113	104
Lockyer	99	106	105	114	109	112
Maritime	–	–	95	–	97	99
Molloy	95	105	98	104	102	96
Mundah	97	104	94	103	98	96
Roe	99	109	102	108	104	102
Yarra	98	103	98	103	102	101
Vlamingh yield (t/ha)	2.83	2.46	3.05	1.51	2.34	2.76

For Agzone map, see Figure 5. SOURCE: DAFWA CVT data 1999-2006.

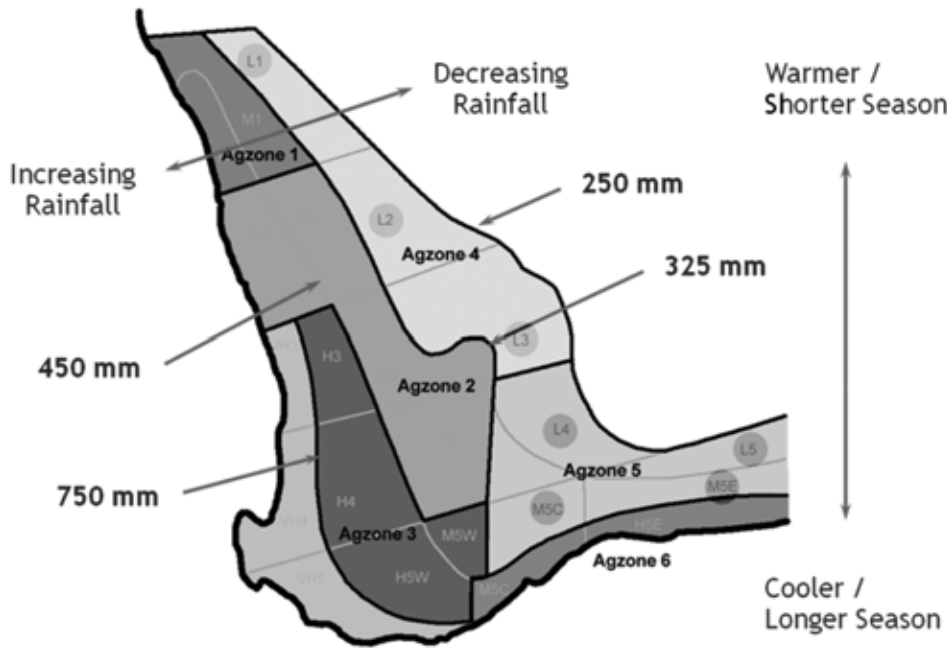


Figure 5. Agzone map for Western Australia showing differences in annual rainfall and growing season across the 6 defined Agzones. Barley is grown in winter (May to October) between the 29° and 35° S parallels (Agzone map courtesy of Jen Garlinge, DAFWA CVT).

yielding than Gairdner (Table 7). Gairdner is the least robust therefore of the three medium spring maturity cultivars.

Very few cultivars with a feed classification out yield Vlamingh by more than 10% (Table 8), and 10% is the minimum yield level differential required for growers to seriously consider switching from growing a malting cultivar like Vlamingh to a feed barley cultivar. If feed barley prices were to remain above AU\$ 300/t, then a yield difference of only 6–7% becomes the critical yield point to switch to feed barley. This is based on an assumption of a malt premium of AU\$ 40/t, only half the Vlamingh grown meeting the malting specifications, and the two crops having the same input costs. Even in this scenario, very few feed barley cultivars would give higher returns than Vlamingh, especially since Vlamingh has a grain plumpness similar to Stirling and a higher probability of malting than Baudin and

Gairdner (Table 15). Agzone 4 would be one environment where feed barley is likely to be a better choice than Vlamingh, due to the low probability of meeting malting requirements.

Grain plumpness

There has been a progressive genetic increase in grain plumpness of medium spring malting cultivars since the focus on breeding for malting quality started in the late 1980s in Western Australia. The introduction of cv. Franklin led to the development of Gairdner and then Baudin. Vlamingh is the first non-semi-dwarf malting cultivar developed for Western Australia within the medium spring maturity group. Vlamingh is a significant improvement in grain plumpness over Baudin and Gairdner (Tables 9, 10, 11 and 12).

Screenings levels in Vlamingh through the 2.5 mm slotted sieve, used on receipt of barley in Western Australia, are equivalent to Hamelin and slightly higher than Stirling

Table 9. Predicted screenings, grain protein and grain brightness for Baudin, Gairdner, Hamelin and Vlamingh relative to Stirling. Analysis is a direct analysis with all five cultivars sown in all 263 trials.

Stirling	Baudin	Gairdner	Hamelin	Vlamingh
Predicted average grain weight (mg, db)				
28	26.2	30.0	28.7	27.9
32	29.9	33.5	32.3	31.4
36	33.7	37.0	35.9	35.0
40	37.5	40.6	39.5	38.6
44	41.2	44.1	43.0	42.2
Predicted screenings (% < 2.5 mm)				
5	14.5	22.6	7.9	8.0
10	19.6	27.0	12.4	12.3
15	24.6	31.4	17.0	16.6
20	29.7	35.8	21.6	20.9
25	34.8	40.2	26.1	25.2
Predicted grain protein (% db)				
8	7.4	7.3	7.8	7.2
10	9.5	9.5	9.8	9.5
12	11.5	11.7	11.8	11.7
14	13.6	13.9	13.8	14.0
16	15.6	16.1	15.8	16.2
Predicted grain brightness (Minolta L)				
54	55.7	56.4	54.9	55.5
56	57.5	58.0	56.8	57.3
58	59.3	59.7	58.8	59.1
60	61.1	61.3	60.7	60.9
62	62.9	63.0	62.6	62.7

SOURCE: DAFWA agronomy and some CVT data 2002–2007. db = dry basis

Table 10. Change in screenings (% < 2.5mm) due to soil group, location and date sown.

	Soil group+ ¹			
	loamy earth		sandy duplex	
	Location: Calingiri — May–Oct rain = 235 mm			
Date sown	22-May-02	11-Jun-02	22-May-02	11-Jun-02
Baudin	27	23	7	10
Gairdner	30	37	20	16
Hamelin	10	23	9	7
Stirling	6	11	2	4
Vlamingh	12	13	3	6
Location: Brookton — May–Oct rain = 277 mm				
Date sown	5-Jun-02	27-Jun-02	5-Jun-02	27-Jun-02
Baudin	13	13	16	25
Gairdner	12	30	18	36
Hamelin	8	17	20	18
Stirling	5	15	9	20
Vlamingh	4	7	12	13
Location: Brookton — May–Oct rain = 263 mm				
Date sown	16-May-02	11-Jun-02	16-May-02	11-Jun-02
Baudin	13	13	16	25
Baudin	39	49	18	18
Gairdner	43	48	27	23
Hamelin	17	30	12	11
Stirling	11	30	5	12
Vlamingh	20	27	15	9

¹ Soil type was assessed according to Schoknecht (2002).

Table 11. Screenings (% <2.5 mm) of five medium spring barley cultivars sown at three plant densities in the presence of a low and medium level of ryegrass weed competition averaged across four locations (Beverley, Calingiri, Gibson and Katanning), but sown at two dates of seeding three weeks apart in 2005.

Date sown		Late May						
Weeds		7 ryegrass plants/m ² Barley density (plants/m ²)			117 ryegrass plants/m ² Barley density (plants/m ²)			
Cultivar	65	107	166	Average	65	103	161	Average
Baudin	5	8	8	7	9	9	12	10
Buloke	11	10	11	10	17	14	14	15
Flagship	8	9	9	9	14	13	12	13
Gairdner	11	12	17	14	16	19	25	20
Vlamingh	4	4	5	4	8	7	6	7
Average	8	9	10	9	13	12	14	13
Date sown		Late May						
Weeds		7 ryegrass plants/m ² Barley density (plants/m ²)			107 ryegrass plants/m ² Barley density (plants/m ²)			
Cultivar	60	98	158	Average	59	97	151	Average
Baudin	15	20	21	18	20	23	24	22
Buloke	24	25	25	25	26	28	28	27
Flagship	16	14	20	17	23	23	27	24
Gairdner	23	30	33	29	33	35	37	35
Vlamingh	12	10	13	12	15	16	17	16
Average	18	20	22	20	23	25	27	25

Table 12. Screenings (% <2.5 mm) of two barley cultivars with and without leaf disease control at three locations for four levels of fertilizer nitrogen application 6 weeks after sowing in 2006.

Baudin Vlamingh				
Location Calingiri — May–Oct. rain = 202 mm				
N applied (kg N/ha)	Nil fungicide	Plus fungicide	Nil fungicide	Plus fungicide
0	23	6	7	5
20	30	8	11	6
40	35	11	10	6
60	39	14	10	6
Location Beverley — May–Oct. rain = 188 mm				
N applied (kg N/ha)	Nil fungicide	Plus fungicide	Nil fungicide	Plus fungicide
0	48	33	11	9
20	57	44	18	15
40	67	54	24	25
60	77	62	31	31
Location Katanning — May–Oct. rain = 186 mm				
N applied (kg N/ha)	Nil fungicide	Plus fungicide	Nil fungicide	Plus fungicide
0	27	28	12	14
20	38	32	12	11
40	39	28	25	18
60	40	38	17	15

(Table 9). Around 15% more Gairdner grain is expected to pass through the 2.5 mm sieve than for Vlamingh when grown under the same management. In comparison, Baudin screenings are between Vlamingh and Gairdner, with around 6–10% more grain expected to pass through the 2.5 mm sieve than for Vlamingh. These differences in screenings are largely accounted for by differences in average grain weight (Table 9) and/or differences in grain shape (Figure 6; Table 3).

Whilst Stirling, Hamelin and Vlamingh have a similar grain shape (Figure 6; Table 3), the average grain weight of Vlamingh grains is 0.5 to 1.5 mg lighter than Stirling grains (Table 9), hence the slightly higher screenings. Screenings in Gairdner are higher because it has a narrow grain shape (Figure 6) despite having heavier grains than the other cultivars.

The benefit of this improved grain shape is greater stability of its screenings across soil types (Table 10), sowing dates (Tables 10 and 11), seeding rates (Table 11) and in response to applied nitrogen (Table 12).

Response to soil type and sowing date

Table 10 highlights research that compares the performance of cultivars on different soil groups within the same paddock. The comparisons presented show differences in the performance of five malting cultivars on two major soil groups differing in textural properties and water holding capacity at three different locations in the medium-rainfall areas of Western Australia. Within a paddock, the research plots (or soil groups) may be as little as 100 m apart, so the differences observed for a sowing date are more likely to be influenced by differences in soil-related properties than by differences in environment (i.e. rainfall). Soil type can have a big impact on cultivar selection for a paddock. Across the three locations in Table 10, Vlamingh was a more stable option (lower screenings) than Baudin and Gairdner as soil type changed in the paddock or if seeding was delayed. Despite the 7–10 day difference in flowering date between Vlamingh and Stirling, Vlamingh generally had higher screenings but they were within the malting specification

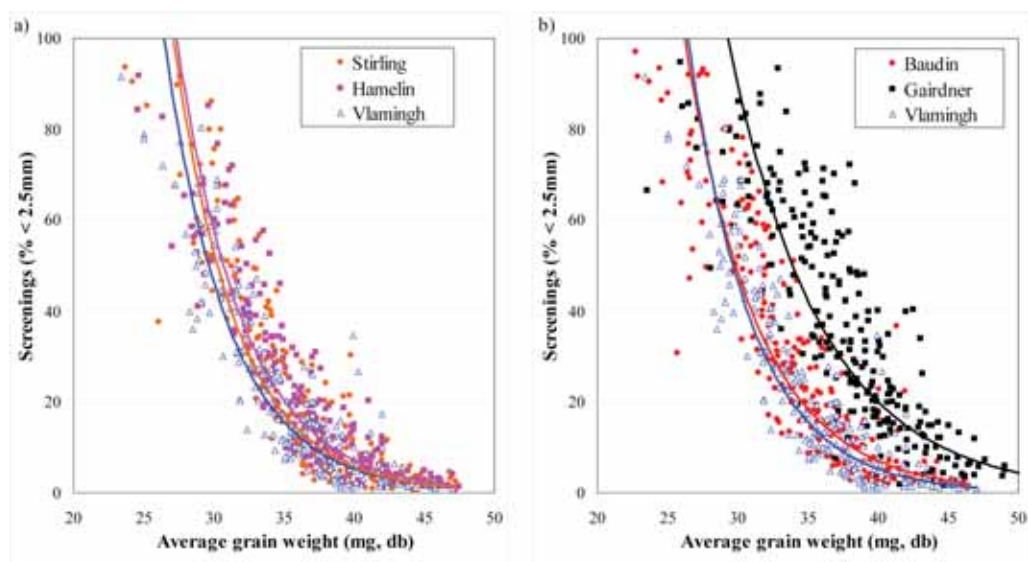


Figure 6. Differences in grain shape between (a) Hamelin, Stirling and Vlamingh; and (b) Baudin, Gairdner and Vlamingh.

window in as many situations as Stirling was.

Table 11 highlights another comparison of the impact of delayed seeding on screenings levels in five medium spring cultivars averaged across four locations in the medium- to high-rainfall environment. Whilst there was a doubling of screenings with a three-week delay in seeding, the screening levels in Vlamingh were lower than Baudin, which in turn were lower than Gairdner. This was compounded by the impact of competition with weeds (in this case rigid ryegrass, *Lolium rigidum* Gaud.). The impact of weed competition was to increase screenings in all cultivars. Vlamingh was again able to meet industry targets of a maximum of 20% screenings. Vlamingh as such will provide growers with greater confidence in being able to meet the 20% screenings limit for delivery as malting in medium-rainfall environments.

Response to seeding rate

The rounder grain shape of Vlamingh reduces the impact that increased seeding rate has on screening levels relative to Baudin and Gairdner barley. Gairdner is acknowledged

as being very sensitive to planting density, especially with delayed seeding (Paynter *et al.*, 1999). Across four locations in the medium- to high-rainfall environment, Gairdner screenings increased as the number of barley plants established increased (Table 11). This was in both the presence of a low level of weed competition (<20 ryegrass plant/m²) and a moderate level of weed competition (100–130 ryegrass plants per m²). In the same situation, screening levels in Vlamingh were unchanged as the number of barley plants sown increased, whilst there was a slight upward trend in Baudin.

Response to applied nitrogen

Over the last five seasons, the receival standards for grain protein have tightened, reflecting international market signals. The most obvious change has been the introduction of a minimum protein level for delivery as malting barley, and the progressive increase in the minimum protein level. Malting barley with grain protein below 9.5% is no longer segregated into malt barley stacks and no longer receives the malting premium.

Significant tonnages of Baudin and Gairdner are delivered each year with grain protein levels below 9.5%. In most cases this is because growers have not applied enough nitrogen to meet the yield potential of these cultivars. As Vlamingh becomes more widely sown in Western Australia there is a risk of low grain protein as growers may adopt the same cautious approach in growing it as they have with growing Baudin and Gairdner barley. As the new medium spring cultivars are higher yielding than the early spring cultivars, they require more nitrogen to meet their demand. There are many nitrogen decision aids available that can assist growers match yield potential with nitrogen fertilizer needs.

One of the risks in applying nitrogen to barley is the delivery of grain with screenings levels above 20%. Screenings in Baudin and Gairdner are both sensitive to applied nitrogen (Paynter, 2005). As nitrogen increases, screenings increase. In situations

where malt barley is likely to be achieved, the risk is managing the level of input required to maximize yield and not blow out screenings. Another risk for Gairdner is growing a tall crop and then having it lodge at harvest. This is also a risk for Vlamingh, but not for Baudin due to its short straw (Table 4).

Our research has shown that in management situations which produce barley between 9 and 11% protein, grain protein levels in Vlamingh will be similar to Baudin and Gairdner and lower than Stirling and Hamelin (Table 9). This is because Baudin, Gairdner and Vlamingh are higher yielding and the lower protein is related to dilution. Where grain protein levels are above 13%, usually due to low seasonal rainfall, the grain protein level of Vlamingh is similar to other cultivars because any yield advantage decreases under these situations.

Our research has also shown that there is a similar impact of nitrogen application on

Table 13. Premium payable for barley grain meeting malt barley receival specifications, premiums for delivery of grain within defined protein window, cultivar premiums and end-point royalty payable on delivery. Receival standards based on 2007-08 Cooperative Bulk Handling Receival specifications except maximum screenings limited to 20%.

Maximum screenings limited to 20 %					
1. Price premium over feed barley (AUS\$/t)					
Malting barley	AUS\$ 40				
<i>Shochu</i> (Stirling only)	AUS\$ 55				
2. Specifications for delivery as malting					
	Malt barley	<i>Shochu</i> barley	basis		
Minimum protein	9.5 %	9.5 %	dry		
Maximum protein	13.0 %	12.5 %	dry		
Maximum screenings	20 %	14 %	% < 2.5 mm		
Minimum brightness	56 L	59 %	Minolta 'L'		
3. Protein window for which a premium is payable					
Minimum protein	10.0 %				
Maximum protein	11.0 %				
4. Premium payable (AUS\$/t) if grain suitable for delivery as malting					
	Baudin	Gairdner	Hamelin	Vlamingh	Stirling
Protein inside protein window	AUS\$ 5.00	AUS\$ 2.50	AUS\$ 5.00	AUS\$ 5.00	AUS\$ 5.00
Cultivar premium	AUS\$ 7.00	AUS\$ 5.00	AUS\$ 7.00	AUS\$ 0.00	AUS\$ 0.00
5. End-point royalty premium (AUS\$/t) payable on delivery as malt or feed					
	Baudin	Gairdner	Hamelin	Vlamingh	Stirling
Delivery as malting	AUS\$ 3.00	AUS\$ 0.00	AUS\$ 3.00	AUS\$ 3.50	AUS\$ 0.00
Delivery as feed	AUS\$ 1.00	AUS\$ 0.00	AUS\$ 1.00	AUS\$ 1.50	AUS\$ 0.00

average grain weight of the three medium spring cultivars. Average grain weight of Baudin, Gairdner and Vlamingh decreases to a similar degree as nitrogen is increased. The impact of nitrogen on screenings is therefore related to differences in the grain shape of the three cultivars. Nitrogen application is less likely to push Vlamingh over the 20% receival limit than it would do with Baudin, as Vlamingh grain has a rounder grain shape (Tables 3 and 9; Figure 6). Table 12 has three

examples of differences in the responsiveness of Baudin and Vlamingh to applied nitrogen as measured by screenings.

Table 12 also shows that it can be critical to reduce the level of disease in the crop canopy using fungicides (i.e. at Calingiri), if the risk of high screenings is to be reduced when using nitrogen to meet yield and protein targets. The impact in Vlamingh is less than that observed for Baudin due to their differences in grain shape and susceptibility to leaf disease.

Table 14. Relative performance (AUS\$ /ha relative to Stirling) of Baudin, Gairdner, Hamelin and Vlamingh when grown under the same management as Stirling in state wide trials over six seasons (2002-2007).

Agzone	Season	Baudin	Gairdner	Hamelin	Vlamingh	No. of observations
	2002	43.36	21.01	19.64	45.92	34
	2003	41.96	- 2.05	29.66	80.54	59
	2004	26.29	- 6.02	- 0.48	29.05	62
	2005	83.71	52.73	38.91	75.30	26
	2006	- 18.91	- 40.08	9.28	- 7.31	56
	2007	5.94	- 26.14	11.39	20.49	26
	AVERAGE	26.05	- 5.07	16.03	38.77	263
	Season	Baudin	Gairdner	Hamelin	Vlamingh	observations No. of
	late May	57.30	25.57	30.58	69.34	83
	early June	31.95	2.91	9.69	35.71	85
	late June	- 7.12	- 34.06	7.82	18.96	73
	early July	- 4.57	- 55.28	12.86	0.94	22
Agzone	Season	Baudin	Gairdner	Hamelin	Vlamingh	observations No. of
Agzone 1	May	71.97	104.81	54.39	145.35	7
	June	91.30	47.60	72.47	99.10	6
Agzone 2	May	67.62	3.31	11.90	95.37	26
	June	- 4.92	- 44.72	- 3.94	21.36	58
Agzone 3	May	74.77	63.76	49.35	57.98	7
	June	30.48	14.38	- 5.54	10.44	23
Agzone 4	May	- 7.71	- 48.94	- 5.47	- 1.07	10
	June	- 28.67	- 47.69	10.09	- 12.10	13
Agzone 5	May	72.27	20.38	44.41	43.93	19
	June	35.93	9.89	27.08	38.17	38
Agzone 6	May	48.18	68.43	50.98	73.48	14
	June	12.01	- 0.87	7.77	52.64	20
	Season	Baudin	Gairdner	Hamelin	Vlamingh	observations No. of
Non-alkaline duplex soils	May	69.81	44.01	17.81	62.79	32
	June	28.72	- 12.61	14.27	36.88	62
Alkaline duplex soils	May	73.43	13.02	48.63	69.15	18
	June	11.16	- 6.63	4.65	28.99	34
Gravels	May	38.89	- 14.01	28.99	71.54	17
	June	- 2.86	- 6.59	0.46	20.95	33
Earths and sands	May	33.69	44.84	37.53	80.31	16
	June	4.50	- 34.97	11.60	15.72	29

¹ Agzones represent different regions for which cultivar performance is evaluated and recommendation made. See Figure 5. 2. Soil type was assessed according to Schoknecht (2002).

Responsiveness to weed competition and herbicides

Vlamingh has good early vigor, which helps with the crop's competitiveness with weeds, although Vlamingh does have a more open canopy before stem elongation than cultivars like Baudin and Gairdner (Paynter and Hills, 2007, 2009). Weed competition trials have shown that Vlamingh was as competitive against rigid ryegrass (*Lolium rigidum* Gaud.) as three cultivars of similar maturity, Baudin, Gairdner and Buloke (Paynter and Hills, 2009). Whilst the effect of weed competition on the yield of Vlamingh was similar to other cultivars, the number of ryegrass weed seeds set under Vlamingh was higher than under

Baudin, but similar to Gairdner (Paynter and Hills, 2007, 2009). The reason for that observation is not clear, but may have been related to the lower plant establishment of Vlamingh across sites. Poorer canopy closure before stem elongation may have also been a contributing factor, but plant height of Vlamingh was superior to cultivars like Baudin. Tiller number in Vlamingh was similar to Gairdner.

Baudin and Hamelin were found to be less competitive against ryegrass at plants densities below 90 plants/m² than they were above 90 plants/m² (Paynter and Hills, 2009). The competitiveness of Vlamingh was not altered by seeding rate in that study.

Table 15. Proportion of grain samples of Baudin, Gairdner, Hamelin, Vlamingh and Stirling meeting malting barley receival standards and of Stirling meeting *shochu* barley receival standards when grown under the same management in state-wide trials over six seasons (2002–2007).

Season	Baudin	Gairdner	Hamelin	Vlamingh	Stirling as malting	Stirling as <i>shochu</i>
2002	29%	18%	38%	44%	41%	29%
2003	47%	22%	59%	61%	64%	25%
2004	27%	21%	48%	40%	48%	21%
2005	58%	46%	58%	46%	46%	12%
2006	14%	9%	21%	18%	20%	7%
2007	23%	12%	38%	42%	38%	23%
Average	32%	20%	44%	41%	44%	19%
Sowing date						
late May	39%	20%	54%	52%	51%	22%
early June	44%	32%	55%	49%	54%	26%
late June	18%	11%	27%	30%	33%	15%
early July	9%	0%	14%	9%	14%	0%

Table 16. Average premium (AUS\$/t) over feed barley for Baudin, Gairdner, Hamelin, Vlamingh and Stirling meeting malting barley receival standards and for Stirling meeting *shochu* barley receival standards when grown under the same management in state-wide trials over six seasons (2002–2007). The maximum premium achievable (AUS\$/t) assumes 100% of the grain meets specification and is based on data in Table 13.

Season	Baudin	Gairdner	Hamelin	Vlamingh	Stirling as malting	Stirling as <i>shochu</i>
2002	13.41	8.31	16.94	16.74	16.47	20.88
2003	21.80	10.21	27.05	23.47	25.76	29.58
2004	12.06	9.68	21.90	15.03	19.35	22.50
2005	26.50	21.35	26.12	17.38	18.46	20.19
2006	5.61	4.06	8.91	5.38	7.86	8.93
2007	9.96	5.29	16.50	15.54	15.38	18.85
Average	14.27	9.14	19.53	15.37	17.49	20.40
Max. premium achievable	49.00	47.50	49.00	41.50	40.00	55.00

However, sowing Vlamingh at the suggested plant density of 120 to 150 plants/m² can increase grain yield potential and reduce the number of weed seeds set in the crop. This is equivalent to a sowing rate of 75–85 kg/ha estimated from an 80% emergence with a seed weight averaging 40 to 45 mg. Vlamingh has a coleoptile length around 70 mm (Table 4), but should be sown at 2–3 cm depth to maximize emergence and competitiveness with weeds. This is especially important as Vlamingh will most likely be sown with chemicals that reduce coleoptile length, such as foliar fungicide seed dressings and the herbicide trifluralin.

The weed competitiveness and grain yield of Vlamingh is reduced when sown at a row spacing above 30 cm (Paynter and Hills, 2007). The development of farming systems for barley that include cultivars like Baudin, Gairdner and Vlamingh based on a row spacing wider than 30 cm are likely to be associated with an increase in weed seed set unless cultural practices that manage weeds inter-row are implemented.

Despite its competitive nature, competition with weeds does reduce the grain yield and increase the screenings of Vlamingh. As few as 100–130 ryegrass plants/m² can reduce grain yield by 20% (Paynter and Hills, 2007, 2009). Screenings increased from 4% to 7% in late-May-sown Vlamingh, and from 12% to 16% in mid-June-sown Vlamingh when the number of ryegrass weeds established increased from <20 plants/m² to between 100 and 130 ryegrass plants/m² across four trials sown in 2005 (Table 11). It is important that Vlamingh crops are sown in paddocks with a low weed burden to give Vlamingh crops every possible opportunity to return high yields and grain that meets receival specifications for malt.

Vlamingh appears tolerant to most of the common herbicides registered for barley when used as per label instructions. Limited testing has seen some evidence of yield reductions occurring when pinoxaden/cloquintocet-

methyl (i.e. Axial) and metsulfuron methyl (i.e. Ally) have been used (Harmohinder Dhammu, DAFWA CVT, pers. comm.).

Where will Vlamingh be grown in Western Australia?

Research trials have shown that Vlamingh is competitive with Baudin and Gairdner for grain yield but has a rounder grain shape. It is also higher yielding than Stirling and Hamelin and has similar grain plumpness. The question is what benefit does this give barley growers in Western Australia? An economic analysis was conducted using data collected from 263 small plot research trials conducted over six seasons from 2002 to 2007, with 75% of the datasets derived from agronomic research trials (usually two or more dates of seeding) and the other 25% from crop cultivar evaluation trials (only one date of seeding), where grain quality data was collected. Grain quality is not routinely measured in crop cultivar evaluation trials, which limit their usefulness for this type of analysis.

Nearly 80% of the small plot trials were conducted in rainfall regions where the average annual rainfall was > 325 mm. The proportion of observations in each Agzone was: Agzone 1 – 5%; Agzone 2 – 35%; Agzone 3 – 13%; Agzone 4 – 10%; Agzone 5 – 24%; and Agzone 6 – 13%. The breakdown across rainfall zones is equivalent to 30% in high rainfall, 50% in medium rainfall and 20% in low rainfall.

Soil types at each location were grouped into four main groups according to major textural properties, after Schoknecht (2002), and the percentage of observations across each of these groups was: non-alkaline sandy and loamy duplex soils – 40%; alkaline sandy and loamy duplex soils – 21%; ironstone gravelly soils – 21%; and earths and deep sands – 18%.

There were significant climatic contrasts between the six seasons in which the small plot trials were conducted. Rainfall patterns recorded in 2002, 2004 and 2006 were below average in most regions of Western Australia, with low spring rainfall a feature of the 2004 season. The rainfall patterns in 2003, 2005 and 2007 were closer to average in most regions of Western Australia. The proportion of observations in each year was: 2002 – 13%; 2003 – 22%; 2004 – 24%; 2005 – 10%; 2006 – 21%; and 2007 – 10%. Trials planted in May formed 32% of the dataset, June 60%, whilst July sowing data formed 8%.

Barley grain received by Cooperative Bulk Handling is classified as malting or feed depending on cultivar, physical quality (hectolitre weight, moisture content, screenings, grain protein, grain brightness, and mould) and level or presence of contaminants. For this analysis, all cultivars compared could be classified as malting if they met the malt barley receival standards. Only three physical quality traits (screenings, grain protein and grain brightness) were used to calculate net farmgate price. The standards applying to the 2007/08 barley receival standards was used as a basis for the economic analysis, except that screenings were limited to 20%, where the 2007/08 standards allowed up to 25% due to the poor seasonal conditions. The maximum grade payable for Baudin, Gairdner, Hamelin and Vlamingh was “malt” and the maximum grade payable for Stirling was “*shochu*”. The assumptions used in the economic analysis are listed in Table 13.

The economic analysis clearly shows that Vlamingh has the potential to be a more profitable option than growing Baudin, Gairdner, Hamelin and Stirling in medium- and high-rainfall areas of Western Australia (Table 14). Vlamingh had higher returns in almost all seasons except 2006, was higher in Agzones 1, 2, 3, 5 and 6, and was higher the earlier the crop could be sown. This was despite the fact that Stirling could be

received as *shochu*, which it did in 19% of observations. Vlamingh had advantages over other cultivars on all soil types despite showing symptoms of boron toxicity on soils where the sub-soil pH >6.5 in CaCl₂. This demonstrates no clear association between boron symptoms and performance.

Across the six seasons, the net returns from growing Vlamingh were AUS\$ 39/ha higher than Stirling, AUS\$ 23/ha more than Hamelin, AUS\$ 13/ha more than Baudin and AUS\$ 44/ha more than Gairdner. The risk to growers in adopting Vlamingh as their medium spring maturity barley is lower than with Baudin and Gairdner. This is clearly evident with the change in relative returns due to sowing date. Where seeding could not occur until late June (as may have been the case in the lower rainfall years of 2002, 2004 and 2006) the sowing of Baudin and Gairdner would have lost growers money relative to switching to sowing Stirling instead (Table 14). The net returns from Vlamingh were still superior to Stirling.

The returns from growing Vlamingh were higher than Baudin and Gairdner because Vlamingh grain met the receival standards for malting barley more often (Table 15). Across the six seasons of trials Vlamingh met malt barley receival standards in 41% of cases, similar to Stirling and Hamelin, but higher than Baudin at 32% and Gairdner at 20%.

The median screenings of Vlamingh over the 263 comparisons was 14.8% compared to Stirling 11.8%, Hamelin 16.4%, Baudin 23.3% and Gairdner 33.5%. Some of the situations where Vlamingh may have been discounted for high screenings were offset by lower grain protein and improved grain brightness. When compared at the same planting and harvesting dates, grain of Vlamingh was similar in brightness to Baudin, slightly darker than Gairdner and brighter than Stirling and Hamelin (Table 9). Differences in grain brightness between the cultivars are more evident in situations

where dockage for grain brightness occurs on receival. Malting grain with a grain brightness below 56 L is paid as feed and if it falls below 51 L it cannot be received. So Vlamingh can be expected to extend the range of situations in which malt barley can be received as malting where Stirling would otherwise be received as feed.

The likelihood of receival of malting for Vlamingh was highest with late May and early June sowing, and decreased as sowing was delayed into late June and July (Table 15). It was higher than Baudin at all sowing dates except for July. It was always higher than Gairdner.

Based on the premiums offered in Table 13 and the proportion of trials that met receival standards in Table 15, Table 16 shows the average premium (AUS\$/t) achieved by each of the five cultivars in relation to that being offered. Across the six years of trials, the average premium achieved by all cultivars ranged from 20% to 45% of what was available. In our research trials, the average premium achieved by Vlamingh was AUS\$ 15.37/t when the malt premium available was AUS\$ 41.50/t (Table 16). With May sowing, the premium was AUS\$ 19.81/t, AUS\$ 18.63/t for early June sowing, AUS\$ 10.57/t for late June, and AUS\$ 1.95 for July sowing.

The malt premium of Vlamingh was similar to Baudin but lower than Stirling and Hamelin. It was, however, higher than of Gairdner. So despite not achieving a higher premium than cultivars like Baudin or Hamelin, it had higher returns per hectare because of its higher grain yield potential in many situations (Tables 7 and 8).

Conclusions

Vlamingh is the newest medium spring malting barley in Western Australia and has favorable agronomic attributes relative to the two other medium spring cultivars received as malting – Baudin and Gairdner (Tables 4, 5, 7 to 12). Unlike Baudin and Gairdner, there is currently no international market

demand for Vlamingh grain. This is because very few markets outside Australia have done commercial brewing with the cultivar. The first shipments of Vlamingh grain would be received by international customers in the 2008 season.

Overall, micro-malting results indicate the malting features of Vlamingh are between that of Gairdner and Baudin (Table 1; Figure 1). It is expected that Vlamingh will suit international markets where lower levels of starch adjuncts are used in the brewing process. This may allow Western Australian barley growers to enter new or different markets than those currently serviced. As a soft-grained cultivar it may also be suitable for the *shochu* market in Japan.

During this period of international market development only a small tonnage of Vlamingh grain will be required. As such, Vlamingh will only be received at selected receival points for the 2008/09 harvest in Western Australia. The number of available receival points is likely then to increase for the 2009/10 harvest.

Growers wishing to grow Vlamingh for the malting market will need to consult Farmnote 289/2008 (Russell *et al.*, 2008). Farmnote 289 contains guidelines on how to grow Vlamingh to meet malt barley receival standards. By following the suggested management protocol, it should ensure that we maximize the quality of grain made available to customers.

Should Vlamingh gain international market acceptance, then research shows it has the potential to be competitive against current malting varieties. In this case, subject to any incentives that favor the production of high extract, high diastase cultivars like Baudin, Vlamingh has the potential to replace Baudin and Gairdner in the areas where they are currently grown and become more widely sown than them. Vlamingh is suggested for sowing in medium- and high-rainfall areas. It is not suggested for normal sowing opportunities in low-rainfall areas due to the risk of running out of soil moisture to fill its grain.



Figure 7 shows the adaptation map for Vlamingh relative to Baudin and Gairdner in Western Australia and clearly shows how the genetic improvement in grain plumpness is resulting in an increasing area of suitability for medium spring cultivars.

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Response to selection for increased malt extract

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Abstract

The level of malt extract remains a critical parameter for the success of new varieties in the malting and brewing industry. Physical and biochemical analyses have identified husk thickness as a key factor influencing malt extract. The potential negative impacts of thin husk, including propensity for skinning damage and pre-harvest sprouting, are outlined in the context of breeding strategies to minimize these risks. Genetic analysis of malt extract within the Haruna nijo \times Galleon mapping population confirmed the association of both traits with the short arm of chromosome 2H, and highlighted the ability of NIRS to detect variation conferred by this locus. Subsequent research focused on the development of NIRS calibrations for husk content and the validation of molecular markers linked to the 2HS locus. Crossing schemes to introgress the thin husk trait across a broad range of germplasm have been executed in conjunction with selection strategies using MAS for allele enrichment in segregating populations and whole grain NIRS screening of early generation fixed lines. The response to genotypic and phenotypic selection for increased malt extract within the University of Adelaide Barley Program is examined over the last ten seasons (1997–2006). The results of the study are discussed in terms of the rate of genetic gain achieved within the breeding program, and the impact of this approach in improving outcomes from wide crossing strategies.

Introduction

Malt extract is the key economic parameter for maltsters and brewers in all markets. High levels of malt extract are therefore desired by the malting and brewing industries. Since the success of Schooner in the mid 1980s, mainstream Australian varieties have failed to match the malting quality standards set by European and Canadian varieties. Subsequently, by the early 1990s, Australia's share of the important Japanese market, for example, had fallen from 33% to 19% (Powell, 1997). This loss in market share can be largely attributed to the lower levels of malt extract in Australian varieties (Roumeliotis *et al.*, 1999).

The Australian barley industry responded to this by implementing a major national research program on the genetics of quality, whilst simultaneously investing in infrastructure for quality evaluation to support an increased focus on breeding for malting quality.

This paper reports on the response to selection for increased malt extract by addressing the impact of improved genetic knowledge, and specifically the implementation of Marker Assisted Selection (MAS) and Near Infrared Spectroscopy (NIRS) selection strategies on breeding for quality, based on a long-term analysis of malt extract. The response to genotypic and phenotypic selection for increased malt extract within the University of Adelaide (UA) Barley Program is examined over

the last ten seasons (1997–2006). The genetic gain achieved for malt extract through the combination of MAS, NIRS and pragmatic breeding is also analyzed. In addition, opportunities for future gains in malt extract are addressed, including the effective deployment of very thin husk, which may potentially offer a pathway to higher levels of malt extract. The potential of the next generation of elite malting quality is discussed through an introduction to the quality profile of the UA Barley Program line WI4262.

Genetic analysis for malt extract

Within the UA Barley Program, molecular mapping efforts have focused on identifying the genetic basis for malting quality within a range of international germplasm. The four loci that have been the most extensively characterized are located on chromosome 1H (Alexis derived), 2HS and 2HL (both Haruna nijo derived) and 5H (Harrington derived). These four now form the basis of routine MAS within the UA Barley Program. The relationship between malt extract and various factors contributing to malt extract was investigated in the Galleon \times Haruna nijo mapping population. The interval map for barley chromosome 2H for Galleon \times Haruna nijo shows that both malt extract and husk content are influenced by the same region, with Haruna nijo alleles conferring high malt extract and low husk content (Figure 1.) This work and subsequent validation studies confirm that the Haruna nijo 2HS locus increases malt extract by conferring decreased husk content.

Impact of marker assisted selection on malt extract quality in Australian germplasm

Routine MAS within the UA Barley Program commenced in 1995, with disease resistance and abiotic stress tolerance being the

predominant target traits at that time. Since then the number of traits under selection and the scale of the Program has increased dramatically, with a total of 45 000 assays across 18 traits completed in 2006. Routine marker screening for malt extract began in 2000. Table 1 shows the number of marker screens carried out between 2000 and 2006, demonstrating a rapid and significant increase.

To determine the impact of MAS on malt extract in Australian germplasm, NIRS whole grain analysis data from F_3 -derived F_4 lines grown in Stage 0 double-row trials was examined. This included all malting-quality germplasm NIRS scanned between 1997 and 2006, with the number of lines ranging in each year from 2 561 to 5 977, with a total of 38 209 lines. The control variety Schooner was included in the control grid each year and this was used as the comparative basis.

Figure 2 shows the frequency of lines in Stage 0 with malt extract superior to Schooner. The plot illustrates that there has been a significant increase in the number of lines with high malt extract between 1997 and 2006. In 1997, only 21% of lines had higher malt extract, but the frequency had increased to 81% in 2006.

The relationship between the intensity of MAS and the malt extract levels across the germplasm base is shown in Figure 3. This plot illustrates the frequency of lines in Stage 0 with malt extract superior to Schooner, overlaid with the corresponding marker screens for malt extract, which were conducted on the preceding complex-cross F_1

Table 1. UA Barley Program marker screens for malt extract between 2000 and 2006.

Year	Number of marker screens for malt extract
2000	889
2001	1395
2002	1994
2003	2617
2004	2411
2005	3772
2006	9347

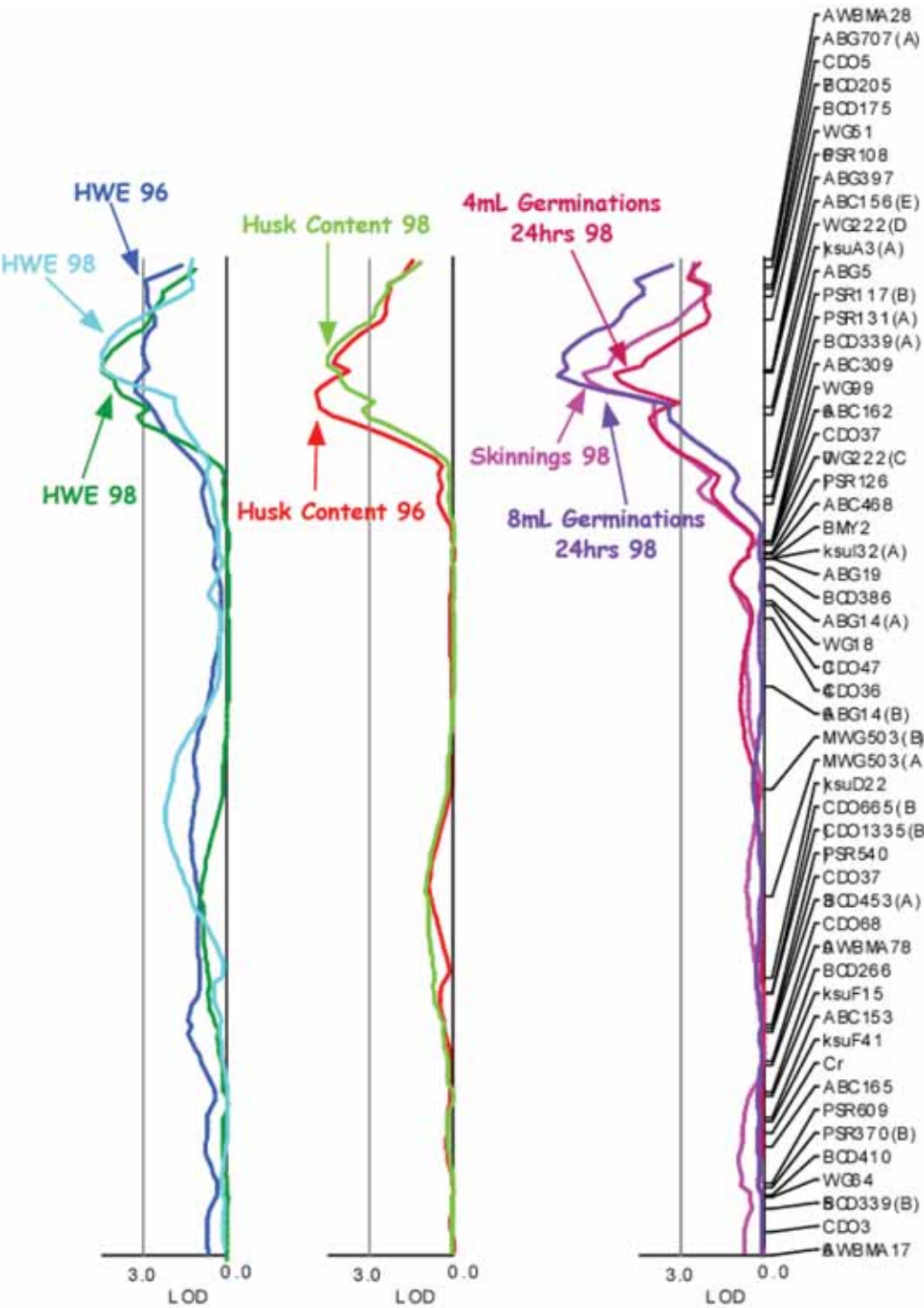


Figure 1. Interval map for barley chromosome 2H, for the Galleon \times Haruna nijo mapping population (Collins *et al.*, 1999).

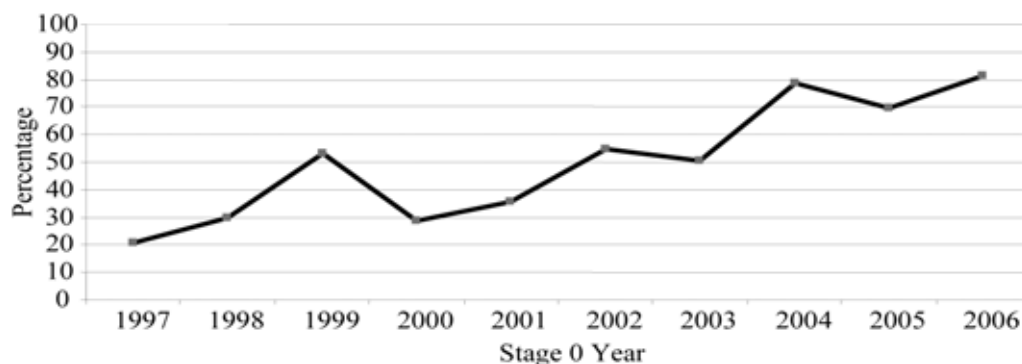


Figure 2. The frequency of lines in UA Barley Program Stage 0 trials between 1997 and 2006 with malt extract superior to Schooner.

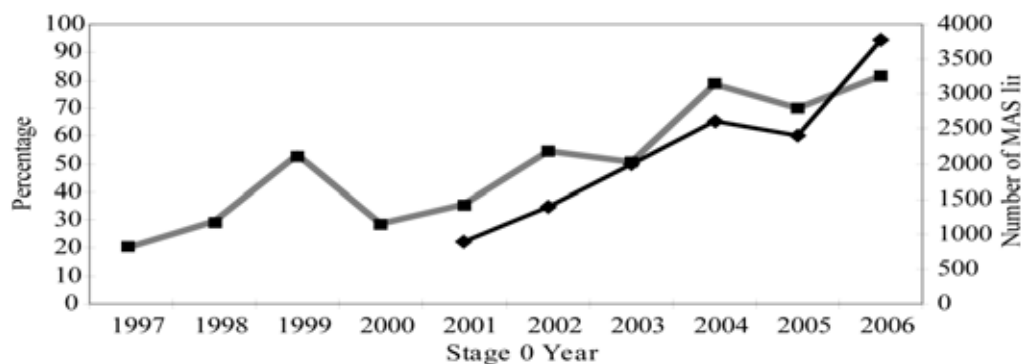


Figure 3. The frequency of lines in UA Barley Program Stage 0 trials between 1997 and 2006 with malt extract superior to Schooner, overlaid with the corresponding marker screens for malt extract, which were conducted on the preceding complex-cross F_1 populations.

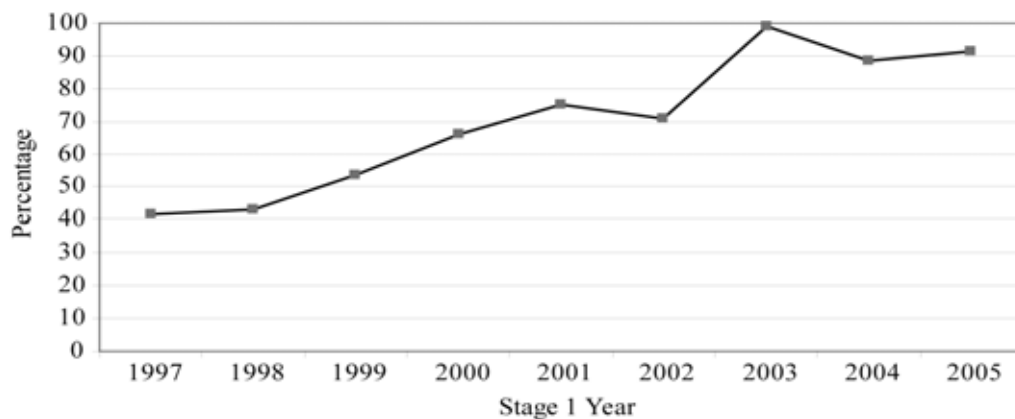


Figure 4. The frequency of lines in UA Barley Program Stage 1 trials between 1997 and 2005 with malt extract superior to Schooner.

populations. As marker screen numbers have increased, so has the frequency of lines with higher malt extract. The close relationship between the level of marker screening and the percentage of lines with malt extract greater than Schooner illustrates the major impact of MAS in selecting for improved levels of malt extract.

Impact of Near Infrared Spectroscopy on malt extract evaluation in Australian germplasm

The second key strategy used to select for increased malt quality is NIRS. This technology is used to predict malting quality in early generation material. Samples are scanned and the results are used as a basis for selecting lines for promotion to Stage 1 yield trials.

NIRS uses both visible and near-infrared light which is reflected in the range 400 nm–2500 nm at 2 nm intervals. The reflected light at known wavelengths indicates the relative composition of the barley. It is a high throughput, non destructive technology. ‘In-house’ calibrations have been developed and used in the UA Barley Program since 1995, with the database now containing over 3 000 samples, and 16 calibrations have been developed since 1995, with both whole-grain and whole-malt applications.

In order to maintain robust calibrations, a representative subset of the season’s samples is added to the database on an annual basis to account for new germplasm and for differing seasonal conditions. In addition, this group of samples acts as a validation set. For malt extract, the correlations between the laboratory results and the NIRS are very high; $R^2 = 0.925$ for whole grain and $R^2 = 0.953$ for whole malt, providing high confidence in the predictive power of the technology.

To assess the impact of NIRS on malt extract in Australian germplasm, malt quality analyses of F_3 -derived F_5 lines from Stage

1 yield trials were examined. This included all malting quality germplasm, micro-malted and analyzed between 1997 and 2005, with the number of lines ranging in each year from 196 to 391, with a total of 2 583 lines. The control variety Schooner was included in the control grid each year, and this was used as the comparative basis.

Figure 4 illustrates the frequency of lines in Stage 1 with malt extract superior to Schooner. The plot shows that there has been a significant increase in the number of lines with higher malt extract between 1997 and 2005. In 1997, only 42% of lines had higher malt extract, but this had increased to 91% in 2005. Importantly, this data validates the selection of lines for promotion using whole-grain NIRS.

The relative effectiveness of NIRS selection across each season is shown in Figure 5. The frequency of lines in Stage 1 trials with higher malt extract than Schooner is overlaid with the corresponding frequency of lines in Stage 0 with malt extract superior to Schooner. These samples had been NIRS scanned the previous year as F_3 -derived F_4 lines. NIRS selection has increased the frequency of lines with high malt extract in all years, with the increase ranging between 16 and 161%, depending on the season.

There are, however, some limitations in using NIRS selection, particularly in seasons resulting in poor physical grain quality. In drought-affected seasons, as was the case in 1999 and 2004, NIRS selection was shown to be less effective, with a lower frequency of lines with higher malt extract than Schooner evident in 2000 and 2005 when samples were micro-malted and analyzed in Stage 1 trials.

Validation of combined MAS, NIRS and traditional selection for malt extract in Australian germplasm

To validate the combined effects of MAS, NIRS and traditional selection for malt

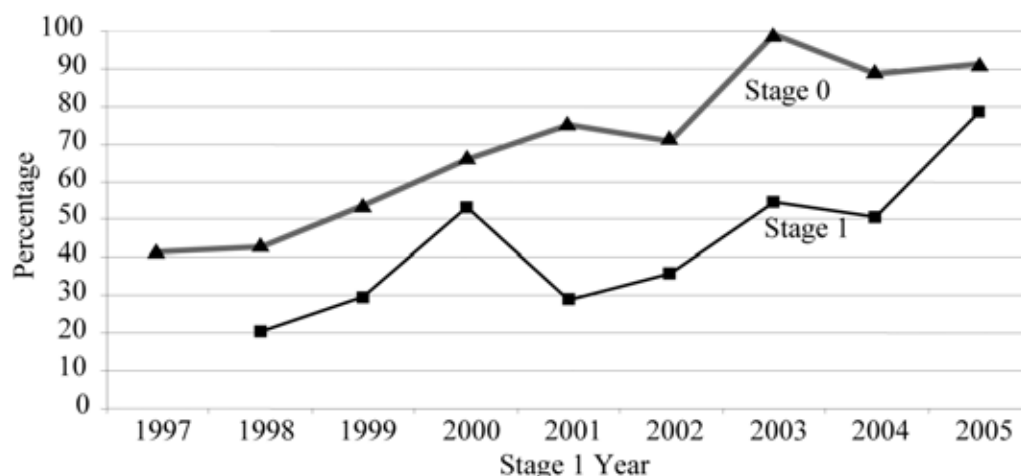


Figure 5. The frequency of lines in the UA Barley Program Stage 1 trials with higher malt extract than Schooner, overlaid with the corresponding frequency of lines in Stage 0 with malt extract superior to Schooner.

extract, the malt quality analysis of advanced lines from Stage 3 replicated yield plots at multiple sites was examined. All malting quality germplasm, micro-malted and analyzed between 1997 and 2005, was assessed, a total of 326 lines. This analysis was based on 22 sites \times season combinations and all analyses were carried out using small-scale EBC standard methods (Roumeliotis and Tansing, 2003). The control variety Schooner was included each year and this was used as the comparative basis.

Figure 6 illustrates the frequency of lines in the UA Barley Program Stage 3 trials with malt extract superior to Schooner between 1997 and 2005. The period 1997 to 1999 is the product of traditional selection alone, with the frequency of lines exhibiting acceptable quality oscillating between 11 and 39%. In addition, these lines were equally variable in their agronomic performance.

The combined impact of MAS and NIRS selection strategies can be measured from 2000 onwards, with the frequency of lines with higher malt extract rising from 15% in 2000 to 96% in 2005. It is also significant to acknowledge that this improvement in malt

quality has been achieved with simultaneous improvements in grain yield and disease resistance.

Genetic gain achieved for malt extract in elite Australian germplasm through the combination of MAS, NIRS and pragmatic breeding

The actual mean malt extract increase in the UA Barley Program, Stage 3 trials is shown in Figure 7. In 2000, from when the impact of combined MAS and NIRS selection strategies can be measured, the mean extract genetic gain was only 0.16%. Since then, there has been a significant increase, with results showing that the mean malt extract levels increased by 2.5% in 2005.

MAS, NIRS and sustained pragmatic selection pressure has increased the frequency of lines with high malt extract across the entire UA Barley Program, and the absolute extract levels in advanced lines have also increased significantly.

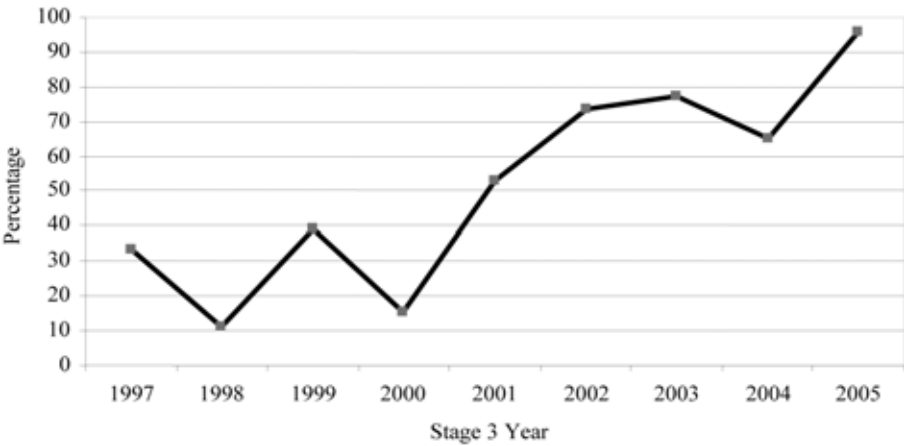


Figure 6. The frequency of lines in the UA Barley Program Stage 3 trials with malt extract superior to Schooner between 1997 and 2005.

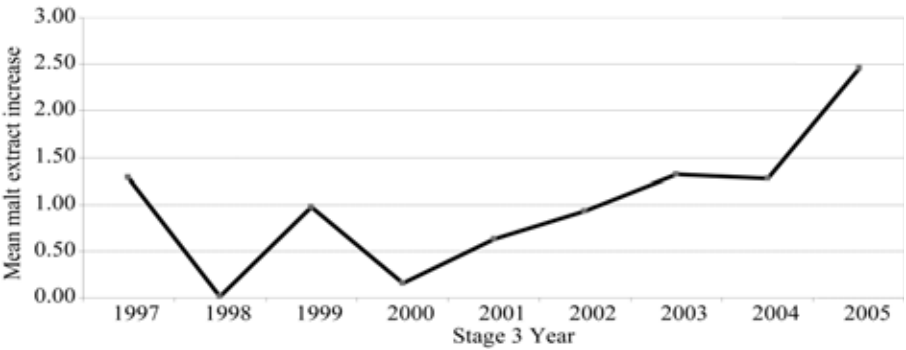


Figure 7. The mean malt extract increase in the UA Barley Program Stage 3 advanced trials, between 1997 and 2005.

Opportunities for future gains in malt extract

There are a number of correlated responses to the selection for increased malt extract levels. The data from this study suggests that grain protein levels are trending lower, but this is not a consistent trend and the results are for the most part inconclusive. In addition, although the data is limited, this study suggests that husk content is trending lower, although the magnitude of the decrease in husk is quite modest.

Of these factors, lower husk content potentially offers a pathway to higher levels of malt extract. The understanding of the genetic

control of husk content has been relatively well characterized, with research showing a strong relationship between high malt extract and thin husk. However, there are a number of potential problems associated with thin husk. As previously reported in Roumeliotis *et al.* (1999), these include an increased tendency for weather damage and pre-harvest sprouting, an increased likelihood of embryo damage and skinning during harvest and subsequent grain handling, and over-modification during the malting process. Husk also plays a part in forming the filter bed during lautering, and low levels may impact on the brewing process. Most of these issues relate to skinning damage

and are amplified by poor husk adherence.

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In a previous study by Roumeliotis *et al.* (2001), 103 lines derived from Dhow within 2 crosses were classified by their husk adherence (excellent, good or poor). In addition, these lines were analyzed for percentage skinning, husk content and malt extract. The results in Table 2 show that although the selections characterized by poor husk adherence had on average 15% higher skinning and 0.8% lower husk content, they only added 0.6% to malt

extract levels. Most importantly, the highest malt extract levels were actually achieved by those lines characterized by good and excellent husk adherence. The key finding is that husk adherence segregates independently, with the results demonstrating that it is possible to breed and select for lines with low husk content and good hull adherence to achieve very high malt extract levels.

Deployment of the thin husk trait within the UA Barley Program has involved a deliberate and systematic crossing and selection approach to further improve malt extract levels. MAS is used to combine the chromosome 2H QTL along with other loci. In 2002, an NIRS calibration was developed to measure husk content. The correlation between the laboratory reference EBC method (EBC, 1998) and the NIRS is $R^2 = 0.829$. All lines NIRS scanned in Stage 0 double-row trials are assessed for husk content. NIRS operators also visually inspect samples for skinning damage. In addition, plot harvester set up is constantly monitored and adjusted using Schooner as the control sample to keep skinning risk constant. Utilizing this coordinated approach, the aim is to increase malt extract levels through exploiting thin husk, without increasing the risk of skinning damage.

The impact of this approach and the initial response to selection for thinner husk can be seen in Figure 8. The plot illustrates absolute mean malt extract in UA Barley Program Stage 0 double-row trials overlaid with absolute husk content, compared with Schooner, between 1997 and 2006. Absolute malt extract levels have risen from 0.92% lower than Schooner in 1997 to 1.18% higher in 2006. Simultaneously, husk content levels have decreased by 0.3% since 2003, and have remained constant for

Table 2. The effect of husk adherence on skinning, husk content and malt extract in a study of 103 selections from Dhow crosses.

Husk adherence	Skinning		Husk Content		Malt Extract		N
	Mean %	Range	Mean %	Range	Mean %	Range	
Excellent	8	2–17	9.76	7.95–11.70	78.2	75.5–81.0	24
Good	13	5–32	9.41	7.24–11.75	78.3	74.8–80.8	55
Poor	23	4–53	8.94	6.35–10.27	78.8	77.1–80.5	24
LSD 5%	3.5		0.47		NS		

NS= not significant

the last three seasons. This initial assessment of the response to selection for thinner husk shows that it has made some impact, with a positive change seen from 2003 onwards.

WI 4262 – The next generation of malt extract

Recent varieties released by the UA Barley Program reflect the advances in malt extract achieved across the germplasm base. Listed in Table 3 are the major varieties released by the UA Barley Program since 1968, their year of release and relative malt extract levels. When Clipper was released in 1968, this variety was producing malt extract levels of 79%. The successful variety Schooner was released in 1983, producing malt extract levels of 80%.

However, despite the releases of Sloop and the cereal cyst nematode (CCN)-resistant version SloopSA in 1997 and 2002,

respectively, malt extract levels had only increased by 0.5%. The release of Dhow in 2002 was a breakthrough in terms of malt extract levels. This is a variety producing very high levels of malt extract (84%), but it was also very thin husked and highly prone to skinning damage. As a result, it never became a mainstream variety. Consequently it is with Flagship released in 2006 and the new elite line WI4262, which is currently undergoing commercial-scale testing, that we can see a new generation of malt quality varieties that reflect this shift in quality and increase in absolute malt extract levels.

WI4262 (Chieftain/VB9624/4/Keel/3/Sahara/WI2723//Chebec/5/BX98A; 080-375) is a CCN-resistant semi-dwarf line with Gairdner maturity. It has good grain size and good resistance to spot form net blotch and scald, and with excellent boron tolerance. Table 4 shows the performance of WI4262 from the 2005 season, expressed as a percentage of Schooner, with analysis carried out by both industry (Joe White Maltings (JWM), Barrett Burston Maltings (BBM), and International Malting Company (IMC)) and the UA Quality laboratories showing comparable results. WI4262 is suited to the domestic market, exhibiting very high extract (83.8%), modest diastatic power, and low fermentability, consistent with the *Sd2L* beta-amylase allele (Table 5).

Table 3. UA Barley Program malt variety releases 1968–2006

Variety	Year of release	% Malt Extract
Clipper	1968	79
Schooner	1983	80
Sloop	1997	80.5
SloopSA	2002	80.5
Dhow	2002	84
Flagship	2006	83
WI4262	Currently in commercial-scale testing	83.8

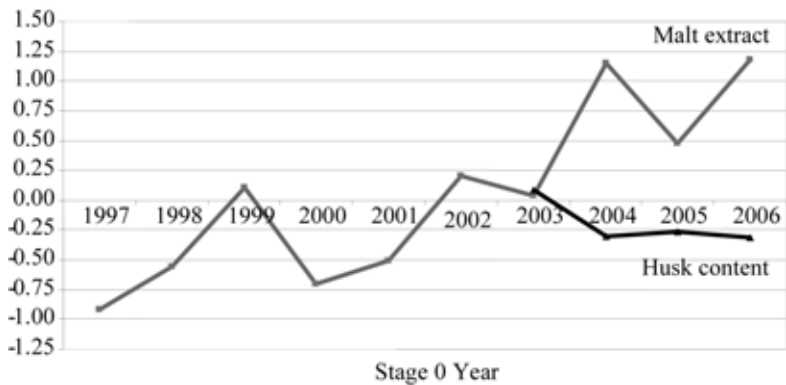


Figure 8. Absolute malt extract and husk content levels compared with Schooner in the UA Barley Program Stage 0 double-row trials, between 1997 and 2006.

Table 4. WI4262 Malt quality data, expressed as a percentage of Schooner.

	Malt Extract	Diastatic Power	AAL	Kolbach Index	Malt Protein
JWM ¹	104	119	95	95	93
BBM ¹	104	129	99	88	95
IMC ¹	107	78	96	90	96
UA ²	107	99	100	91	89

¹ Industry laboratory data from 2005 season Stage 3 samples from Brentwood, mean of 2 replicates. ² UA data from 2005 season Stage 3 samples from Yeelanna, Swan Hill, Clinton and Weetulta, mean of 2 replicates. AAL = Apparent Attenuation Limit.

Table 5. WI4262 UA Quality Laboratory data, 2003–2005 seasons.

Varieties	% Malt Extract	Diastatic Power (μ moles maltose equiv/min/gm)	% AAL	% Kolbach Index	% Malt Protein
WI4262	83.8	367	82.7	41.0	9.84
Schooner	78.3	361	82.5	45.1	10.74
% of Schooner	107	102	100	91	92

All malt quality parameters were assessed using standard analytical methods (Roumeliotis and Tansing, 2003). AAL = Apparent Attenuation Limit

Conclusion

Improvement in malt extract is an important goal of the UA Barley Program, since high malt extract levels are required by the malting and brewing industries. Both MAS- and NIRS-based selection have increased the frequency of lines with high malt extract. In addition, the combined impact of MAS, NIRS and pragmatic breeding has increased the absolute levels of malt extract in advanced lines. The initial response and impact of effective deployment of very thin husk suggests that it may offer the possibility of developing varieties with further improvements in malt extract levels. The recently released variety Flagship and WI4262, currently undergoing commercial-scale testing, signal a new generation of malting lines, reflecting the shift in quality and increase in absolute malt extract levels.

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Production of doubled-haploid malting barley (*Hordeum vulgare* L.) population for breeding and genetic studies in Turkey

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Abstract

In this research we aimed at integrating anther culture methodology with a traditional barley breeding program to improve new winter malting barley cultivars suitable for different agro-ecologies of Turkey. For this reason, 13 different F_1 combinations derived from highly diverse origins were planted in the early spring of 2007, and then several spikes from these crosses were collected and stored under laboratory conditions. In all, 750 anthers were dissected and plated in culture medium and 303 green plantlets were produced. A total of 170 plantlets were transplanted into compost to obtain mature fertile plants for seed setting. The highest rate of DH barley plants was obtained from Tarm \times Angora, crosses with 24 plants, while the lowest rate was obtained from Asahi 5/2* Aleli// Tokak/3/Tokak/4 /Karatay-94 crosses, with two plants. However, 133 additional plantlets were lost during transplanting. This first attempt clearly showed us that there was no interaction between genotypes and media conditions used in this study. This is very encouraging for accelerating barley breeding procedures and further genetic studies in this area. In the second step of this project, the first barley mapping population was going to be produced in Turkey for mapping of some abiotic and biotic traits by using this DH population and marker development tightly linked for these traits.

Introduction

We aimed at producing doubled-haploid (DH) barley lines by anther culture to improve high quality malting barley cultivars suitable for different ecologies of Turkey, and to produce mapping populations to be used in QTL analysis of malting and some agronomic traits. Integration of anther culture technique with conventional cultivar improvement will result in speeding up the breeding of malting barley cultivars at the Central Research Institute for Field Crops (CRIFC).

Materials and methods

A series of crosses designed to meet Turkish malting barley requirements were produced (Table 1) and subjected to the production of DHs by anther culture, as described by Cistue *et al.* (1999) and Castillo *et al.* (2000). After 0.7 M mannitol pre-treatment to anthers, three different culture media (induction, regeneration, shooting) were used. All these stages are shown in Figure 1. Liquid nutrition medium containing ficoll was used to induce embryogenesis. A mapping population of barley DH lines was developed from the cross between Tarm-92 and Angora. Tarm-92 is a two-row feed barley selected from the cross Tokak \times 4875 landrace. It is resistant to cold and drought, susceptible to scald (*Rhynchosporium secalis*) and leaf stripe (*Pyrenophora graminea*), and

Table 1. Efficiency of double-haploid production in 18 barley crosses.

Cross	No. of anthers cultured	No. of plantlets	Plantlets per 100 anthers	Plantlet deaths	No. trans- planted
ANGORA/TARM	100	150	150	20	130
ZEYNELAGA/TARM-92//TARM	20	3	15	1	2
TODOR/AYDANHANIMA//AYDANHANIM	40	20	50	–	20
YEA557.6/YEA422.1//80-5042/3/TOKAK/4/TOKAK					
/5/ZEYNELAĞA	40	12	60	3	9
CRYSTAL/AYDANHANIM	40	30	100	14	16
RUENA7ORZA-96	40	22	55	12	10
MINORI - MUGI J 830/ZEYNELAĞA	40	17	42.5	6	11
KT 2171/ZEYNELAĞA	40	30	75	12	18
BELLISIMA/KARATAY-94//KARATAY-94	40	16	40	10	6
ASAHI 5/2*ALELI//TOKAK/3/TOKAK/4/KARATAY-94	60	9	15	7	2
BARKE/ZEYNELAGA//ZEYNELAGA/3/ANADOLU-98	40	20	50	–	20
OGLON/ÇATALHÖYÜK	40	61	68	21	40
STANDER/EFES-98	60	20	33.3	8	12
4848(SANTIÁN)/ZEYNELAĞA//TARM-92	40	2	10	2	–
ZEYNELAGA/BÜLBÜL-89//YESEVİ-93	40	1	5	1	–
FLAMENCO/NOVATOR//AYDANHANIM	30	8	40	8	–
MORA/ESCOBA//MORADILLA/ESCOBA/3/TARM-92					
/4/TARM-2/5/ZEYNELAĞA	20	2	10	2	–
HSPON3435/ÇATALHÖYÜK//ÇATALHÖYÜK/3/					
ÇATALHÖYÜK/4/ÇATALHÖYÜK	20	1	5	1	–
TOTAL	750	424	-	92	296

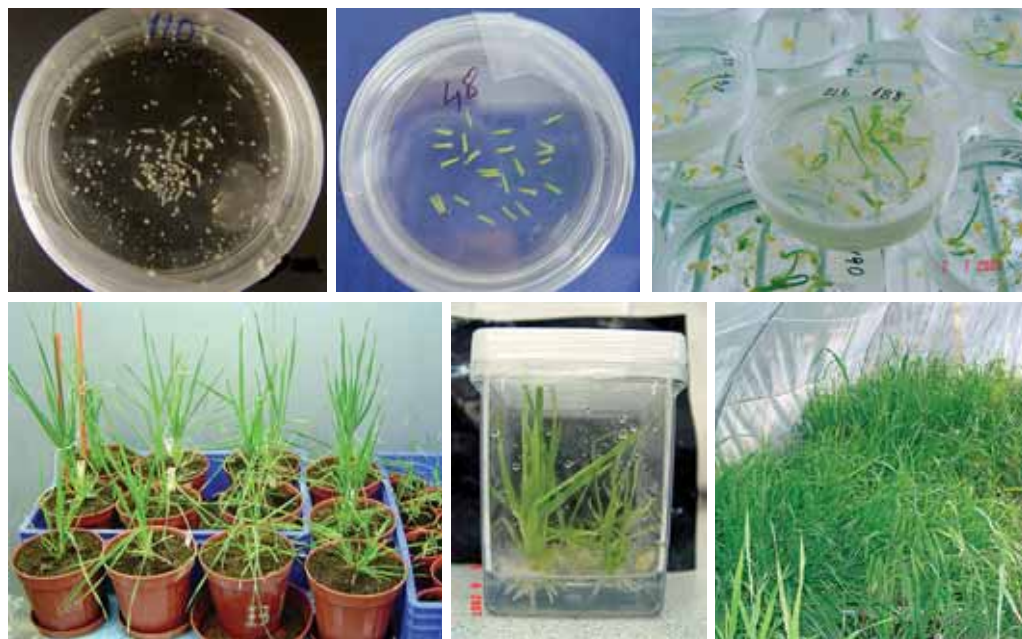


Figure 1. Different stages of doubled-haploid barley production.

to lodging. Angora is semi-dwarf two-row malting barley. This cultivar is tolerant of zinc deficiency and boron toxicity, which affect the crop in dry areas. Angora was developed from the cross (Triax \times 818) \times (Malta \times Ungar) \times (line no. 818 \times Sultan). It is susceptible to cold and drought, resistant to powdery mildew, lodging, and moderately resistant to scald (*Rhynchosporium secalis*) and leaf stripe (*Pyrenophora graminea*). This cultivar is susceptible to zinc deficiency and boron toxicity, which affect the crop in dry areas.

Results and discussion

The 13 different F_1 combinations derived from highly diverse origins were planted in early spring of 2007, and then several spikes from these crosses were collected, from which 750 anthers were dissected and plated in culture medium, resulting in the production of 320 green plantlets (Table 1). A total of 424 plantlets were transplanted into compost to get mature fertile plants for seed setting, and although 92 plantlets were lost during transplanting, the remaining 296 plants were fertile and able to produce seeds. The highest rate of DH barley plants was obtained from the Tarm 92 \times Angora cross,

with 130 plants, while the lowest rate was obtained from the Asahi 5/2* Aleli//Tokak/3/Tokak/4/Karatay-94 cross, with only two plants.

Conclusion

This first attempt clearly showed us that there was minor interaction between genotypes and media conditions used in this study. This is encouraging for accelerating barley breeding procedure. In the second step of this study, the first barley mapping population (Tarm 92 \times Angora) will be produced in Turkey for malting and some agronomic traits by using this DH population.

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Comparative analysis of the genetic relatedness of barley varieties by simple sequence repeat markers

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Abstract

In this study, the genetic diversity and relationships among 40 elite barley varieties were analyzed based on SSR genotyping data. A total of 85 alleles were detected by 35 SSR loci selected 5 each from 1–7 linkage groups, and 79 alleles were polymorphic. At each locus, 2 to 5 alleles were amplified. A cluster analysis based on the genetic similarity coefficients was carried out, and the 40 varieties investigated were classified into four subgroups, and seven malting barley varieties developed in China fell into the same subgroup at the level of GS 0.62. The convincing evidence of narrow genetic background of barley germplasm used in China indicated the importance and urgency of increasing diversity of germplasm in China's malting barley breeding. Two candidate loci associated with malting quality traits were found from the comparative analysis of connecting the mapping information of SSR markers used in this study with the results of QTL identifications previously reported.

Introduction

Barley (*Hordeum vulgare* L.) is a major cereal grain grown for malt, the primary ingredient for beer production. The improvement of malting barley varieties is a complex process involving the manipulation of as many as 22 malting quality traits, such as malt extract yield, kernel plumpness, enzyme activity for starch modification and percent grain

protein, along with many agronomic traits, including grain yield, lodging resistance and seed shattering (Rasmusson and Phillips, 1997). The complexity of malting barley improvement has led barley breeders to work within narrow gene pools (Wych and Rasmusson, 1983; Horsley *et al.*, 1995). Therefore, the modern varieties are becoming more genetically homogeneous and more vulnerable to pathogens and adverse environmental conditions (Asins and Carbonell, 1989). This has prompted a search for new sources of variation that might be useful in plant breeding programs, and many national and international organizations have stressed the need for the collection, conservation and use of the cultivated species and the endemic varieties (Brown *et al.*, 1990). The total number of barley accessions in genebanks, including redundant materials, is estimated to be about 260 000. Only a small portion of these accessions has been characterized at the phenotypic level, and an even smaller portion has been characterized at the genotypic level (Matus and Hayes, 2002).

Traditionally, morphological traits, cytological characters, biochemical tests and pedigree information have been used to study genetic diversity and classification of barley germplasm. However, such methods are always associated with various limitations and are insufficient to reveal the whole information within barley resources (Yuan *et al.*, 2000). Many types of molecular markers, including RFLPs, RAPDs, AFLPs

and SSRs have been used to characterize barley germplasm (Liu *et al.*, 1996; Russell *et al.*, 1997a, b; Shi *et al.*, 2004). The SSR marker system, an excellent molecular marker system with the advantages of being co-dominant, abundant, highly reproducible, highly polymorphic, and easy to assay, has been used in many types of genetic analysis, such as the construction of linkage maps, analysis of genetic diversity of germplasm, and identification of molecular markers for marker assisted selection (Liu *et al.*, 1996; Matus and Hayes, 2002; Saghai-Marooof *et al.*, 1994; Marcel *et al.*, 2007). Saghai-Marooof *et al.* (1994) began to search all barley sequences in the GenBank and EMBL databases for tandem di- and tri-nucleotide repeats, and selected four SSR-containing genes to assess the extent of genetic variation in 104 cultivated barleys worldwide and 103 wild genotypes from Israel. Later, Russell *et al.* (1997b) used 11 SSR markers to distinguish 24 barley genotypes representing 23 varieties and a breeding line in official trials. Struss and Plieske (1998) investigated the genetic diversity and its relationships in wild barley genotypes, landraces and cultivated varieties with 15 SSR markers. Ivandic *et al.* (2002) also examined the allelic variation of genetically mapped SSRs in 39 wild barley genotypes from the Fertile Crescent. In China, Feng *et al.* (2003) used SSR markers to study the genetic diversity and geographical differentiation of 50 accessions of two-row wild barley genotypes from Tibet. But little is known about the genetic diversity and relationships among Chinese elite malting barley varieties identified using SSR markers.

Liu *et al.* (1996) initiated mapping of 45 SSRs on to a RFLP map. Subsequently, a second-generation linkage map of barley using only SSR markers was constructed (Ramsay *et al.*, 2000). Meanwhile, the physical mapping of the seven barley chromosomes was published and a severe suppression of recombination around the

centromeres leading to extensive clustering of markers in genetic linkage maps was observed (Künzel *et al.*, 2000; Künzel and Waugh, 2002). Quantitative trait locus (QTL) analyses of malting quality have been performed in recent years on a number of crosses derived from different germplasm sources originating from North America, Europe and Australia, and great advances in knowledge have been gained (Marcel *et al.*, 2007; Marquez-Cedillo *et al.*, 2000; Hoffman and Dahleen, 2002; Langridge and Barr, 2003; Li *et al.*, 2003).

Breeding good malting barley varieties to meet the dramatically increasing demand for beer production is becoming a big challenge for barley researchers and breeders in China. However, the genetic bases and relationships among different genotypes selected for malting barley breeding are still unclear. In this study, we used 35 mapped SSR loci selected to include 5 from each of the seven linkage groups to survey 40 barley accessions, including some parental lines of several mapping populations and elite genotypes of interest to our malting barley breeding program. The objectives of this study were (i) to distinguish these genotypes, (ii) to estimate the genetic diversity and relationship within these barley resources, and (iii) to provide useful information for the conservation of genetic resources and the enhancement of malting barley breeding.

Materials and methods

Plant materials

Forty barley varieties were chosen for the study (See Table 1). Among these varieties, 12 are malting barley varieties recently released in China, and 28 are cultivated barley varieties collected from different countries, including some parental lines of several mapping populations and some commercial varieties imported to China as malt barley. All varieties were grown on the farm of Zhejiang Academy of Agricultural

Table 1. The cultivars and lines investigated in this study.

No.	Name	Origin	Row type	No.	Name	Origin	Row type
1	Franka	Germany	6	21	Encore	Canada	6
2	Yangnongpi 4	China	2	22	Proctor	Britain	2
3	Excel	Canada	6	23	TR306	Canada	2
4	Amji Nijo	Japan	2	24	Triumph	Germany	2
5	Stander	America	6	25	Steptoe	America	6
6	Legend	Canada	6	26	Kym	Britain	2
7	Ci4196	China	2	27	Ganpi 4	China	2
8	Zhedar 1	China	2	28	Schooner	Australia	2
9	Gold Promise	Britain	2	29	Metealfe	Canada	2
10	Nudinka	Germany	2	30	Copeland	Canada	2
11	ZJU 8	China	2	31	Esterel	France	6
12	Supi 3	China	2	32	Kendall	Canada	2
13	Daner	China	2	33	Franklin	Australia	2
14	Kenpi 8	China	2	34	Viviane	Canada	6
15	Hua 30	China	2	35	Ganpi 3	China	2
16	Zhepi 8	China	2	36	Xiumai 3	China	2
17	Baudin	Australia	2	37	Kippen	Canada	6
18	Sloop	Australia	2	38	Kings	Canada	2
19	Harrington	Canada	2	39	Island	Canada	2
20	Stirling	Australia	2	40	Grant	Canada	6

Sciences in 2005. Each variety was grown in a three-row plot of 2 m row length and 0.25 m between rows, with 100 vigorous seeds sown in a line and the basically the same agronomic management was applied as in local barley production. The experiment was arranged as a randomized complete block design with three replicates.

SSR analysis

DNA was extracted from leaf tissue of 3-week-old seedlings based using a modified CTAB method described by Stein *et al.* (2001). Thirty-five SSR loci distributed throughout the seven linkage groups with known map locations were selected after searching the GrainGene database. Information on primer sequences and PCR conditions for each set of primers are available at www.genetics.org/cgi/content/full/156/4/1997/DC1, and all primers were synthesized by Hangzhou Biotechnology Co. Ltd.

Polymerase chain reactions (PCRs) were performed in Bio-Rad Research My Cycler™ Thermocyclers. DNA fragments were amplified in a 15 µL reaction mix containing 75 ng of template DNA, 1× PCR buffer (Mg²⁺ free), 0.375 U of Taq DNA

polymerase, 300 µM of dNTPs, 2.25 mM of Mg²⁺, and 0.75 µmole/L of forward and reverse primers. PCR conditions for SSR primers Nos. 1 to 17 was as following: one cycle of 9°C for 3 min, 58°C for 1 min, 72°C for 1 min, followed by 30 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. The reaction was ended with a 5 min extension at 72°C. PCR conditions for the remaining SSRs was: one cycle of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The reaction was ended with a 5 min extension at 72°C. The PCR products were detected on 6% polyacrylamide gels. The electrophoreses were performed at 90 W for 2 hours in 1×TBE buffer, and the gels were stained with the method described by Bassam *et al.* (1991).

Data analysis

The presence or absence of a particular band amplified from each SSR pair used in this study was scored as 1 and 0, respectively. The coefficient of genetic similarities (GS) were calculated according to Nei and Li (1979):

$$GS = 2N_{ij}/(N_i + N_j)$$

where N_{ij} is the number of band types

presented in both genotypes i and j , N_i is the number of band types presented in genotypes i , and N_j is the number of band types presented in genotype j . Based on the similarity matrix, a dendrogram showing the genetic relationships between genotypes, was constructed using the unweighted pair group method with arithmetic average (UPGMA) in the software NTSYS-pc version 2.01 (Rohlf, 1998).

Comparative analysis

By using the SSR-based high-density linkage map constructed by Ramsay *et al.* (2000) as the reference, a comparative analysis was made to link the mapping information of SSR markers used in our study with agronomic and malting quality QTL data and locations obtained from the studies previously reported (Liu *et al.*, 1996; Struss and Plieske, 1998; Ramsay *et al.*, 2000; Marquez-Cedillo *et al.*, 2000; Hoffman and Dahleen, 2002; Emebiri *et al.*, 2004; Zale *et al.*, 2000).

Results

Allelic variation of SSR

PCR products were amplified from the 40 barley accessions with all 35 SSR primer pairs and polymorphic fragments were generated from 28 primer pairs. A total of 85 alleles were detected, among which 79 alleles (92.9%) were polymorphic with a mean of 2.4 alleles per locus.

Genetic similarity

All the 85 loci were used to calculate the coefficient of genetic similarity (GS) among the 40 accessions. The largest genetic variation was found among all barley genotypes, with the GS value ranging from 0.39 to 0.98. The GS value ranged from 0.45 to 0.88 within Chinese genotypes, and from 0.39 to 0.98 within foreign genotypes, suggesting that there was a higher genetic diversity in the foreign gene pool.

Cluster analysis

The relationships between accessions were estimated by a UPGMA cluster analysis of GS matrices (Figure 1). All 40 barley genotypes were discriminated successfully by SSR markers. Forty genotypes were classified into two groups (labeled as I and II) at the level of GS 0.57. Group I included six accessions, while group II consisted of 34 accessions. At the level of GS 0.62, group II was further classified into two subgroups (labeled as IIa and IIb), containing 8 and 26 varieties respectively.

Twelve Chinese malting barley varieties were clustered into different groups, two in group I, seven in subgroup IIa and three in subgroup IIb. It is interesting to note that the varieties included in the same group share one or more common breeding ancestors based on the evidence of pedigree information. For example, seven Chinese malting barley varieties and one Japanese variety, Amji Nijo, clustered into subgroup IIa, showing that they are very closely related. Amji Nijo, introduced as a malting barley into China in the early 1980s, was the parent that contributed most to the development of the Chinese varieties. Similar cases were found for Ganpi 3, Ganpi 4 and Kenpi 8, in subgroup IIb, which also included most of the European varieties. Hungarian barley resource had been used as the parental lines when developing these varieties (Wang *et al.*, 2003). Two Chinese varieties, Zhedar 1 and Ci4196, were classified into group I, together with 4 Canadian genotypes, because these two varieties had been adopted by Canadian breeders in the 1990s.

Comparative analysis

The framework genetic map was generated from the comparative analysis conducted to link the SSR markers used in this study with agronomic and malt quality QTL data and locations obtained from the studies previously reported (Figure 2), with 23 SSR markers mapped on an SSR-based linkage

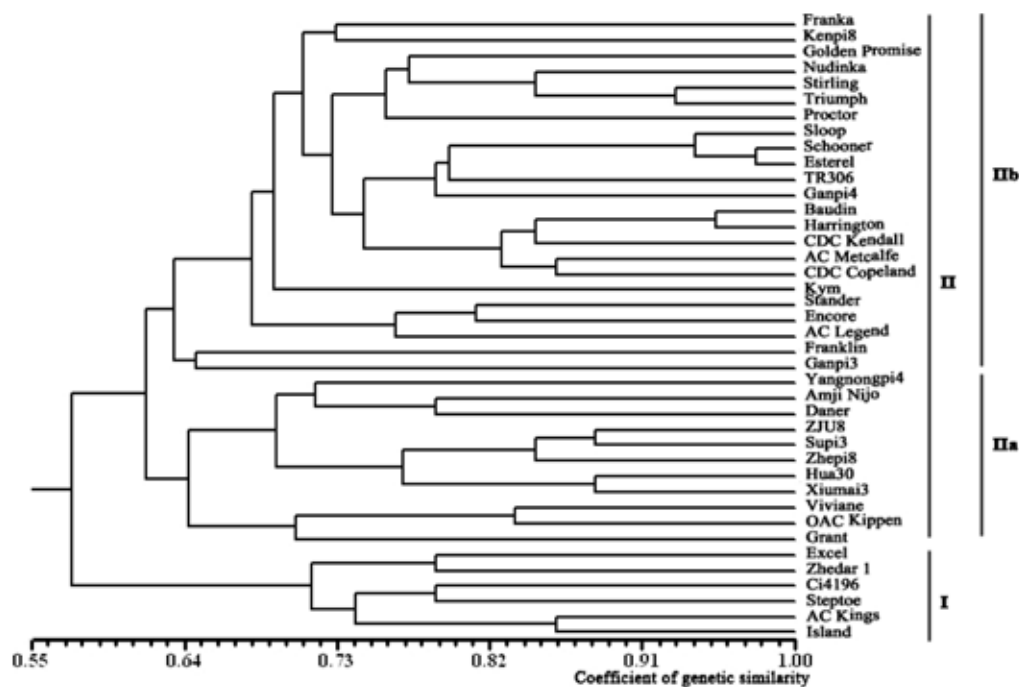


Figure 1. Dendrogram of 40 barley varieties based on the UPGMA method

map (Ramsay *et al.*, 2000), and several SSR markers were associated with QTLs for agronomic and malt quality traits (Figure 2). Two SSR markers were mapped to chromosome 1, and the marker *EBmac0501* was associated with QTLs for malt extract and diastatic power. Four SSR markers were mapped on chromosome 2, the marker *Bmac0134* was linked to QTLs for malt extract, barley beta-glucan and leaf rust. Five SSR markers were mapped on Chromosome 3, and *Bmag0067* coincided with QTLs for barley beta-glucan, barley beta-glucanase and malt viscosity, and marker *Bmag0854* was associated with QTLs for alpha-amylase, finished malt beta-glucanase, beta-amylase and scald. Three SSR markers were on both arms of chromosome 4. Marker *HVM40* was linked to QTLs for alpha-amylase, starch, grain protein, test weight, beta-amylase, malt extract and diastatic power. Marker *Bmag0375* was associated with QTLs for alpha-amylase and viscosity. There were QTLs

for grain yield and fine-coarse difference on chromosome 5 at marker *Bmac0306*. QTLs in the region of chromosome 6 at *Bmag0807* were associated with grain protein, diastatic power, alpha-amylase and malt extract. Three SSR markers were on chromosome 7, but none was associated with the QTLs for the agronomic and malt qualities. The marker data of *Bmac0006* indicated that eight malting varieties, namely Yangnongpi 4, Amji Nijo, Daner, ZJU 8, Supi 3, Zhepi 8, Hua 30 and Xiumai 4, showed the same banding pattern. The marker data of *EBmac0501* showed the same result, while six Canadian varieties, namely Stander, Encore, Legend, Viviane, Kippen and Grant, also had the same banding pattern.

Discussion

Assessment of the extent of genetic variability within barley germplasm is fundamental for barley breeding and the conservation of

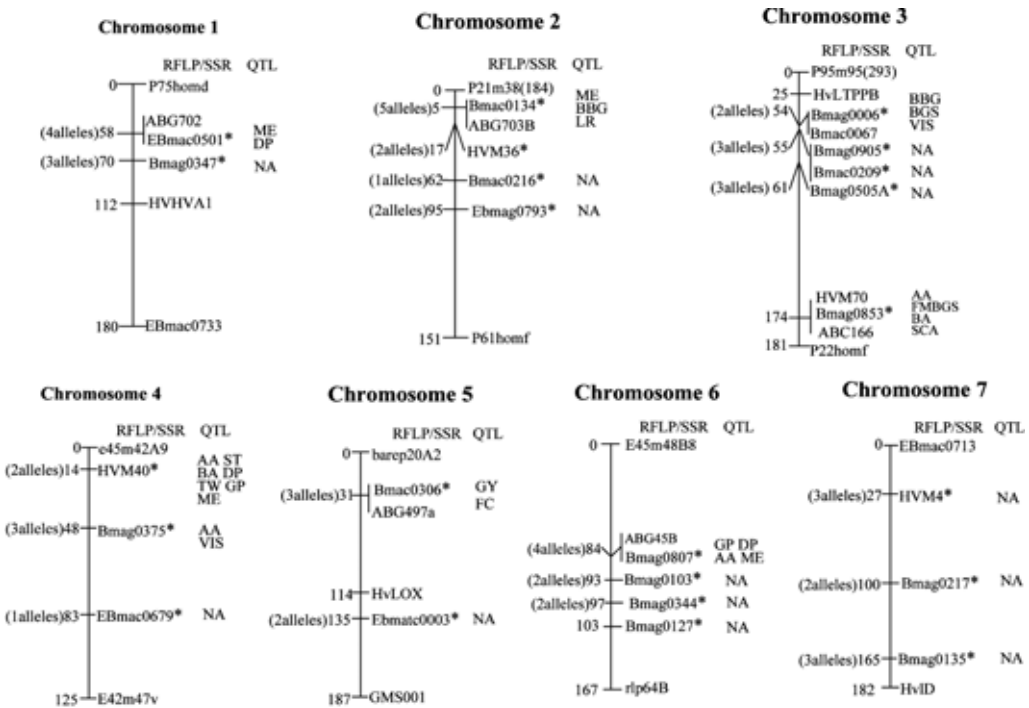


Figure 2. Map positions of SSR markers (*) on barley chromosomes and associated QTLs from the summary of QTL studies. Key to the QTL abbreviations is as follows: AA = alpha-amylase; BA = beta-amylase; BBG = barley beta-glucan; BGS = beta-glucanase; GP = grain protein; ME = malt extract; FC = fine-coarse difference; DP = diastatic power; VIS = viscosity; ST = starch; TW = test weight; GY = grain yield; and LR = leaf rust.

genetic resources, as it could provide a general guide for choosing appropriate parental lines to make suitable cross combinations for particular breeding purposes. The evaluation of genetic variation among barley resources from different countries has been reported (Asins and Carbonell, 1989; Matus and Hayes, 2002; Russell *et al.*, 1997b; Shi *et al.*, 2004; Struss and Plieske, 1998; Ivandic *et al.*, 2002; Feng *et al.*, 2003; Zhang *et al.*, 2005; Hou *et al.*, 2005; Pejic *et al.*, 1998). Since China has been identified as one of the genetic diversity centers of barley and rich in both landrace and cultivated barley, several studies have been conducted to evaluate the genetic relationships among different barley populations for different purpose (Yang *et al.*, 2003). The genetic diversity of 67 barley genotypes selected from the eastern of China

was analyzed by using 30 RAPD markers, and a total of 223 alleles were detected, among which 130 alleles (58.3%) were polymorphic (Shi *et al.*, 2004). It was also reported that there were 153 alleles based on 28 RAPD markers assayed in 38 malting barley varieties from China, and only 91 (59.4%) alleles were polymorphic (Zhang *et al.*, 2005). By using 22 RAMP markers, the genetic variation of 60 barley accessions from China was investigated, 116 alleles were detected, and 98 alleles (84.48%) were polymorphic (Hou *et al.*, 2005). In this present study, a total of 85 alleles were detected by 35 SSR loci selected in such a way as to have 5 loci for each of the 7 linkage groups, and 79 alleles (92.9%) showed polymorphism. It indicated that the SSR marker system is more efficient for assessing genetic variation, detecting a

higher level of polymorphism than the ones detected by other markers.

Pejic *et al.* (1998) reported that the information on polymorphism would be sufficient if more than 70 alleles were detected. To ensure the efficiency and representation of the genetic information among genotypes, Shi *et al.* (2004) suggested that more than two SSR loci each from seven linkage groups should be selected for assaying the genetic diversity. Saghai-Marooft *et al.* (1994) reported that there was an average of 18 alleles per locus based on four SSR loci assayed in the 207 genotypes. The highest number of alleles per SSR locus found in barley was 37, based on HVM4 assayed on 104 accessions of *H. vulgare* subsp. *vulgare* and 103 accessions of *H. vulgare* subsp. *spontaneum*. Other investigators have reported lower average numbers of alleles per locus in barley, ranging from 1 to 15 (Struss and Plieske, 1998; Feng *et al.*, 2003; Becker and Heun, 1995; Dávila *et al.*, 1999). We found the lowest number of alleles per locus in 40 barley accessions based on 35 SSR loci analyzed, with a range from 2 to 5. It is suggested that the number of alleles detected would depend, in large part, on the population size of the barley germplasm sampled, different map locations of SSR loci and different methods for discriminating the amplified products. Since the SSR loci selected were relatively evenly distributed along the whole barley genome, the information about genetic relationship revealed by this study should be more representative and meaningful.

Genetic relationships were found to be very close among the Chinese malt barley varieties selected in this study. The narrow genetic background of malting barley germplasm was revealed by the results of cluster analysis, showing that most of malt barley varieties developed in China fell into the same subgroup. In contrast, the foreign genotypes investigated in this study had a higher genetic diversity and broader genetic

base. Therefore, in order to avoid negative effects of low genetic diversity, extensive adoption of elite genotypes with different origins as parental lines for malting barley breeding in China should be an efficient way to implement the strategy of widening the genetic base of malt barley germplasm proposed by Chinese barley breeders at the end of the last century (Sun, 1998; Zhang, 1999).

QTLs are often clustered, so a discriminating marker could be located in a region associated with a number of traits (Marquez-Cedillo *et al.*, 2000; Hoffman and Dahleen, 2002; Emebiri *et al.*, 2004; Zale *et al.*, 2000). Emebiri *et al.* (2004) reported that the SSR marker *Bmag0067* on chromosome 6 was in a region associated with beta-glucan, beta-glucanase and malt viscosity. Marquez-Cedillo *et al.* (2000) also found that agronomic and malting quality QTLs were clustered. The RFLP marker *ABG463* on chromosome 5 was in the region associated with alpha-amylase, diastatic power and malt extract. In our study, the results of comparison analysis showed that the SSR marker HVM40 on chromosome 4 was in the very region associated with alpha-amylase, starch, grain protein, test weight, beta-amylase, malt extract and diastatic power. The marker data of *Bmac0006* indicated that eight malting barley varieties, namely Yangnongpi 4, Amji Nijo, Daner, ZJU 8, Supi 3, Zhepi 8, Hua 30 and Xiumai 4, showed the same allele, as the same band pattern was observed after gel differentiation of generated PCR products. The marker data of *EBmac0501* showed that six Canadian varieties, Stander, Encore, Legend, Viviane, Kippen and Grant, also had the same allele. So it is likely that the allele of *EBmac0501* is associated with malt extract and diastatic power, and the allele of *Bmac0067* is associated with barley beta-glucan, barley beta-glucanase and malt viscosity. Further validations are needed to confirm these candidate regions of key QTLs for the characteristics of malting quality in barley.

Acknowledgements

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Session 7

Barley uses – Food

Genetics of (1,3;1,4)-beta-glucan synthesis in barley: applications in food, feed and beer

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Abstract

Cellulose synthase-like *CsIF* genes have been implicated in the biosynthesis of (1,3;1,4)-beta-D-glucans in barley. Of the seven *HvCsIF* genes, four have been mapped to a single locus on chromosome 2H, in a region corresponding to a major QTL for grain (1,3;1,4)-beta-d-glucan content. The other *HvCsIF* genes map to chromosomes 1H, 5H and 7H, and in two cases the genes are close to other QTLs for grain (1,3;1,4)-beta-d-glucan content. Two peaks of transcription are apparent in the starchy endosperm of developing grain. One occurs just after endosperm cellularization, while the second occurs much later in grain development. Marked varietal differences in transcription of the *HvCsIF* genes are observed during endosperm development. Given the commercial importance of barley (1,3;1,4)-beta-d-glucans in stock feeds and in malting and brewing, the observation that only two genes are transcribed at high levels in developing grain is of potential relevance for the future manipulation of grain (1,3;1,4)-beta-d-glucan levels.

Introduction

The cell walls of the starchy endosperm of barley grain are relatively uncomplicated with respect to their composition. They consist of approximately 70% by weight of (1,3;1,4)-beta-d-glucan and about 20% arabinoxylan, with small amounts of cellulose, glucomannan, protein and other minor components (Fincher and Stone, 2004). Walls generally constitute considerably less than 10% of the grain, while the (1,3;1,4)-beta-d-glucan component represents 2-10% of barley grain, depending on the variety and environmental conditions during grain development, and arabinoxylans represent 4-8% of the grain (Fincher and Stone, 2004). Nevertheless, the walls and their constituent polysaccharides affect many quality characteristics of the grain, whether the quality is related to food, feed or the malting and brewing industries.

Although the chemical structures of most wall polysaccharides have been determined (Fincher and Stone, 2004), there is relatively little information on the enzymes involved in their biosynthesis. It has been suggested

that type I polysaccharide synthases, which include enzymes with an action pattern based upon the iterative transfer of glycosyl residues from sugar nucleotide donors onto main chain backbones of wall polysaccharides (Farrokhi *et al.*, 2006) are responsible for the synthesis of the backbones of wall polysaccharides such as cellulose, arabinoxylans, xyloglucans, (1,3;1,4)-beta-d-glucans or glucomannans (Doblin *et al.*, 2002). The type I polysaccharide synthases are encoded by a large multigene family known as the cellulose synthase (CesA) superfamily (Richmond and Somerville, 2000). Sub-groups within this gene superfamily include the cellulose synthase sub-family (CesA) and cellulose synthase-like (Csl) sub-families A–H, each of which consists of multiple genes. In rice there are 37 Csl genes, while in *Arabidopsis* there are 30 (Hazen *et al.*, 2002; Somerville *et al.*, 2004).

Burton *et al.* (2006) used a comparative genomics approach to show that a cluster of the monocot-specific *CsIF* genes in rice is located in a genomic region corresponding to one containing a major quantitative trait locus (QTL) for grain (1,3;1,4)-beta-d-glucan content in barley. When the rice *CsIF* genes were expressed in *Arabidopsis*, (1,3;1,4)-beta-d-glucan was subsequently detected in walls of the transgenic *Arabidopsis* lines. On this basis, it was concluded that the rice *CsIF* genes encode polysaccharide synthases that are essential for the synthesis of the (1,3;1,4)-beta-d-glucans of monocot cell walls, although the participation of other enzymes or ancillary proteins could not be precluded (Burton *et al.*, 2006).

Here, the *CsIF* gene subfamily of barley is described in detail. Seven *HvCsIF* genes and their corresponding cDNAs have been sequenced, and the corresponding genes have been assigned positions on genetic maps from a Clipper × Sahara mapping population (Burton *et al.*, 2008). Transcriptional activities of individual members of the gene subfamily have been compared in a number

of organ and tissue extracts from barley, using quantitative-PCR, and patterns of gene transcription have been monitored during endosperm development in barley.

Results

The barley *CsIF* gene family

Seven near-full-length cDNAs were isolated from various cDNA preparations and the seven corresponding genes were obtained by screening BAC libraries or by PCR amplification from genomic DNA preparations, using gene-specific primers (Burton *et al.*, 2008). The nomenclature of the barley *HvCsIF* genes has been based on the nomenclature of their likely orthologues from rice and, overall, the barley and rice orthologues have similar genome locations, similar nucleotide sequences and similar intron-exon arrangements.

Sequence identity values of 53–69% were observed between the individual barley *HvCsIF* genes. The *HvCsIF7* gene has a single intron, but all the other barley *HvCsIF* genes contain two introns towards the 5' ends of the genes. The locations of the introns are conserved both in the barley gene family and in the *OsCsIF* gene family from rice. The introns in the barley genes range in size from 138 bp to over 5000 bp (Burton *et al.*, 2008).

Mapping the barley *HvCsIF* genes

The positions of the *HvCsIF* genes were mapped using RFLP and SNP genotyping (Figure 1). The *HvCsIF9* gene was mapped to the short arm of chromosome 1H, near the centromere, *HvCsIF6* mapped to a position near the centromere of chromosome 7H, and *HvCsIF7* was located on the long arm of chromosome 5H (Burton *et al.*, 2008). No recombination was observed between the *HvCsIF4*, *HvCsIF8* and *HvCsIF10* genes, all of which mapped to the centromeric region of chromosome 2H (Figure 1). The *HvCsIF3* gene could not be mapped directly

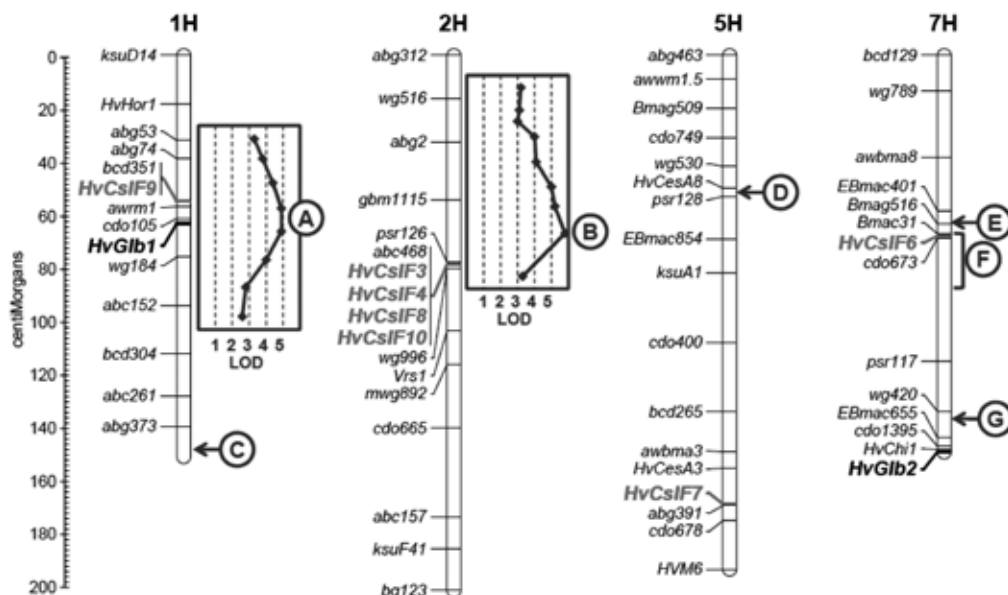


Figure 1. Genetic maps of barley chromosomes 1H, 2H, 5H and 7H showing the positions of barley *HvCslF* genes mapped in a Clipper \times Sahara population (from Burton *et al.*, 2008).

but, because it is on the same barley BAC as *HvCslF4*, it must also be at this position near the centromere of chromosome 2H.

Approximate positions of previously reported QTLs for grain (1,3;1,4)-beta-d-glucan content are also shown in Figure 1 and are based on alignment of the Clipper \times Sahara map with genetic maps from other populations. Graphs A and B show statistically significant test statistics (LOD) values reported by Han *et al.* (1995) based on mapping in a Steptoe \times Morex population (Figure 1). The letters C, D and E show the locations of markers at which Molina-Cano *et al.* (2007) detected QTLs in a Beka \times Logan population, F shows the location of a QTL reported by Igartua *et al.* (2002) in a Derkado \times B83-12/21/5 population, and G shows the location of a marker at which Kim *et al.* (2004) detected marker-trait association in a Yonezawa Mochi \times Neulssalbori population (Figure 1).

Transcript profiling in developing barley endosperm

The transcription patterns for individual members of the *HvCslF* gene family during the development of barley endosperm were determined by Q-PCR. The experiments were performed on two barley varieties, namely the elite malting variety Sloop and the hullless barley Himalaya. Transcripts of the *HvCslF9* gene peaked about 8 days after pollination (DAP), and appeared to be much more abundant in the variety Sloop, and transcripts of *HvCslF9* decreased quickly thereafter to very low levels (Figure 2; Burton *et al.*, 2008). Relatively higher levels of *HvCslF6* mRNA were detected throughout endosperm development but there was a marked increase in abundance of this mRNA late in grain development (Figure 2). Again, differences between the varieties Sloop and Himalaya were observed. Transcript levels of other members of the *HvCslF* gene family were

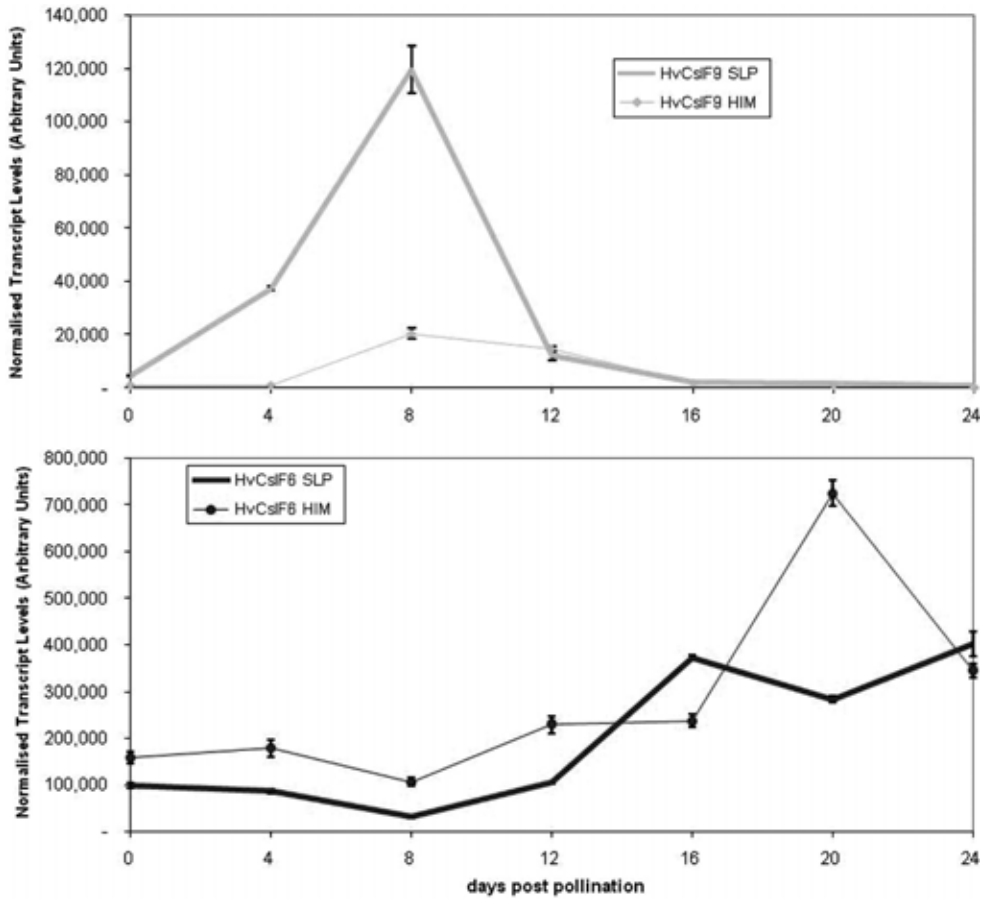


Figure 2. Normalized transcript levels of the barley *HvCsIF9* (upper panel) and *HvCsIF6* (lower panel) genes in developing endosperm at various times post pollination. Levels of mRNA are presented as number of copies per microtitre, after normalization. Error bars indicate standard deviations for each mRNA. The two datasets are for the variety Sloop (SLP) and Himalaya (HIM) (From Burton *et al.*, 2008).

much lower, but detectable (data not shown). Because a major QTL for (1,3;1,4)-beta-d-glucan content of barley grain has been reported close to the gene encoding (1,3;1,4)-beta-d-glucan endohydrolase isoenzyme EI (Han *et al.*, 1995), levels of transcripts for the corresponding gene, designated *HvGlb1*, were monitored during endosperm development. A sharp peak in mRNA abundance for *HvGlb1* was seen 12 DAP in the variety Sloop, but lower levels were detected in Himalaya (data not shown).

Properties of proteins encoded by the *HvCsIF* genes

The amino acid sequences deduced from the cDNAs revealed that the *HvCsIF* genes encode family GT2 glycosyl transferases (Coutinho *et al.*, 2003) with 810–947 amino acid residues. Amino acid sequence identity values ranged from 40–63%. The putative catalytic site residues D, D, D, QxxRW (Doblin *et al.*, 2002) were evident (Figure 3), but no obvious signal peptides or other

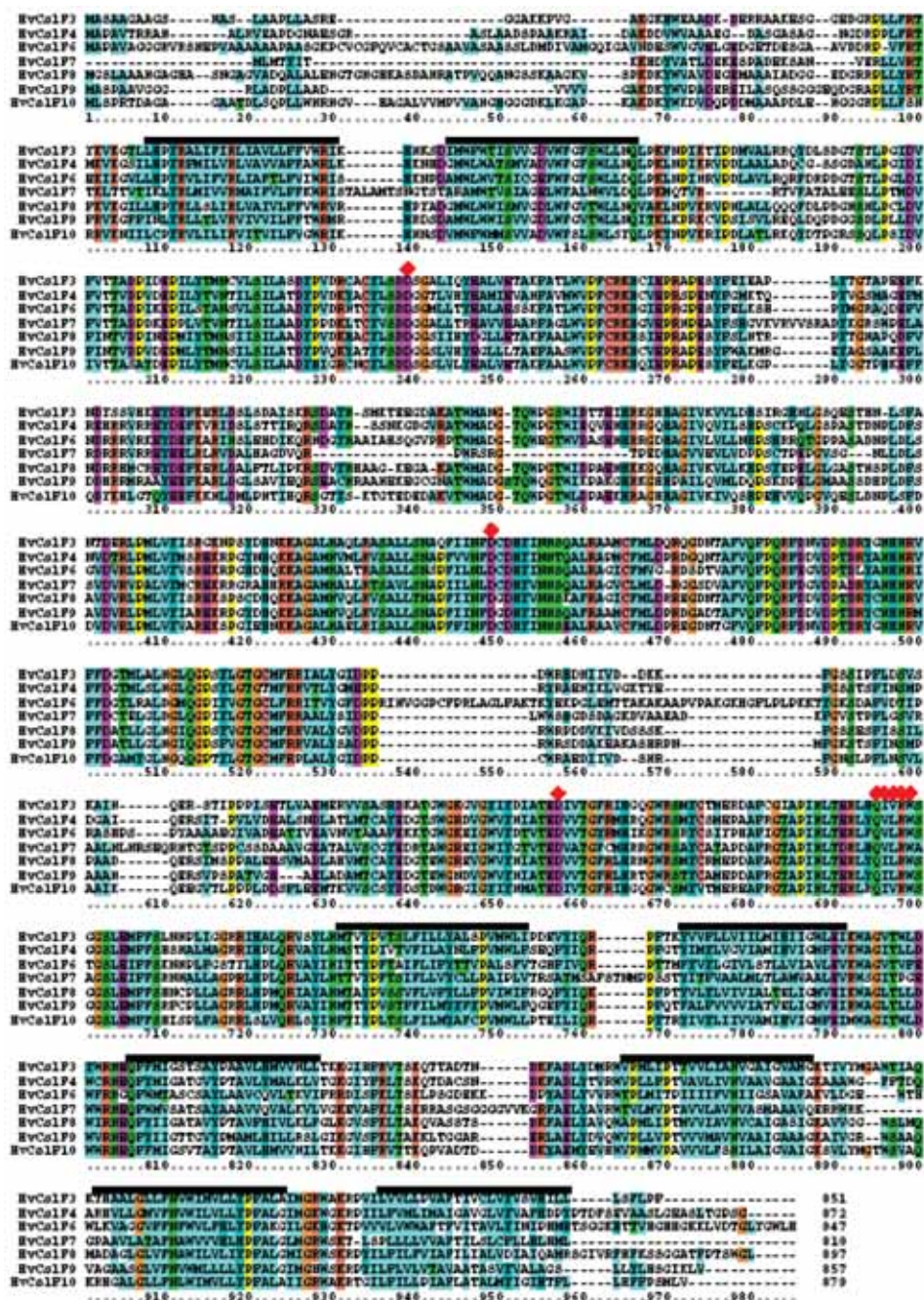


Figure 3. Alignments of deduced amino acid sequences of the *HvCsIF* proteins. Hydrophobic residues are shown in blue, polar residues in green, acidic residues in purple, basic residues in brown, glycine residues in light brown and proline residues in yellow. The positions of transmembrane helices predicted by the program TMHMM are indicated by solid black bars above the sequence and the D, D, D, QxxRW putative catalytic motif is indicated with red diamonds.

common peptide motifs were detected. Eight trans-membrane helices (TMHs) were detected, with two located towards the NH₂-terminal region of the proteins and six located towards the COOH-terminus (Figure 3).

Discussion

Cellulose synthase-like CslF genes have been implicated in the biosynthesis of (1,3;1,4)-beta-d-glucans in barley (Burton *et al.*, 2006). Here, four of the seven *HvCslF* genes have been mapped to a single locus on chromosome 2H, in a region corresponding to a major QTL for grain (1,3;1,4)-beta-d-glucan content (Figure 1; Burton *et al.*, 2008). The other *HvCslF* genes map to chromosomes 1H, 5H and 7H, and in two cases are close to other QTLs for grain (1,3;1,4)-beta-d-glucan content (Figure 1).

The nucleotide sequences of the barley *HvCslF3*, *HvCslF4*, *HvCslF8* and *HvCslF10* genes in the cluster on chromosome 2H reveal identities in the range 53–69% (data not shown). This shows that, if the genes arose by duplication of a common ancestral gene in that region of the genome, there has been a high degree of sequence divergence. Similarly, the transcription patterns of the genes are divergent (data not shown). Transcript levels for the four genes are relatively low during endosperm development, compared with those for *HvCslF6* and *HvCslF9*, which may mean that *HvCslF* transcript levels are not always indicative of the activity of encoded enzymes or other cellular components that are required for (1,3;1,4)-beta-d-glucan synthesis in the grain (Burton *et al.*, 2008).

Overall, there was a high level of correspondence between published QTL map positions and the map positions of the *HvCslF* genes. Apart from the QTL on chromosome 2H discussed above, the other major QTL for (1,3;1,4)-beta-d-glucan content in barley grain identified by Han *et al.*, (1995) was on chromosome 1H and accounted for about 15% of the variation in grain (1,3;1,4)-beta-d-glucan content. In the

Clipper × Sahara population used here, the *HvCslF9* gene mapped near the centromere of chromosome 1H (Figure 1). In the same region Han *et al.*, (1995) detected overlapping QTLs for (1,3;1,4)-beta-d-glucan content in un-germinated barley grain (Figure 1), for malt (1,3;1,4)-beta-d-glucan content and for malt (1,3;1,4)-beta-d-glucan endohydrolase activity. They suggested that all of these QTLs may be attributable to *HvGlb1*, which encodes barley (1,3;1,4)-beta-d-glucan endohydrolase isoenzyme EI (Slakeski *et al.*, 1990). However, it is possible that (1,3;1,4)-beta-d-glucan content in grain is influenced by the expression of both *HvCslF9* and *HvGlb1* during grain development. In this situation, one would expect to see transcripts for both genes in the developing endosperm, as observed here (data not shown).

The most abundant mRNA transcripts in developing barley endosperm are those of the *HvCslF6* and *HvCslF9* genes (Figure 2). Transcriptional activities of the two genes vary both with respect to the amount of mRNA and to the timing of transcription (Burton *et al.*, 2008). Transcription of the *HvCslF9* gene peaks at 8 DAP, at a stage when cellularization of the endosperm is complete and starch deposition has commenced. (1,3;1,4)-beta-d-Glucan is detectable in endosperm walls from 5 DAP onwards (Wilson *et al.*, 2006). In contrast, *HvCslF6* transcripts are detected at much higher levels throughout endosperm development (Figure 2). Variations in transcript levels of the *HvCslF6* and *HvCslF9* genes were observed between different barley varieties (Figure 2), consistent with the fact that QTLs for (1,3;1,4)-beta-d-glucan content have been detected in chromosomal regions where these genes are located. Transcript levels of the *HvCslF9* gene are much higher in the malting quality variety Sloop than in the hullless variety Himalaya during the cellularization stage of initial wall development (Figure 2), while levels of mRNA from the *HvCslF6* gene are higher in Himalaya than in Sloop

during most of endosperm development, and particularly at 20 days post-pollination (Figure 2). The *HvCslF6* transcript levels are consistent with the generally higher levels of (1,3;1,4)-beta-d-glucan in Himalaya, which are typically 6% by weight, than in Sloop, where 3-4% by weight (1,3;1,4)-beta-d-glucan is normally found in mature grain (A.J. Box, R.A. Burton, H.M. Collins and S.A. Jobling, unpublished data). It is important to note that transcript abundance might not necessarily be a good indicator of enzyme activity or of (1,3;1,4)-beta-d-glucan deposition in the wall. The *HvCslF* enzymes encoded by low abundance mRNAs might be just as important as, or even more important than, the *HvCslF6* and *HvCslF9* enzymes for the deposition of (1,3;1,4)-beta-d-glucan in developing grain (Burton *et al.*, 2008).

To confirm the participation of *HvCslF* genes in (1,3;1,4)-beta-d-glucan synthesis, we are attempting to manipulate levels of (1,3;1,4)-beta-d-glucan, in both vegetative tissues and grain, through up- and down-regulation of *HvCslF* genes in transgenic barley. Initial results indicate that levels of (1,3;1,4)-beta-d-glucans in walls can indeed be manipulated in this way (data not shown). Altering the levels of (1,3;1,4)-beta-d-glucan in walls of barley and other cereals could find applications in human and animal nutrition, or in the malting and brewing industries. Barley (1,3;1,4)-beta-d-glucans appear to be beneficial to human health, where they represent soluble dietary fiber and appear to reduce the risks of colorectal cancer, high serum cholesterol and cardiovascular disease, obesity and non-insulin-dependent diabetes (Brennan and Cleary, 2005). At the same time, (1,3;1,4)-beta-d-glucans are considered to be anti-nutritive in feed formulations for monogastric animals and have undesirable effects in cereal processing applications such as malting and brewing (Brennan and Cleary, 2005). In the case of the malting and brewing industries, high levels of (1,3;1,4)-beta-d-glucans in malt are associated with filtration

difficulties in the brewery and in some cases the precipitation of insoluble aggregates in the final beer.

The availability of information on the *HvCslF* gene family, together with the transcriptional profiles presented here and by Burton *et al.*, (2008), have identified *HvCslF6* and *HvCslF9* as potential target genes for manipulation of (1,3;1,4)-beta-d-glucan levels in barley grain. In addition, it should now be possible to exploit this information in breeding programs, either through a transgenic approach, or through the analysis of natural variation in *HvCslF* gene structure, *HvCslF* gene transcription rates and (1,3;1,4)-beta-d-glucan levels in mapping and mutant populations, or in germplasm collections.

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Barley for food: traits and improvements

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Abstract

Barley is an ancient cereal grain and has evolved from a food to a feed and malting crop. However, barley food use today remains important in some cultures around the world, particularly in Asia and Africa. Renewed interest in barley food use centers around beta-glucan effects on lowering blood cholesterol, blood pressure, and glycaemic index. Whole-grain barley foods seem to be associated with increased satiety and weight loss. Breeding for food quality (processing, nutrition) has been minimal. A host of physical and chemical properties are important for barley food processing and products. Several traits of importance studied over time include hullless and protein, lysine, starch, and beta-glucan content. Traits of more recent or continued interest include beta-glucan, vitamin E derivatives, grain and product color, and grain hardness. Color is related to polyphenol content and polyphenol oxidase activity. Grain products made from barley with the proanthocyanidin-free trait are brighter white than those made from normal barley grain. Kernel hardness can affect grain processing and end-use products. Recent studies indicate the broad range in and complexity of inheritance of beta-glucan content and kernel hardness. There is great potential to improve barley for food use and to utilize barley in a large number of cereal-based products as a substitute partially or wholly for currently used cereals such as wheat, oat, rice, and maize.

Introduction

Barley (*Hordeum vulgare* L.) is an ancient and important cereal grain crop. It ranks fifth among all crops in annual dry matter production in the world today (129×10^6 t, 2002–2006 mean) behind maize (*Zea mays*, 610×10^6 t), wheat (*Triticum* spp., 551×10^6 t), rice (*Oryza sativa*, 429×10^6 t), and soybean (*Glycine max*, 180×10^6 t), and ahead of sugar cane (*Saccharum* spp., 93×10^6 t), potato (*Solanum tuberosum*, 60×10^6 t), and sorghum (*Sorghum bicolor*, 50×10^6 t), (FAO, 01/2008). Barley is one of the first agricultural domesticates, along with wheat, pea (*Pisum sativum*), lentil (*Lens culinaris*), goat (*Capra aegagrus hircus*), sheep (*Ovis aries*) and cattle (*Bos taurus*), dating from at least 10 000 years ago in the Fertile Crescent of the Middle East. Barley was presumably first used as human food, but eventually evolved primarily into a feed, malting, brewing and distilling grain due in part to the rise in prominence of wheat and rice. In recent times, about two-thirds of the barley crop has been used for feed and one-third for malting, with only about 2–3% used directly for food. Barley is arguably the most widely adapted cereal grain species, with production at higher latitudes and altitudes and farther into deserts than any other cereal crop. It is in extreme climates that barley remains a principal food source today, e.g. Himalayas, Ethiopia and Morocco. Barley is also important in the human diet in East Asian countries such as Japan and Korea.

Previously, barley was used as food in the Middle East, northern and eastern Europe and western Asia (Chattersjee and Abrol, 1977; Ryu, 1979; Newman and Newman, 2006).

Barley was recognized early on as a good tasting, high-energy food. Given what we know today, the major advantage of incorporating barley into various food products and their consumption stems from barley's potential health benefits. Effectiveness of barley beta-glucans in barley food products in lowering blood cholesterol (Newman *et al.*, 1989; Behall *et al.*, 2004), and glycaemic index (Wood *et al.*, 1990; Cavallero *et al.*, 2002) has been well recognized in numerous publications and widely accepted. Barley is a rich source of tocopherols, including tocopherols and tocotrienols, which are known to be capable of reducing serum LDL cholesterol through their antioxidant action (Qureshi *et al.*, 1986, 1991). The recent approval of barley soluble beta-glucan health claims by the FDA for lowering blood cholesterol level could further boost the food product development of barley and consumer interest in eating those food products. Furthermore, the diverse genetic background of barley, which leads to wide variation in grain chemical composition and physical characteristics, subsequently delivers a great potential for using barley in various foods. Barley may be classified as hulled or hulless, two-row or six-row, spring or winter, malting or feed, and further by composition of endosperm starch (normal, waxy, high amylose), beta-glucan, and proanthocyanidin levels (high, low, none).

Historically, barley as an important food source in many parts of the world declined particularly in the 19th and 20th centuries. Better product quality and mouth-feel of food products prepared from wheat and rice compared with barley considerably decreased the use of barley as food. Accordingly, in contrast to wheat, there has been little improvement in food processing and product

development of barley. No significant efforts or attempts to systematically breed and develop varieties for food uses have been devoted to barley. The quality requirements of barley for food use have not been well established, making it difficult for food manufacturers to select raw materials suitable for use in specific food products.

For food uses, barley grain is first abraded to produce pearled or pot barley, and further processed to grits, flakes and flour. Pearled barley, grits or flour have been used in the preparation of many traditional dishes in Russia, Poland, Tibet, Japan and India (Chatterjee and Abrol, 1977). Pearled barley is used as a rice substitute and for production of soy paste and soy sauce in Korea (Ryu, 1979). Hulless barley has been particularly important in Himalayan and East Asian countries. In Western countries, pearled barley is used in breakfast cereals, stews, soups, porridge, bakery flour blends and baby foods (Bhatty, 1993). In Middle Eastern and North African countries, barley is pearled and ground, and used in soups, flat bread and porridge (Bhatty, 1993).

Barley flour, prepared from pearled grain through hammer milling or roller milling, can easily be incorporated into wheat-based products, including bread, cakes, cookies, noodles, biscuits, and extruded snack foods at levels from 15 to 30% (Newman and Newman, 1991). Newman *et al.* (1990) prepared 100% barley flour muffins of acceptable quality, which were only slightly inferior in volume, density and moisture to wheat flour muffins. Some cookie and brownie recipes can be substituted with 100% barley flour and maintain acceptable quality and flavor (Ullrich, unbiased personal experience!).

A review of physical and chemical traits of barley grains related to processing, food product, and nutritional quality, as well as prospects for barley improvement are presented. Emphasis is on grain hardness, carbohydrate and color traits.

Physical characteristics of barley grain

Barley grain that is clean, bright yellow-white, plump, thin hulled, medium hard and uniform in size is generally suitable for food uses and preferred for pearling (Pomeranz, 1974). The shallow crease of the barley grain is an advantage in the production of pearled barley that is used as a rice extender. Barley grain with a shallow crease allows even removal of bran layers and crease during pearling with minimum loss of endosperm, improving the appearance of pearled grains and making them similar to polished rice in appearance. Harder endosperm texture could be an advantage during the de-hulling and pearling processes by minimizing the loss of endosperm. At the same time, grains of harder endosperm texture may demand more mechanical energy for grinding and milling, and more broken kernels may result.

Clean, white and uniform-sized grain are common physical characteristics required for the initial processing of many cereal grains, including wheat, rice, oat, maize and barley. Considering general preferences by consumers and food manufacturers for bright white color of pearled grain and milled flours, whiteness of products is one of the most critical traits of barley for food uses. Uniformity, as well as size and shape of barley grains, as in other cereal grains, influence the efficiency of hulling, pearling and milling processes, and determine the yield of resulting products. A thin hull is easier to remove, leading to the increased yield of de-hulled grain.

Grain hardness

Grain hardness may be measured by particle size index, pearling index, time to grind, sound or energy of grinding, starch damage, near-infrared analysis (NIR), and crushing or slicing of kernels (Halverson and Zeleny, 1988). Kernel hardness is considered one of the major factors affecting the processing and product quality of wheat (Pomeranz and

Williams, 1990) and extensive study has set standards for cereal hardness in general. There is a general agreement that the adhesion between the starch granules and the protein matrix in the endosperm mainly determine the grain hardness of wheat (Darlington *et al.*, 2000). The presence of friabilin on the surface of starch granules in soft wheat leads to a weak association between starch granules and the protein matrix, resulting in soft endosperm texture. In hard wheat there is a tight adhesion between starch granules and the protein matrix, due to the lack of friabilin. It is generally agreed that puroindolines are the major components of friabilin in wheat (Greenwell and Schofield, 1986; Morris *et al.*, 1994; Oda and Schofield, 1997), and close proximity of the puroindoline genes to the hardness locus on chromosome 5D of bread wheat (Sourdille *et al.*, 1996; Giroux and Morris, 1997) further support the control of wheat grain hardness by puroindolines. Kernel hardness influences particle size of wheat flour and starch damage during milling, and consequently affects processing and product quality. Similarly, kernel hardness of barley could have a large influence on milling by affecting particle size, the energy needed to crush grain, and starch damage in flour.

The significance of grain hardness on malting quality of barley has been well recognized. Allison *et al.* (1976) reported that malting barley varieties are classified as soft, whereas non-malting varieties are considered hard. Fox *et al.* (2007a) noted that because barley varieties and breeding lines have been initially bred for malt production, and accordingly selected for softer textured endosperm, barley is generally softer than wheat. Grain hardness, as measured by milling energy, is negatively correlated with hot water extract and endosperm modification of malt, significantly affecting the malting quality of barley (Allison, 1986; Swanston and Taylor, 1988; Swanston, 1995; Brennan *et al.*, 1996). Kernel hardness also affects pearling in that increased kernel hardness

means increased pearling time and energy expended (Edney *et al.*, 2002).

While no or little friabilin in barley starches was observed by Morrison *et al.* (1992), the presence of wheat friabilin homologues in barley, though smaller in amount than in wheat, was reported by Jagtap *et al.* (1993) using a highly specific monoclonal antibody. Darlington *et al.* (2000) also showed the occurrence of a similar level of friabilin in starch granules isolated using dry sieving from both hard and soft endosperm barley. Their subsequent study reports the presence of both hordoindoline (puroindoline homolog of wheat) a and b in the mature barley endosperm, with hordoindoline b being the major isoform. Furthermore, there was a lack of a clear relationship between the presence of hordoindoline a and grain texture (Darlington *et al.*, 2001).

Gautier *et al.* (2000) identified hordoindolines and mapped them to the short arm of chromosome 5H. Likewise, Beecher *et al.* (2002), investigating the influence of hordoindoline variation upon grain hardness in the Steptoe \times Morex cross, reported that the most significant quantitative trait locus (QTL) for hardness is located on the distal end of the short arm of chromosome 5H, explaining 22% of the single kernel characterization system (SKCS) hardness differences. It has also been reported that the hordoindoline genes share the same 5H chromosome region with QTLs for grain texture, as measured by milling energy and malt extract (Thomas *et al.*, 1996; Mather *et al.*, 1997; Beecher *et al.*, 2001).

Darlington *et al.* (2001), using molecular marker analysis of hordoindolines, failed to show any differences between three soft- and three hard-textured barley cultivars in amino acid sequence. However, Beecher *et al.* (2001) observed substantial allelic variation in barley for both hordoindoline a and hordoindoline b sequences. By sequencing single nucleotide polymorphisms (SNPs) of three hordoindoline genes, Fox *et al.*

(2007b) showed no clear relationship with grain hardness, malt or feed quality. Despite a large variation in grain hardness of barley genotypes, the changes in hordoindoline gene sequences showed no association with grain hardness. There appears to be very little variation in hordoindoline genes among barley breeding populations, and the hordoindoline genes may actually account for only a small portion of variation in grain hardness.

Based on hardness determination of a large number of barley breeding lines and varieties grown at multiple locations, Fox *et al.* (2007b) reported significant influences of both genotype and environment on grain hardness, and that environmental conditions that increased grain protein also increased grain hardness. A study of 959 barley breeding lines and check cultivars from 10 breeding programs in the USA through the USDA-CREES sponsored Barley Coordinated Agricultural Project (Barley CAP) revealed a wide range of 30 to 92 SKCS units of grain hardness (Nair *et al.*, 2010).

Other structural and compositional characteristics of barley endosperm could contribute to grain hardness. Properties of protein, starch and beta-glucan, and their interactions and packing during grain filling, could make potential contributions to grain hardness in barley. In contrast to wheat, barley contains high levels of beta-glucan, which constitutes 75% of barley endosperm cell walls (Fincher, 1975; Fincher and Stone, 1986; Henry, 1987) and forms the inner layer of the cell walls (Bamforth and Kanauchi, 2001). Henry and Cowe (1990) reported positive relationships between grain protein and beta-glucan content and grain hardness of barley.

Obviously, our understanding of barley grain hardness and factors contributing to grain hardness are limited. After all, there has been little need or demand for the consideration of grain hardness in the conventional uses and processing of barley.

With increased interest in using barley in various food products, demanding more intensive and complex processing, it is crucial to produce and supply barley of proper grain hardness. For the development of barley varieties of proper hardness characteristics, the following questions need to be answered:

- What are the relative contributions of genotype and environment on grain hardness of barley?
- How is the structure of the endosperm cell and cell walls related to grain hardness?
- What is the significance of the quantity and quality of major grain constituents, including starch, protein and beta-glucan on the variation of barley grain hardness?

Grain color

Generally, sound barley grain has a bright light-yellow or off-white color. However, barley grain is frequently discolored by fungal infections in humid conditions or abnormal phenol metabolism during the grain filling period, which lowers malting quality of barley (Anderson and Banttari, 1976; Li *et al.*, 2003). There is genetic variability for discoloration tolerance among barley varieties (Miles *et al.*, 1987; Young, 1997; Edney *et al.*, 1998) and multiple genes control tolerance to grain discoloration (Li *et al.*, 2003).

Given barley's diversity, grain color can vary from light yellow to purple, violet, blue and black, which is mainly caused by the level of anthocyanins in the hull, pericarp and/or aleurone layer. Anthocyanin colored grain types, although produced in small amounts, are often used for making specialty foods due to their colorful appearance (Abdel-Aal *et al.*, 2006). Highly colored grain is also receiving much attention for applications in functional foods due to their antioxidant health-enhancing potential activities (Satue-Gracia *et al.*, 1997; Nam *et al.*, 2006; Philpott *et al.*, 2006). However, the majority of barley produced possesses bright, light-yellow grain color, which is generally preferred

for malting and brewing purposes. Color is generally irrelevant for feed uses, but could be a factor in animal fat and egg yolk color.

Because barley grain is first pearled to remove the hull and pericarp, and often further ground to produce flour for various food applications, white color is preferred. Considering the general preferences by food processors and consumers for bright white-colored pearled grain and flour, it is important to have light-white colored grain absent of any discoloration or pigmentation. Additionally, hullless barley kernels with a shallow crease and round shape are advantageous in the production of clean, bright white pearled grain. Color is revisited below under chemical composition.

Chemical composition of barley grain

Whole barley grain is composed of about 65–68% starch, 10–17% protein, 4–9% beta-glucan, 2–3% free lipids and 1.5–2.5% minerals (Czuchajowska *et al.*, 1998; Quinde *et al.*, 2004). Total dietary fiber ranges from 11 to 34% and soluble dietary fiber from 3 to 20% (Fastnaught, 2001). Hullless or de-hulled barley grain contains 11–20% total dietary fiber, 11–14% insoluble dietary fiber and 3–10% soluble dietary fiber (Fastnaught *et al.*, 1996; Fastnaught, 2001). Pearling reduces insoluble fiber, protein, ash and free lipid content, but increases starch and beta-glucan content by the removal of outer layers, including hull, bran (pericarp, testa) and germ (Quinde *et al.*, 2004; Quinde-Axtell *et al.*, 2006). Composition and physical characteristics of barley grain have large influences on processing properties and product quality of foods prepared from or incorporated with pearled barley and/or barley flour. Barley grains of different types often possess quite different physical and chemical characteristics. Hullless barley, because of little or no effort needed to remove the hull during threshing or processing, appears to be more suitable for processing

and uses for human consumption than hulled barley. Waxy starch endosperm genotypes not only deliver unique physical properties to food products incorporated with or prepared from those genotypes, but also contain greater grain protein and beta-glucan than those of regular starch endosperm. However, because of the fact that human consumption of barley and barley containing food products has been relatively insignificant in comparison with other cereal grains, development of new processing and food products has been neglected and, furthermore, there has been very little concern or effort to define quality requirements of barley for food uses. Accordingly, we have very little understanding of the food quality traits of barley, and there have been few guidelines or protocols for systematically breeding barley varieties for human consumption. The significance of beta-glucan and tocopherols for human nutrition is well known, but little is known about the functional properties of beta-glucan for making food products. Some of the traits preferred for specific food applications are known through investigations on incorporating barley into wheat-based food products.

Amylose content of barley starch

Amylose content of barley starch varies from 0% in zero-amylose waxy, up to 5% in waxy, 20–30% in normal and as high as 45% in high-amylose barley (Merritt, 1967; Henry, 1988; Bhatta and Rossnagel, 1997). Barley, as well as wheat and maize starch, with reduced amylose content exhibits lower pasting temperature, and greater hot-paste viscosity, swelling power, granule fragility and freeze-thaw stability than starch with higher amylose content (Zheng and Sosulski, 1998). Accordingly, barley grains and flours of different starch amylose contents exhibit a wide range in processing properties, as well as product quality.

High- or normal-amylose barley compared with waxy barley produced superior white salted noodles (Lagassé *et al.*, 2006) and exudates (Baik *et al.*, 2004b). In contrast, waxy hulless barley compared with normal or high amylose barley produced superior tortillas and puffed products (Ames *et al.*, 2006) and ultra-low-fat pork bolognas (Shand, 2000). Hulless waxy barley is generally preferred to normal barley as a rice extender or substitute in Japan and Korea, because of faster water imbibition during cooking, faster cooking time, and texture similar to cooked rice.

Beta-glucans

Mixed linked (1→3),(1→4)beta-D-glucans constitute approximately 75% of the barley endosperm cell walls, along with 20% arabinoxylans and protein (Fincher and Stone, 1986; Henry, 1987). The beta-glucans in the endosperm cell walls appear to be covalently bonded to protein, forming large molecules of 107 Da (Forrest and Wainwright, 1977). Both beta-glucans and arabinoxylans are negative determinants of malting and brewing quality (Lusk *et al.*, 2001).

Barley grain usually contains 2–10% beta-glucan (Henry, 1987; Fastnaught, 2001). However, a beta-glucan-rich hulless, waxy barley isolate “prowashonupana” (high protein, waxy, short awn nude ‘Compana’) may contain as much as 15–18% beta-glucan (Åman and Newman, 1986; Andersson *et al.*, 1999; Fastnaught, 2001). Because of the usual association of the waxy starch gene (wax) with high beta-glucan content, waxy endosperm types generally show greater beta-glucan content than barley types with normal starch (Ullrich *et al.*, 1986; Newman and Newman, 1991). Beta-glucan content of barley grain is mainly determined by genetic factors (Powell *et al.*, 1985) and less by environmental factors during the grain filling period (Henry, 1986; Stuart *et al.*, 1988). However, Fastnaught *et al.* (1996), Pérez-Vendrell *et al.* (1996) and Yalçın *et al.* (2007)

noted significant influences of both genotype and environment on beta-glucan content of barley grain. Greenberg (1977) estimated that beta-glucan content is controlled by two to three dominant genes. Analysis of the Steptoe \times Morex mapping population revealed three QTLs: one on chromosome 1H and two on chromosome 2H, each explaining 5 to 20% of the variation (Han *et al.*, 1995), while a similar analysis of a Beka \times Logan population identified QTLs on chromosomes 1H, 5H and 7H, explaining 8 to 21% of the variation (Molina-Cano *et al.*, 2007).

Health benefits of barley beta-glucans, including reduction in blood cholesterol and glucose, and weight loss by increased satiety and control of type-2 diabetes, have been well recognized and proven through both animal experiments and human clinical trials as cited above. At the same time, the functional properties of beta-glucans in food processing and end-use quality of barley are little known, with the exception of malting and brewing. A close relationship between total beta-glucan content and grain hardness determined using a Brabender hardness tester (Henry, 1985) and thicker endosperm cell walls in high beta-glucan barley (Andersson *et al.*, 1999) may indicate a significant role for beta-glucan in grain hardness variation. Positive relationships of beta-glucan content with water uptake of grain during cooking and crumb moisture content of bread incorporated with 30% barley flour, and negative relationship with hardness of cooked noodles incorporated with 30% barley flour, was reported by Baik *et al.* (2004a).

Color of barley grain food products

Color and appearance serves as indicators of food product wholesomeness and quality, and are the first factors considered by consumers before purchase and consumption. Consumers demand and prefer a specific color for each food product. If the food product loses or deviates from the expected color, it

is immediately rejected regardless of other quality characteristics. Bright white color is generally preferred by consumers for the majority of cereal grain-based food products. Bright white or yellow color is favored for many Asian noodles. Polyphenol oxidase (PPO) activity has been largely implicated in the discoloration of noodles (Baik *et al.*, 1995; Zhao and Seib, 2005).

Graying and dark color development in pearled and cooked barley as a rice extender or substitute, in iron-fortified infant cereal made with barley flour (Theuer, 2002), and various wheat-based products such as leavened and unleavened breads, pastas and Asian noodles, incorporated with barley (Knuckles *et al.*, 1997; Başman and Köksel, 1999; Marconi *et al.*, 2000; Quinde *et al.*, 2004; Izydorczyk *et al.*, 2005; Ereifej *et al.*, 2006; Erkan *et al.*, 2006; Lagassé *et al.*, 2006) has been a concern of food manufacturers and one of the major obstacles preventing the development of barley-based or barley-incorporated food products. The undesirable dark discoloration reported in these products as observed in white salted noodles incorporated with barley flour could result from enzymatic or non-enzymatic reactions, or a combination. Non-enzymatic browning results from polymerization of endogenous phenolic compounds and from the Maillard reaction. Enzymatic browning is the discoloration that is caused mainly by PPO activity.

Barley grains contain a much greater amount of phenolic compounds (0.2–0.4%) than other cereal grains (Bendelow and LaBerge, 1979). Barley grain phenolics are composed of polyphenols, phenolic acids, proanthocyanidins (PAs) and catechins, and are mostly present in the hull, testa and aleurone (Nordkvist *et al.*, 1984). PPO is found throughout the barley kernel (Jerumanis *et al.*, 1976) and may contribute to the oxidation of phenolic compounds, inducing dark-gray color in barley food products, as observed in many fruits and vegetables, as well as, Asian noodles. Large

variation in both total polyphenol content and PPO activity among barley genotypes has been shown (Quinde *et al.*, 2004). Total polyphenol content was about 0.04% in hulled PA-free genotypes, while it ranged from 0.13 to 0.22% in hulled PA-containing and hulless genotypes. Dimeric PAs are the major phenolic compounds of barley grain, while monomeric PAs, including catechin, appear to be more effective substrates of PPO for discoloration of barley flour dough than dimeric and trimeric PAs (Quinde-Axtell and Baik, 2006).

Quinde-Axtell *et al.* (2005) determined the food discoloration potential of different classes and genotypes of barley and evaluated the relationships between chemical constituents of grain and dark color development in cooked pearled barley grain, barley flour paste and dough. Differences in the brightness of cooked grain, flour paste and dough were evident among all types of barley. Hulled PA-free genotypes generally produced brighter (greater L^* , a spectrophotometer measure unit) cooked grain, gel and dough than PA-containing and hulless genotypes. They found negative relationships between the total polyphenol content of grain and the brightness of cooked grain, paste and dough, whereas there was no relationship between PPO activity and brightness of cooked grain, paste or dough. Initially, the primary interest in the proanthocyanidin-free trait was for brewing quality. Beer made from PA-free barley malt eliminates the need to stabilize beer against permanent and chill haze (Wettstein *et al.*, 1985). However, it turns out that this trait is also a significant positive factor in white color formation and stabilization in barley food products.

The effects of genotype on dough brightness, total polyphenol content and PPO activity appeared to be much greater than those of environment (Quinde-Axtell *et al.*, 2005), indicating that through genotypic improvement, discoloration of barley food

products can be effectively controlled. However, barley grown in low-precipitation environments tends to have higher total polyphenol content, lower PPO activity, and darker color of flour dough than barley grown in other environments. The above discussion about color issues in barley food products notwithstanding, the current trend to incorporate increasing levels of whole grain foods into the diet for health benefits and taste should reduce color preference biases.

Conclusions

Barley is one of the most ancient crops, and it has evolved through domestication to its status today as a major world crop, based on acreage and production. Its evolution has carried it from principally a food crop to mainly a feed and malting/brewing/distilling crop. Whereas relatively little barley is used directly for food today (discounting the role of beer in the human diet), it has great potential to reclaim some of its prominence as a food grain, largely due to its high nutritional value. Barley grain provides low fat, highly digestible carbohydrates (mainly starch) for energy, relatively well-balanced protein to meet amino acid requirements, minerals, vitamins, especially vitamin E and other antioxidants, primarily polyphenolics, and insoluble and soluble fiber with general and specific health benefits. The endosperm cell walls are uniquely rich in beta-glucans that positively affect serum cholesterol and glucose levels, that in turn affect cardio-vascular health and diabetes control, respectively. The USA Food and Drug Administration recently issued a health benefit endorsement for barley based on beta-glucan effects on lowering blood cholesterol. High fiber and/or other components also have a satiety effect, which can positively affect weight control, as well as speed up passage of food in the colon.

Whereas, much is known about the nutritional and health benefits of barley

consumption, much less is known about the functionality of barley grain components in terms of processing and food product and ingredient development. Aspects of the physical and chemical nature of the kernel,

including fiber, color, texture and hardness, present opportunities and challenges for use of barley for food. Barley is more difficult to mill into flour than wheat, for example. Color development and dynamics make barley more

Table 1. Barley grain traits for food uses.

Grain Traits	Significance/Role in	Desired Quantity or Quality	Some Key References
Physical			
Grain Size and Shape	Malting, pearling, milling	Plump, uniform, round	Pomeranz, 1974
Color	Malting, pearling, milling	Bright yellow-white	Li <i>et al.</i> , 2003
Hull	Malting, pearling, milling	Thin or absent for food uses	Pomeranz, 1974
Crease	Pearling, milling	Shallow depth	Jadhav <i>et al.</i> , 1998
Bran	Pearling, milling	Non-shattering, tough & leathery	Jadhav <i>et al.</i> , 1998
Flake formation	Roller milling	Absent	Jadhav <i>et al.</i> , 1998
	Malting, pearling, milling, flour particle size, water imbibition, cooking	Soft for malting and milling; Hard for pearling; Unknown for other food uses	Allison <i>et al.</i> , 1976; Allison, 1986; Swanston and Taylor, 1988; Swanston, 1995; Brennan <i>et al.</i> , 1996; Edney <i>et al.</i> , 2002
Chemical			
Protein	Foam production/stability in beer; essential amino acids	10–12% protein for brewing, high lysine proportion	Newman and McGuire, 1985; Munck, 1992; Doll <i>et al.</i> , 1974; Ullrich and Eslick, 1978
Amylose	Cooking; physical properties of processed products	Low for rice extender, noodles and tortilla; high for extrusion	Zheng and Sosulski, 1998; Baik and Czuchajowska, 1997; Lagassé <i>et al.</i> , 2006; Baik <i>et al.</i> , 2004b; Ames <i>et al.</i> , 2006
Soluble beta-glucan	Cholesterol, serum glucose, diabetes and body weight reductions; food processing and product quality; brewing process	High content for nutritional aspects; little unknown functionality for food uses; low content for brewing	Qureshi <i>et al.</i> , 1986; Newman <i>et al.</i> , 1989; Wood <i>et al.</i> , 1990; Bamforth and Barclay, 1993; Fastnaught, 2001; Cavallero <i>et al.</i> , 2002; Behall <i>et al.</i> , 2004.
Insoluble fiber	Satiety / weight control / food passage in colon	High content for high satiety / weight loss / increase of faecal bulk/ constipation alleviation	Liljeberg <i>et al.</i> , 1999; Lazaridou and Biliaderis, 2007
Tocols	Cholesterol reduction; antioxidant	High	Qureshi <i>et al.</i> , 1991; Peterson and Qureshi, 1993
Polyphenol oxidase	Food color	Absent	Jerumanis <i>et al.</i> , 1976; Quinde <i>et al.</i> , 2004; Quinde <i>et al.</i> , 2004; Quinde-Axtell and Baik., 2006; Bendelow and LaBerge, 1979; Nordkvist <i>et al.</i> , 1984
Polyphenols	Food color, Haze in beer	Absent	

difficult to work with than rice, for example. Nevertheless, barley can substitute partially or wholly for wheat in many unleavened baked products, for cooked polished rice, and for oats and maize in cold and hot breakfast cereals. In fact, there is a trend for increased use of barley in particular in whole grain bread and breakfast cereal products.

Although, there is much to learn about barley food quality traits, there is enough information, including genetic information, to significantly improve barley for food use through breeding (Table 1). Some information about genetic control of beta-glucan and protein levels, kernel color and hardness, waxy and high-amylose starch exists and can provide breeding targets. Genetic improvement could involve more drastic changes than discussed above, especially through transformation. Barley development or malting (germination) processes could turn barley into a “chemical biofactory” to over-produce pharmaceuticals or nutraceuticals, or even naturally occurring biochemicals such as lysine, vitamins, etc. In this situation the barley grain or plant would be fractionated to concentrate target chemicals, natural or transformed.

Barley is secure in uses for feed, malting, and food, and the potential is great to improve barley for all these uses. The future of barley for use in food products is increasing and very promising.

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Food quality evaluation of an international collection of barley landraces

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Abstract

Barley has been a staple food since its domestication 10 000 years ago in the Fertile Crescent. Traditionally, it has been utilized in various ways, particularly in marginal regions, according to the local culture, with typical breads, drinks and dishes often prepared from local barley landraces. This project consists of assessing food quality richness of these landraces through the evaluation of 584 barley landraces from 42 countries, evaluated in two different environments and assessed for several food quality traits. These traits were beta-glucan content (ranging from 4.93 to 7.67%), protein content (7.18 to 20.23%), hardness (very hard to very soft), husk percentage (0 to 46%), aleurone color (white, green, blue or black) and 1000-kernel weight (19.3 to 64.5 g). Principal components analysis associated with 3D categorized plots applied to the landrace data revealed groups with specific quality characteristics associated with specific barley food products.

Introduction

Historically, barley had a reputation for building strength, and it was used by the gladiators of the Roman Empire in their training diet in the form of bread (*pulmentum*). It has been the energy food of the masses, especially in regions characterized by harsh living conditions and low-output production systems typical in developing countries. Preferentially, the local landraces are used

for traditional recipes (e.g. *Aballagh* in Morocco, *Tsangpa* in Tibet, *Machica* in Ecuador, *Gofio* in Canary Islands and *Giotta* in Switzerland). However, barley is still rarely used for food purposes when one compares global production and trade (Figure 1). Since the finding of barley health benefits linked to barley's content of soluble dietary fiber (beta-glucan) that reduces risk of coronary heart disease, elevated blood cholesterol and glucose levels, and reduces obesity via increased satiety, the use of barley grain, rather than wheat and rice (Figure 2), as a food or food ingredient has increased gradually over time in developed countries through the development of various food barley products made from improved varieties. From these products, which are barley flakes, pearl and pot barley, barley grits, waxy barley, hullless barley and barley flour, several dishes and drinks are prepared (porridge, granola, muesli, cookies, muffins, desserts, salads, soups, puddings, stews, casseroles, pancakes, breads, pasta, biscuits, pizza crusts, coffee, tea, etc.). Recipes for several dishes having barley as a main ingredient can be freely obtained online. Barley landraces are the result of natural and farmer selection pressure over many generations and are still preferred to improved varieties for making traditional foods, mainly in harsh regions, because probably the selection was in favor of food quality. Thus, assessing a collection of international barley landraces to eventually find promising examples for food uses has a major importance.

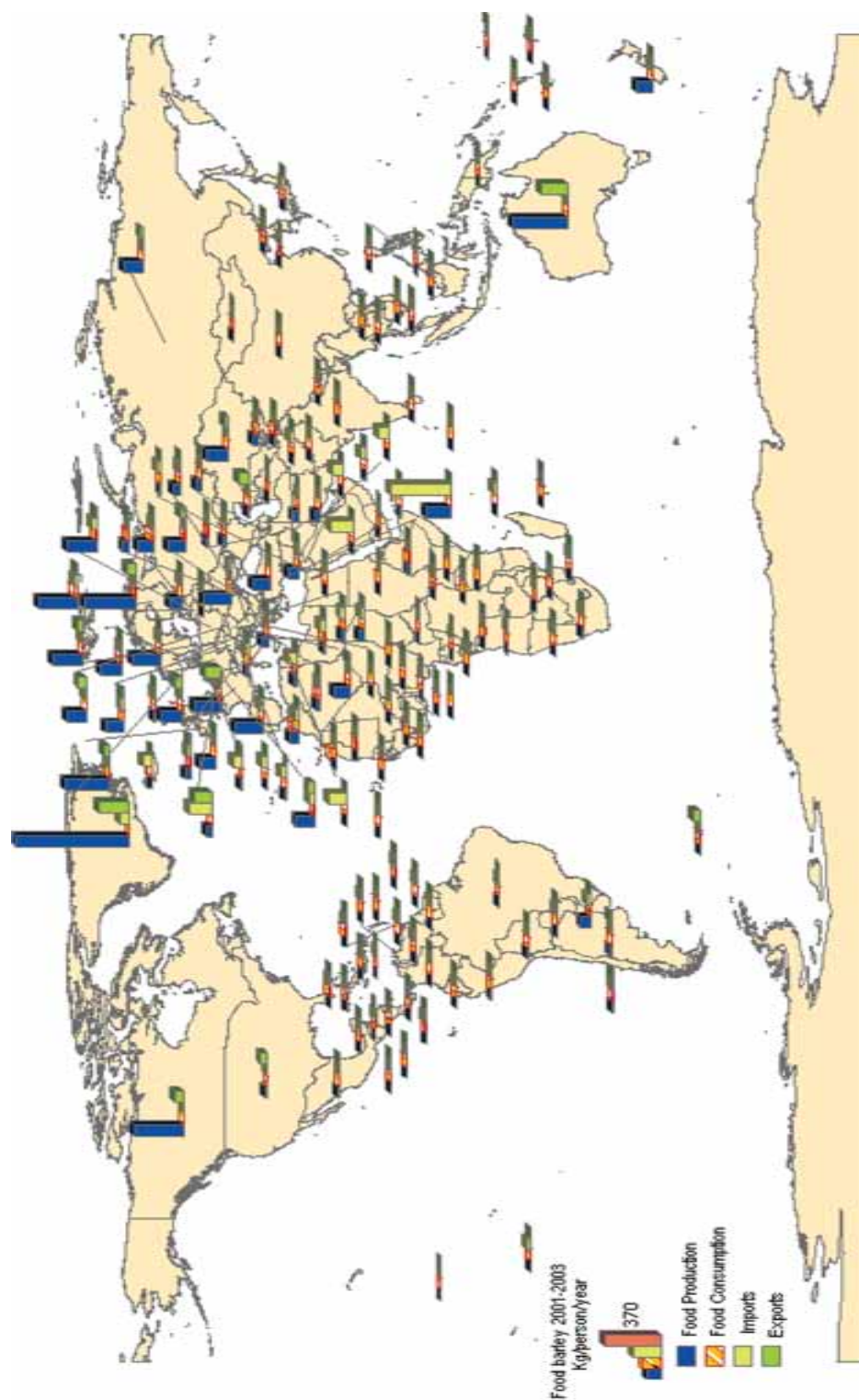


Figure 1. Worldwide food barley situation 2001–2003 in kg/person/year (FAO Data from http://www.fao.org/es/ess/faostat/foodsecurity/index_en.htm).

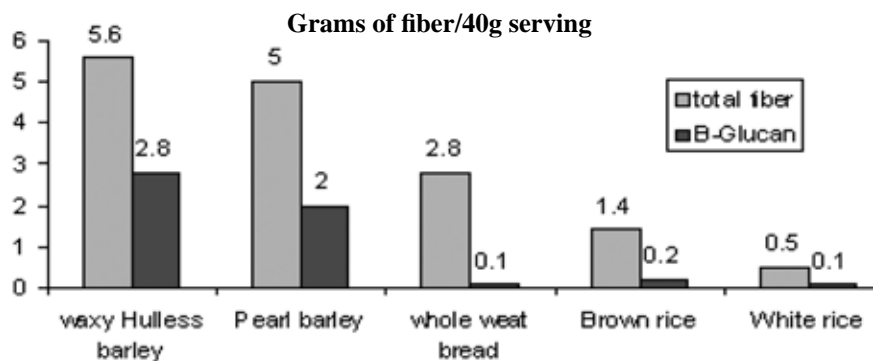


Figure 2. Amount of fiber and beta-glucan serving per product (Conway, 2007).

Materials and methods

The 584 barley landraces investigated were from ICARDA’s gene bank and represented an international selection of germplasm with various morphological, agronomic and physiological characteristics. They were split into two trials, using spatial analysis for an unreplicated design (Row × Column design), and implemented at two of ICARDA’s experiment stations (Tel Hadya, a favorable site, and Breda, an unfavorable site). The trials were:

- Diverse Barley Germplasm (DBG): 246 entries from 42 countries, including 36 accessions of *Hordeum spontaneum* and 4 improved varieties used as repeated checks.
- Landrace collection (LAND): 338 landraces from 34 countries, with 4 improved varieties as repeated checks.

Sample origin and their number are shown in Figure 3.

Several quality parameters have been evaluated for the whole set, namely: husk%, estimated by de-hulling 20 g of barley grains with the barley pearler, 1000-kernel weight (TKW) using grain counting apparatus; aleurone color (Aleur. C.) scored visually from pearled grain; hardness (Hard) by shaking 5 g of barley flour in sieve shaker of 200 mesh sieves; protein content (P%); and beta-glucan content (BG%), extracted from the calibration of Near Infrared spectrometry for this parameter (Table 1).

The data were analyzed using spatial analysis for an unreplicated design with Genstat software, and the Best Linear Unbiased Predictors (BLUPs) were calculated. The subsequent statistical

Table 1. Range and evaluation tools of the screened quality parameters.

	Husk %	Aleurone color	TKW	Hardness	P %	BG %
Class 1	<5 Very Low	White	15–25 g Very Small	30–40% Very Hard	<9 Very Low	<5 Very Low
Class 2	5–10 Low	Green	26–35 g Small	41–50% Hard	9–11.5 Low	5–5.5 Low
Class 3	10–15 Medium	Blue	36–45 g Medium	51–60% Medium	11.6–13.5 Medium	5.6–6 Medium
Class 4	15–20 High	Brown	46–55 g Large	61–70% Soft	13.6–15.5 High	6.1–6.5 High
Class 5	>20 Very High	Black	> 55 g Very Large	> 70% Very Soft	15.6–17.5 Very High	6.6–7 Very High
Class 6					>17.5 Extra High	>7 Extra High
Tools	Barley pearler	Visual on pearled barley grain	Seed counter	Sieve shaker with 200 mesh sieves	Near Infrared spectrometry	Near Infrared spectrometry

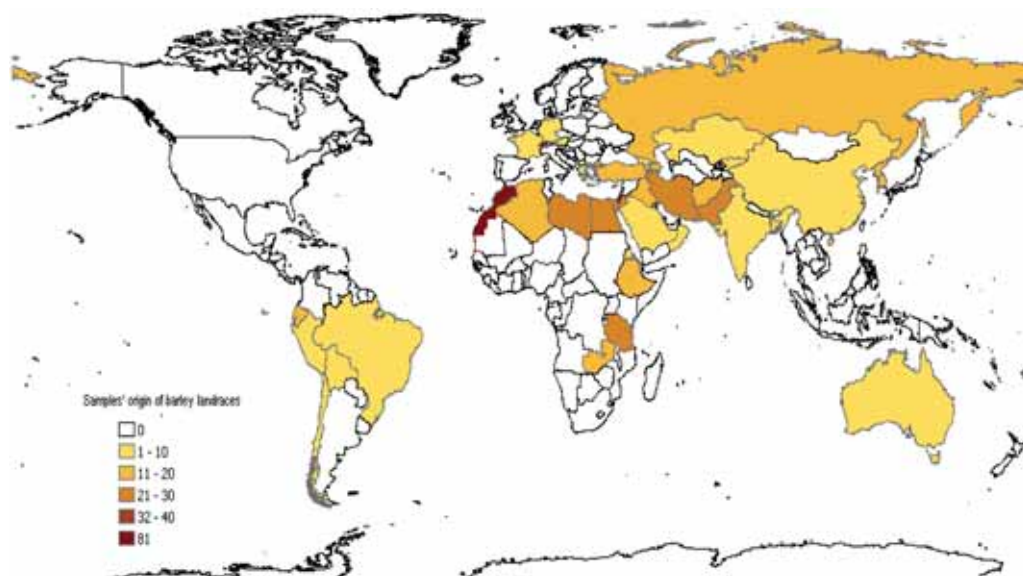


Figure 3. Origin of barley landraces samples used in the evaluation.

analyses were conducted on the BLUPs, using SPSS and PAST for analysis of variance and principal component analysis, while STATISTICA software was used for simultaneously graphing all the quality parameters.

Results and discussion

The mean values at both experiments stations are shown in Figure 4. As depicted, the mean values for husk percentage and protein content were high on the unfavorable site, unlike TKW. Hardness and beta-glucan content differed slightly between the two environments. In the unfavorable environment, less rainfall associated, with high temperature, affected grain filling, which led to low TKW negatively correlated with protein content. High husk percentage values in the raw data were mainly observed in the wild species *H. spontaneum*.

Analysis of variance of the quantitative data of the five quality parameters for LAND and DBG materials showed significant differences between sites for all the quality parameters (Table 2).

Based on Table 1, the numbers of samples for class of the quality parameters are presented in Table 3. Out of the 584 samples, 11 samples of LAND material have medium hard kernels, while the rest have hard kernels. Protein content was mostly low to medium in both materials. Beta-glucan content had as values medium, high and very high, and thus favorable for food.

With PAST software, the two principal axes of the principal component analysis for LAND and DBG quantitative data (Husk%, Hard, TKW, P% and BG%) explained respectively 96.03 and 98.13% of the variance (Figures 5 and 6).

As shown in Figures 5 and 6, hardness, protein and beta-glucan content grouped together with a significant number of samples far from husk% (undesirable for food consumption). Unfortunately, it is difficult to group the individuals according to their quality parameter class. As all the samples for both DBG and LAND were hard to medium hard, considering the parameter (hardness) as a fixed factor, the 3D categorized plots of STATISTICA software illustrated in Figures 7 and 8 depicted the combination of the five

Table 2. Analysis of variance of the five quality parameters across sites.

	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
DBG material	Husk%	4682.507	1	4682.507	2394.457	0.000
	Hardness	48.442	1	48.442	49.354	0.000
	TKW	2164.862	1	2164.862	109.339	0.000
	Protein%	1104.437	1	1104.437	4517.770	0.000
	beta-glucan%	8.319	1	8.319	1023.179	0.000
	beta-glucan%	8.319	1	8.319	1023.179	0.000
LAND material	beta-glucan%	8.319	1	8.319	1023.179	0.000
	Husk%	226.265	1	226.265	7.275	0.007
	Hardness	23.188	1	23.188	861.542	0.000
	TKW	255.615	1	255.615	5.653	0.018
	Protein%	332.052	1	332.052	146.444	0.000
	beta-glucan%	54.354	1	54.354	1017.241	0.000

Table 3. Distribution of barley samples according to the appropriate class for LAND and DBG materials.

Class	Dependent						Type III Sum of					
	Aleur. C.	Husk%	Hard	TKW	P%	BG%	Aleur. C.	Husk%	Hard	TKW	P%	BG%
1	131						93	10		3		
2	1	7	327	31	196		39	1	246	53	64	1
3	164	328	11	265	142	1	87	84		131	139	92
4	15	3		42		336	9	117		56	30	142
5	27					1	18	34		3	12	11
6											1	
Total	338	338	338	338	338	338	246	246	246	246	246	246

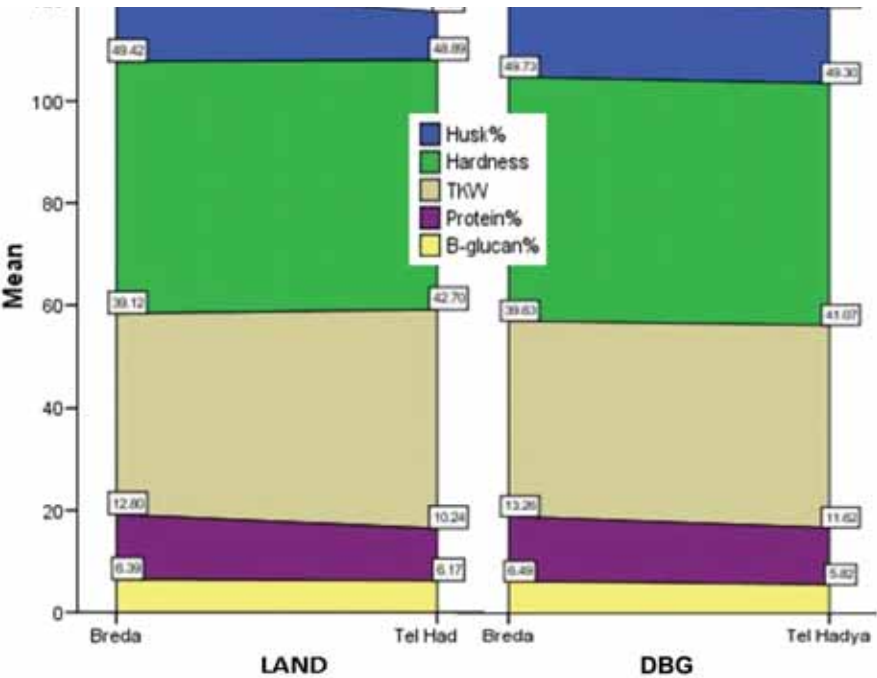


Figure 4. Range values of LAND and DBG materials in both Tel Hadya and Breda experiment stations.

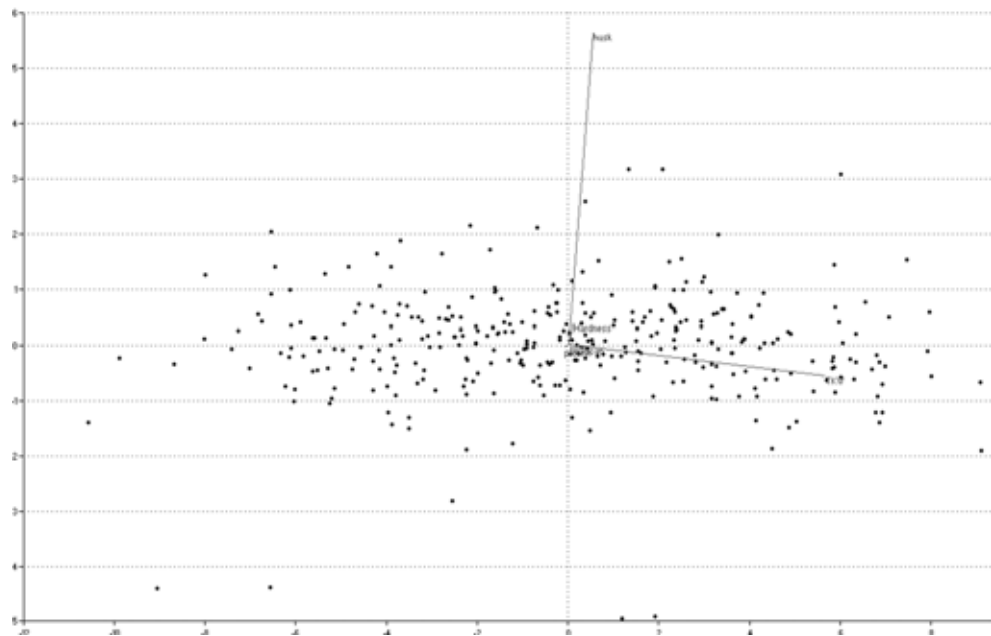


Figure 5. Principal Component Analysis of LAND material.

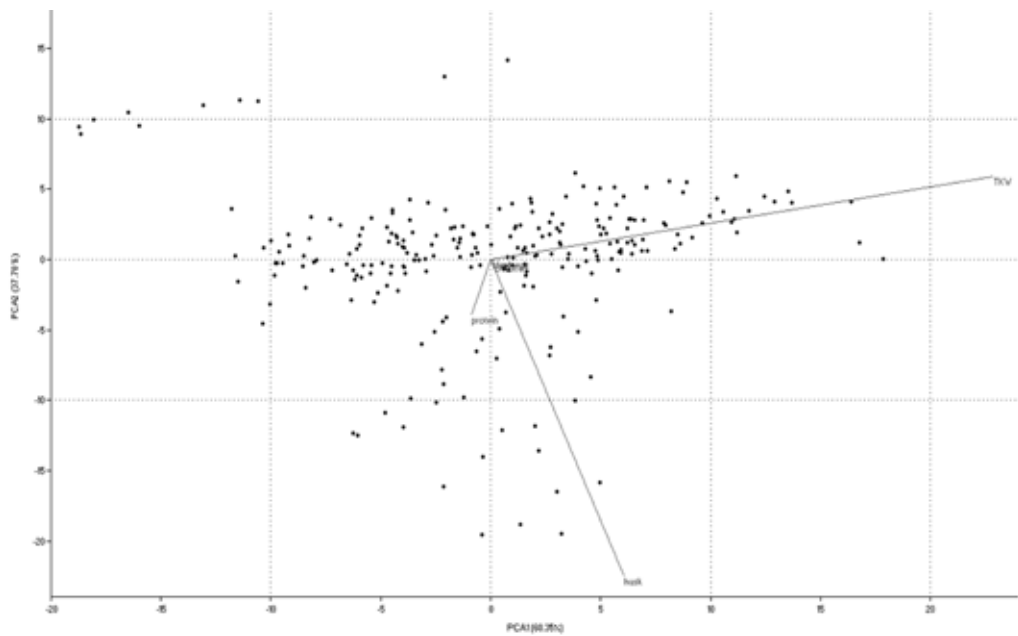


Figure 6. Principal Component Analysis of DBG material.

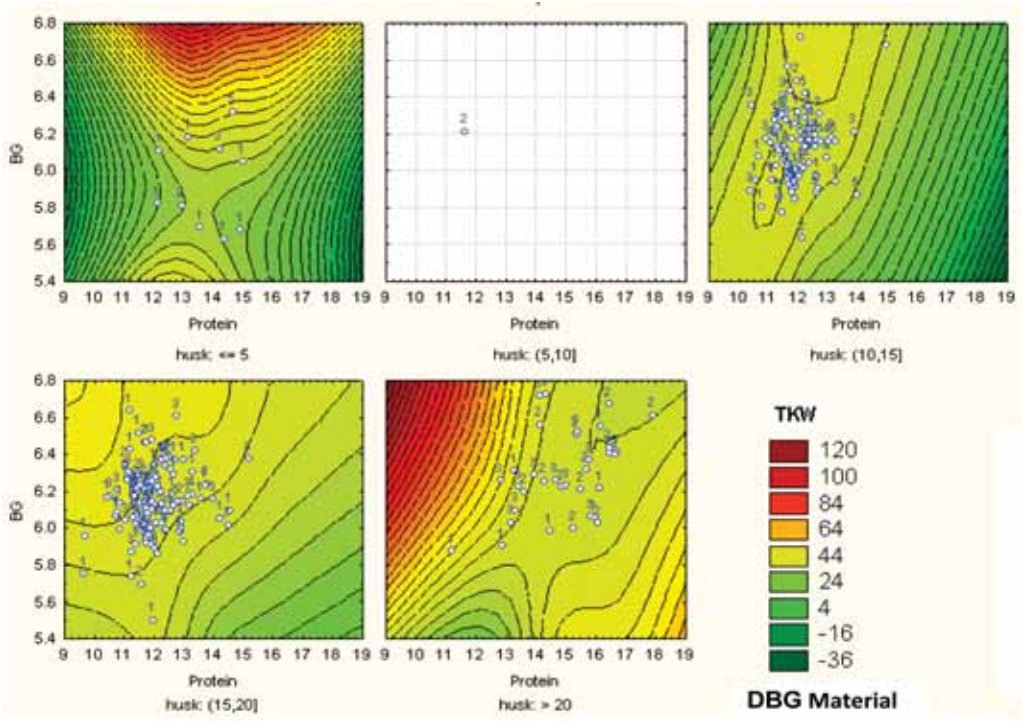


Figure 7. Categorized plot of the quality parameters of DBG material.

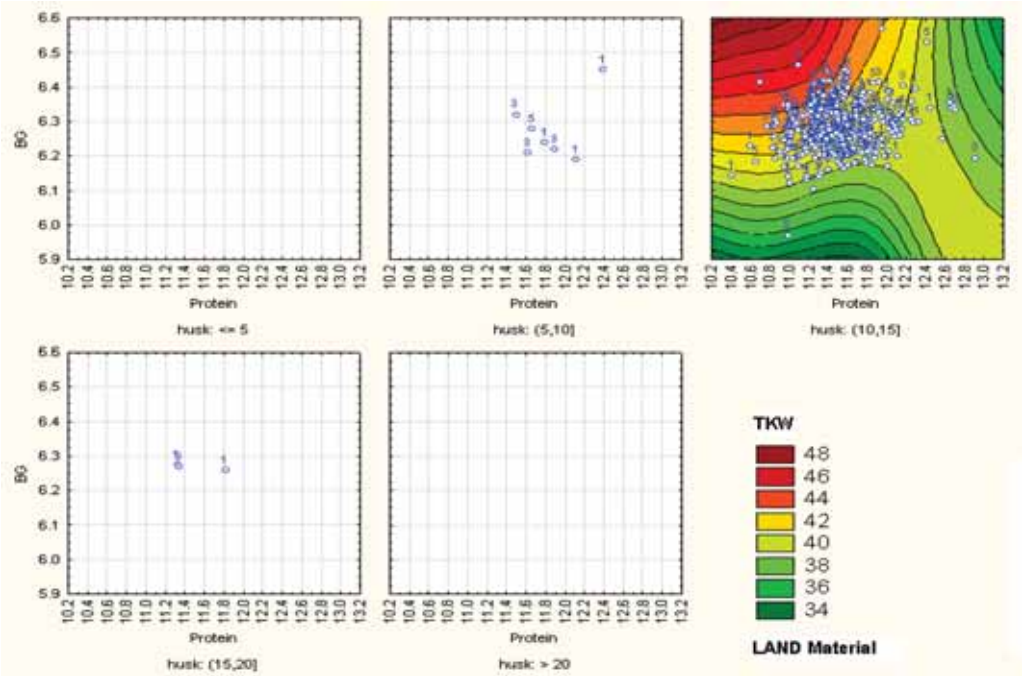


Figure 8. Categorized plot of the quality parameters of LAND material.

quality parameters (Husk%, Aleur. C. showed as class numbers, TKW, P% and BG%) in order to disclose the appropriate samples suitable for barley products. As an example and according to MacGregor and Bhatti (1993), pearled barley is defined as low husk, medium to hard kernel, TKW of 45 g, white aleurone with a protein content of 10% and 5% of beta-glucan content. We can observe that in the two first graphs in both Figures 7 and 8 (husk<10%), 4 samples respond to the criteria listed above in DBG material and one sample in LAND material.

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Effect of beta-glucans and starch type on glycaemic response after consumption of barley bread

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Abstract

Barley is the world's fourth most important cereal after wheat, rice and maize. This cereal can be moreover appreciated for its potential beneficial properties when utilized as "functional" food. "Functional foods" may provide health benefits after consumption of appropriate quantities in a normal diet. Barley contains beta-glucans, polysaccharides that are very useful in reducing post-prandial glycaemic index and blood cholesterol. The aim of the research was optimization of the enrichment process for beta-glucan content and the identification of barley genotypes that best adapt to the enrichment process, with the prospect of producing enriched bread and to test the *in vivo* biological properties of this new food. Hulled, hullless and waxy barley genotypes were compared. Among the barleys tested, hullless, in particular waxy, genotypes accumulate more beta-glucans, and the enrichment process could separate a beta-glucan-rich flour fraction. This aspect could be exploited to develop food with a reduced glycaemic index.

Barley as functional food

Barley is the world's fourth most important cereal after wheat, rice and maize. Barley is commonly used for animal feed and malting production, but in recent decades this cereal has become increasingly appreciated for its potential beneficial properties when utilized as "functional" food. The term "functional

food" implies a food that may provide health benefits after its consumption of suitable quantities in a normal diet. Cereals can be easily used as ingredients for the production of "functional foods", as they contain several groups of compounds of strong nutritional importance (mainly vitamins, fitosterols, antioxidants, soluble fiber; Liu, 2007). Barley in particular is rich in soluble dietary fiber, thanks to its (1→3)-(1→4) mixed linked beta-glucans; beta-glucans are non-starchy polysaccharides that represent the major structural components of endosperm cell walls (MacGregor and Fincher, 1993). The consumption of barley can reduce the rate at which glucose is released to the blood (Björck *et al.*, 2000) causing a reduction in the Glycaemic Index (GI). The GI is a measure of the increment in blood glucose concentration that occurs after ingestion of food rich in carbohydrates. A diet with a low GI, based on the consumption of whole cereals, with high levels of dietary fiber (particularly soluble fiber), is associated with reduced risk of development of type 2 diabetes and coronary heart disease (Frost *et al.*, 1999; Liu *et al.*, 2000; Liu, 2002). Bread is generally considered high glycaemic food (Fardet *et al.*, 2006) because of its high starch and low fiber contents, but incorporation of beta-glucans derived from barley can be useful to reduce its glycaemic response (Cavallero *et al.*, 2002).

The level of beta-glucans varies among genotypes (mostly from about 2% to 10%;

Baik and Ullrich, 2008). Hulless barleys often have high beta-glucan contents (Bhatty, 1992) and are mainly used as human food because of ease in processing and their edibility.

Barley genotypes also vary in the amylose/amylopectin ratio; both waxy starch (with up to 100% amylopectin) and high-amylose (over 35%) varieties are known. Interestingly, these genotypes with anomalous starch composition contain higher beta-glucan levels (Baik and Ullrich, 2008; Izydorczyk and Dexter, 2008).

The objective of the research was the optimization of the enrichment process for beta-glucan content and the identification of barley genotypes that best adapt to the enrichment process (Ferrari *et al.*, 2009) with the target of producing enriched bread and testing the *in vivo* biological properties of this new food.

Beta-glucan enrichment

In recent decades several researcher groups have investigated how to enrich flours in terms of beta-glucans. Barley fractions enriched in beta-glucans can be obtained by extraction with aqueous solvents, as reported by Cavallero *et al.* (2002): they obtained an enriched fraction by water extracting the barley flour coarse fraction with subsequent centrifugation and freeze-drying of the supernatant. However, such processing would be highly energy-intensive, and sometimes they require the use of non-edible solutions, like NaOH or organic solvents, residues of which are prohibited in human foods. Another way to increase beta-glucan content is milling and sieving of flours to discriminate beta-glucan rich fractions (Bhatty, 1992; Sundberg and Åman, 1994; Wu *et al.*, 1994; Knuckles and Chiu, 1995; Yoon *et al.*, 1995; Kiryluk *et al.*, 2000; Andersson *et al.*, 2000; Izydorczyk *et al.*, 2003). Air classification was sometimes reported to be more effective (Wu *et al.*, 1994; Vasanathan and Bhatty, 1995; Andersson *et al.*, 2000; Wu and Doehlert, 2002), but several steps had to be used to

reach a good beta-glucan increment in the flour.

At the CRA-GPG laboratories, a beta-glucan enrichment system was optimized, based on micronization of barley grains and subsequent air classification of flours, to improve both beta-glucan concentration and flour yield, with few air classification steps.

By means of an industrial apparatus, it was possible to operate multiple micronizations (fine milling technique, in which grains vortically rotate with strong collisions, disaggregating to very refined flour) and an air classification (turbo separation, based on particles' dimensions and specific weights). The apparatus sorted the flour into two portions: a coarse fraction (CF) and a fine fraction (FF). For every flour fraction, total beta-glucans were determined.

This enrichment method is based on a series of sortings of micronized flour (Figure 1), with a progressive increase in the yield of a selected fraction; a curve for beta-glucan enrichment vs. yield can then be calculated and the most suitable combination of yield and beta-glucan content can be chosen.

We tested this approach on different barley varieties (hulled, hulless and waxy) (Table 1).

As shown in Table 1, winter genotypes had better production, but the highest concentration of beta-glucans were reached by two spring hulless barleys (Priora, with normal starch, and CDC Alamo, with waxy starch). All genotypes were processed as reported in Figure 1 and the beta-glucan enrichments obtained are reported in Table 2. With this enrichment process we were able to separate out barley flour fractions with twice the beta-glucan concentration of the grain (Table 2).

The flour yield of the enriched fraction was good (about 30%) and similar among all the genotypes tested. The best results were obtained by cultivars CDC Alamo and Priora, whose enriched flour fractions had 15.7% and 11.2% beta-glucans, respectively, and gave a good flour yield (about 30%; Table 3).

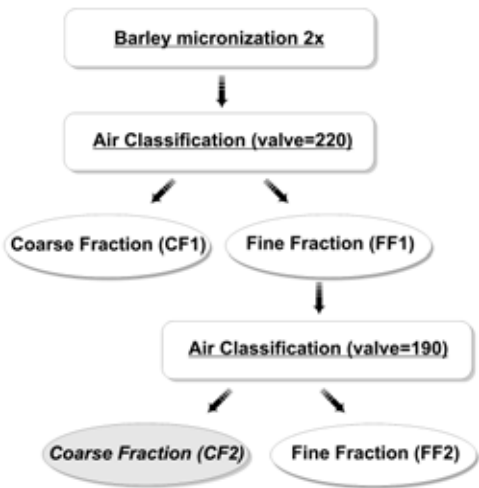


Figure 1. Flow-sheet of the enrichment process optimized for cv. Priora. The final enriched fraction is colored.

Air classification of barley flour has been reported in the literature, but most of the processes proposed require many cycles to reach a good beta-glucan enrichment, and in some cases with a only a low yield. Andersson *et al.* (2000) used air classification with seven different barley cultivars. In most cases, the beta-glucan content was doubled, but the flour yield was low, less than 20%. A better result was obtained with the hulless waxy cultivar Prowashonupana, which is a barley genotype that, although of poor agronomical performance, has a very high beta-glucan level.

However, the air classification system adopted by Andersson *et al.* (2000) was not very effective; the enriched flour fraction had a yield of about 33%, but despite the very high initial beta-glucan content (17%), it was only enriched to a content of about 22%.

Table 1. Production and beta-glucans content of the barley genotypes tested in the work. Values are means over three years (Fiorenzuola d’Arda, Italy, 2003–2005).

Genotype	Sowing	Caryopsis	Production (t/ha)	Beta-glucans (%)
Zacinto	autumn	Naked	5.2	3.8
Aiace	autumn	Hulled	6.9	4.6
Nure	autumn	Hulled	6.4	3.5
FIOR 7344	autumn	Naked	6.1	4.1
Rondo	spring	Naked	2.2	5.1
Priora	spring	Naked	2.4	5.4
CDC Alamo	spring	Naked	2.6	7.8
Tidone	spring	Naked	3.6	3.9
LSD _(0.05)			1.3	0.8

Table 2. Beta-glucan enrichment and flour yield of the barley genotypes processed using the protocol shown in Figure 1.

Genotype	Beta-glucan content (%)	
	Micronized flour	Air classified flour
Zacinto	3.9 ± 0.1	7.6 ± 0.3
Aiace	4.6 ± 0.1	7.4 ± 0.2
Nure	3.4 ± 0.1	6.7 ± 0.3
FIOR 7344	4.1 ± 0.1	9.0 ± 1.4
Rondo	5.1 ± 0.4	7.7 ± 0.3
Priora	5.4 ± 0.1	11.2 ± 0.6
CDC Alamo	7.8 ± 0.3	15.7 ± 0.3
Tidone	3.9 ± 0.0	6.1 ± 0.1

Table 3. Beta-glucan and flour yield of barley cultivars CDC Alamo and Priora. CF1 and CF2 are the coarse fractions from the first and second air-classification, respectively; FF2 is the fine fraction from the second air classification.

Genotype	Flour fraction	Yield (%)	Beta-glucans (%)
CDC Alamo	Micronized	—	7.8
	CF1	10.4	11.1
	CF2	28.4	15.6
	FF2	61.2	2.3
Priora	Micronized	—	5.4
	CF1	16.5	8.1
	CF2	29.8	11.2
	FF2	53.7	3.0
LSD _(0.05)			0.7

In contrast, Wu *et al.* (1994) obtained, from Prowashonupana (17.4% beta-glucans), a fraction with 31% beta-glucans and a yield of 62%, but de-fatting with hexane was required before processing the flour. The same authors used air classification to obtain an enriched flour fraction with 14.6% beta-glucans and a flour yield of 27.3%, using the hullless high beta-glucan (8.0%) barley CI 4362. This latter enrichment is similar to those presented in this work, though Wu *et al.* (1994) used four air-classification steps to reach the same results, while we used only two steps. Vasanthan and Bhatta (1995) obtained final coarse fractions with 13–24% beta-glucans. However, they obtained the best result (enriched flour with 23.8% beta-glucans) with the waxy cultivar SB89528, which gave a yield of only 7.6%. Similarly, a flour fraction with 13.1% beta-glucans and 10.4% yield was obtained with the normal-starch barley cv. Condor.

Optimization of roller milling and sieving using high beta-glucan varieties allows one to obtain fiber-rich fractions with yields >20% and beta-glucan contents >15% (Izydorczyk *et al.*, 2003). These results are similar to those obtained from air classification. It therefore seems that in the continuous process of technical improvement, both sieving and air classification are tending to produce similar results. The critical point is the fine milling of the grain: once flour milled to a sufficiently fine degree has been obtained, both techniques

appear to be able to separate out comparable beta-glucan enriched fractions.

Perspectives

As evident from our research, waxy barley is associated with a high level of beta-glucans and the enrichment process described above could separate out a beta-glucan-rich flour fraction. This could be exploited to develop food with a reduced glycaemic index. In particular, the enriched flour fraction could be used in a blend with wheat flour to obtain bread with a high concentration of beta-glucans. The separation of a flour fraction rich in beta-glucans allows use of smaller quantities of barley flour (which could make worse the rheological behavior of the blend during bread making), but at the same time providing a high concentration of beta-glucans. In the literature, a waxy barley (cv. Prowashonupana) was used, which had a very high beta-glucan concentration, and it was effective in reducing the glycaemic index of bread. However, this barley cultivar has poor yields. The naked waxy barley used in this work, in contrast, had good production performance (see Table 1), although its beta-glucan content was lower than cv. Prowashonupana. At the same time, the starch quality, i.e. the amylose/amylopectin ratio, is another factor that could affect the glycaemic response. In fact, in the literature,

it is reported that waxy starch causes greater glycaemic response than high-amylopectin starch (Kabir *et al.*, 1997). It would be very interesting to assess if this trait rather than the beta-glucan concentration could have more influence on the Glycaemic Index of food made with waxy barley.

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Barley breeding and its potential impact on human health and nutrition

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Abstract

The frequency of heart disease, colorectal cancers, type II diabetes and obesity are steadily increasing in westernized societies, and also rising in developing countries. Such diseases are causing major public health problems, which are becoming a high priority concern for governments worldwide. A viable approach for improving public health is to make appropriate changes to food products, to give substantiated health benefits while retaining consumer appeal. Consequently, cereal grains have become prime candidates for such modifications. Numerous studies have indicated small alterations in grain composition (e.g. starch, beta-glucan and fiber) can deliver significant health benefits once integrated into food. Processing techniques such as pearling, milling and flaking allow barley to be utilized in a variety of food products. Availability of hulless barley can provide manufacturers with a fast cooking grain (whole or pearled), excellent pearling and flaking quality and a higher soluble fiber (or beta-glucan) content. Hulless barley breeding at the Waite Campus specifically aims to develop hulless types with these high value-added traits for the food industry. We have compared the physical grain quality, pearling, baking and flaking quality of Australian waxy hulless barley with other hulless and covered barleys. These quality parameters have been investigated to a lesser extent for high amylose hulless barley. The quality attributes of these hulless barleys will be discussed in the context of potential products and market opportunities. Recent progress in improving the agronomic

performance of hulless barley to support economically viable crop production will also be presented.

Introduction

The frequency of type II diabetes, heart disease, colorectal cancers, and obesity are steadily increasing in westernized societies, and also rising in developing countries. Such diseases are causing major public health problems, resulting in these issues becoming a high priority for governments worldwide. A viable approach for improving public health is to make appropriate changes to food products to give substantiated health benefits while retaining consumer appeal. Consequently, cereal grains have become prime candidates for such modifications. Numerous studies have indicated small alterations in grain composition (e.g. starch, beta-glucan and fiber) can deliver significant health benefits once integrated into food (Regina *et al.*, 2007). Consequently, as dietary fiber is identified as being an important component of the human diet, the potential of barley as a food grain is beginning to be recognized. The Food and Drug Administration's final ruling on 22 May 2006 supports this by adding barley as an additional source of beta-glucan.

Barley contains two types of starch: amylose and amylopectin. Three main groups of barley types have been identified with respect to their amylose content: Low amylose or 'waxy' (0–10%), normal amylose (~25%) or high amylose (>35%). The waxy gene, *wx* (formerly called *glx* or *wx*) is located on chromosome 7HS. The waxy starch phenotype is conferred by various mutations

at this locus that either preclude transcription or encode an inactive form of the starch synthase enzyme. The high-amylose gene, *amol* is located on chromosome 1H. The genes controlling both these starch mutants are recessive in their expressions.

The endosperm of waxy barley has an inherently bright white appearance, which is a desirable feature for pearled barley and products derived from milling. In addition, the changes that waxy starch undergoes when it is heated with water are responsible for the unique character of many of our foods. Some examples are the viscosity and mouth-feel of gravies, and texture of some confectionaries, pie fillings, and, to a lesser extent, baked products. Furthermore, waxy barley starch gelatinizes at lower temperatures and has better freeze-thaw stability.

Compared with other cereals, barley contains high levels of beta-glucan, which are important contributors to dietary fiber. The waxy gene tends to be associated with elevated levels of (1→3, 1→4)-beta-D-glucan. High beta-glucan content in food has been shown to have numerous health benefits, including lowering blood cholesterol and reducing colon cancer. High fiber content in foods also gives the consumer a feeling of 'full-ness' aiding in satiety (Kim *et al.*, 2006), and, as a consequence, may be useful in weight control.

Equally, hulless barley with high amylose starch also has the potential to make an important contribution to improving human health, particularly the prevention and management of type II diabetes, heart disease, colon and rectal cancers, obesity, constipation and diverticular disease. In Australia, the benefits of foods enriched with non-starch polysaccharides (NSP) and resistant starch (RS) to the general population have been highlighted over the past 20 years, and are slowly gaining consumer acceptance (Topping *et al.*, 2003). Initially, these foods were enriched with high-amylose maize starch; however, the scope can be broadened to develop additional products with whole-grain ingredients.

The University of Adelaide Barley Program aims to develop hulless barley types for the food industry (waxy and high-amylose types), the malting and brewing industries, various industrial and commercial uses (e.g. low and high amylose) and as feed for monogastric animal (normal starch).

The hulless waxy line WI3693 was developed from a three-way cross between Azhul, a waxy hulless cultivar (University of Arizona, USA) and two cereal cyst nematode (CCN)-resistant, covered feed types, cv. Barque and cv. Keel (South Australian cultivars). Older waxy cultivars tend to be low yielding, susceptible to disease and have poor straw strength, but WI3693 is a medium-height plant that is lodging resistant, has bright white grain and straw at maturity, has a mid-season maturity in a Mediterranean environment, and also has resistances to CCN and spot form of net blotch (SFNB).

The normal-starch line WI3930 was developed from a three-way cross between a hulless barley selection CIMMYT 42002 from CIMMYT/ICARDA, Mexico, and the South Australian covered feed cultivars Galleon (CCN resistant) and Skiff (CCN susceptible). WI3930 has a similar plant type to Skiff and is medium to early in a Mediterranean environment. WI3930 is a semi-dwarf plant type with improved lodging and straw strength resistances and better head retention than Torrens. WI3930 has a higher yield potential than Torrens in all agro-ecological zones of South Australia. WI3930 is moderately resistant to CCN, leaf scald and shows adult resistance to leaf rust, but is susceptible to SFNB.

Currently, the Barley Program is assessing three high-amylose genotypes (High Amylose-11, High Amylose-58 and High Amylose-126) developed from a two-way cross between SES15-30, a high-amylose, covered, 2-row genotype (from Scotland, UK) and a CCN-resistant, hulless, 2-row feed type (from South Australia). All three lines showed improvements in grain yield when compared with SES15-30. These genotypes have a short to medium plant height, are

lodging resistant and are medium to late maturing in a Mediterranean environment.

This paper describes the progress made for some products made from waxy hulless barley, normal-starch hulless barley, and to a lesser extent, high-amylose barley, and their quality attributes in relation to food acceptability and health.

Methods

Barley samples

All barley samples were obtained from the University of Adelaide Barley Program. The sample of 'Euro' Oats was obtained from the Oat Breeding Program, SARDI, Waite Campus, South Australia. Hulless barleys can be susceptible to embryo damage during harvesting. Consequently, gentle harvesting techniques were adopted, resulting in 10–50% of the husk remaining attached to the grains. These samples were de-hulled before processing as described below.

Beta-glucan analysis

Total mixed linkage beta-glucan was measured using the mixed-linkage beta-glucan assay procedure (McCleary Method), Megazyme, Ireland.

Total starch analysis

Total starch was measured using the total starch assay procedure (amyloglucosidase/alpha-amylase method (AA/AMG)), Megazyme, Ireland.

Amylose/Amylopectin analysis

Amylose/amylopectin ratios were measured using the amylose/amylopectin assay procedure (K-AMYL), Megazyme, Ireland.

RVA viscosity analysis

A pasting profile was generated for seven barley genotypes with the Rapid Visco Analyser (RVA, AGT Laboratories, South Australia). Flour (4 g) was suspended in 25 ml of distilled water using the standard 13 min profile.

Pearled barley

Four barley genotypes (WI3693 – waxy, hulless; Torrens; WI3930 – normal, hulless; and Schooner – normal, covered) were grown in three replicated trials at four South Australian sites (Brinkworth, Clinton, Yeelanna and Weetulta) during 2004. Barley samples (40 g) were de-hulled using a laboratory-scale oat de-huller (except for covered barley). Each de-hulled sample was pearled in a Satake grain testing, mill model TM-05, until 20% (hulless types) or 35% (Schooner) of kernel was removed (between 2 and 7 minutes, depending on sample). The sound-kernels measurement was calculated as a percentage of whole pearled grain retained on a 2.0 mm screen. New MG staining was performed using the method of Dr T. Omori (SANWASHURUI Co. Ltd., Japan, pers. comm.).

Whiteness (L-value) of pearled samples were measured with a Minolta colorimeter using the CIE $L^*a^*b^*$ color score. Barley flour colors were also assessed on the whole grain and pearled samples by cooking 10 g of flour sample in 90 ml of R.O. water for 5 minutes. The cooked slurry was transferred to a Petri dish and allowed to cool for 30 minutes. Whiteness (as described above) of the gels was measured at zero hours and 24 hours later. The stability of the gel color was expressed as the difference between the two L-value readings.

Tocol analysis of pearled flour

Supercritical Fluid Extraction was used to extract oil from waxy pearled flour (WI3693; Charlick, South Australia; 2003; 20% flour removed) as described by Washington and Box (2004). Results are expressed as percentage of dry weight of pearled flour. Analysis of tocopherols and tocotrienols was performed at the National Measurement Institute, Australia, as described in (Washington and Box, 2004). Results are expressed as $\mu\text{g}/100\text{ g}$.

Omega-3 and Omega-6 analysis of barley flour

Analysis of total fatty acids extracted from 100% barley flour (Torrens, Schooner, WI3930, WI3693, and three high-amylose lines), WI3693 pearled flour (66%, 70% and 77%) and the byproducts of these pearled samples, was performed by the Child Nutrition Research Centre, Waite Campus, South Australia. Omega-3 and Omega-6 results are expressed as % distribution.

Flaked and rolled barley

Samples (500 g each) of WI3693, cv. Morrell (both grown at Charlick in 2003) and oat cv. Euro were rolled at BRI (Sydney, NSW, Australia). Prior to rolling, the samples were de-hulled (described above) and conditioned to a moisture content of 25% approximately 12 hours before processing. Once conditioned, the grain was steamed at atmospheric pressure for 35 minutes. The steamed grain was then immediately passed through smooth flaking rolls on a Vario roller mill. The material was passed through the rolls for a single pass and another sample was passed through the rolls for two passes to produce a thinner flake. The gap on the mill was set to approximately 0.01 mm. The flakes were then dried at 125°C for 15 minutes and allowed to cool to room temperature before packing. Screenings were measured on 50 g of each flaked sample sieved (3 times) over 1.7 mm, 2 mm and 3.86 mm Endecott sieves. The product retained on the sieve was weighed and recorded as a percentage of the whole sieved sample. Whiteness (L-value) was measured as described above.

Milling and baking

Samples of WI3693 and Morrell were de-hulled prior to milling in a laboratory-scale Buhler mill (AGT laboratories, Australia). Samples were not conditioned prior to milling. Moisture content was approximately

10%. Flour products were reconstituted to produce whole grain flour. The total mixed linked beta-glucan of Morrell flour was 4.1%, and of WI3693 flour was 6.7%. Three types of pita breads were made at Regency TAFE, Australia, containing either 50% waxy barley (WI3693) flour + 50% bakers flour; 50% hullless normal barley (Morrell) flour + 50% baker's flour; or 100% baker's flour.

Results

Agronomy

Introduced hullless lines are generally poorly adapted to many agro-ecological regions in Australia. Therefore, our biggest challenge has been to develop hullless lines that are more comparable to Australian locally adapted cultivars. In 1992, cv. Namoi was released as an Australian hullless cultivar, but suffered from poor yields. Cv. Torrens was released in 2001, with a strong focus on market development for hullless barley. Torrens averages 4% higher yields than Namoi, with higher yield potential in most agro-ecological regions of South Australia. However, its average yield potential compared with cv. Schooner is 92%. The University Barley Program has now also targeted the development of hullless barley adapted to higher rainfall areas. In 2005, WI3930 yielded up to 8% and 10% higher than Schooner and Torrens, respectively. However, in 2006 and 2007, much of the Australia's cereal production belt received below average rainfall, and consequently, in 2007, WI3930 yielded up to 20% lower than Schooner, but 11% higher than Torrens (Figure 1). Pure seed production of the hullless feed barley line WI3930 commenced in 2005, with potential release in 2009. WI3930 has improved grain yield and feed quality compared with Torrens. The 2007 drought conditions also affected the grain yields of WI3693, which yielded 35% and 4% less than Schooner and Torrens, respectively.

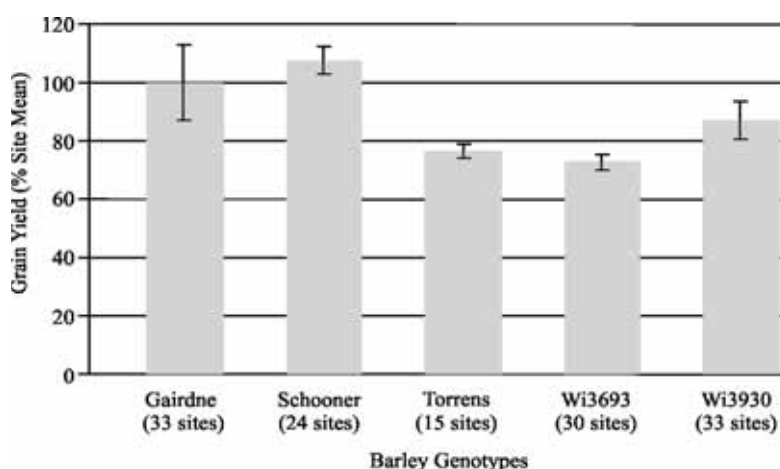


Figure 1. Summary of yield potential of WI3693 and WI3930 in comparison with Gairdner, Schooner and Torrens in 2007 National Variety Trials.

Plant type comparisons

A large number of introduced hulless lines from Canada and Mexico have the propensity to be very tall, resulting in severe lodging, stem break and head loss, culminating in up to 50% reduction in grain yields. To reduce the severity of these negative traits, semi-dwarf alleles (*sdw*) have been introgressed, resulting in the development of WI3930. WI3930 has a semi-dwarf habit and is resistant to lodging, stem break and head loss (Table 1). In comparison, Torrens has a medium plant height and is susceptible to head loss, but has better resistance to lodging and stem break compared with Schooner (data not shown).

Disease resistance

Table 2 provides a summary of the disease ratings of WI3930 and other major barley varieties. These ratings are based on observations made in field trials and field and laboratory disease screening nurseries by the Field Crops Pathology Unit (SARDI) and the University of Adelaide.

Kernel color

There is a large environmental influence on kernel color, with Clinton and Brinkworth being the best locations for producing the whitest grain for WI3693, as shown by their lowest L-value (Figure 2). Furthermore, color differences may be explained through the spike

Table 1. Summary of agronomic field observations from 2007 University of Adelaide Stage 3 Trials in South Australia.

Genotype	Glenthorne			Moyhall				Strathalbyn
	Height type	Head loss (heads/m ²)	Lodging*	Height type	Juvenile Lodging*	Lodging prior to harvest*	Stem break**	Plant height (cm)
Gairdner	Semi-dwarf	0	9.0	Semi-dwarf	9.0	8.0	9.0	—
Schooner	Medium	4	8.5	Medium	7.5	6.3	7.7	56
WI3693	Medium	2	8.0	Short - medium	9.0	6.0	7.5	—
WI3930	Semi-dwarf	2	8.5	Semi-dwarf	9.0	7.7	8.8	40

* lodging assessed on a scale of 1–9, with 9 = upright; 1 = fallen over. **Stem break assessed on a scale of 1–9, with 9 = zero; 1 = 100% breakage.

Table 2. Disease ratings for WI3930 compared with Torrens and Schooner.

Genotype	Leaf Scald	Powdery Mildew	Leaf Rust	Spot form of net blotch	<i>Pratylenchus neglectus</i>		CCN
					resistance	tolerance	
Schooner	MS/S	S	S	MS/S	MR/MS	MI	S
Torrens	MS	MR/MS	MR/S	MS	MR	I	R
WI3930	MR	MS	R (<i>Rph 2</i>)	S	na	na	MR

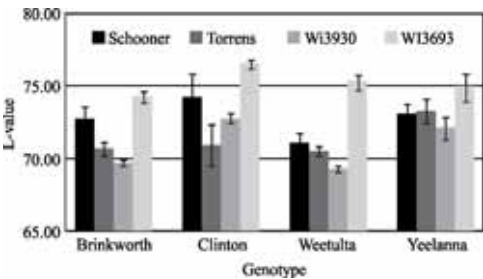


Figure 2. Color measurement (L-value) from South Australian trials (2004; 4 locations; 3 replicates).

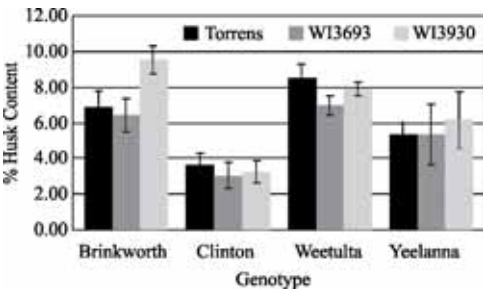


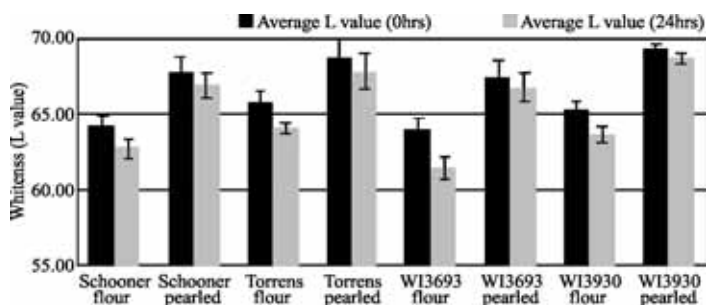
Figure 3. Husk content of hulless genotypes grown at four locations in South Australia during 2004.

morphology. The developing hulless grain can often get too large for the surrounding palea and lemma. Consequently, there can be a gap of a few millimetres between the palea and lemma and so the grain can be exposed, resulting in ‘sunburn’, a problem that has not been observed in wheat, triticale or oats. It is difficult to compare whiteness values between covered and hulless genotypes due to the component of the grain being measured, for example, husk in covered and aleurone in hulless. The issue of threshability in hulless genotypes further complicates this. For example, the husk content results in Figure 3 indicate that WI3930 has the highest husk content (9.52% at Brinkworth), resulting in a low L-value (69.31), compared with a low husk content at Clinton (3.25%), ensuing a higher L-value reading of 72.75.

Although kernel color has no effect on feed value, in the food industries the product must be aesthetically pleasing to the purchaser of the product if it is to be successfully marketed. White-color barley flour is generally preferred to a dark color. Dark discoloration of barley when used, for example, as a rice extender or in baby food

preparation, is a serious concern to food industries and can be a significant factor preventing the integration of barley in foods. Barley contains numerous polyphenols, proanthocyanidins and catechins, which are distributed in the husk and aleurone layer. Numerous studies have illustrated the role of polyphenols in brewing, particularly their implication in haze formation in beer. However, the relationship between barley polyphenols and the discoloration of their food products has not been investigated, mainly due to the comparative insignificance of barley as a food ingredient. Figure 4 compares the whiteness and stability of color of cooked pearled barley flour to cooked whole grain barley flour in a 24 hour period. As expected, the cooked pearled barley flours produced whiter gels than the cooked whole grain barley flours, due to the removal of the husk and aleurone layers and, hence the elimination of the polyphenols. The whiteness of the all gels (from pearled and whole grain) did deteriorate after the 24 hour period, with the WI3693 flour having the lowest L-value at 24 hours (61.50). Torrens had the whitest gels after the 24 hour period with a color reading

Figure 4. Gel color measurements (L-value) from South Australian trials (2004; 4 locations; 3 replicates).



of 64.13. Furthermore, WI3693 flour was the least stable for whiteness, producing a difference of 2.48 units compared with 1.43 units for Schooner flour. WI3930 pearled was the most stable for whiteness with a difference of 0.59 units compared with 0.85 units for both Schooner and Torrens pearled flours.

More recently, we have been using germplasm from Canada (for example, TR118) to improve kernel color. Figure 5 provides a 2006 comparison of kernel color before and after pearling of WI3693 with a TR118 derived line. The latter produced a pearled grain that was 2.62 and 2.55 L units whiter than WI3693 and Schooner, respectively.

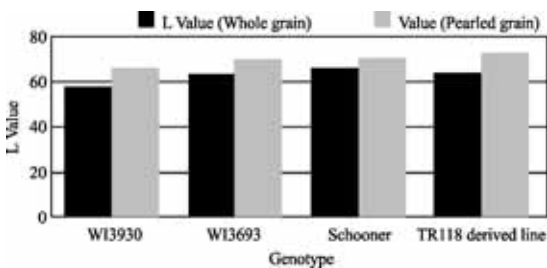


Figure 5. Comparison of kernel color before and after pearling for genotypes grown at Glenthorne, South Australia, during 2006.

Beta-glucan analysis

The results for WI3693 and the high-amylose lines were up to 3.4% higher than results obtained for normal starch, hullless and covered genotypes from Strathalbyn, South Australia, in 2005 (Figure 6).

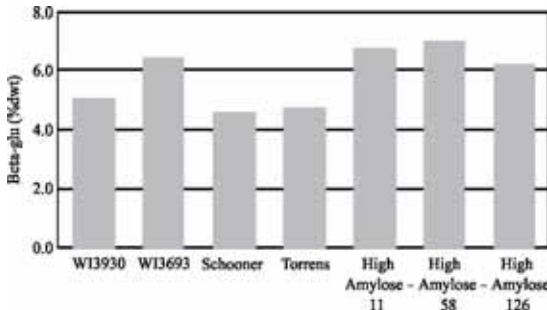


Figure 6. Beta-glucan analysis (one location, one replicate).

Total starch analysis

The results indicate in general that those genotypes with the novel starch traits have starch contents between 6.3 (high amylose lines) and 11.8 (WI3693)% lower when compared to the normal starch, covered genotypes (Figure 7).

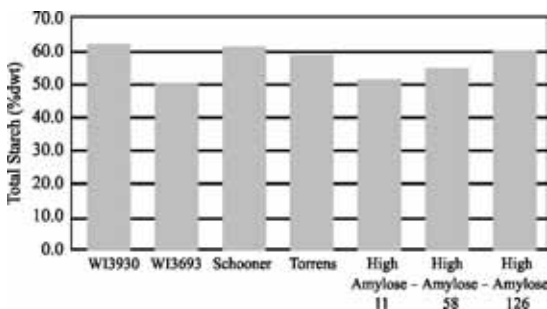


Figure 7. Total starch analysis (one location, one replicate).

Amylose/Amylopectin analysis

As expected, WI3693 (waxy starch) had 18.6% less amylose than Schooner

(Figure 8). The high-amylose genotypes had 15.4% more amylose than Schooner. What we did not expect, was the lower amylose contents for WI3930 (13.6% lower) and Torrens (8.9% lower) when compared with Schooner. Both these genotypes are hullless with a normal starch type, and therefore approximately 25% amylose content would be expected for these lines.

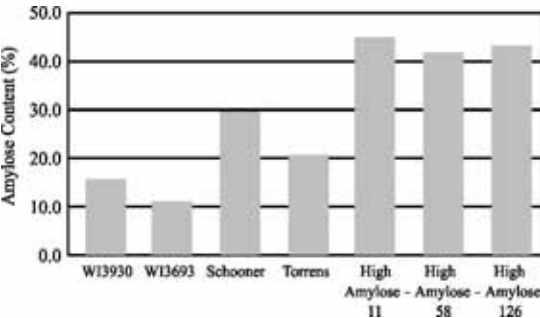


Figure 8. Amylose/amylopectin analysis (one location, one replicate).

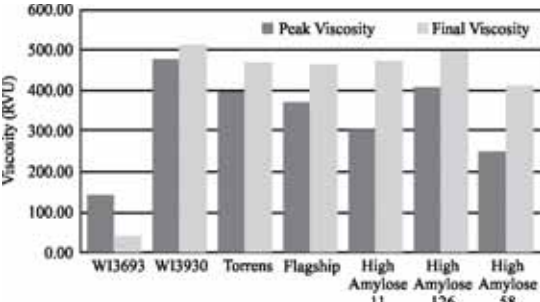


Figure 9. RVA viscosity profiles for seven barley genotypes.

RVA viscosity analysis

Figure 9 shows peak and final viscosity profiles of the seven barley genotypes assessed. All genotypes, except WI3693, produced higher final viscosities. WI3930 produced the highest viscosities (478.08 and 515.58 RVA) with WI3693 (143.83 and 41.83 RVA) producing the lowest. These low viscosities indicate that WI3693 may have suffered weather damage prior to harvesting. This observation could be substantiated by WI3693’s having a very low peak time (3.47 minutes) compared to an average of 6.30 minutes for the other six genotypes.

Pearled barley

To calculate the amount of pearling required to compare hullless and covered barleys, new MG stain was used to determine the quantity of aleurone (blue) lost and endosperm (pink) retained. To achieve a pearling equivalent of 65% in the covered type (Schooner) (Figure 10a), we concluded that the hullless type needed to be pearled to 80% or 20% loss (Figure 10c). Schooner pearled to 80% still retained significant amounts of husk and aleurone (Figure 10b).

Environment plays a significant role in pearling quality; so four diverse locations and three plot replicates were analyzed. WI3693 performed well in pearling

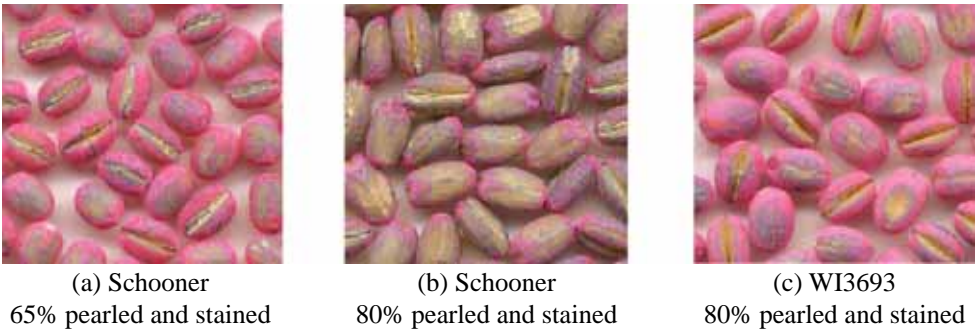


Figure 10 (a), (b) and (c). Pearled barley stained with new MG stain.

trials as assessed by percentage of sound kernels remaining after pearling (Figure 11). At all sites WI3693 had more than 80% sound kernels after pearling, with the highest percentage sound kernels (93.84%) observed at Yeelanna. This was higher than or equivalent to the percentage of sound kernels of both Torrens and Schooner. In addition, pearled barley whiteness (L-value) was measured (Figure 2). Again, WI3693 was brighter than Torrens and Schooner, with an average L-value of 75. Furthermore,

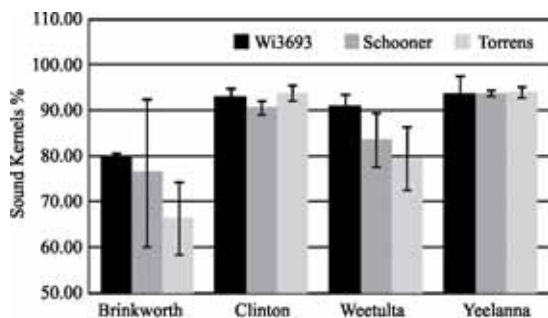


Figure 11. Pearling quality as expressed as % sound kernel from South Australian trials (2004; 4 locations; 3 replicates).

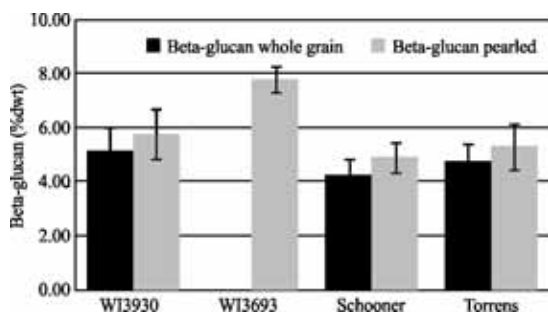


Figure 12. Comparison of beta-glucan contents before and after the pearling process.

beta-glucan was measured before and after pearling (Figure 12), with all genotypes having up to 0.65% higher in the pearled samples.

Tocol analysis of pearled flour

Oil analysis of flour from WI3693 (Charlick, South Australia, 2003) pearled to remove 20% of the outer layer exhibited a high oil content of 11.2% when extracted using supercritical fluid extraction. Analysis of tocals demonstrates that significant concentrations of these valuable antioxidants (Washington and Box, 2004) can be extracted from this waste product of pearling (Table 3).

Omega-3 and Omega-6 analysis of barley flour

The level (%) of Omega-3 fatty acids (Figure 13a) is very low, with the majority of the genotypes assessed having between 4.45 and 5.01%, regardless of their starch composition. The results observed for the pearled flour and by-products of WI3693 were most interesting. The by-products of the pearling process appear to be Omega-3-rich, with the 22.38% by-product having the highest quantities (4.65%). However, these levels were lower than the levels observed in whole grain sample of WI3693. The opposite trend was observed for the Omega-6 fatty acids (Figure 13b), with the pearled flour being a richer source of this class of fatty acids. A greater variability in the amounts of Omega-6 fatty acids was observed, with the high-amylose lines exhibiting up to 4% more than the other genotypes assessed.

Table 3. Oil and Tocol content of WI3693

Sample	Treatment	Oil content		Tocol content mg/100 g								Total T:T3
		% dry weight	α -T	β -T	γ -T	δ -T	α -T3	β -T3	γ -T3	δ -T3	T+T3	
WI3693	20% pearled flour	11.2	47	2.4	11	7.9	190	7.7	80	3.5	350	1:4

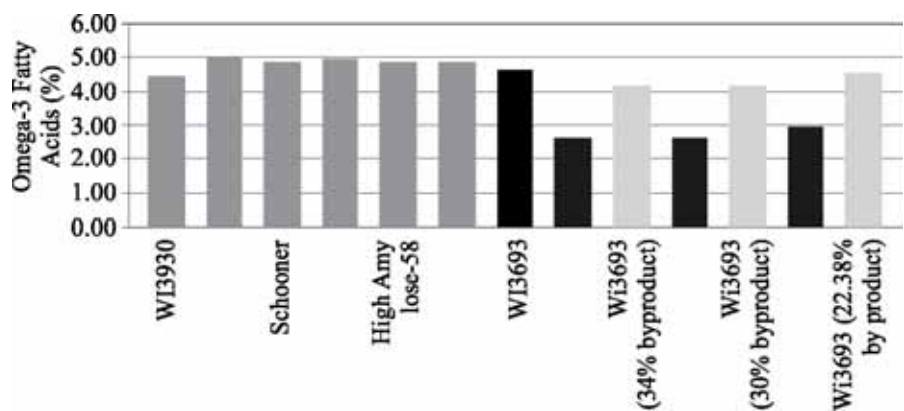


Figure 13a. Omega-3 fatty acid analysis.

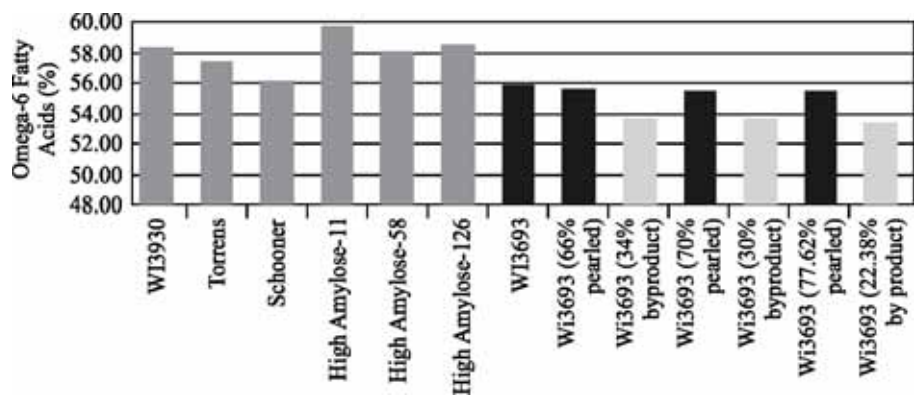


Figure 13b. Omega-6 fatty acid analysis.

Flaked and rolled barley

Rolling of WI3693 produced a thin white flake similar in color to Morrell (Figure 14a), but marginally darker than the oat product (Figure 14a). WI3693 flakes were the only sample to be retained on all screens, with greater than 80% efficiency (Figure 14b). Morrell performed satisfactorily, with only 60% of the ‘pass 2’ flakes retained on the 3.86 mm screen. The oat sample performed poorly, with very high screenings for all sieves. The poor performance of oats was probably the result of the extreme rolling conditions compared with industry practice. However, it does demonstrate the resilience of WI3693 and Morrell during flaking.

Barley pita breads

Not surprisingly, 50% barley pita breads were darker than those made with 100% baker's flour (Figure 15a). Visually, the 50% WI3693 pita had slightly better color than the 50% Morrell pita. It also pocketed better and staled more slowly than the 50% Morrell pita. To investigate whether lipoxygenase could improve WI3693 dough color, 2% soy flour (containing natural lipoxygenase) was added to the WI3693 flour (100%) dough. Color measurements show that dough color can be improved by the addition of a natural lipoxygenase (Figure 15b).

WI3693 pita bread had higher moisture, fat, ash and beta-glucan content than the



Figure 14a. Visual comparison of barley flakes.

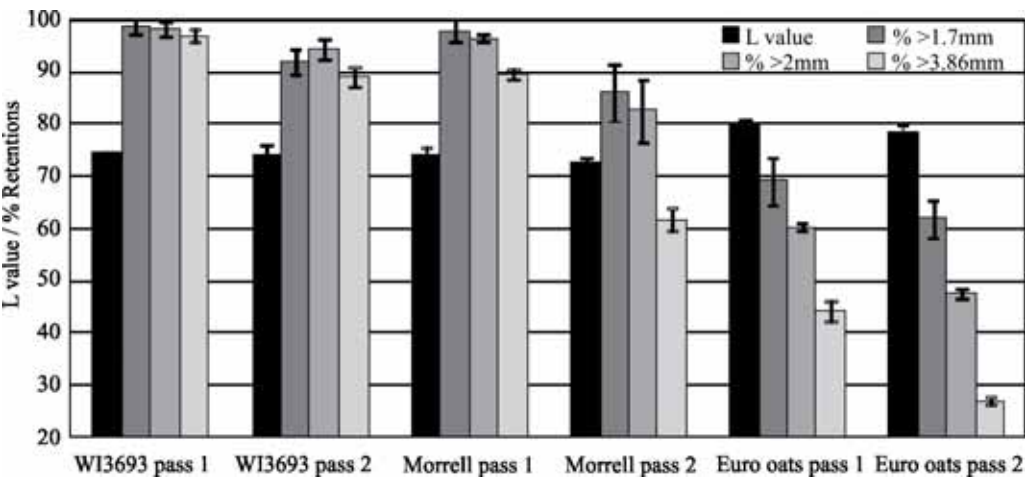


Figure 14b. Comparison of whiteness (L-value) and % retention of barley and oats flakes.

other two pita breads (Table 4). However, the carbohydrate and starch content were lower. Both barley pita breads had similar fiber content, which was almost twice as much as that of the baker's flour control bread; however the beta-glucan component was significantly higher in the WI3693 pita. The higher moisture is favorable to the industry due to lower product cost per volume. The higher fiber, beta-glucan and lower

carbohydrate content of the barley pita breads provide significant health advantages.

Conclusions

The University of Adelaide Barley Program aims to develop hulless barley types for the food industry (waxy and high-amylose types), the malting and brewing industries, various industrial and commercial uses (e.g. low- and



Figure 15a. Visual comparison of pita breads.

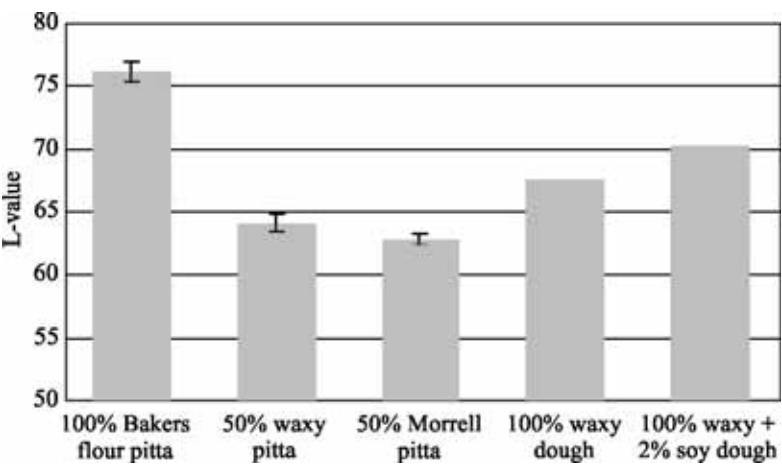


Figure 15b. Whiteness score of pita breads and waxy barley flour dough (L-value). Color measurements were taken on the inside of the pita pocket.

Table 4. Carbohydrate and beta-glucan analysis of pita breads.

	50% Waxy barley flour	50% Normal barley flour	100% Bakers' flour
Moisture %	41.6	40.5	35.6
Fat %	2.7	2.4	2.0
Protein (N × 5.7) %	8.6	8.4	8.6
Ash%	1.7	1.6	1.5
Total Dietary Fiber %	5.8	5.8	2.8
Carbohydrate (db) %	39.6	41.3	49.5
Total Starch %	37.5	39	47
Resistant starch %	ND	ND	ND
Total beta-glucan %	3.94	2.76	0.43

high-amylose) and as feed for monogastric animal (normal starch).

Our biggest challenge has been to develop hulless lines that are more comparable to Australian locally adapted cultivars. As a result, the University of Adelaide Barley Program released cv. Torrens in 2001 with a national, co-ordinated approach as a first step to supporting developments similar to those observed in the Canadian pig industry. Compared with older hulless cultivars, Torrens has improved grain yield, agronomy and feed quality. The Barley Program has now also targeted the development of hulless barley adapted to higher rainfall areas. Pure seed production of the hulless feed barley line WI3930 commenced in 2005, with potential release in 2009. WI3930 has improved grain yield, foliar disease resistance and feed quality compared to Torrens.

In this paper we have compared advanced germplasm, Torrens, WI3930 and WI3693 with cv. Schooner, but the same breeding principles have been applied to the development of other hulless germplasm throughout all the stages of the program. The objectives of the barley program are to improve yield, stability and adaptation; improve straw strength; reduce lodging, stem break and head loss; improve grain plumpness; decrease the effects of harvest damage on germination rates; and make selections for improved kernel color.

Hulless barley also has potential in the malting and brewing industries. Preliminary research with Joe White Maltings (Stewart *et al.*, 2004) indicates hulless barley has potential in the production of malt extract for beer. The challenge with this germplasm development has been in assessing those breeders' lines that offer a wide variety of quality traits. We have shown results for Torrens and WI3930 that indicate potential for a diverse range of industries, including feed and food. The future for the development of hulless barleys is looking very favorable. We have described the nutritional benefits

of the waxy, hulless line WI3693. Results indicate a grain that is fiber-rich, low in fat and potentially with low GI. In addition, it also displays excellent physical properties in pearling, flaking and pita bread production compared to normal hulless genotypes and oats.

WI3693 whole milled flour incorporated at 50% into pita breads produced an acceptable product that pocketed well with a fiber and beta-glucan content more than 2 times and 9 times, respectively, than that of the wheat control. The high moisture content of this product is also attractive to manufacturers, who can save on ingredient costs. The high beta-glucan content of waxy dough results in a higher moisture uptake. There was also an indication that the waxy product staled more slowly than the normal starch barley product, although staling occurred even more slowly in the wheat control. The issue of staling needs to be further investigated. The addition of 2% soy flour showed an improvement in the pita bread color.

Barley has been shown to have a very low Glycaemic Index (GI) compared with other grains (Arndt, 2006). Waxy hulless barley could offer a low cost, value-added ingredient to the health-conscious food producer and consumer. Other foods and products made from WI3693 are currently under investigation.

Finally, we also described the preliminary results obtained for the high-amylose types. Initial results indicate that these lines are comparable to normal types for 1000-kernel weights, grain protein, and kernel color (data not shown). They also have the added nutritional benefit of having at least a 2% higher beta-glucan level than Schooner. High-amylose hulless barley also has the potential to make an important contribution to improving human health, particularly the prevention and management of diabetes, coronary heart disease, obesity, colorectal cancers, constipation and diverticular disease. There is still a large amount of research to be

done to improve the agronomy and product development of high-amylose barleys and make them more competitive with high-amylose maize.

Acknowledgements

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Determination of iron and zinc content in food barley

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Abstract

The main objective of this study was to identify sources of high iron and zinc content in landraces and hulless barley grain to be used in developing new barley germplasm with improved value for human nutrition. A collection of 333 landraces from 34 countries was grown for three consecutive years at ICARDA's research station located near Tel Hadya, in Syria. In the third year, the collection was also planted at a second ICARDA research station, near Breda. The samples were analyzed at Waite Analytical Services, University of Adelaide, Australia. A spatial analysis was carried out using GENSTAT. The heritability of iron content in the grain was 0.589 in Breda and 0.562 in Tel Hadya; the heritability of zinc content was 0.639 and 0.605 in Breda and Tel Hadya, respectively. Six landraces were identified with consistently higher iron across environments, ranging from 38.8 to 51.6 ppm. Three of these entries also have high zinc content, ranging from 37.3 to 42.5 ppm. Moroccan landraces are a very promising source of high iron and zinc content in the grain. Ethiopian, Russian and Ecuadorian landraces also have a few promising sources. During 2007, ICARDA's micronutrient laboratory applied a dry-ashing method for the determination of iron and zinc content in hulless barley. A total of 1087 breeding lines were grown at Breda in 2005 and analyzed for iron and zinc. The iron content ranged

from 22.6 to 36.7 ppm while zinc content ranged from 19.98 to 49.72 ppm. The variety Atahualpa confirmed its high iron and zinc content: it ranked first for iron and second for zinc (43.9 ppm). Both iron and zinc content were highly and positively correlated with protein content (0.56 and 0.74, respectively). The correlation coefficient between iron and zinc content was 0.64. Sample preparation for barley grain analysis is discussed for reducing contamination.

Introduction

Barley is a major staple food in several regions of the world, such as in some areas of North Africa and Near East, in the highlands of Asia, in the Horn of Africa, in the Andean countries and in Baltic States. The annual consumption of food barley in the above countries ranges between 2 and 36 kg/person (1999–2002 statistics) (Grando and Gomez Macpherson, 2005).

The main objective of this work was to identify sources of high iron and zinc content in landraces and hulless barley grain to be used in developing new barley germplasm with improved value for human nutrition.

Barley landraces screening

A collection of 333 landraces from 34 countries was grown for three consecutive years (2003–2005) at ICARDA's research station located near Tel Hadya (TH) in Syria. In the third year the collection was also planted at a second ICARDA research

station, near Breda (BR). The samples were dispatched to Waite Analytical Services, University of Adelaide, Australia, for micronutrient analysis, where the wet ashing method is used: all samples are digested with nitric/perchloric acid, then analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (Ciros, Spectro Analytical Instruments, Cleve, Germany) for 20 elements, including Fe, Zn, Al and Ti (Health Canada, 1985; Zarcinas *et al.*, 1987). In the third year, two repeated checks (Arta and Rihane-03) were added, and a row and column design was used. A spatial analysis was carried out using Gen Stat. The BLUPs of the third year and the unadjusted values of

the first and second year were used to carry out Genotype \times Environment analysis using GGEbiplot software.

The heritability of iron content in the grain was 0.589 in Breda and 0.562 in Tel Hadya; the heritability of zinc content was 0.639 and 0.605 in Breda and Tel Hadya, respectively. Correlation coefficients between iron content in the four environments (Table 1) ranged from 0.241 (Tel Hadya 2003/Tel Hadya 2005) to 0.394 (Tel Hadya 2003/Tel Hadya 2004). In the case of zinc content (Table 2) the lowest correlation coefficient was between Tel Hadya 2003 and Breda 2005; the highest correlation was between the two locations planted in 2005.

Table 1. Correlation coefficients between grain iron contents in different environments.

	Tel Hadya 2003	Tel Hadya 2004	Tel Hadya 2005
Tel Hadya 2004	0.394		
Tel Hadya 2005	0.241	0.339	
Breda 2005	0.364	0.359	0.376

Table 2. Correlation coefficients between zinc contents in different environments.

	Tel Hadya 2003	Tel Hadya 2004	Tel Hadya 2005
Tel Hadya 2004	0.321		
Tel Hadya 2005	0.319	0.319	
Breda 2005	0.306	0.352	0.480

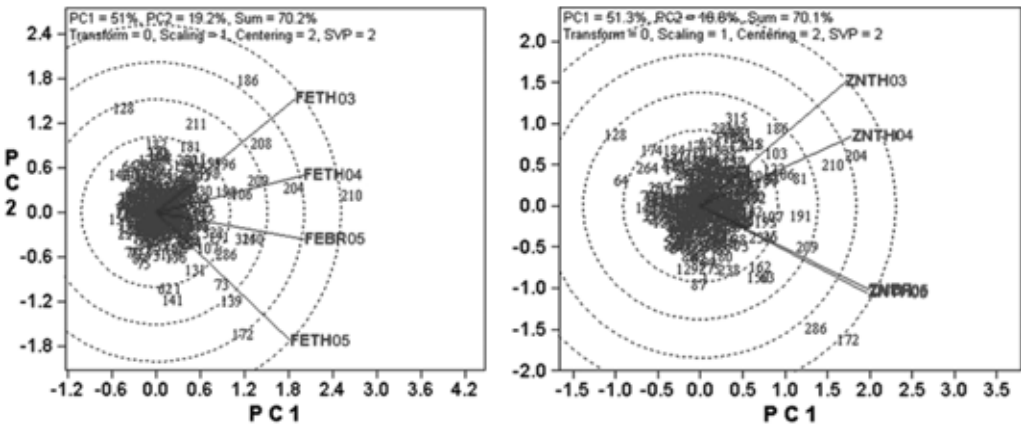


Figure 1. Bi-plots for iron (left) and zinc (right) content in the grain of 333 landraces tested in four different environments.

As shown by the bi-plots for both iron and zinc content (Figure 1), six landraces were identified with consistently higher iron across environments, ranging from 38.8 to 51.6 ppm, and three of these entries were associated with high zinc content, ranging from 37.3 to 42.5 ppm. Moroccan landraces are very promising sources of high concentrations of iron and zinc in the grain. Ethiopian, Russian and Ecuadorian landraces also have a few promising sources (Table 3).

Effect of pearling on removing of iron contamination in barley grain

A set of 10 grain samples were selected randomly from the landraces grown in 2005. The samples were handled with special care during head collection and hand threshing using powder-free nitrile gloves to avoid any contamination. Seeds of the same entries were sampled after harvesting by a plot combine, with sub-samples pearled by Seedburo

barley pearler for 20 second. The samples were analyzed by Waite Analytical Services, University of Adelaide, Australia, for micronutrient analysis. Results indicated that barley grains were naturally contaminated by dust in the field (Table 4), the plot combine had no significant contamination effect, and the pearling of barley grain for 20 second is recommended to remove the existing contamination (aluminum values of less than 3 mg/kg is considered as natural background level). No significant effect of dust contamination was observed on zinc level in grain.

Hulless barley screening

During 2007, ICARDA's micronutrient laboratory applied a dry-ashing method (Health Canada, 1985) for the determination of iron and zinc content in hulless barley. The method consists of preparing an ash in a furnace at 500°C; nitric acid is used to decompose the organic matter, then the inorganic residue is dissolved in an

Table 3. Barley landraces with higher iron and zinc content (mg/kg).

Entry #	Origin	FeTH03*	FeTH04	FeBR05	FeTH05	ZnTH03*	ZnTH04	ZnBR05	ZnTH05
81	Ecuador	36.3	24.1	30.3	25.5	45.0	34.4	35.8	37.0
106	Ethiopia	47.8	45.0	34.1	28.3	39.2	38.3	32.7	35.7
191	Morocco	44.6	32.8	34.4	30.1	41.4	33.3	31.8	42.8
204	Morocco	63.3	48.9	35.2	33.8	46.4	46.6	35.8	41.2
208	Morocco	67.1	39.6	34.3	28.7	38.8	30.7	26.9	32.1
209	Morocco	48.2	42.6	46.2	24.6	34.2	38.6	45.2	32.7
210	Morocco	67.1	63.7	37.9	37.9	43.6	44.0	31.8	42.4
250	Russia	49.7	30.4	45.6	30.2	44.1	24.5	31.6	31.3
Mean		38.1	27.0	29.9	24.5	33.6	26.8	30.0	31.6
Minimum		25.9	16.5	21.7	12.1	25.1	18.0	21.5	17.7
Maximum		67.4	63.7	48.6	39.4	49.5	46.6	49.0	50.2

* = Hand threshed, unpearled samples.

Table 4. Effect of pearling barley grain by a Seedburo barley pearler on removing Fe contamination in barley grain. Values represent the mean of 10 samples.

	Fe (mg/kg)	Zn (mg/kg)	Al (mg/kg)	Ti (mg/kg)
Hand threshed	30.18	34.84	9.75	0.65
Combine	31.9	35.28	9.17	0.60
Combine + 20 sec pearling	24.2	32.4	2.44	0.09

appropriate volume of diluted hydrochloric acid. The extract is analyzed by flame atomic absorption spectrophotometer (PerkinElmer 373, USA).

A total of 1087 breeding lines grown at Breda in 2005 were analyzed for iron and zinc. The iron content ranged from 22.6 to 47.1 ppm while zinc content ranged from 19.98 to 49.72 ppm. The variety Atahualpa confirmed its high iron and zinc content: it ranked first for iron and second for zinc (43.9 ppm).

Both iron and zinc content were highly and positively correlated with protein content (0.56 and 0.74, respectively). The correlation coefficient between iron and zinc content was 0.64.

Conclusions

Moroccan barley landraces are a very promising source of high iron and zinc content. The hullless variety Atahualpa confirmed its high iron and zinc content: it ranked first for iron and second for zinc.

Under field conditions, natural contamination with iron occurs in barley grain by dust. Grain pearling eliminated the iron contamination from soil dust.

Acknowledgements

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Nutritional characteristics and properties of barley cultivars grown in Korea

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Abstract

Barley is one of the earliest cultivated cereal grains in the world. There are a variety of ways in which barley is used as food in India, China, Japan, Korea and the West Asia-North Africa region. Recently, barley has been gaining renewed interest for food due to its hypocholesterolaemic effect and other desirable nutritional and functional characteristics. There are some barley varieties meant for special purpose; colored (purple, green, black lemma) and waxy barley with high beta-glucan for functional food and low phenol content to prevent the discoloration during storage of cooked barley. Recently, we have developed Zasoojeongchal, with purple lemma, and Jinjuchal, with low discoloration after cooking. In this study, we focused on the improvement of nutritional characteristics and properties of barley for functional food. A set of 27 naked and 25 covered barley cultivars grown in Korea were tested and characterized for their total phenol, protein and soluble beta-glucan content. Results showed that total beta-glucan ranged from 3.86% to 9.72% in naked barley and from 4.13% to 7.39% in covered barley. The highest total beta-glucan content was obtained in Doowonchapssalbori and Seodunchalbori, a naked and covered barley cultivar, respectively. Total phenol content ranged from 0.17 to 0.3% in naked barley lines and from 0.15 to 0.28% in covered barley lines.

Introduction

Barley (*Hordeum vulgare* L.) is the forth most important cereal in the world in terms of total production, after wheat, rice and maize. It is mainly utilized in the malting and brewing industry and for animal feed, with a small proportion is used for human consumption. Taste and appearance factors, along with its poor baking quality, have limited the use of barley as human food. However, in recent years there has been a growing research interest in the utilization of barley in a wide range of food applications. Beta-glucan is one of the functional compounds of barley, and beta-glucans not only influence nutritional value and functional properties of food, but are also beneficial to human health. The insoluble fraction in cereal grain contains a large proportion of cellulose and has beneficial effects in the gastrointestinal tract. The soluble fractions contain mostly pectin, arabinoxylan and beta-glucan. Barley contains high levels of soluble dietary fiber, particularly mixed linkage (1,3)-(1,4)-beta-D-glucans. Barley is unique among cereals in having high concentrations of beta-glucan, which is known to have a cholesterol-lowering effect, to regulate blood glucose level and insulin response in diabetics, and even to reduce cancer risk. It is important to distinguish between two types of beta-glucans and examine their ratios in barley cultivars, because of the very different physiological effects of soluble and insoluble dietary fiber.

The limited use of barley as food is probably due to undesirable color and unfamiliar flavor of barley-based food products. The gray color of barley food products may be non-enzymatic or enzymatic. Non-enzymatic browning results from the polymerization of endogenous phenolic compounds. In recent years, there have been significant breeding activities on hulless barley in various countries, including Korea.

In this study, we analyzed the content of total and soluble beta-glucan in various barley cultivars and investigated the ratios of soluble beta-glucan to total beta-glucan. Total phenol, proanthocyanidin content and whiteness of cooked barley during storage were measured to evaluate the relationship between discoloration potential and phenol or proanthocyanidin content in barley. We introduce two new cultivars, which were developed in Korea in 2006 and 2007.

Materials and methods

A set of 27 naked barley and 25 covered barley cultivars, obtained from the Honam Agricultural Research Institute, Rural Development Administration, Jeollabuk-do, South Korea, were used in this research. One proanthocyanidin-free cultivar (Radiant), Zasoojeongchal, which has purple lemma, and Jinjuchal, characterized by low discoloration after cooking, were also used in this research. Fifteen naked barley lines were investigated. All barley samples were planted on 26 October 2005 in Iksan, a southern province of Korea. Plot size was 1.5 m × 6 m, seeding rate was 160 kg/ha. Each experiment utilized a completely randomized design replicated four times.

Most of the varieties were local commercial varieties. The whole grains were ground by a Retsch centrifugal mill (Model Dm/Zm 100) with 0.2 mm sieve. The mixed beta-glucan contents of the barley samples were determined using a Megazyme beta-glucan assay kit (Megazyme International Ireland

Ltd., Ireland). Soluble beta-glucan was extracted using 5 ml H₂O from 0.5 g ground sample at 38°C for 2 hours. Total phenol content was quantified by the Folin-Denis method. Proanthocyanidin was assayed by Vanillin reaction. Protein and amylase content were determined by Elementar Analyzer System (US/Vario Macro) and a colorimetric method. The change of whiteness of cooked barley during storage was checked with a colorimeter. Cooking conditions were that 10 g of polished grains were boiled in 30 ml water at 100°C for 40 min.

Results and discussion

Beta-glucan

Total beta-glucan content among 27 naked barley was the highest, 9.72, in Doowonchapssalbori (Table 1). Total beta-glucan content ranged 3.86 to 9.72, and soluble beta-glucan ranged from 2.36 to 5.56. Soluble beta-glucan as a proportion of total beta-glucan ranged from 48.3 to 72.12%. The highest total beta-glucan content among 25 covered barley was 7.39, in Seodunchalbori, and the range was 4.13 to 7.39. Soluble beta-glucan ranged from 2.68 to 5.20. Soluble beta-glucan as a proportion of total beta-glucan ranged from 64.63 to 79.39%. Total beta-glucan in waxy cultivars showed higher values than in non-waxy, and polished grain had a higher content than whole grain (Table 2).

Total phenol and proanthocyanidin

Total phenol and proanthocyanidin content are important factors in discoloration (Figure 1). Whole barleys showed higher total phenol and proanthocyanidin content than polished barleys, although we did not find any relationship between proanthocyanidin and total polyphenol content. The total phenol content of proanthocyanidin-free lines in whole and polished barley was 0.146 and 0.130% and proanthocyanidin content was 0.004%, which is about 6.0 times lower than in polished barleys.

Table 1. Variation in content of total, insoluble, and soluble beta-glucan (%) and in soluble beta-glucan as percent of total beta-glucan of covered and naked barley cultivars in Korea.

Covered barley	Total	Insoluble	Soluble	Soluble, % of total	Naked barley	Total	Insoluble	Soluble	Soluble, % of total
Olbori	5.53	1.55	3.98	71.90	Baegdong	5.76	2.16	3.60	62.49
Nagyeoung	5.35	1.42	3.93	73.54	Songhag	5.24	1.46	3.78	72.12
Seodunchal	7.39	2.26	5.13	69.39	Saessal	6.31	2.65	3.66	57.95
Daeyeon	5.22	1.49	3.73	71.37	Nulssal	3.86	1.50	2.36	61.22
Daebaeg	4.40	1.22	3.18	72.26	Kinssal	5.34	2.08	3.26	61.02
Mirag	4.75	1.29	3.45	72.75	Naehanssal	5.20	2.02	3.19	61.26
Keunal	5.70	2.02	3.69	64.63	Hinssal	4.48	1.72	2.75	61.52
Sanglog	4.71	1.55	3.16	67.15	Hinchalssal	8.05	4.16	3.89	48.30
Daegin	4.57	1.20	3.37	73.79	Olssal	4.55	1.99	2.56	56.24
Saeal	4.33	1.28	3.05	70.48	Saechalssal	7.45	3.72	3.73	50.05
Keunalbori 1	5.25	1.67	3.59	68.25	Chunchussal	4.69	1.88	2.81	59.87
Geungang	4.13	1.45	2.68	64.97	kwanghwalssal	4.41	1.51	2.90	65.74
Taepyeong	5.67	1.26	4.41	77.71	Kanghossal	5.01	1.92	3.09	61.65
Kang	4.40	1.31	3.09	70.18	Daehossal	5.34	1.75	3.59	67.18
Tapgol	5.33	1.31	4.02	75.40	Jaegangssal	4.84	1.57	3.27	67.56
Saeol	4.37	0.90	3.47	79.39	Jinmichapssal	6.56	2.95	3.61	55.01
Chal	7.27	2.53	4.73	65.16	Donghossal	4.70	1.66	3.04	64.65
Saekang	5.04	1.50	3.54	70.19	Saenulssal	4.28	1.26	3.02	70.55
Al	4.41	1.50	2.91	66.04	Pungsanchalssal	5.73	2.80	2.92	51.03
Oweol	4.92	1.14	3.78	76.83	Namhossal	6.84	2.50	4.34	63.47
Alchan	6.20	1.31	4.89	78.88	Hobanchalssal	6.38	2.40	3.99	62.45
Paldo	5.95	1.61	4.34	72.94	Saehanchalssal	5.89	1.85	4.04	68.57
Milyangket	5.27	1.32	3.96	75.02	Donghanchalssal	6.49	3.27	3.22	49.59
Taegang	5.88	1.75	4.13	70.25	Cheonghossal	4.28	1.49	2.79	65.18
Gwangan	6.99	1.79	5.20	74.35	Duwonchapssal	9.72	4.16	5.56	57.24
					Jaechalssal	6.00	2.68	3.33	55.41
					Dasongssal	6.53	2.54	3.99	61.07

Cvs in blue are waxy types.

Table 2. Variation in content of total, insoluble, and soluble beta-glucan (%) and insoluble beta-glucan as percent of total beta-glucan of whole and polished grain in barley cultivars.

	Cultivar	Total	Soluble	Insoluble	Soluble, % of total
Olbori	Whole	5.37	3.62	1.75	67.36
	Polished	5.86	3.63	2.23	61.90
Seodunchal*	Whole	6.97	4.56	2.41	61.90
	Polished	8.73	5.24	3.49	60.01
Saessal	Whole	5.45	3.33	2.12	61.09
	Polished	5.76	2.75	3.01	47.69
Saechalssal*	Whole	6.54	4.02	2.52	61.54
	Polished	7.20	3.73	3.47	51.82
LSD(5%)		0.51	ns	0.41	

* = waxy barley, ns= not significant.

Total phenol content was between 1.9 and 3.1 mg/g in whole barley and between 1.1 and 2.7 mg/g in polished barley. Proanthocyanidin content was between 1.00 and 2.21 mg/g in whole barley and from not detected (n.d.) to 1.05 mg/g in polished barley (Table 3). Whole barley had

higher total phenol and proanthocyanidin content than polished barley. Total phenol content was positively correlated with proanthocyanidin content. The whiteness of polished barley was between 33.2 and 39.5, except line 14, which had a value of 18.4. The whiteness of cooked barley during storage

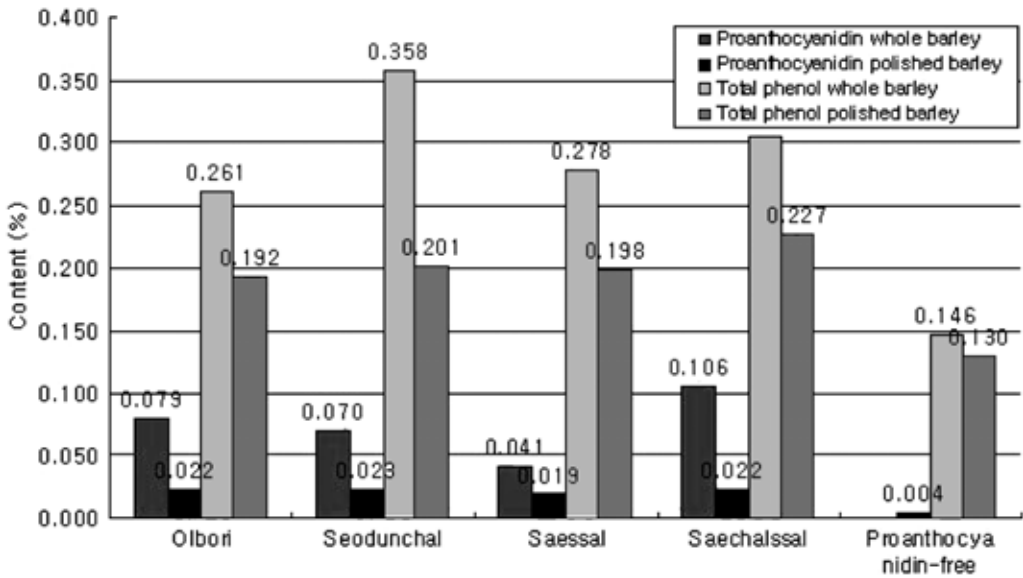


Figure 1. Total phenol and proanthocyanidin content of whole and polished barley.

Table 3. Total phenol and proanthocyanidin content of whole and polished barley.

	Proanthocyanidin(mg/g)		Total phenol(mg/g)		Whiteness		
	Whole	Polished	Whole	Polished	0 hr	24hr	53hr
NR1	1.30	0.33	2.3	2.1	34.5	20.7	15.4
NR2	1.72	0.55	3.1	2.3	36.5	19.8	15.4
NR3	1.43	0.66	2.8	2.4	35.0	20.1	15.0
NR4	1.47	0.51	2.7	2.2	36.6	20.7	15.0
NR5	1.41	0.46	2.6	2.5	37.0	20.8	16.8
NR6	1.38	0.53	2.5	2.4	34.5	20.5	15.2
NR7	1.74	0.63	2.6	2	36.0	18.7	14.3
NR8	1.00	0.24	1.9	1.6	39.5	23.9	20.3
NR9	1.35	0.39	2.5	2.2	33.2	18.5	14.7
NR10	1.75	0.74	3.4	2.3	35.8	16.9	13.2
NR11	1.29	0.66	2.4	1.9	35.3	19.9	14.8
NR12	1.32	0.36	2.8	2.7	37.2	20.3	17.2
NR13	1.18	0.38	2.4	2.2	34.9	23.3	18.7
NR14	2.21	1.05	2.5	1.8	18.4	15.0	11.1
NR15	1.48	0.60	2.5	2.4	33.2	19.0	15.1
Radiant	n.d	n.d	1.1	1.1	37.1	34.8	29.8

n.d. = not detected

slowly decreased to 53 hour. Radiant was a covered barley cultivar developed in the USA and has low total phenol content. The whiteness of Radiant (a proanthocyanidin-free cultivar) barley decreased slightly with increasing storage time. There was a strong positive correlation between total phenol and proanthocyanidin content and a negative

correlation between whiteness of cooked barley after 24 hr and proanthocyanidin content (Figure 2).

Zasoojeongchal

Zasoojeongchal is a new, colored naked barley cultivar developed from a cross between HB83072, a disease resistant and

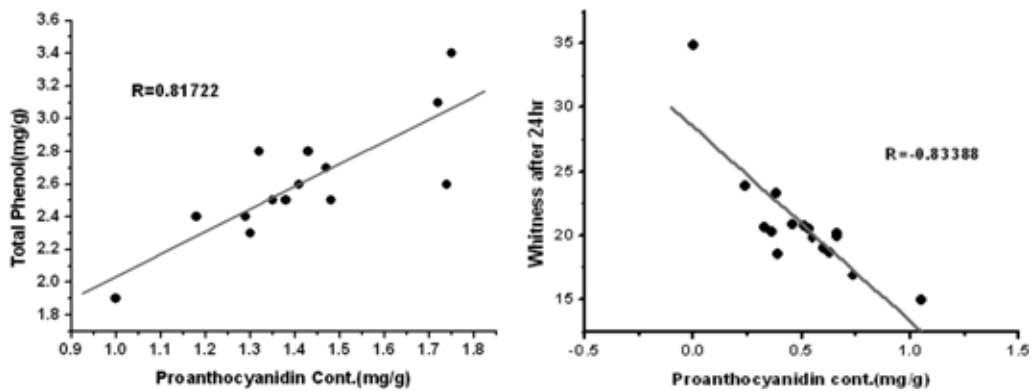


Figure 2. The relationship of proanthocyanidin content with total phenol and proanthocyanidin (left), and Whiteness of cooked barley after 24 hr storage (right).

Table 4. Pearling and cooking characteristics of cultivar Zasoojeongchal.

Cultivar	Anthocyanin content (ug/g)	beta-glucan content (%)	Amylose content (%)	Grain pearling whiteness	Cooking quality	
					Water absorption (%)	Expansion (%)
Zasoojeongchal	15.2	6.6	5.7	31.6	246	403
Saechalssalbori	0	5.8	5.1	37.2	243	412

Table 5. Pearling and cooking characteristics of cv. Jinjuchal in comparison with cv. Saechalssalbori.

Cultivar	Total phenol (mg/g)	Proanthocyanidin (mg/g)	Protein (%)	Whiteness	Beta-glucan (%)	Amylose (%)	Absorption (%)	Expansion rate (%)
Jinjuchal	5.9	1.00	11.9	36.1	8.4	6.0	249	401
Saechalssalbori	9.8	1.72	12.7	38.2	6.6	5.4	242	425

good quality line, and Dokusima mochi hadaka, a colored and naked barley with waxy endosperm developed by the Honam Agricultural Research Institute (HARI), National Institute of Crop Science (NICS), Rural Development Administration (RDA) in 2006. The new cultivar Zasoojeongchal had 75 cm culm length, 5.4 cm spike length, and it showed weak winter hardiness. It has a higher beta-glucan content, anthocyanin content, water absorption rate and expansion rate than the check cultivar, Saechalssalbori.

Jinjuchal

Jinjuchal is a new naked and waxy barley cultivar developed from a cross between Jinmichapssal, with strong winter hardness, good cooking quality and high whiteness, and Suweon 333, developed by the Honam Agricultural Research Institute (HARI), NICS, RDA in 2007. The new cultivar Jinjuchal had 82 cm culm length and 4.6 cm spike length, with higher beta-glucan content, whiteness after cooking, and lower

total phenol content than the check cultivar, Saechalssalbori. The whiteness and lightness decreased with storage time after cooking, but Jinjuchal's decrease rate was less than the check cultivar, Saechalssalbori.

Conclusion

- Waxy cultivars contained more total beta-glucan than non- waxy, and polished grain had higher content of beta-glucan than whole grain. Total beta-glucan content was the highest (9.72) in Doowonchapssalbori. Soluble beta-glucan as a proportion of total beta-glucan ranged between 48.3 and 79.4%. There was a strong positive correlation between total beta-glucan and soluble beta-glucan.
- Whole barley grains showed higher total phenol and proanthocyanidin content than polished barley grains. Whole barley had higher total phenol and proanthocyanidin content than polished barley. There was a strong positive correlation between total phenol and proanthocyanidin content and a negative correlation between whiteness of cooked barley after 24 hr and proanthocyanidin content.
- Cv. Zasoojeongchal is a new, colored naked barley cultivar developed by the Honam Agricultural Research Institute (HARI), NICS, RDA in 2006. It is a six-row waxy barley, 75 cm tall with a spike 5.4 cm long. It has higher anthocyanin content, greater water absorption rate and greater expansion rate than control, cv. Saechalssalbory.
- Cv. Jinjuchal is a new, naked waxy barley cultivar developed in 2007. The new cultivar is 82 cm tall with a spike 4.6 cm long. It has higher beta-glucan content, whiteness after cooking, and lower total phenol content than cv. Saechalssalbory. The whiteness and lightness decreased according to storage time after cooking, but this decrease in Jinjuchal was smaller than in the control.

Session 8
Barley uses – Feed

Breeding barley for end-use quality. Will it change barley marketing?

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Abstract

- NIRS is a rapid and cost-effective way to screen for feed quality in both research and commercial samples.
- We can build a quality standard that will market feed grains on end-use quality.
- We can build better barley for feed that will increase feed efficiency and reduce environmental impact.
- Two-row or six-row barley does not ensure better quality.
- Simply measuring test weight (bushel weight) and kernel weight (seed plumpness) do not define nutritional quality.

Introduction

The most limiting and expensive traits to select for, whether you are breeding new varieties or marketing/buying product, are the quality traits. However, these are often the most economically important traits, determining the end use of our grain. Quality traits are not simply inherited; they have other physiological effects on the seed or final product and are significantly affected by the environment. The variation between samples of barley for any one trait is between 20 and 40%. This variability is due to both genetic and environmental factors. Simply measuring test weight (bushel weight) and kernel weight (seed plumpness) do not define nutritional quality.

Phenotyping breeding populations

Over the last 40 years, our research program has looked at many different techniques to rapidly screen breeding populations for different perceived or real economic quality traits. Most techniques are too expensive or unreliable to screen large numbers of samples. In about 1978 we began to look into Near-Infrared Reflectance Spectroscopy (NIRS) technology. However it took until 1995 to see the technology mature to the point where it could be used for screening breeding populations for a significant number of quality parameters. This was due to advancement in both scanning monochromator technology and desktop computer capability. Modern NIRS is accurate, repeatable and rapid, as well as non-destructive, allowing breeding programs to screen large numbers of lines for multiple characteristics simultaneously.

NIRS technology uses electromagnetic radiation to measure energy absorption of hydrogen-containing molecules, which produces a 'fingerprint' or spectrum of the sample. This spectral data is then matched with data from traditional wet chemistry analyses and/or animal performance data to produce a calibration equation. The NIRS equation can then be used to predict feed quality characteristics such as protein and energy in unknown samples, all in just a matter of minutes. Therefore, with this technology the breeder is able to rapidly

phenotype genetic populations for many quality traits simultaneously. At the present time, the Field Crop Development Centre (FCDC) in Lacombe, Alberta, Canada, is running over 40 000 samples through the laboratory in a 4-month period, and screening for over 25 quality characteristics related to malting, feed and food quality (Table 1).

Measuring end use quality

Measuring and breeding for end-use quality has long been the norm for malting barley and bread wheat. However, feed grains have not been measured for quality and are primarily

used in animal diets based on price and perceived average quality characteristics for the species. Reports from the maize industry in the USA indicate that the variability for Digestible Energy (DE) in corn is 7 to 8% (FeedInfo News Service, 31 May 2005). We have found the variability in Canadian barley to be between 25% and 40%, depending on the quality trait. This has tremendous economic impact on the efficiency of feed use in barns and feedlots. Figure 1 is based on data from the breeding program over years and locations and compares the range and mean for DE and % Protein Digestibility (PD) in a pig. This

Table1. Statistics for NIRS equations developed at the FCDC to predict barley quality characteristics used for screening in the breeding program.

Equations for Barley					Equations for Malt Barley				
Constituent		Minimum	Maximum	SEC*	R ²	Constituent		Minimum	Maximum
Hulled Protein	%	8.10	17.70	0.17	0.95	Conagra Protein	%	8.40	17.59
Hulless Protein	%	8.35	18.58	0.30	0.98	Fine Extract	%	70.00	85.50
Protein Digestibility	%	39.90	97.70	1.62	0.91	Diastatic Power	DP	11.00	352.00
Energy Digestibility	%	68.00	92.10	1.44	0.92	Alpha Amylase	DU	5.00	103.20
Digestible Energy Content	kcal/kg	1845	3742	56	0.92	Total Malt Protein	%	8.19	18.75
Gross Energy	kcal/kg	2665	4237	21	0.90	Soluble Malt Protein	%	2.66	8.31
Lysine	%	0.36	0.54	0.05	0.97	Wort B-Glucan	mg/l	18	1200
Starch	%	50.30	72.09	1.26	0.91	Friability	%	1.95	100.00
B-Glucan	%	2.68	7.03	0.23	0.91	Viscosity	cP	1.40	2.77
Pentosan	%	2.78	6.66	0.18	0.89	Homogeneity	%	29.60	100.00
Ash	%	1.32	3.06	0.07	0.92	Skinning Potential	%	0	97
Lipid	%	1.45	3.71	0.09	0.92				
Total Fibre	%	7.99	23.34	0.45	0.95				
Soluble Fibre	%	2.30	8.25	0.27	0.93				
Grain Color	Minoita	35	56	1.04	0.91				
Pearl Color	Minoita	21	29	0.45	0.89				
Pearl Rating		2	9	0.36	0.91				

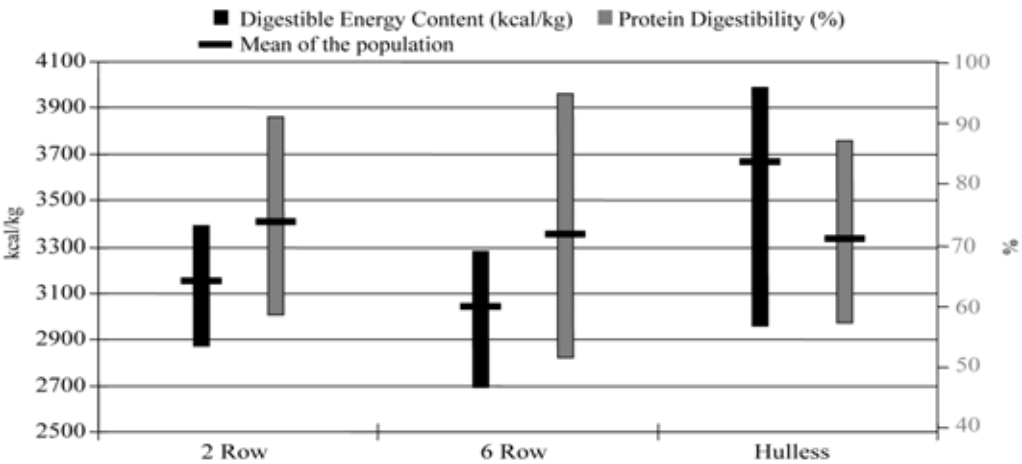


Figure 1. Range of swine DE Content (kcal/kg DM) and PD (%) in two row, six-row and hulless barley populations from the FCDC breeding program over years and locations.

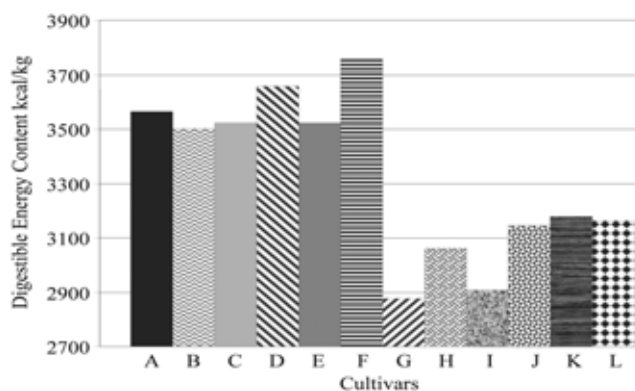


Figure 2. Differences between cultivars in swine DE content (kcal/kg DM), as predicted by FCDC NIRS model, of barley grown in an advanced yield trial at Lacombe in 2006.

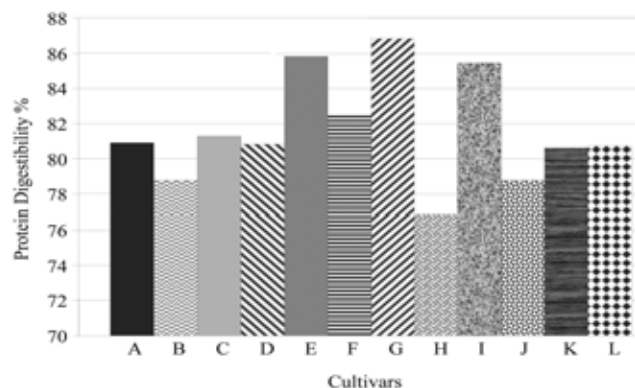


Figure 3. Differences between cultivars in swine PD (%), as predicted by FCDC NIRS model, of barley grown in an advanced yield trial at Lacombe in 2006.

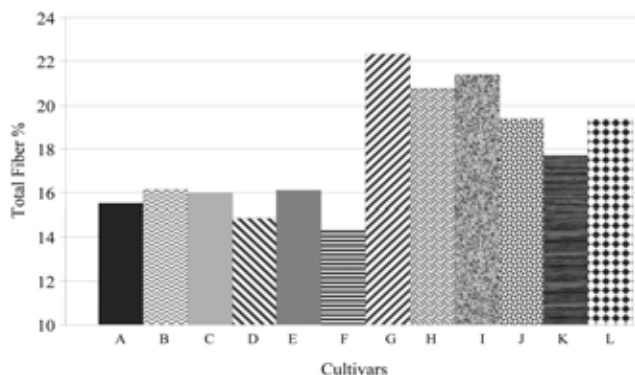


Figure 4. Differences in cultivars in total Dietary Fiber (%DM) content, as predicted by FCDC NIRS model, of barley grown in advanced yield trial at Lacombe in 2006.

gives a good indication of the genetic variability available to the breeder.

Information in the Figures 2, 3, 4 and 5 is based on advanced yield trial material grown in one location in 2006. Cultivars A through F are hulless lines and lines G through L are hulled lines. There are two-row and six-row types in both sets. What is important in this set of figures is not only the variability we see for the four traits measured but also the interactions between the traits. On an economic basis, cultivar F would be the best line for feeding swine.

The biggest problem in measuring feed quality is that it is not defined and differs depending on the species and maturity of animals being fed. The second-biggest problem is that the error in animal research is often too great to define the sample's characteristics. If you couple that with the sample size needed for animal trials and the cost of these trials, we are limited to bench-top techniques that we hope have a relationship to animal performance. As of yet, there are no standardized, in vitro techniques used worldwide for establishing animal feed quality.

Another large source of error in developing NIRS technology for feed quality is in the selection of the sample sets that are used to develop and test the calibration. The variability in a commercial sample of cv. Metcalfe barley compared with a genetically pure sample from a randomized controlled experimental plot is shown in Figure 6.

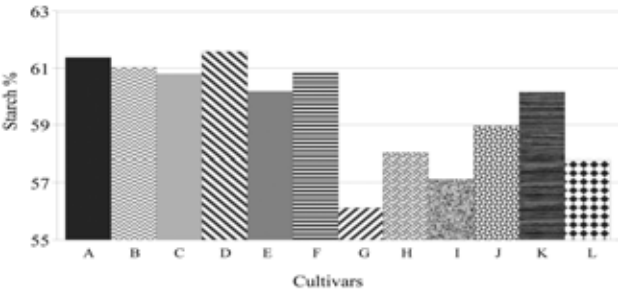


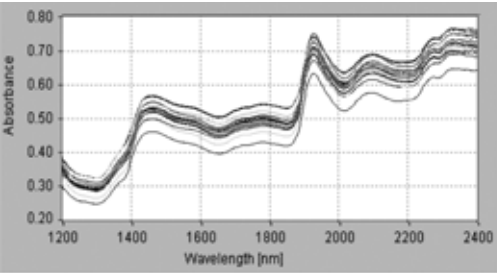
Figure 5. Differences between cultivars in Starch (%DM) content, as predicted by FCDC NIRS model, of barley grown in advanced yield trial at Lacombe in 2006.

This represents how environment can introduce variation within a sample thereby affecting the commercial use of NIRS technology. Therefore, depending upon the trait and the genetic and environmental influence on that trait, a special set of samples that contain the genetic and environmental variability represented in a breeding population may be required for the development of a calibration. In fact, a different set of samples may be required for every trait. Good examples are beta-glucan and amino acids in barley. For this we would recommend using the same population building techniques that would be used for building a Molecular Marker population;

that is, making several crosses between high and low lines and building a calibration sample set from randomly derived F₅ lines grown at several locations.

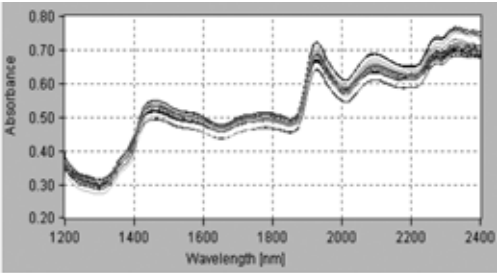
Our goal is to develop a set of in vitro techniques that can be used to develop robust calibrations for as many feed quality characteristics as possible. These calibrations can be used to phenotype both research and commercial samples. This should lead to further definition of the genetics controlling these quality characteristics and the development of varieties with greater economic feed value.

One of the questions that must be asked is, ‘Will this methodology lead to significant improvement in quantitatively inherited traits?’ Since 1990, FCDC has made significant progress in screening genotypes for swine energy and protein digestibility, as shown in Figure 7. We have not as yet, tried to determine how many genes are involved in this improvement or if the genes are markable. We suspect that it is similar to breeding for yield in that you can make more progress selecting for the yield than you can by selecting for individual components of yield.



Commercial sample of AC Metcalfe – 16 scans

Protein Content (%)	
Min	11.46
Max	13.58
Difference	2.12
Ave	12.31
StdDev	0.6449



Breeding sample of AC Metcalfe – 16 scans

Protein Content (%)	
Min	11.02
Max	12.24
Difference	1.22
Ave	11.65
StdDev	0.2830

Figure 6. NIR spectral and protein determinations showing the variation within a commercial and a genetically pure sample of AC Metcalfe barley.

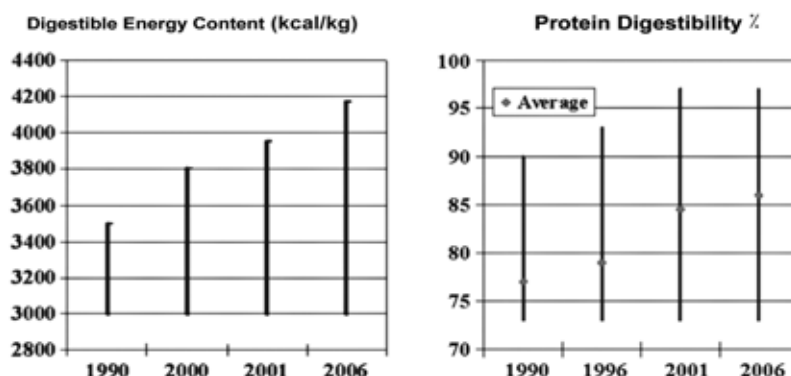


Figure 7. Range of hulless barley germplasm for DE content (kcal/kg) and PD (%) as affected by selection using NIRS between 1990 and 2006

Table 2. Comparative economic value of hulled and hulless barley samples with different kernel weight and test weight. Values in Canadian dollars (C\$).

Barley type	TKW (g)	Test weight (kg/hL)	Protein (%)	Protein digestibility (%)	Gross energy (kcal/kg)	Digestible energy (kcal/kg)	Value of grain (C\$/t)
6-row feed	43.6	52.1	11.7	75.6	4000	2700	120.00
6-row feed	47.7	52.4	10.9	70.8	4000	2900	140.23
6-row feed	37.8	49.1	12.6	64.4	4000	3000	155.80
2-row feed	47.1	54.8	13.0	68.1	4000	3000	155.80
6-row hulless	32.2	64.6	13.2	75.3	4100	3600	227.39
2-row hulless	49.6	65.5	14.3	76.3	4100	3600	227.39

TKW = 1000-kernel weight. For grain value comparison, canola oil (pure DE source) at C\$ 880 per tonne was used. Energy values rounded to nearest 100 kcal/kg.

Commercializing NIRS technology

We have begun the process of transferring the NIRS technology used in genetic development to commercial partners, thereby allowing for the definition of the major quality components of feed ingredients in a standard format. This will allow both feed and livestock producers to price ingredients according to their true feed value.

Ultimately the use of NIRS technology should result in considerable monetary savings for livestock producers and feed manufacturers by providing quick reliable analysis of feed quality components. These savings could be achieved through formulation of diets more closely related to the actual nutrient requirement of the animal (decreasing

safety margin factors) and/or through better animal performance. Feed grain producers will also benefit from NIRS technology by using the grain analysis to market their grain according to its specific quality traits, and hence being paid for actual feed grain quality. This is demonstrated in Table 2 that shows the comparative economic value of barley in swine diets by the addition of canola oil to bring the DE content up to 3600 kcal/kg, i.e. equal to maize. It is very evident from this comparison that kernel weight and test weight do not affect economic value.

Last but not least, it will define for the plant breeder quality traits of economic importance and the tools to phenotype these traits, thereby developing greater economic and nutritional value in the crop.

Further reading

- Field Crop Development Centre publications can be accessed on our Web site at: [http://www1.agric.gov.ab.ca/\\$department/deptdocs.nsf/all/fcd5464](http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/fcd5464)
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Barley QTLs: A compilation of known loci and the discovery of a new QTL for dry matter digestibility (DMD)

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Abstract

Quantitative trait loci (QTLs) in barley have been reported for nearly two decades, describing genomic regions influencing morphology, agronomics, quality and disease resistance. In 2001, Hayes and co-workers published a 'Summary of Published Barley QTL Reports', comprising over 700 QTLs. With the support of the USBGMP, these QTLs have been validated in the literature, assigned Plant Ontology values, supplemented with additional statistics when possible, given a descriptive name to include the trait, population and barley chromosome (e.g. QHd.StMo-1H describes a heading date QTL in the Steptoe \times Morex population on 1H) and entered into GrainGenes. An accompanying worksheet providing all QTL records, data type descriptions and templates for new data is available online (wheat.pw.usda.gov/ggpages/barleyQTL_workbook.xls). QTLs published since 2001 are now being curated, with the aim of the GrainGenes database becoming current in 2008. QTLs are also being placed on a consensus map, available on CMap, for comparative mapping. Our laboratory is also discovering new QTLs for barley forage and straw digestibility, using the bovine rumen as a model for pre-treatment in an ethanol fermentation production stream. Within the Barley World Core Collection,

we found that forage digestibility ranges from 60 to 90%. Association analysis using results from an Illumina GoldenGate® assay revealed known QTLs (heading date, plant height, etc.) as well as QTLs for digestibility.

Part 1.

The barley community curation workbook

Quantitative trait loci (QTLs) have been described for barley since 1992, when agronomic studies found QTLs for height and heading date (Hackett *et al.*, 1992) and seed dormancy (Ullrich *et al.*, 1992). Since then, hundreds, perhaps thousands of QTLs have been mapped in barley for agronomic characters, quality-related traits, and biotic and abiotic stress responses. By the late 1990s, several barley labs were mapping QTLs, when publications peaked at about 50 titles per year and has remained constant. Throughout this time, QTL discovery for other small grains (wheat, maize and rice) followed a similar course (Figure 1).

From the beginning, the wheat community compiled published QTLs in the Catalogue of Gene Symbols for Wheat (McIntosh *et al.*, 2006) and systematically named the QTLs to include a basic symbol "Q", locus symbols

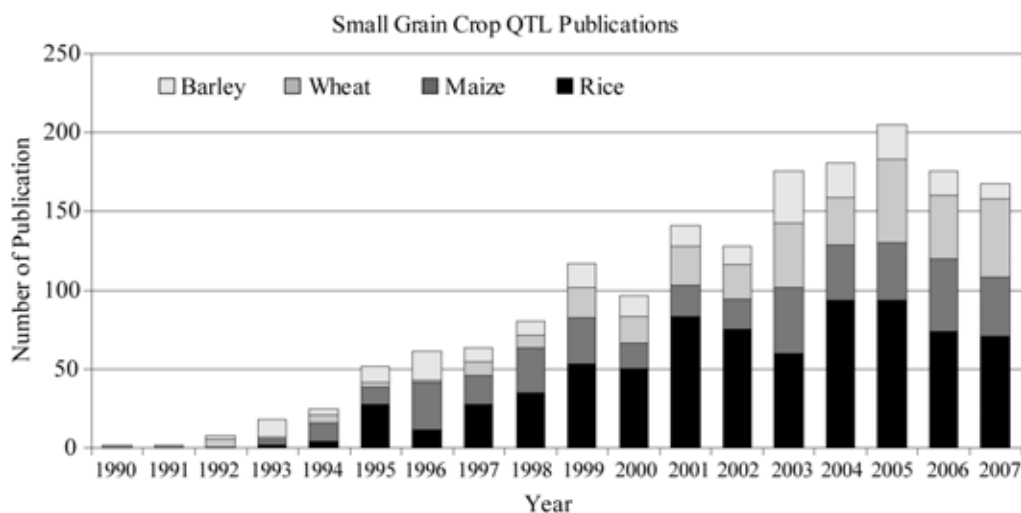


Figure 1. Results of Google™ Scholar (scholar.google.com) Title Search (ex. 'barley OR *Hordeum*' AND 'QTL or quantitative trait')

(trait abbreviation and lab designator), and chromosome. For example, QYld.psr-7B is a yield QTL, mapped at the John Innes Centre, on chromosome 7B.

By 2006 there was still no systematic naming scheme universally adopted by the research community to name barley QTLs, and no mention of QTLs appeared in the "Rules for Nomenclature and Gene Symbolization in Barley" (Franckowiak and Lundqvist, 2007). Some authors mimicked the wheat naming scheme, while others just designated the QTL by a trait name or environment. In 2006 at the USDA/ARS Western Regional Research Center in Albany CA, Victoria Carollo, Tom Blake, Patrick Hayes and Olin Anderson met to plan the Barley Community Curation Workbook (BCCW), and devised a new nomenclature for barley QTLs. Barley QTL names would consist of a "Q", a trait abbreviation, the population abbreviation, and chromosome. For example, QAa.StMo-2H is a QTL for alpha-amylase activity mapped in the Steptoe \times Morex population on chromosome 2H. Although similar to the wheat model, the barley QTL name replaces the laboratory designator with an abbreviation for the parent

population used in QTL discovery. This is both more descriptive and less political.

Summaries of barley QTLs have not, to date, been cataloged in an ongoing effort like that achieved by the Wheat Gene Catalog. In 2000, Zale *et al.* published a summary of 168 barley malting quality QTLs representing 19 malting traits in 9 mapping populations, and placed these onto consensus RFLP maps. In 2001, the North American Barley Genome Mapping Project (NABGMP) conducted a multi-institutional effort to summarize what was then all published QTLs, producing a catalog of 748 individual QTL records with significant markers, BIN assignment, references, and associated traits (Hayes *et al.*, 2001).

In 2006, the BCCW project was launched, with two main goals: (1) to provide templates for the barley research community to contribute QTL-related data to GrainGenes (Carollo *et al.*, 2005; <http://wheat.pw.usda.gov>) and (2) to provide a spreadsheet to track the curation of the 748 QTLs summarized by the NABGMP into GrainGenes records, followed by addition of QTLs published after 2001 as new QTLs are curated. The BCCW is updated frequently and freely downloadable

as an Excel workbook from the GrainGenes Website (<http://wheat.pw.usda.gov/ggpages/barleyQTLworkbook.xls>) (Figure 2).

The BCCW is an Excel workbook comprising 8 worksheets. The Introduction page describes the project, encourages community participation and provides instructions for data submission. The “QTL Worksheet Original» is a compilation of 748

QTLs catalogued by Hayes *et al.* (2001). This worksheet contains the basic information, such as references, associated markers, chromosomes, parents, traits, etc., and has been enriched with new names (synonyms) and Trait Ontology terms.

The workbook has four template worksheets that provide a simple mechanism for new data submission. These are entitled:

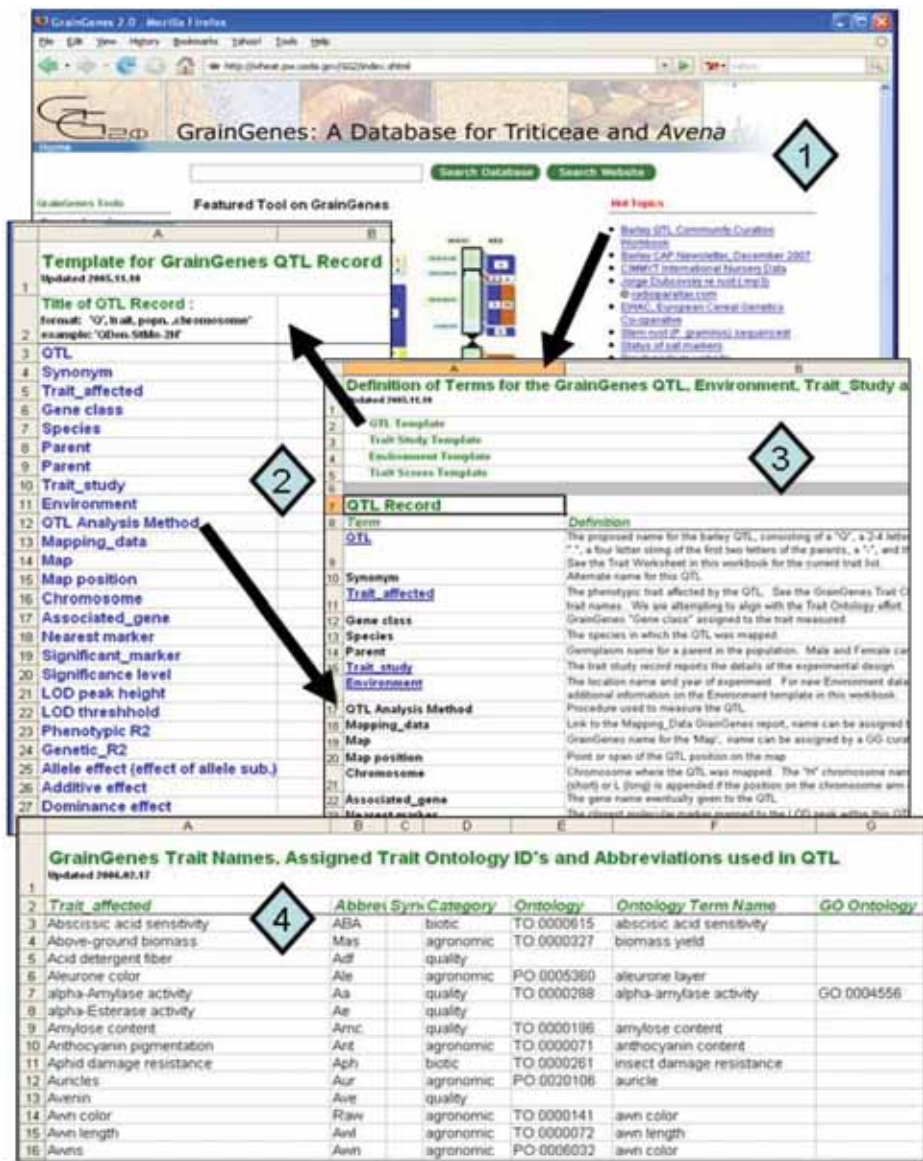


Figure 2. Overview of the elements of the BCCW worksheets.

QTL; Trait Study; Environment; and Trait Scores. Templates each have a column of all possible data types for that record, which are hyperlinked to the ‘Terms Defined’ worksheet (Figure 2). The “Terms Defined” worksheet provides terms used in the templates, definitions and examples of data. Where appropriate, examples are hyperlinked to GrainGenes records. The “Traits” worksheet lists all traits, abbreviations, and Trait Ontology numbers for the new Barley QTL and existing traits already in GrainGenes.

The “Loaded onto GrainGenes” worksheet contains all of the new information for curated QTLs that were loaded onto GrainGenes as database files. This file is especially useful as one can sort the QTLs by significant markers to discover seemingly unrelated QTLs mapping to the same position in the barley genome.

Curation involves assigning a new name to the locus in a consistent and intuitive manner, as described above. When multiple QTLs from the same population are mapped to the same chromosome (about 200 to date), numbers are appended to the QTL name (ex. QHt.HaMo-2H.2). In some cases, these are from different publications, and the oldest described QTL will be given the “.1” extension. If two QTLs are from the same publication, the paper is first referenced to see if the authors assigned a number, if not, a number is appended to the QTL name (example QGpc.StMo-3H.1, QGpc.StMo-3H.2) as the community has traditionally assigned marker names, i.e. starting from the telomere of the short arm and working toward the telomere of the long arm.

All QTLs are validated in the original reference and associated data is edited when necessary, specifically with respect to significant molecular markers and enhanced with additional statistics (LOD scores, phenotypic R^2 , allele (additive) effect and significance level) and other information (QTL analysis method, higher scoring parent, additional references, traits and comments)

wherever possible. Nearly all of the original 748 QTLs have now been curated, with the exception of QTLs that were only mapped to AFLP markers, resulting in ca. 560 new barley QTL records in GrainGenes as of January 2008.

As an extension of the BCCW, four QTL consensus map sets are being assembled on a ‘backbone’ barley consensus map. Separate map sets will display QTLs for agronomic traits, quality traits, biotic stress responses and abiotic stress responses, and should be available in the summer of 2008. The ‘backbone’ maps, originally constructed by Rostoks *et al.* (2005) and available on GrainGenes as the Map_Set ‘Barley, Consensus 2005, SNP’ has been selected to provide the backbone maps for the QTL consensus maps. The 1230 loci on these maps, including single-nucleotide polymorphisms (SNPs), RFLPs, microsatellites and mapped genes, match many of the markers used for mapping QTLs. For QTLs with significant markers not found on the ‘Barley, Consensus 2005, SNP’ maps, further work can be done using the CMap map comparison tools to approximate the location of the QTL. The rationale for placement will be noted in the GrainGenes record as a Comment.

Some might argue that one enormous map set would be a more useful tool for comparing QTLs. Since GrainGenes displays maps using the CMap software, users can easily align maps with common markers and could align (for example) all four 1H QTL consensus maps to discover overlapping QTLs across broad categories. As QTLs are curated, they are assigned a trait that will eventually designate the appropriate consensus map. Table 1 lists all traits curated in barley as of January 2008, and the number of QTLs for that trait. Note that the same QTL mapped in different populations will be counted as a separate QTL record so the number of actual regions in the barley genome influencing the trait may be fewer.

Table 1. Traits of the barley QTLs in GrainGenes as of January 2008.

Abiotic Stress	No. of QTLs	Agronomic	No. of QTLs
ABA response at germination	6	Crossability with wheat	4
Boron sensitivity	8	Crown fructan content	1
13C content	4	Days to heading	49
Salt sensitivity at germination	5	Days to maturity	3
Salt sensitivity in seedlings	10	Dormancy	5
Winter survival	4	Germination speed	7
Biotic Stress	No. of QTLs	Head shattering	4
Reaction to leaf rust	3	Height	18
Reaction to net blotch	13	Leaf tip necrosis	14
Reaction to powdery mildew	7	Lodging	11
Reaction to <i>Pyrenophora graminea</i>	4	Stem breaking	4
Reaction to scald	8	Basal Internode length	2
Reaction to spot blotch	2	Shoot differentiation	1
Reaction to stem rust	4	Straw diameter	3
Quality-Related	No. of QTLs	Straw length	3
Alpha-Amylase activity	24	Top internode length	3
Beta-amylase	1	Kernel length	2
Beta-glucan (barley)	3	Kernel plumpness	7
Beta-glucan (malt)	10	Kernel weight	8
Beta-glucan (wort)	1	Kernels per spike	5
Beta-glucanase activity	5	Plant grain weight	11
Beta-glucanase activity (green malt)	5	Plant weight	3
Beta-glucanase activity (kilned malt)	6	Spike grain weight	14
Diastatic power	28	Spike length	3
Extract viscosity	3	Spikes per area	4
Fine coarse difference	7	Test weight	28
Malt extract	39	Yield	32
Milling energy	2		
Soluble/total protein	9		
Wort clarity	1		
Wort viscosity	1		
Grain nitrogen	11		
Grain protein content	23		
Nitrogen 15 content	4		
Nitrogen content	4		

Part 2.

The discovery of a new QTL for dry matter digestibility (DMD)

Introduction

Apparently new traits can be in part composed of well-known genes that affect phenotype pleiotropically. Straw and forage digestibility are traits that are growing in importance as our economies demand transition from grain-based ethanol production to ethanol

production from agricultural waste. Effective QTL curation can identify these well-known genes that affect morphogenesis and help the researcher focus more directly on newly discovered genes with desirable characteristics.

Straw is an abundant by-product of small-grain production systems. Barley, wheat and oats produce nearly as much straw as grain; however across North America’s small-grains growing states, the market for straw is generally far less than the amount produced and baled. Aged, decaying piles of baled straw are commonly seen along the rural highways in the grain producing regions.

Annual forage crops, including barley, are well adapted to dryland cropping systems in the Northern Great Plains and are currently produced on 100 000 ha in Montana (Lenssen, 2005). Hooded or awnless hay barley varieties are among America's most popular annual forages. The Montana State University barley improvement program began development of barley varieties with improved straw fermentability and improved forage quality in 2006. In this report we discuss our initial findings.

The fermentable biomass of forage and straw is lignocellulose, which contains three major groups of polymers: cellulose, hemicellulose and lignin (Kuhad and Singh, 1993). Pre-treatment of lignocellulosic biomass is currently necessary to render cellulose and hemicellulose more accessible for further breakdown into component sugars for ethanol fermentation. Cellulose and hemicellulose can be released from lignin physico-chemically by acid, base, high temperature, and pressure treatments (Mosier *et al.*, 2005) or biologically by microbial communities, such as those populating the bovine rumen. Following release from lignin, enzymatic hydrolysis of cellulose and hemicellulose can be performed prior to or simultaneously with fermentation (Wright *et al.*, 1987).

Physico-chemical pre-treatment involves extremes of pH and temperature (Huang and Li, 1998; Saha *et al.*, 2005). This can be expensive, and toxic byproducts of lignin degradation (e.g. furfurals) can interfere with downstream fermentation. We expect microbial pre-treatment systems to be more environmentally benign and less costly.

Cell wall lignin makes up 25 to 33% of the dry weight of most plant biomass (Boerjan *et al.*, 2003). Lignin limits access to the cellulosic polysaccharides during processing, inhibits the fermentation process and creates a large co-product/waste stream during fermentable sugar production (Grabber *et al.*, 1998). Lignin reduction has been proposed

as a primary method to improve fermentation efficiency of wheat and barley straw, making them more attractive as a source for production of fermentable sugars (Carpita, 1996).

Significant work has been done to improve feed stocks to contain less lignin (Hu *et al.*, 1999; Koonin, 2006) and to increase ethanol yield during pentose or mixed hexose/pentose fermentation (Dien *et al.*, 2003; Becker and Boles, 2003; Jeffries, 2005). Several research groups have focused on finding or designing enzymes or organisms capable of more efficiently hydrolyzing lignocellulose at neutral or slightly acidic conditions so that pH re-adjustment would not be necessary (Kulkarni *et al.*, 1999; Huang *et al.*, 2005; Walker *et al.*, 2006). In addition, quite extensive work has been conducted exploring rumen (bovine, bison, deer, elk) and termite gut flora for efficient communities and organisms for effective lignocellulose conversion (Sahu *et al.*, 2004; Dröge *et al.*, 2006).

One traditional method to reduce plant lignin percentage, thereby improving cellulose yield, revolves around the utilization of naturally-occurring plant variation to increase *in rumen* digestibility (Figure 3). The MSU barley improvement program has effectively utilized the bovine rumen as a cost-effective bioassay to improve the feed quality of barley varieties (Blackhurst *et al.*, 1999; Bowman *et al.*, 2001). *In rumen* digestibility was successfully utilized in the development and release of 'Haxby' and 'Valier' barleys, dryland feed barley varieties with improved rumenal digestion characteristics.

The USDA Spring Barley World Core Collection (WCC) provides an excellent germplasm resource that enabled identification of lines containing novel alleles that dramatically improved the feed value of barley grain (Bowman *et al.*, 2001). This collection of 1917 *Hordeum vulgare*, *H. bulbosum* and *H. spontaneum* accessions is a subset of the 28 000 barley accessions



Figure 3. The *in rumen* dry-matter digestibility (DMD) assay at Montana State University.

collected worldwide and maintained by the National Small Grains Collection (NSGC) in Aberdeen, Idaho, USA. These lines represent a vast array of genetic resources, most of which are untapped for barley improvement.

Barley genetic analysis enjoyed a technological breakthrough in 2006. Previously, our laboratory and others successfully utilized single nucleotide polymorphisms (SNPs) as genetic markers (Shin *et al.*, 1990; Kanazin *et al.*, 2002). Following this initial effort, a large international collaboration, partly funded by the US National Science Foundation program, developed several thousand additional SNP markers that were incorporated into a high-throughput, massively parallel SNP detection platform manufactured by Illumina Corp. This platform simultaneously assays 1536 loci. We selected 96 lines from the WCC to represent as much diversity as possible. These 96 lines were analyzed using the Illumina GoldenGate® SNP platform. We performed association analyses (Flint-Garcia

et al., 2003) on these 96 lines utilizing the *in rumen* dry matter digestibility datasets, our field performance datasets and the SNP full-genome haplotypes.

As we found in our search for genes controlling useful grain quality variation, the most interesting lines (those with >80% forage digestibility) derive from germplasm resources that have not been utilized by modern plant breeders. The genes contributing to better forage digestibility are almost certainly not represented in today's cultivars. In 2008, we plan to utilize the Illumina genotyping platform again to assay genotypes of the most digestible and the least digestible lines from the WCC. We will then continue to correlate digestibility within allele state at each of these chromosomally-mapped genes to identify SNP polymorphisms that are genetically linked to genes contributing to variation in straw and forage digestibility, by using association analysis techniques.

Association analysis, also known as association mapping, is a method that relies

on linkage disequilibrium to discover the relationship between phenotypic variation and genetic polymorphisms (Brescghello and Sorrells, 2006). Association analysis should enable identification of key genes for transfer to modern, high yielding barley varieties. Of 100 association analysis citations identified in a recent search, only a few were performed in plants (Flint-Garcia *et al.*, 2003; Wilson *et al.*, 2004; Szalma *et al.*, 2005; Brescghello and Sorrells, 2006) while most involved human genetics studies. Strength of association between a single nucleotide polymorphism (SNP) or local haplotype and a gene modifying the phenotypic value of a trait of interest can be modified by several factors. These include the frequency and magnitude of the effect of the gene, the frequency and distribution of alleles at nearby loci, and the magnitude of linkage disequilibrium around the gene, which can, both individually and in combination, affect the ability to detect association between markers and traits (Zondervan and Cardon, 2004).

Methods

Plant samples

In 2006, we grew the entire USDA Barley World Core Collection (WCC) at the Montana State University A.H. Post Research Farm. In 2007, we grew two sets of the WCC from grain harvested from spring varieties from 2006, both under irrigation and in a dryland nursery. Switchgrass (*Panicum virgatum*) and *Miscanthus* sp. were grown in a greenhouse at the MSU Plant Growth Center. Barley straw was collected post-harvest from both nurseries, and wheat straw was collected from twelve known cultivars post-harvest at A.H. Post Research Farm.

Forage samples were collected at flowering, dried, and milled. In 2006, forage samples were collected from all entries of the WCC. In 2007, forage samples were only taken from about 120 entries of each of the tails of the DMD% distribution from the 2006

harvest, as well as the 96-member subset previously genotyped using the Illumina genotyping assay. Straw samples from a selection of wheat cultivars and all entries in the barley WCC from both irrigated and dryland nurseries were collected after plants had senesced completely. Miscanthus (n=8) was collected after one month (juvenile) and one year post-emergence from the rootstock (adult) from green tissue and dried. Switchgrass samples (n=38) were taken approximately one year after germination and were flowering. All forage and straw samples were milled in a Wiley laboratory mill to pass through a 2 mm screen.

Nylon mesh bags (Ankom) were filled with 0.5 g of the milled forage or straw, heat-sealed and assayed using the bovine rumen dry matter digestibility (DMD) method at the MSU Oscar Thomas Nutrition Center. This method involves putting 150 nylon bags (including one blank) into a large mesh bag and placing it into the rumen of a cannulated cow for 48 hours. (Figure 3) Samples are then removed, rinsed completely, dried for 48 hours at 60°C, reweighed and %DMD calculated.

Genotyping

The WCC was grown in the greenhouse and plants were harvested about 5–7 days after germination. Samples were frozen in liquid nitrogen and ground with a bead-beater. DNA was extracted from all 1917 barley lines using the Qiagen DNeasy Plant Mini Kit. Genotyping of a 96-member subset of these lines was performed using the Illumina assay at the USDA Genotyping Center in Fargo, ND, USA, using the Barley Pilot OPA1 primer set (T.J. Close, R. Waugh, K. Sato and A. Graner, pers. comm.).

Association analysis

With genotypes resulting from the Illumina Assay, we performed an Association Analysis using the TASSEL software developed by Ed Buckler's lab (USDA-ARS,

www.maizegenetics.net) for DMD values, as well as agronomic characters. Over 1100 of the loci detected by the Illumina Assay were previously mapped using the Steptoe \times Morex, Barke \times Morex and Oregon Wolfe Barley model mapping populations. Using the ‘Barley, Pilot OPA1 Consensus’ map available on GrainGenes, we produced a preliminary genetic map marking the locations of genes affecting plant height, heading date and forage digestibility.

Results

Barley forage dry matter digestibility (DMD) values from the World Core Collection (WCC) were consistent within accessions and varied from 58% to 91%. A 96-member subset of the core was selected to maximize diversity in 2006. This subset, the Core of the Core, was genotyped using the Illumina assay and the barley Pilot OPA1 primer set. The distribution of digestibility in this small subset of the WCC reflected well the range

of DMD values observed across the entire WCC (Figure 4). The highly and poorly digestible lines selected to represent the ‘tails’ of the DMD distribution from the 2006 dataset demonstrated the stability of forage digestibility over locations and years. Note that many of the very highly digestible forage samples from 2006 (>85% DMD) were actually winter barleys and did not produce seed to plant in 2007 (Figure 4)

Compared with the forage grass species evaluated, barley forage gave the highest overall levels of %DMD, with an average of 76.5%. The juvenile tissue (harvested one month after emerging from the rootstock) of one *Miscanthus* accession (V564-7-1) was highly digestible, at 78%, but this was reduced to 50.4% by maturity. Among *Miscanthus* accessions, digestibility in juvenile tissue varied from 56% to 78%, but dropped to 35% to 63% in mature tissue (one-year old). Switchgrass accessions had DMD values ranging from 57% to 68%. Barley straw accessions had DMD values ranging from

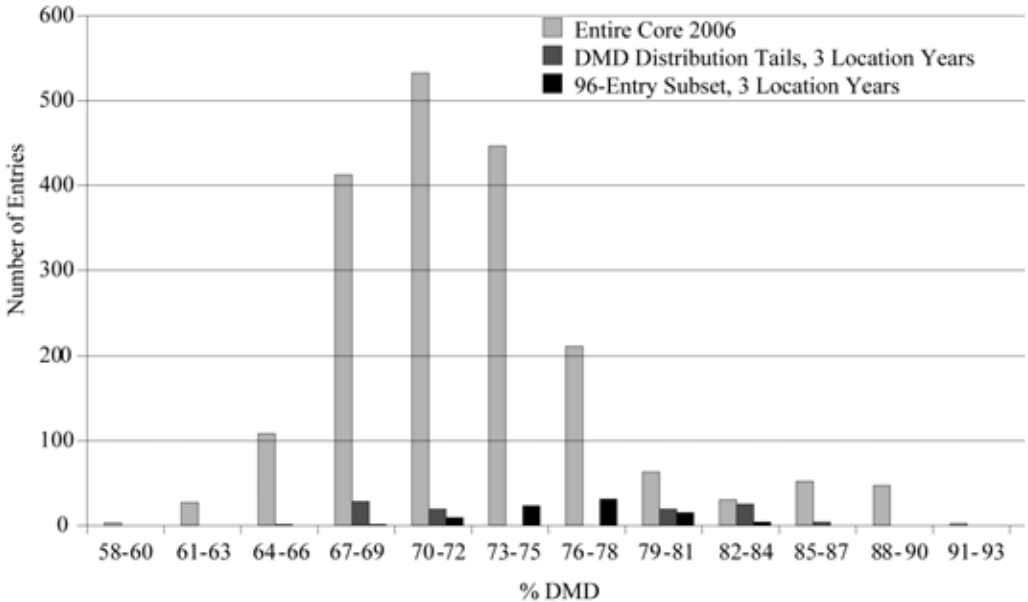


Figure 4. Dry Matter Digestibility (% DMD) in the Barley World Core Collection Forage using the *in rumen* assay.

40% to 75%. Wheat straw had DMD values ranging from 40% to 54% and we found no indication that solid stem vs. hollow stem or red vs. white varieties influenced DMD (Table 2; Figure 5).

An association analysis using the Illumina data generated from the 96-member subset of the WCC using previously mapped barley loci (Barley, Pilot OPA1, Consensus) identified putative genes for percent dry matter digestibility (%DMD) (Figure 6) as well as heading date, plant height, awn length, peduncle length and lodging (data not shown). Some of the association analysis-derived QTLs co-localized with well-known genes affecting plant morphogenesis. Others

did not. This result points toward candidate QTLs that might influence lignocellulose degradability, rather than leaf-stem ratio.

Conclusions

Barley straw is an excellent candidate as a cellulosic ethanol feedstock, and barley forage could easily be made a more easily digested cattle feed. In this report we mapped and marked genes impacting forage digestibility. We are currently developing validation populations to determine the value of these association analysis-derived QTL for variety improvement. Association analysis utilizing a broad germplasm array and

Table 2. DMD values for different barley samples and other species.

Crop	No. of entries	Minimum DMD%	Maximum DMD%	Average DMD%
Barley forage 2006	1862	58.3	90.7	73.2
Barley forage 2007, Dryland	314	67.5	88.5	78.9
Barley forage 2007, Irrigated	328	67	87.5	77.5
Barley straw	31	40.1	75.1	56.1
Wheat straw	10	40.6	54.7	50.1
Miscanthus sp. (juvenile)	8	57.3	77.7	62.4
Miscanthus sp. (adult)	8	35.6	62.3	50.4
Switchgrass	38	56.5	67.2	62.3

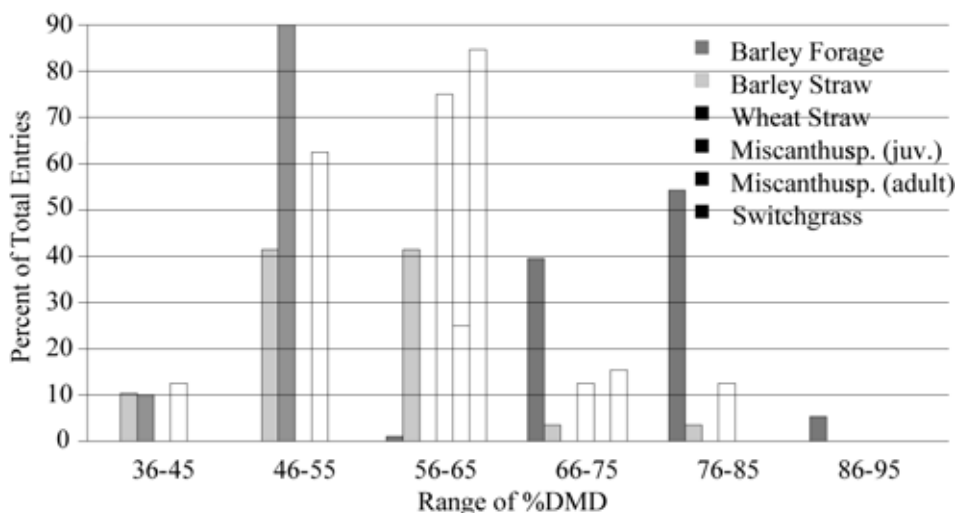


Figure 5. % DMD using the *in rumen* assay on barley forage and straw, wheat straw, juvenile and adult *Miscanthus* species and switchgrass. Since the sample numbers in the collections vary widely, data is shown as a percentage of total entries.

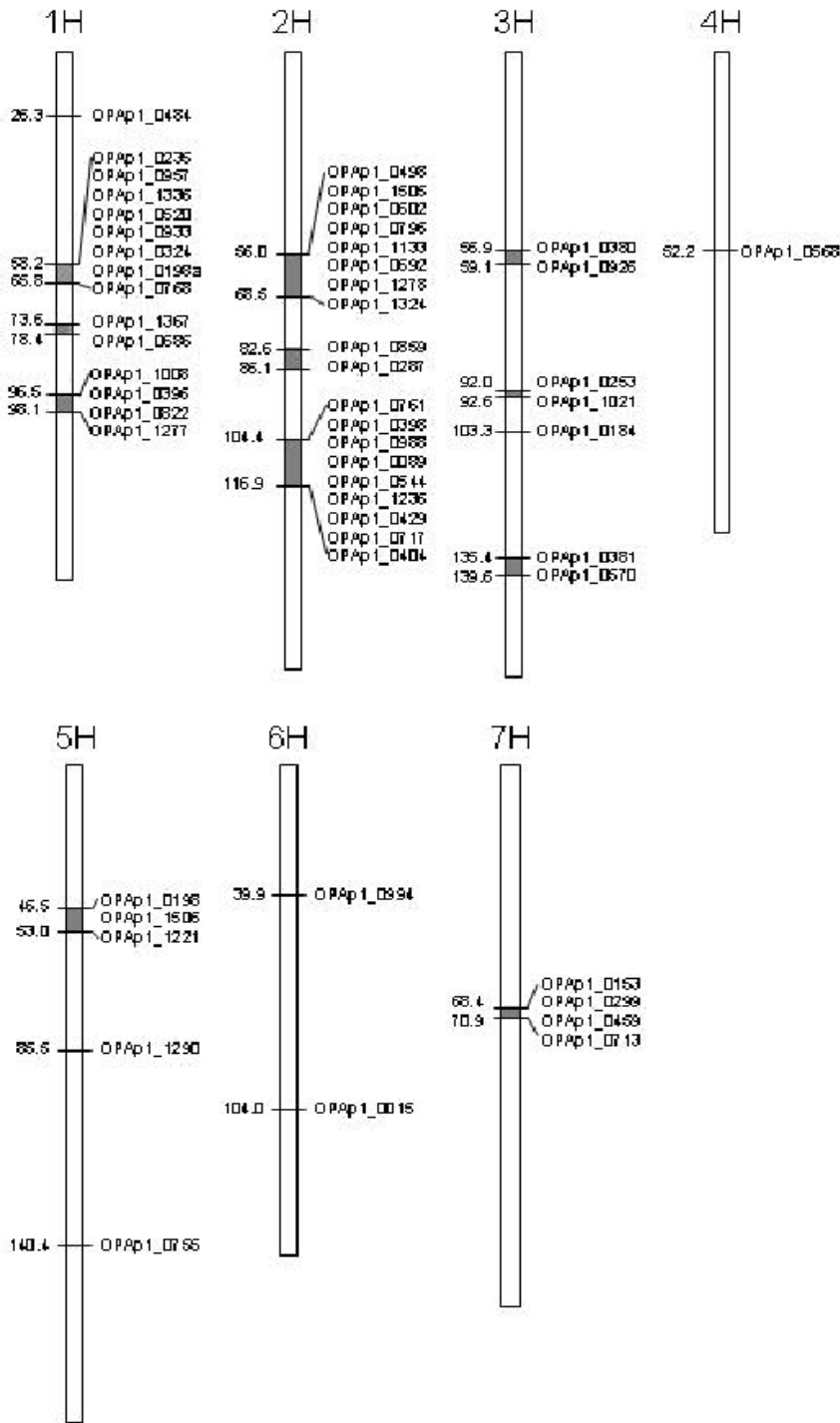


Figure 6. Genetic map of the OPA1 loci that are highly correlated ($P \leq 0.001$) with dry matter digestibility (%DMD).

dense, informative genome-wide haplotypes appears to be a promising route to gene discovery, and may help to identify parents for classic QTL analysis studies. In this study we identified 18 candidate QTL that impinge on forage digestibility. Some of these are well-known and affect forage digestibility by modifying plant growth and development. The earliness per se gene on chromosome 2H is one of these, affecting overall development and altering forage quality.

The BCCW summarizes QTLs, such as the %DMD QTL, that will be discovered and refined through efforts such as ours. When these and other data are catalogued onto spreadsheets and placed onto consensus maps we will be better able to identify the genes we wish to assemble into promising new barley varieties.

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Prospects for barley in augmenting forage resources in arid and semi-arid regions in India

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Abstract

Barley (*Hordeum vulgare* L.) is an important cereal crop, next to wheat in acreage and production in Rajasthan. The addition of barley to the crop portfolio is due to monsoon irregularities or frequent drought, resulting in inadequate supply of irrigation water to *Rabi* crops (In India, the *Rabi* season starts from second fortnight of October and ends by March/April. Main crops grown during *Rabi* are wheat, barley, brassicas, chickpea, oat and berseem). In recent years, severe drought has been reported in the Northern Plains of India, particularly Rajasthan, resulting in the migration and loss of livestock in search of green fodder in the November to January period. Barley, being a fast growing crop with high biomass, has been recognized as a potential forage resource. Barley cultivation can be more productive and profitable if the cost of production is further reduced by developing efficient dual-purpose barley varieties. Sowing in the second fortnight of October will provide a harvest of green forage at 50–55 days to feed livestock during the lean period. Systematic research efforts pyramiding all the desirable traits through breeding approaches are in progress. The advanced lines were tested over different environments with the objective of evaluating the promising genotypes for green forage yield at first cut of 50–55 days and the grain yield from the regenerated crop. The genotypes RD 2670, RD 2715, RD 2035

and RD 2552 gave promising results as dual-purpose barley. These findings may help in planning for more rigorous evaluation in augmenting forage resource availability in problematic areas.

Introduction

In arid and semi-arid regions of India, animal husbandry occupies an important role, which is increasing with increasing aridity. These regions have a climatic classification primarily based on indices of atmospheric moisture, and are essentially water-deficit areas. In India, the arid zone covers 10% of the total land area, largely confined to western states, viz. Rajasthan and Gujarat. The semi-arid zone occupies >30% of total land area, largely confined to southern states and western parts of north-western states (Table 1).

Rajasthan sprawls over the major part of North West India between 23°30' and 30°12' N latitude and 69°30' and 78°17' E longitude, and has the highest proportion of its area (73.6%) as arid and semi-arid, followed by Gujarat (29.5%) and Andhra Pradesh (21.5%).

The arid and semi-arid regions are exclusively rainfed, and rainfall is generally low and the rainy season is short. The rainfall in the semi-arid region ranges from 250 to 500 mm/yr. In arid regions the rainfall varies from 100 mm/yr in the western-most part of Jaisalmer district in Rajasthan to 400 mm/

Table 1. Extent of arid and semi-arid zones in different states of India.

State	Area (million km ²)			As % area of state
	Arid	Semi-arid	Total	
Andhra Pradesh	0.022	0.138	0.182	21.5
Gujarat	0.064	0.090	0.154	29.5
Haryana	0.013	0.027	0.040	6.8
Karnataka	0.009	0.139	0.148	17.6
Madhya Pradesh	—	0.059	0.059	6.2
Maharashtra	0.001	0.189	0.190	20.2
Punjab	0.016	0.032	0.048	8.3
Rajasthan	0.195	0.121	0.316	73.6
Tamil Nadu	—	0.095	0.095	10.0
Uttar Pradesh	—	0.064	0.064	6.7

yr at the eastern fringe along the Aravali hill range. The rainfall is highly erratic and unpredictable. Approximately 95% of the rainfall is received during monsoon (June–September) and the coefficient of variability ranges from 40 to 60%. High evaporation (1500–2000 mm/year) coupled with low rainfall causes results in severe aridity.

Forage availability and demand

Rajasthan, with its very high proportion of arid and semi-arid areas, has a big gap between demand and supply of forage. A high degree of fluctuation is observed in forage production, which is mainly associated with the amount and distribution of rainfall.

A total supply of 58.7 million tonne of forage has been available in the state, but the total demand for fodder for livestock has been 64.5 million tonne. Therefore, there has been

a deficit of about 9% in terms of fodder for animals if we take into account all the sources of fodder availability. The forage comes from two sources, namely crop residues and fodder crops, and it was only 48.7 million tonne, with a deficit of 24.5% fodder during a year with normal precipitation (Table 2). The deficit could be higher as per the statistics of 100 calendar years that clearly indicate the state could hardly receive eight normal crop seasons with normal distribution of rainfall and rest of the years had either low and erratic rainfall, or drought and famine.

Barley as a forage resource

Fodder occupies an important place in livestock feed. Rajasthan has a large area (342 239 km²) with 54.67 million livestock (Anon., 2003). The socio-economic status of farmers is poor and the primary livelihood

Table 2. Estimate of total forage production and requirement (million tonne; 2003).

	Sources			Total
	Crop residues (A)	Fodder crops Semi-arid (B)	Fodder from forest and waste land (C)	
Forage Availability	18.6	30.1	10.0	58.7
Requirement				64.5
Deficit — Considering A+B sources				15.8 (24.5%)
— Considering A+B+C sources				5.8 (9%)

of the people is animal husbandry. Realizing the shortfall in forage availability, barley is an important crop that can be used as an alternative forage resource in water-deficit areas and salt-affected soils. The crop occupied an area of nearly 0.23 million ha, producing 0.60 million tonne grain, with a yield of 2.54 t/ha during Rabi 2006-07. The crop has low input requirements and plasticity of adaptation under varied agroclimatic situations, and has expanded to non-traditional areas of Rajasthan, southern Haryana, western Gujarat and western Uttar Pradesh. The crop diversification in these areas is a response to depleting water resources, monsoon irregularities and frequent drought conditions, resulting in inadequate supply of irrigation water to Rabi season crops.

In recent years it has been observed that severe drought in the drier parts of the northern plains, particularly in Rajasthan and western Haryana, resulted in the migration and loss of livestock in search of green fodder in the period from November to January. Berseem (*Trifolium* spp.), oats (*Avena sativa*) and sugarcane tops that have been widely used as green fodder require frequent irrigation and they cannot be grown when water is scarce. Barley, being a fast growing crop with high biomass in the early stages and requiring less water, has been recognized as a potential forage resource in such conditions (Figure 1).

Experimental findings

Plants having desirable traits, namely early growth habit, high biomass and disease resistance, were selected from various crosses through traditional pedigree methods. The 22 advanced bulks and entries from different All India Coordinated Research Project (AICRP) centers were evaluated in replicated trials with a plot size of 5 m × 1.38 m at four locations (Avikanagar, Bikaner, Hisar and Ludhiana) as part of the collaboration within the All India Coordinated Wheat and Barley Research Project and the AICRP on Forage Crops during Rabi 2003-04 in the Northwestern plain zone (Rajasthan, Haryana, Punjab and western Uttar Pradesh) in India. A basal dose of 20 kg N and 30 kg P₂O₅ was applied before sowing, and additional Nitrogen was applied as two doses, i.e. 20 kg N at the 1st irrigation and the remaining 20 kg N was applied after the first cut for green fodder yield at 50–55 days after sowing, followed by irrigation to regenerate the crop for grain purposes. In the experiment, entries with good yield for green fodder and grain yield were observed with satisfactory levels for both traits. The promising entries identified in the trials were repeated during Rabi 2004-05 and 2005-06 with a doubled plot size (5 m × 2.76 m) at the same locations.

The results over locations and years clearly indicated that there is a large variability



Figure 1. Barley as a forage crop in arid and semi-arid areas of India.

among the lines for green fodder yield and grain yield from the regenerated crop. Based on three years' observations, three lines, RD2670, RD2035 and RD2552, were found suitable for utilization as dual-purpose barley. All varieties gave comparable green fodder and grain yield (Table 3).

In another experiment conducted for two years, 2005-06 and 2006-07, in the central zone (CZ) (Gujarat, Madhya Pradesh, Western Rajasthan and Uttar Pradesh) of India, similar varietal behavior was observed in respect to both the traits (Table 4).

From the results (Tables 3 and 4) of the trials in two different zones, i.e. NWPZ and CZ, it is concluded that efficient dual-purpose barley varieties are available and can be sown to harvest green fodder (23 to 25 t/ha), with a follow-on grain yield of 2.0 to 2.5 t/ha with two to three irrigations.

Effect of sowing time on green fodder yield

Barley was sown on three different dates for two consecutive years to study the effect of sowing time on green fodder yield. The crop

sown in the 42nd meteorological week (15–22 October) gave the maximum green fodder. Any delay in sowing has been associated with decreasing yield (Table 5), which is a result of the curtailed growing cycle.

It is suggested that early sowing of barley would maximize the harvest of green fodder to feed the livestock during the lean period.

Nutritive value of barley in comparison to oat

Green forage quality was comparable between the oats and barley cereals at 50–60 days of crop growth.

Barley demonstrated a clear superiority, had better forage quality parameters and should thus be preferred to oat (Table 6). It makes good animal feed, which is about 70% of the total barley grain production and goes to poultry, sheep, goat and swine because it improves firmness and quality of meat in comparison with other feeds.

Conclusion

The livestock population in the country is increasing with changes in the composition

Table 3. Promising barley varieties for dual-purpose use over the years in the North Western Plain Zone (NWPZ) of India.

Variety	Green fodder (t/ha)			Grain yield (t/ha)			Mean yield (t/ha)	
	2003–04 (4)	2004–05 (3)	2005–06 (3)	2003–04 (4)	2004–05 (3)	2005–06 (3)	Fodder	Grain
RD2670	33.40	24.00	14.70	1.93	2.82	3.03	24.94	2.72
RD2035	33.80	22.70	16.80	1.78	2.59	3.13	25.39	2.64
RD2552	31.60	22.00	14.40	2.13	2.53	3.61	23.57	2.88
Varietal Range	16.3–38.3	19.9–24.0	13.2–16.8	0.81–2.36	2.53–2.82	3.03–3.70		
CD	33.0	17.0	17.0	1.3	1.5	2.0		

Figures in parentheses are the number of locations each season; CD = Critical Difference

Table 4. Promising barley varieties for dual purpose in the central zone (CZ) of India.

Varieties	Green fodder (t/ha)		Grain yield (t/ha)		Mean yield (t/ha)	
	2005–06 (3)	2006–07 (3)	2005–06 (3)	2006–07 (3)	2005–06 (3)	2006–07 (3)
RD2715	12.80	21.45	1.69	2.87	17.12	2.28
RD2035	10.60	20.18	1.58	2.74	15.39	2.16
RD2552	11.00	18.49	1.20	2.53	14.74	1.86
Varietal Range	0.95–12.8	18.31–21.45	0.76–1.91	2.06–2.87		
CD	14.0	19.1	1.9	3.8		

Figures in parentheses are the number of locations each season; CD = Critical Difference

Table 5. Barley sown in different sowing dates.

Sowing time	Green fodder yield (t/ha)		Mean yield (t/ha)
	2002–03	2003–04	
15–22 October	35.89	23.72	29.81
1–7 November	29.83	23.89	26.86
15–22 November	26.89	15.44	21.17
CD	2.97	2.00	

CD = Critical Difference

of species, demanding more forage. The regular supply of forage required for livestock poses a severe challenge to those who rely on livestock husbandry as their primary means livelihood, especially in the arid and semi-arid regions lacking adequate water resources. It is necessary to overcome the gap between forage availability and demand, which is a recurrent problem in the water-deficit zones, such as Rajasthan. This is exacerbated by monsoon irregularities or frequent drought, resulting in inadequate supply of irrigation water for Rabi season crops.

Barley, being a fast growing crop with high biomass and low input requirements can increase the forage availability to feed livestock during lean periods. Research efforts have aimed to develop dual-purpose promising genotypes for cultivation in problematic areas to provide green forage and a subsequent grain crop from regeneration. The participatory approach of

Table 6 .Forage quality parameters for barley and oats.

Parameter	Barley	Oat
Dry matter %	18	22
Crude protein %	6.6	6.4
Crude fiber %	31.1	28.7
Total ash %	10.6	9.3
Digestible crude protein%	4.3	2.9
Total digestible nutrients%	59.2	52.4
Neutral detergent fiber	44.7	39.5
Hemi-cellulose	9.7	6.8
Cellulose	28.52	26.88

all the stakeholders in the various farming systems supported by institutional backing is needed to ensure barley as alternate forage resource in adequate quantities for livestock population of the country in general and for the arid and semi-arid regions in particular.

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Defining the phenome as a physiochemical near-infrared spectral response interface for genes, mutants and environment by a barley endosperm model

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Abstract

The importance of gene interaction (pleiotropy) has been highly underestimated due to a lack of an overview of the whole phenotype, now possible by Near-infrared Spectroscopy (NIRS). The complete causal expression of an endosperm mutant—the phenome—can now be represented as a whole NIRS fingerprint of the seed in an isogenic background. Pleiotropy of a gene can be defined by the differential spectrum between the mutant and the parent. In analogy, a changed gene background for a gene or genotype obtained by breeding and the environmental response of a genotype can be defined as whole differential spectra. They are physiochemically evaluated as fingerprints by consulting the spectroscopic literature and validated by correlations to chemical analyses through multivariate data analysis (chemometrics). The mutant- and genotype-specific NIR spectral fingerprints are surprisingly reproducible when compared in the same environment. The practical use of NIRS by “Data Breeding” as visualized in a PCA score plot for selection of an improved gene background for starch expression in a mutant was demonstrated.

Genotype classification by near-infrared spectroscopy and principal component analysis

Breeding for quality implies access to inexpensive and reliable screening methods for chemical and physical variables. The fact that near-infrared data in the range of 800–2500 nm after calibration to classical methods (Williams and Norris, 2001) can be used as a “multi-meter” for a wide range of parameters, immediately replaces with just one measurement, plus calibration checks, tens of analyses previously made using 10 different instruments. The required software, calibrated to the classical analyses, is usually supplied by the instrument manufacturer and based on chemometric pattern recognition data models (Martens and Næs, 1989) such as neural nets and Partial Least Squares Regression (PLSR).

Today, plant breeders and the cereal industry routinely utilize Near-infrared Transmission (NIT) spectrometers for estimation of chemical composition in substances such as water, protein, starch and malt extract. The instrument directly

measures the intact seed sample in the range 800–1050 nm in seconds. In this investigation we use a Visual - Near-infrared Reflectance (NIR) Instrument 400–2500 nm for more detailed information. It is not yet acknowledged by plant breeders outside the cereal laboratories that a PCA classification on seed NIT and NIR spectra is an efficient tool in breeding for quality at the genotype level and for characterization of genetic and technological quality complexes.

The textbook on Near-infrared Analytic Technology edited by Williams and Norris in 2001 just mentions the PCA option on a few pages, without reference to plant breeding. However, Campell *et al.* (2000) demonstrated that NIT data evaluated by PCA can classify a range of single- and double-mutant maize endosperm genotypes. Discriminate PLSR was used by Wang *et al.* (1999) to predict the number of dominant R alleles in single wheat kernels by VIS-NIRS, and by Delwiche *et al.* (1999) to identify different wheat-rye translocation lines by NIRS. We demonstrated in 2001 the usefulness of PCA on NIRS for classification of genetic and environmental differences in barley endosperm mutant material (Munck *et al.*, 2001). We were surprised by the genotype-specific patterns of the spectral NIRS patterns behind the different positions of the samples in a PCA score plot. In this paper we summarize our

recent research (Munck 2003, 2005, 2007, 2009; Møller Jespersen and Munck, 2009; Munck *et al.*, 2004; Jacobsen *et al.*, 2005).

Materials and methods

Three data sets of endosperm-mutant genotypes and normal barley controls (Munck *et al.*, 2004) are introduced with spectroscopic and chemical analyses. The first data set (Figures 1, 2, 3 and 5) consists of 23 samples of 20 barley genotypes grown in the field in 2000. The lines are classified in Figure 2 as normal (N); protein mutants (P), which are 20–45% richer in lysine and moderately lower in starch (5–10%); and carbohydrate mutants (C), low or very low in starch (10–40% less than the control) and with a moderate increase in lysine (5–10%). The P mutant Risø genotypes are the alleles *lys3a* (mutant 1508), *lys3b* (mutant 18), *lys3c* (mutant 19) and *lys4d* (mutant 8). Mutant *lys3m* induced in Minerva originates from Carlsberg. Lysimax and Lysiba are starch and yield improved recombinants from crosses with *lys3a* and normal barley from Carlsberg. The C mutants are the Risø mutants, mutant 16 and *lys5f* (mutant 13) in Bomi and *lys5g* (mutant 29) in Carlsberg II. Mutants 95 and 449 are Perga (of Italian origin) mutants. w1 (line 1201) and w2 (line 841878) of unknown origin were imported

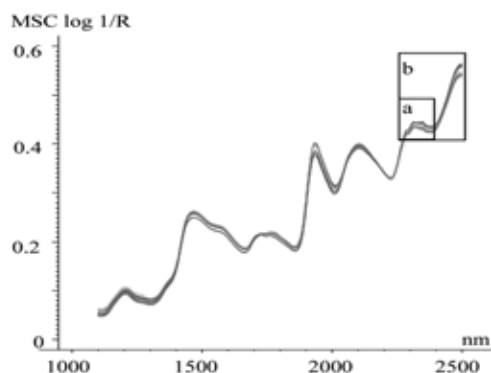


Figure 1. MSC log 1/R NIRS Spectra 1100–2500 nm of the 23 barley samples in Material 1.

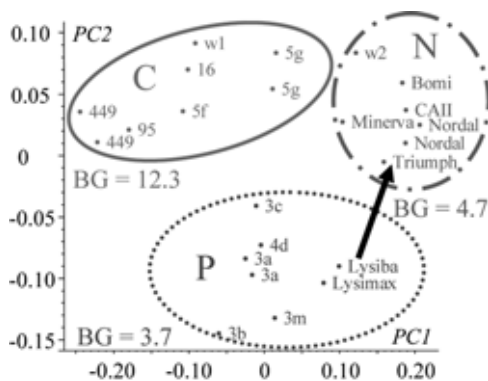


Figure 2. PCA score plot of the spectra in Figure 1; see discussion in text.

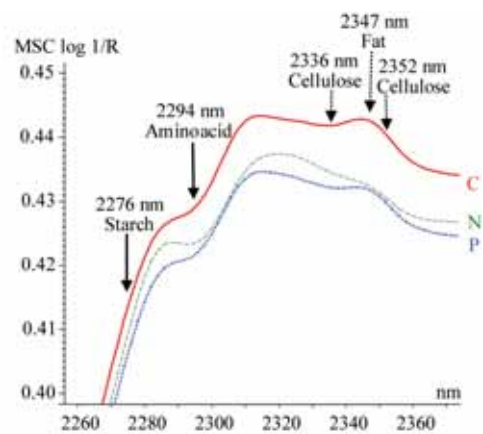


Figure 3. Mean spectra 2260–2360 nm of clusters N, P and C from the PCA in Figure 2.

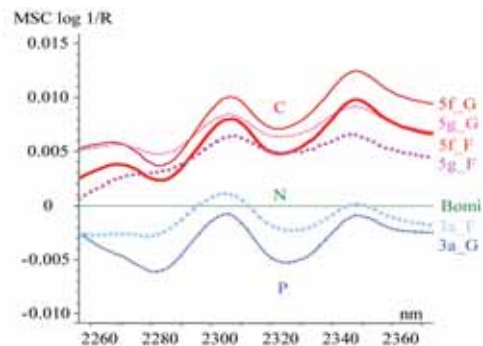


Figure 4. Differential NIRS mutant spectra 2260–2360 nm of *lys5f*, *lys5g*, *lys3a* to Bomi grown in greenhouse (G) and field (F). For chemical composition, see Table 1.

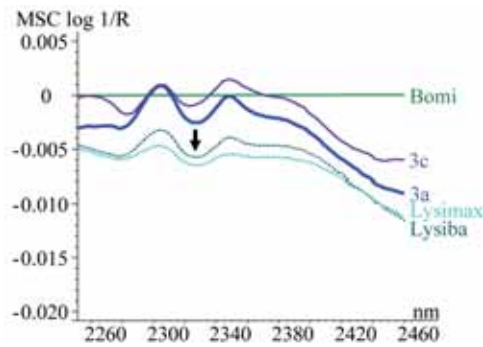


Figure 5. Differential NIRS mutant spectra 2260–2480 nm to Bomi for *lys3a*, *lys3b*, Lysimax and Lysiba.

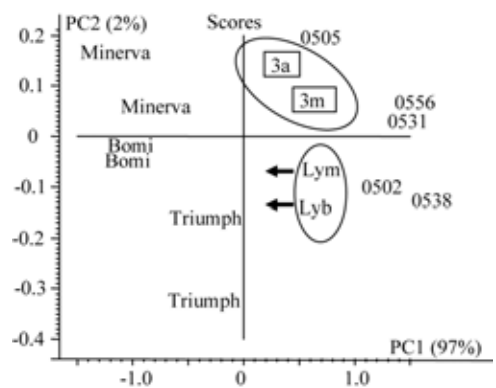


Figure 6. PCA score plot for 15 barley visual-NIRS spectra (400–2500 nm) MSC log 1/R 400–2500 nm demonstrating a case of “data breeding”, as discussed in the text.

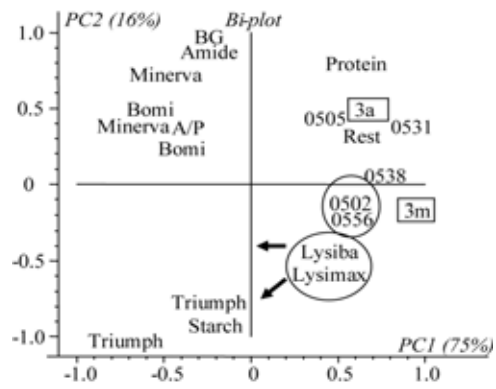


Figure 7. PCA bi-plot for the chemical composition of 15 barley varieties.

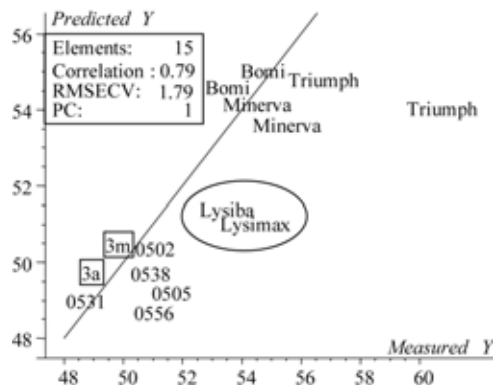


Figure 8. PLSR correlation plot for prediction of starch in Table 2.

Table 1. Chemical composition (as % DM) of barley mutants and isogenic Bomi control grown in greenhouse (G) and field (F), shown as spectra in Figure 4.

		DM%					
		BG	Starch	Protein	Amide	A/P	Fat
Bomi_G	N	6.8	48.8	14.6	0.38	16.2	1.7
Bomi_F	N	4.9	53.6	11.5	0.29	15.8	1.9
3a_G	P	3.6	41.6	16.6	0.29	10.9	3.5
3a_F	P	3.1	48.5	12.7	0.23	11.4	2.6
5g_G	C	13.5	44.7	16.1	0.39	15.1	2.3
5g_F	C	8.9	47.0	11.8	0.26	13.8	2.3
5f_G	C	20.0	30.5	15.7	0.37	14.7	3.7
5f_F	C	16.5	33.0	14.5	0.31	13.4	3.8

to the Carlsberg collection and assigned as waxy mutants. Only w2 was waxy (amylose 4.2%). The second data set (Figure 4; Table 1) consists of four genotypes, namely, Bomi and mutants *lys5f*, *lys5g* and *lys3a* grown in the field (from data set 1) and in the greenhouse. The third data set (Figures 6, 7 and 8; Table 2, from Møller, 2004b) displays 15 barley lines grown in the field; the N (normal) lines Bomi, Minerva and Triumph, the P (protein) mutants *lys3a* and *lys3m* and the breeding lines between *lys3a* and normal barleys, Lysimax and Lysimba (positive seed quality and starch selection) and the unselected *lys3a* breeding lines 502, 505, 531, 538 and 556. The chemical and NIR spectral analysis (on milled flour retained on 0.5 mm sieve) was carried out by a Foss-NIR Systems (USA) 6500 instrument, as described by Munck *et al.* (2004). The raw spectra were multiplicative scatter corrected (MSC) and presented as log 1/R intensity. Chemometric pattern recognition analysis was performed

using Principal Component Analysis (PCA) for classification and Partial Least Squares Regression (PLSR) for prediction, according to Martens and Næs (1989).

Recognizing sample- and genotype-specific NIRS patterns in barley

The NIR spectra from the 23 barley seed samples from Material 1 in Figure 1 depict 1400 wavelength variables with a seemingly narrow variation between samples in absorption value MSC log1/R. Classical statistics of variance based on distributional assumptions cannot extract information from a whole intercorrelated data matrix with thousands of wavelength variables per sample. For this purpose, there is a need for self-calibrating multivariate chemometric data models, such as PCA for classification and PLSR for prediction, both based on latent variables. The PCA (PC1 to PC2) in Figure 2 classifies the spectra in Figure 1 into three distinct populations: normal (N) barley, endosperm mutants with a high lysine percentage in protein (P) and carbohydrate mutants (C). The C barley lines, such as Risø mutant 16, *lys5g* and *lys5f*, which are mutations in the AGP-ase mechanism, have been used by the biochemists to study starch synthesis.

It was therefore surprising when we found (Munck *et al.*, 2004) that these mutants and three others, namely mutant 95, mutant 449 and the w1 (line 1201), all in the C cluster

Table 2. Chemical composition of the 6 normal and *lys3a* genotypes presented in Figures 6, 7 and 8.

	Normal	Group 1	Group 2	Group 3	Group 4
Sowing time	n = 6	Lysiba, Lysimax	502, 556	505, 531, 538	<i>lys3a</i> , <i>lys3m</i>
Protein (P)	11.3 ± 0.4	11.7 ± 0.1	11.7 ± 0.1	12.6 ± 0.2	12.5 ± 0.2
Amide (A)	0.28 ± 0.03	0.21 ± 0.007	0.21 ± 0.007	0.22 ± 0.02	0.23
A/P	15.5 ± 0.9	11.0 ± 0.3	10.9 ± 0.4	10.7 ± 0.8	11.4
Starch (S)	54.6 ± 2.5	52.6 ± 0.5	50.0 ± 0.1	49.4 ± 1.5	48.7 ± 0.2
Beta-glucan (BG)	4.7 ± 1.1	3.1 ± 0.1	3.1 ± 0.2	3.1 ± 0.3	2.8 ± 0.5
Rest (100-P+S+BG)	29.5 ± 1.8	32.7 ± 0.5	35.3 ± 0.3	34.9 ± 1.8	36.1 ± 0.5

(Figure 2) compensated for the loss in starch by overproducing beta glucan (BG). The mean BG value for the C (12.3%), P (3.7%) and N (4.7%) classes are marked in Figure 2. Obviously, there is a strong pleiotropic regulative effect where mutations in starch metabolism may channel glucose from alpha- to beta-glucan production. Normally, PCA classifications, which are useful for an overview of large (spectral) data sets, are published in literature without inspecting the underlying data structure. We generated the mean spectra for the C, P and N genotypes and visually screened the whole spectra. Marked differences were found in the narrow area 2260–2360 nm (Figure 3; marked “a” in Figure 1) of chemical and genetic significance. The near-infrared transmission (NIT) and reflectance (NIR) spectra represent in principle a physico-chemical fingerprint containing repetitive information on the propensity of chemical bonds.

A trained spectroscopist can from the first, second or MSC derivatives of the log1/R NIRS data directly explore specific chemical differences between samples and deduce destructive analyses for verification. The P and C genotypes have a characteristic peak in the area from 2336 to 2352 nm, which in spectroscopic literature is associated with cellulose (2336 and 2352 nm) and fat (2347 nm). The substantial increases of the fat components anticipated from the spectral patterns of *lys3a* and *lys5* barleys are verified by chemical analyses in Table 1.

Further studies (Jacobsen *et al.*, 2005; Munck, 2005, 2007, 2009) demonstrate that the spectral patterns have not only a chemical interpretation, but also a genetic significance as a phenomenological trait expressing the whole active genome as a pattern of chemical bonds represented by the spectral phenome (Munck *et al.*, 2004; Munck, 2007). The reproducibility, fine-tuning and informative capacity of NIRS spectra are indeed impressive. The MSC log1/R absorption range is 0.04 units for classification of C,

P and N barleys in Figure 3. However, the range needed is 100 times less for classifying the C versus P+N groups in the 1890–1920 nm area for dry matter (DM) content within the narrow response of 89–93% DM (Munck, 2007). A high BG content of the C group conditions a mean difference in DM of 1.5% between these groups.

The precision of NIRS allows individual barley samples (genotypes) to be differentiated by their spectral patterns. This is demonstrated by the spectra in Figure 4, with chemical evaluation shown in Table 1. Four samples representing three mutants *lys3a*, *lys5f*, *lys5g* and the normal control Bomi, grown in the field are selected from Material 1 and compared with the corresponding genotypes grown in greenhouse. Figure 4 displays the differential spectra where the mutant spectra are subtracted from those of the Bomi control. A well-conserved genetic pattern is demonstrated in the spectral area 2260–2360 nm for the two different environments. There is some offset and a minor effect on the spectral form due to the environment. Bomi is near isogenic for the *lys3a* and *lys5f* mutants. The differential spectrum to Bomi of these mutants constitutes a spectral representation of pleiotropy involving all expression effects of the mutant at the level of chemical bonds (Table 1) in the endosperm (Munck, 2007; Jacobsen *et al.*, 2005). While protein alone has a low power in discriminating between the genotypes (Table 1), the amide-to-protein (A/P) index clearly separates the high-lysine P mutant *lys3a* from the others. At the same time, the BG-compensated starch mutants (C) show a very high level of BG (16.5–20% DM in *lys5f*) when Bomi is 4.9–6.8 % DM and *lys3a* is reduced to 3.1–3.6%. There is a corresponding reduction in starch from 48.8–53.6% DM in Bomi down to 30.5–33.0% DM in *lys5f*. It is thus the pleiotropic differences in expression of chemical composition between the mutants in Table 1 that explain the unique spectral patterns of the same samples displayed in Figure 4.

“Data Breeding” for complex quality traits by NIRS selecting improved segregants from a PCA score plot

We will now test if an improvement in breeding for plump starch rich seeds in high-lysine *lys3a* barley at Carlsberg 1973–1988 can be followed by NIRS technology to be further exploited for other purposes in plant breeding. In the PCA score plot from Material 1, the improved *lys3a* genotypes Lysiba with starch (S 52.2%) and Lysimax (S 52.9%) are classified between the original *lys3a* (S 48.5%) mutant and the normal barley Triumph, high in starch (S 58.5%), indicating a change in chemical composition.

The differential spectra 2260 to 2360 nm to Bomi (S 53.5%) of the improved Lysiba and Lysimax genotypes and of the *lys3a* (BG 3.1%) and *lys3c* (BG 6.4%) allele are presented in Figure 5. The spectral differences between the alleles *lys3a* and *lys3b* are mainly due to the difference in BG. The spectra from the starch-improved *lys3a* lines are moved downwards to the baseline and the mutant characteristics are flattened out. The area between the *lys3a* spectrum and the Lysiba/Lysimax recombinants marked by the arrow gives a spectral representation of 15 years of breeding work to improve seed quality. Table 2 outlines the chemical composition (six variables) of material 3, with falling starch content from 54.6 to 48.7% consisting of: normal barley, improved *lys3a* breeding lines (group 1), unselected recombinants (groups 2 and 3) and original mutants (group 4).

The PCA biplot in Figure 7 gives a convenient overview of how the chemical analyses influence genotype classification that is comparable with the corresponding VIS-NIRS (400–2500 nm) PCA classification plot in Figure 6. In the bi-plot in Figure 7 the variable “Starch” is positioned near Triumph, indicating a high level of starch in this cultivar. The move in both PCAs

(Figure 6 and 7) of the Lysiba and Lysimax improved genotype from the position of the original low starch *lys3a* mutant towards the high-starch variety Triumph is clearly demonstrated in both PCAs. The NIRS and chemical data sets are combined in the PLSR starch prediction plot in Figure 8. This is how NIR and NIT spectroscopy are utilized today by plant breeders for chemical prediction. Because NIRS gives a total estimate of the chemical composition of a barley sample in a PCA score plot it is now possible empirically, by comparison with a high-quality genotype, to evaluate and to select the whole expression of the genotype on the spectral level in a cross-breeding program by “data breeding”.

A complex quality trait such as nutritional value (described here) or malt quality (Møller 2004a; Møller Jespersen and Munck, 2009) can thus be represented as a whole spectral fingerprint by NIR and NIT spectroscopy. This can be done early in the breeding program without chemical analysis, subsequently ending with a confirmation of the quality by a chemical and pilot malting evaluation of the final varieties. It is clear that NIR and NIT spectroscopy combined with PCA and PLSR data analysis (chemometrics) is a revolution in cost-effective breeding for quality. It reflects in a reproducible way the above-described changes in chemical composition down to 1–2 percentage points of each component by PLSR and summarizes by PCA and visual inspection of spectral intervals subtle differences in quality characteristics as a whole for each genotype and sample representing patterns of chemical bonds.

We conclude that in genetics and plant breeding there is a need for a new multivariate way of thinking (Munck, 2005, 2007, 2009) in re-defining the biological individual, or zygote. By introducing the spectral phenotype, the self-organizing (endosperm) tissue can now be considered as a response interface at the level of chemical bonds for the genome. It is, as demonstrated here, read as a pattern

by spectroscopy, classified by multivariate analysis and interpreted by genetics, chemistry and technology.

Classical genetic variance statistics is focused on individual genes and traits, assuming more or less free distribution. The current Quantitative Trait Loci (QTL) analysis aims at revealing complex genome-phenome-quality relationships. It combines trait and genome information and has until recently employed the traditional analysis of variance, despite the fact that variables in most gene, trait and quality complexes are strongly dependent on each other. This is why QTL analysis has such a mixed reputation. Using multivariate pattern recognition analysis, PCA and PLSR can overcome this problem, as recently demonstrated by Bjørnstad *et al.* (2004) with PLSR. A new high-precision QTL analysis at the spectral phenome level for use by plant breeders will be possible when comparing (by PCA) and combining (by PLSR) NIRS data with RFLP and snip data. It is likely that NIR and NIT fingerprinting can function as a stand-alone analysis, if genetically (by DNA), chemically and technologically defined controls are provided.

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Grain quality in Western Canadian hulled two-row barley

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Abstract

Two years of quality data from multiple Alberta sites run by the Field Crop Development Centre (FCDC) were analyzed to establish baselines for feed, food and malting quality of recently released western Canadian hulled two-row barleys. Over 7 environments (location and years), 49 samples of malting barley and 60 samples of feed barley were analyzed to compare quality traits between the two types. No differences between the types were found for the feed and food traits of protein, protein digestibility, lysine, digestible energy, beta-glucans (grain), pentosans, ash, or lipids. However, total and soluble fiber contents of the malting types were lower than for the feed types, and starch content was higher. For malt traits, fine extract, diastatic power, alpha-amylase, soluble malt protein, friability and homogeneity were higher in the malting types than the feed types, while malt beta-glucan, viscosity and turbidity were lower. So, while malting traits differed between the two types, feed or food quality traits did not, except for some potentially positive changes in starch and fiber content, depending on the end use. The complexity of requirements for different classes of livestock and for food means that there is a need for a wide range of material. Environmental effects also create a wide range of expression for all quality traits that may make selection difficult as we do not clearly understand the heritability of some of these food and feed quality traits.

Introduction

Breeding efforts in western Canada for feed barley have concentrated on improving yield (grain and biomass), disease resistance, and tolerance to stress (lodging resistance with semi-dwarf genes; drought tolerance). Most concentrated efforts to improve food quality in western Canadian barleys focused on hulless types with high beta-glucan, like CDC Rattan from the University of Saskatchewan/Crop Development Centre (US/CDC) in Saskatoon and a high-amylose type from the Agriculture and Agri-Food Canada (AAFC) program at the Brandon Research Centre (BRC). Canadian malting barleys are renowned for their high diastatic power and good modification, with cultivars like Harrington (US/CDC) and AC Metcalfe (AAFC-BRC). The purpose of this paper was to look at the most recently released hulled two-row material that had been entered into the Alberta Cereal and Oilseed Regional Variety Trials to establish feed, food and malting quality baselines.

Materials and methods

Twenty quality traits were measured using NIRS calibrations developed by FCDC (Temelli and Helm, 1999; Helm *et al.*, 2000; Oatway and Helm, 2002). The traits were: protein (%), protein digestibility (%), digestible energy content (kcal/kg), lysine (%), starch (%), beta-glucan (grain, %), pentosan (%), ash (%), lipid (%), total fiber

(%), soluble fiber (%), fine extract (malt, %), diastatic power (°L), alpha-amylase (DU), soluble malt protein (%), beta-glucan (malt, ppm), friability (%), viscosity (cps), homogeneity (%), turbidity (NTU). Two years, 2004 and 2006, were selected for analyses. There were 16 entries in the 2004 trial and 15 in the 2006 trial, with only 7 entries common to both trials (Table 1). Samples were from four locations (Calmar, Lacombe, Olds, Trochu) in 2004 and three locations (Calmar, Lacombe, Trochu) in 2006.

The varieties were classed as Feed or Malting types based on their recommendation for registration or entry into the Prairie Recommending Committee on Oat and Barley Cooperative Trials. Using SASTM, data were analyzed with Proc Mixed to determine

if the contrast of Feed versus Malting type was significant. Data were analyzed with Proc Univariate to determine the effects of years on the two types.

Results

When comparison of malting with feed types was made for feed and food quality traits, only starch, total fiber and soluble fiber contents differed between the two types (Table 2). However for the nine malt quality traits measured, the malt types were significantly different from the feed types.

Year effects were significant for most traits, with the effects being similar for both barley types. For starch, levels were higher in 2004 than in 2006 for both types, with the variation in this trait being similar for

Table 1. Cultivars and advanced lines of two-row hulled barleys grown in Cereal and Oilseed Variety Trials in 2004 and 2006 in Canada.

Cultivar	Breeding Institute	Type (Feed or Malting)	2004	2006
AC Metcalfe	AAFC-BRC	Malting	Yes	Yes
B-2316	BARI	Malting	No	Yes
B-2657	BARI	Malting	No	Yes
Calder	AAFC-BRC	Malting	Yes	No
CDC Copeland	US-CDC	Malting	Yes	No
CDC Cowboy	US-CDC	Feed	No	Yes
CDC Dolly	US-CDC	Feed	Yes	No
CDC Helgason	US-CDC	Feed	Yes	No
CDC Kendall	US-CDC	Malting	Yes	No
CDC Select	US-CDC	Malting	Yes	No
CDC Trey	US-CDC	Feed	Yes	Yes
Conlon	UND	Malting	No	Yes
Conrad	BARI	Malting	No	Yes
Formosa	CMS	Feed	No	Yes
Harrington	US-CDC	Malting	Yes	Yes
McLeod	WPB	Feed	Yes	Yes
Merit	BARI	Malting	Yes	No
Newdale	AAFC-BRC	Malting	No	Yes
Niobe	AF-FCDC	Feed	Yes	No
Ponoka	AF-FCDC	Feed	Yes	Yes
Rivers	AAFC-BRC	Feed	Yes	No
Seebe	AF-FCDC	Feed	Yes	Yes
TR04719	WPB	Feed	No	Yes
Xena	WPB	Feed	Yes	Yes

Key to breeding institutes: AAFC-BRC = Agriculture and Agri-Food Canada Brandon Research Centre (Brandon, Manitoba, Canada); US-CDC = University of Saskatchewan Crop Development Centre (Saskatoon, Saskatchewan, Canada); UND = University of North Dakota (Fargo, North Dakota, USA); CMS = C&M Seeds (Palmerston, Ontario, Canada); WPB = Western Plant Breeders (Bozeman, Montana, USA); BARI = Busch Ag. Resources Inc. (Boulder, Colorado, USA); AF-FCDC = Alberta Agriculture and Food Field Crop Development Centre (Lacombe, Alberta, Canada).

Table 2. Quality traits of feed and malting hulled two-row barleys grown in Alberta, Canada, in 2004 and 2006.

Quality Trait	Significance of contrast of Malting vs. Feed Types	Feed Types			Malting Types		
		Max.	Min.	Mean	Max.	Min.	Mean
Protein	NS	15.19	6.69	10.28	14.40	6.90	10.20
Protein Digestibility	NS	81.6	60.0	68.5	78.9	61.5	69.2
Digestible Energy Content	NS	3302	2920	3110	3309	2967	3117
Lysine	NS	0.79	0.49	0.62	0.76	0.51	0.63
Starch	***	61.5	57.6	59.4	62.2	57.7	59.8
Lipid	NS	2.77	2.15	2.51	2.92	2.24	2.53
Beta-Glucan (Grain)	NS	4.77	1.98	3.37	4.87	2.11	3.32
Pentosan	NS	6.00	4.79	5.44	6.10	4.80	5.45
Total Fiber	**	22.9	18.8	20.6	22.2	18.3	20.1
Soluble Fiber	***	5.64	3.26	4.45	5.02	3.28	4.11
Ash	NS	2.76	1.62	2.13	2.68	1.63	2.13
Fine Extract	***	81.7	74.0	78.9	82.4	77.3	80.0
Diastatic Power	***	196	116	151	208	118	160
Alpha-amylase	***	75.1	45.3	60.1	77.9	51.4	65.2
Soluble Malt Protein	***	6.92	4.21	5.68	7.35	5.02	6.31
Beta-glucan (Malt)	***	593	10	321	506	23	247
Friability	**	95	35	69	98	40	72
Viscosity	***	1.57	1.21	1.39	1.51	1.19	1.37
Homogeneity	***	99.8	80.3	90.9	99.7	88.1	94.9
Turbidity	*	6.06	3.01	4.79	6.33	3.06	4.57

, *, NS = Where the contrast of malt type versus feed type using Proc Mixed of SAS was significant at $P < 0.01$, 0.001 or not significant at $P > 0.05$, respectively.

both types within years (Figure 1). Total and soluble fiber levels were higher in 2004 than in 2006 for both types, but the malting types tended to have less variation in these traits than the feed types (Figures 2 and 3). Fine extract, alpha amylase and malt beta-glucans were higher in 2006 than in 2004 with the malting types having higher levels and less variation in this trait than the feed types (Figures 4, 5 and 6). While diastatic power levels were higher and turbidity was slightly lower within years for the malting than the feed types, these traits had more variability within the malting types than the feed types (Figures 7 and 8). Soluble malt proteins were lower in 2006 than in 2004, with the malting types having more variability in 2004 and less in 2006 than the feed types (Figure 9). Friability and homogeneity were lower in 2006 than in 2004, with the malting types having less variability than the feed types (Figures 10 and 11). Viscosity was higher in 2006 than in 2004, but variability was similar between years and types (Figure 12).

Discussion

The malting barley type was generally consistent in having better malt quality traits than the feed types, but the very interesting thing about these results is that they illustrate that malting types are more consistent in their expression of malting quality traits. The higher starch and lower fiber contents of the malting types in comparison with feed types reflect the push to improve extract levels in the malting types. It appears that the extra extract in two-row hulled malting barley in western Canada is coming through higher starch levels and decreased soluble fiber levels (although pentosans and beta-glucan levels of the grain were not different between the two groups). It may be that further improvements in extract levels of malting barley could be accomplished by decreasing protein levels, especially of soluble proteins, as this trait seems to be relatively high in this group of malting barleys and has been seen as a problem in some malting barleys, with

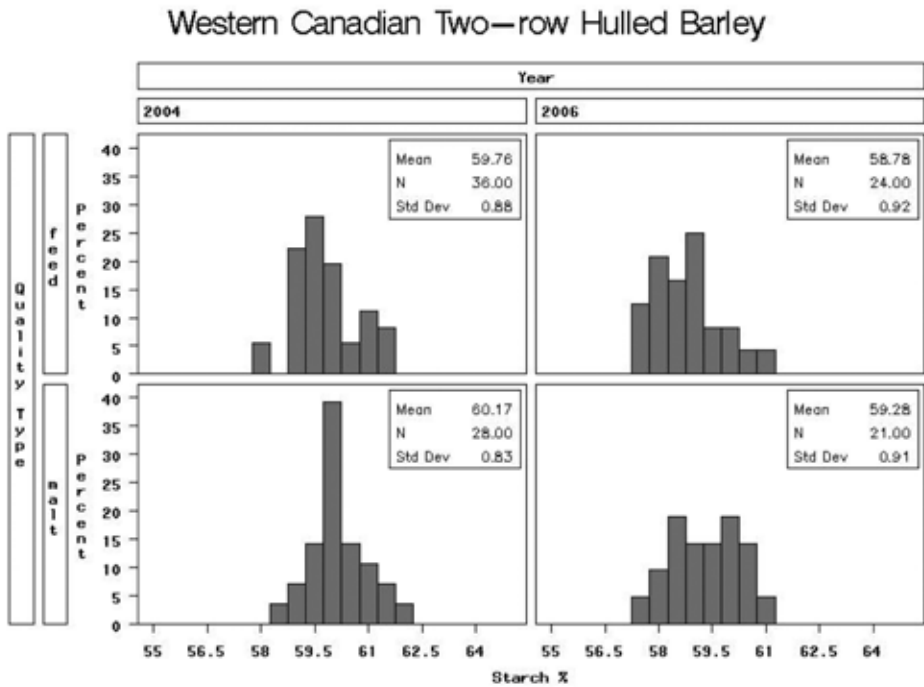


Figure 1. Starch content of the grain for western Canadian two-row hulled barley.

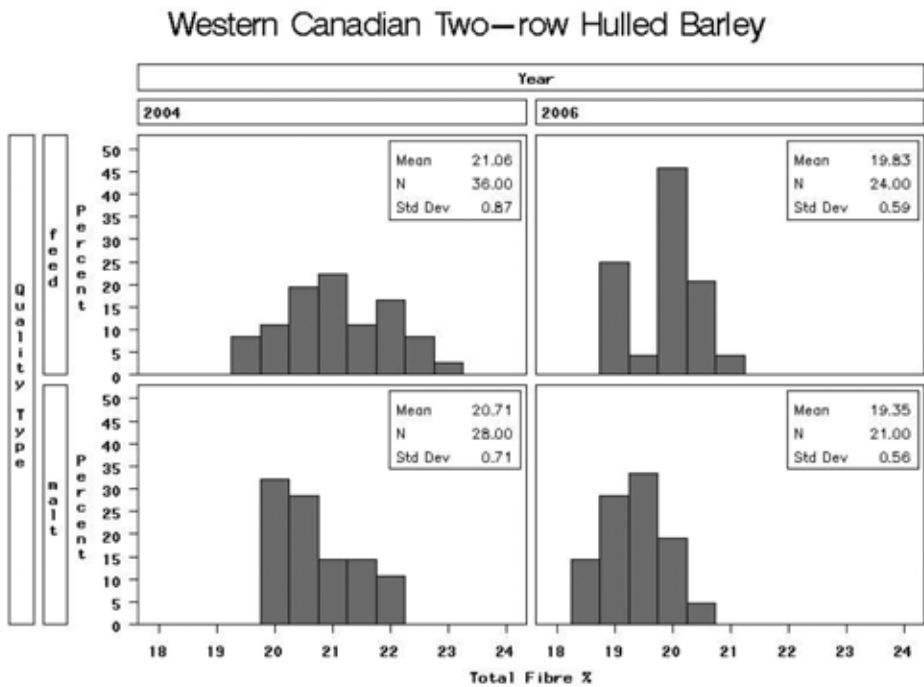


Figure 2. Total fiber content of the grain of western Canadian two-row hulled barley.

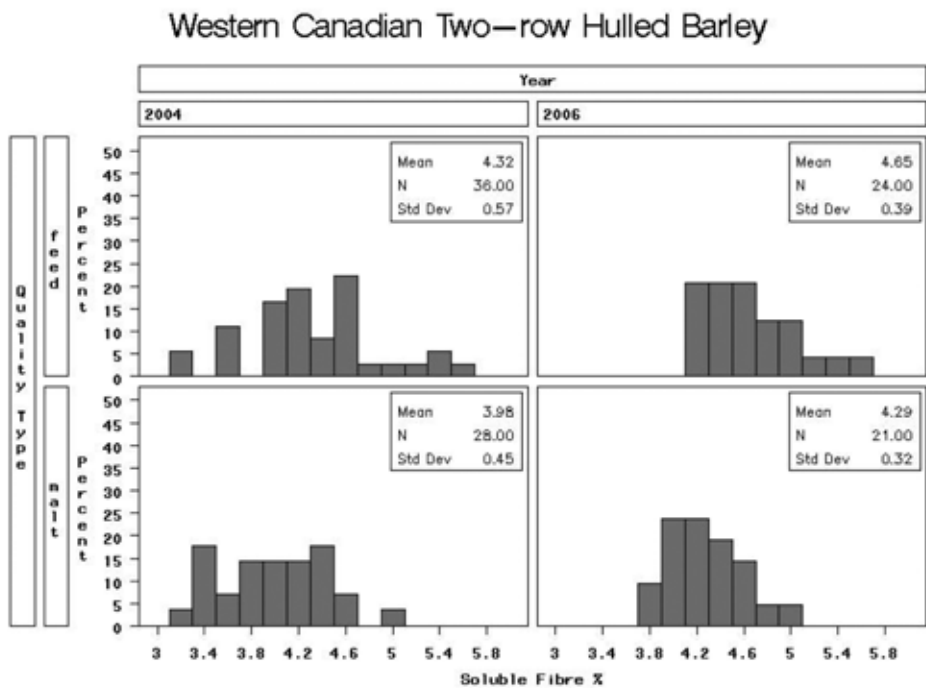


Figure 3. Soluble fiber content of the grain of western Canadian two-row hulled barley.

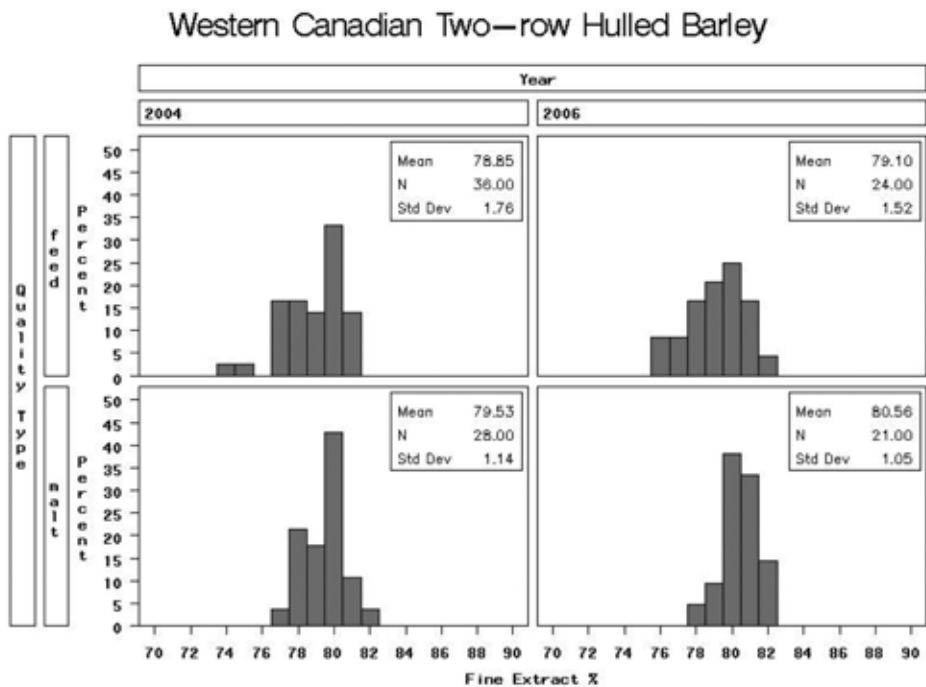


Figure 4. Fine extract of the malt of western Canadian two-row hulled barley.

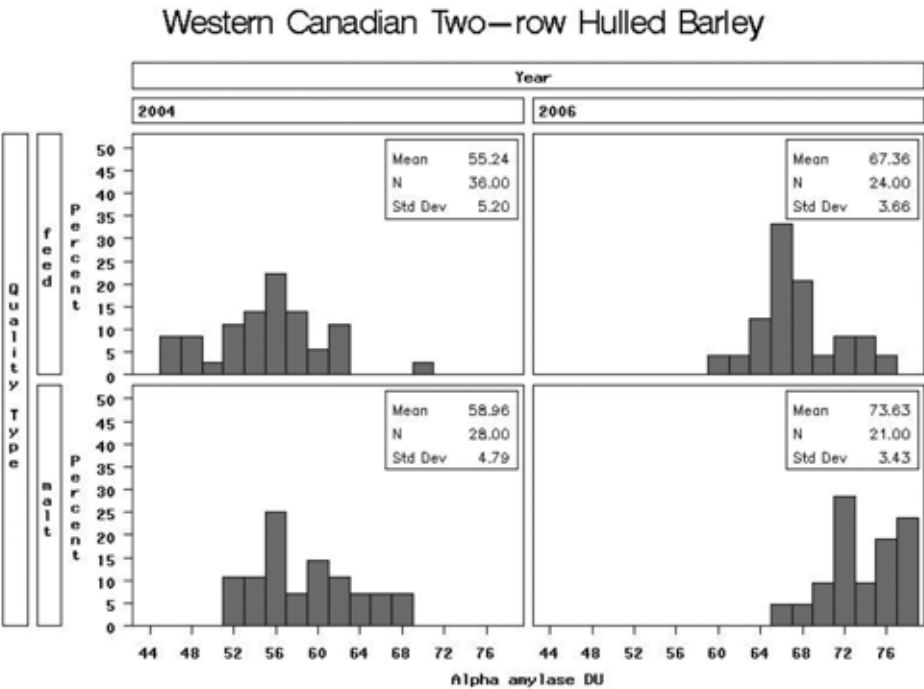


Figure 5. Alpha amylase content of the malt of Western Canadian two-row hulled barley.

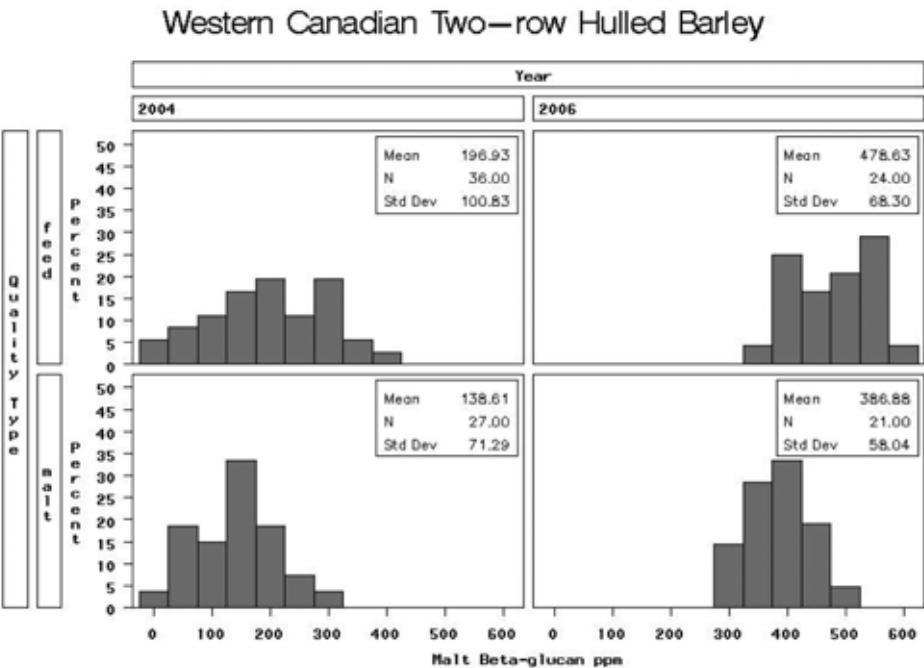


Figure 6. Beta-glucan content of the malt of western Canadian two-row hulled barley.

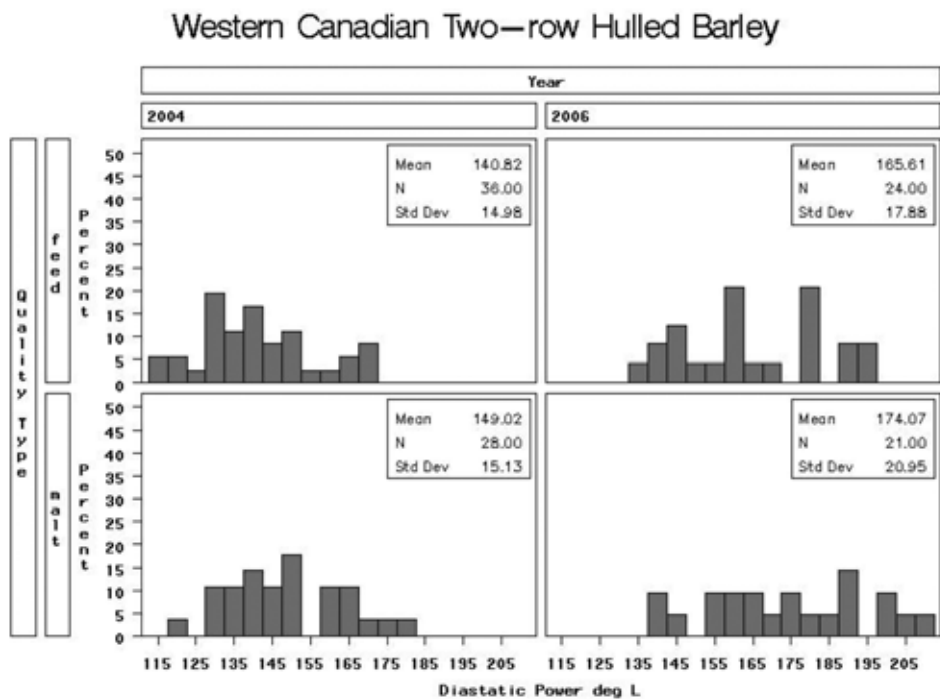


Figure 7. Diastatic power of the malt of western Canadian two-row hulled barley.

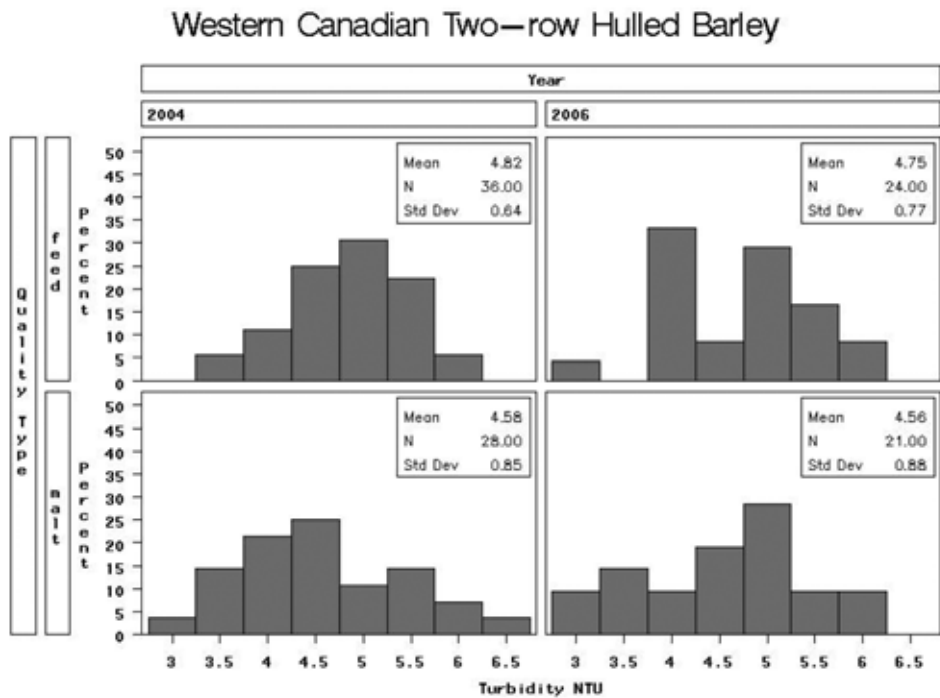


Figure 8. Turbidity of the malt of western Canadian two-row hulled barley.

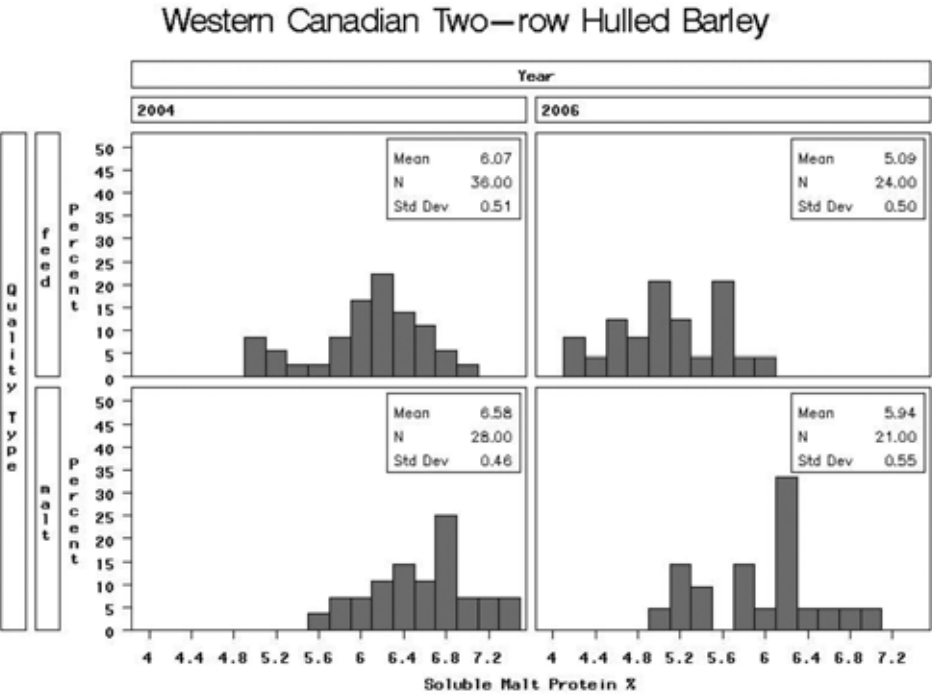


Figure 9. Soluble protein of the malt of western Canadian two-row hulled barley.

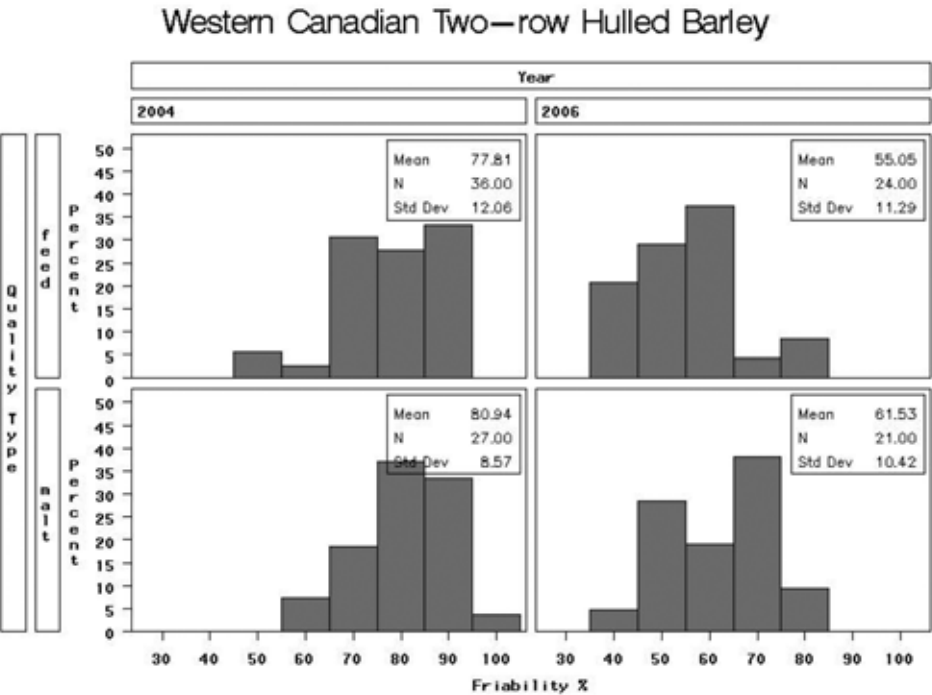


Figure 10. Friability of the malt of western Canadian two-row hulled barley.

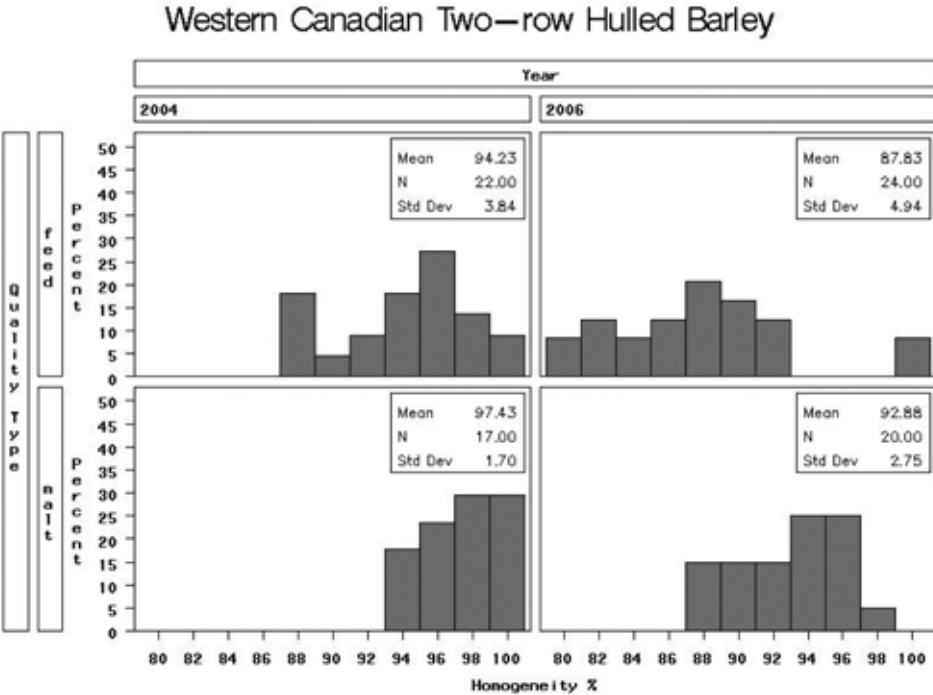


Figure 11. Homogeneity of the malt of western Canadian two-row hulled barley.

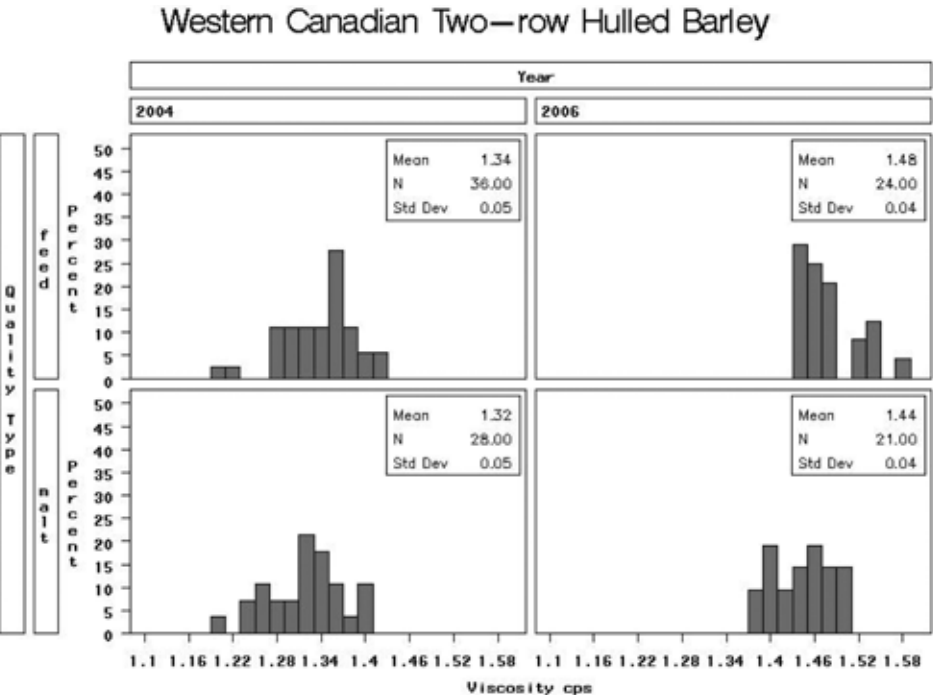


Figure 12. Viscosity of the malt of western Canadian two-row hulled barley.

the American Malting Barley Association suggesting 5.6% as the maximum level for two-row malting barley.

The lack of differences in feed traits between the malting and feed types except for starch and fibers was not surprising given that most attention for feed quality has been paid to test weights and percent plumps, two traits important to processing. Protein digestibility ranged from about 60 to 80% and the range was very consistent between years, so the question arises about how much of this range is genetic and how much is environmental. The same kind of observation can be made for digestible energy content. Therefore, if we are to make any kind of change in energy density of two-row hulled feed barley, we need to determine if there is indeed a genetic component.

As for the food traits, the health claim that barley is a good source of dietary fiber may be compromised in the malting types, although pentosan and beta-glucan levels were no

different between the feed and malting types.

As we seek to make further improvements in feed, food and malting quality, we will need to determine if there are true genetic components to quality traits, or if meeting quality requirements may be through better adaptation leading to less undesirable variation in desired quality traits.

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New barley cultivars with improved morphological characteristics for whole crop forage in South Korea

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Abstract

In South Korea, the domestic consumption of barley as a cereal crop has been decreasing since the 1980s. It has been considered that winter-season crop production could enhance the global competitiveness of the domestic livestock industry by providing better quality fodder to livestock and enhancing field use rate. Therefore, the purpose of barley cultivation for cereal food production has been recently replaced by the production of forage barley. Consequently, the area of barley cultivation for forage is markedly increasing. Forage barley is cultivated especially as forage for cattle. While any type of barley can be used as forage for feeding cattle, forage barleys deliver a higher dry matter yield than conventional feed barley. We have developed forage barley cultivars with cattle-preferred characters such as smooth awn, hood and auricleless types. Cvs. Wooho, Yuyeon, and Dami have smooth awns, hood type spike, and an auricleless plant. We also developed rough awn type barley cultivars, such as Yongyang, Sunwoo, Sangweon, and Soman, showing early-maturity and high yields of about 10 to 12 t/ha of dry matter (average 33 t/ha fresh matter yield) that have been evaluated for forage quality, showing good silage quality (TDN, ADF, NDF, CP, etc.) for whole-crop barley use. Based on these results, it is concluded that barley with smooth awns, hooded, and auricleless could be suitable sources in breeding for whole-crop forage use.

Introduction

In South Korea, the area of barley cultivation is about 60 000 ha. Domestic consumption of barley as a food crop has decreased since the 1980s. Moreover, the Korean government has announced that the government's purchasing price of barley will be reduced annually and the purchase will be terminated by 2012. At the same time, the percentage of domestic fodder, which is mainly produced from rice straw and roughage, was estimated to be about 82% of the requirements in 2007. Good quality fodder is only 31% of the total. In Korea, it has been considered that winter-season crop production could enhance the global competitiveness of domestic livestock industry by providing better quality fodder to livestock and improving land use. Barley cultivation for whole-crop silage (WCS) in the winter-season rice fields can be considered a promising way to enhance feed supply. WCS production can be an efficient way to use farm products as livestock feed, and it can also contribute to increase farm income. Therefore, barley cultivation as cereal food has been recently displaced by WCS production. Consequently, the area of barley cultivation for WCS is increasing markedly in Korea and the area planted in rice fields in the winter season reached 15 000 ha in 2007. The Ministry of Agriculture in Korea has announced plans to promote the extension of the area of barley cultivation for WCS to 100 000 ha by 2015, which should account for 25% of the total fodder

supply. The characteristics of barley varieties required for WCS are not the same as those required for cereal food. For example, high biomass of whole plant, including leaves, culms and grain, is more important than grain weight compared with the varieties grown for food (Sakai *et al.*, 2003). To reduce chemical use during cultivation and to increase the productivity of barley, multiple resistances to disease, especially barley mosaic virus and mildew, are also necessary, together with proper weed control.

Honam Agricultural Research Institute (HARI), part of the National Institute for Crop Science (NICS), has been conducting research programs for breeding WCS barley varieties (HARI, 2002–2007). The primary purpose of these programs is to develop barley varieties suitable for WCS production, with high productivity and low production cost. As a result of our breeding program, seven varieties for WCS production have been developed since 2002; three varieties, Youngyang (2002), Sunwoo (2002) and Sangwon (2004), show high productivity; two varieties, Woocho (2005) and Yuyeon (2006), have ruminant-palatable awns; Dami (2007) is an auricleless phenotype; and Soman (2006) has a shortened growth period, which is appropriate for the cropping system in central South Korea with rice cultivation. In this study, we describe the breeding process and main traits of these WCS cultivars in Korea.

Material and methods

Rough awn type

Cv. Youngyang was developed from a cross in 2002 between cv. Bunong and the elite breeding line Millyang55. Bunong [Hagane(Sammok/Heejin6)/Buheung (Jeraebag/ Suwon18)] has high productivity in forage yield. Millyang55, an elite breeding line with the pedigree [Olbori(Millyang6/Hagane)/Malesteril recurrent selection (81RSPYB15-283-2)], is early-heading. An

elite line, YB3882-3B-17-1-3-1, was selected in 1999 and designated Millyang111.

Cv. Sunwoo was developed from a cross between germplasm line P71523, with good plant type and early-maturing, and the elite breeding line Suweon234. Suwon234 [Kangbori/(Bengei/Ginomeo)] is cold tolerant and lodging resistant. An elite line, SB890278-B-30-4-3-O, was selected in 1999 and designated Suwon366.

Cv. Sangwon was developed from a cross between the germplasm line 72sel and the elite breeding line SB86659-B-YB-22 [(SB82583 (Dong1/XV-Buheung/Millyang12)/SB73243 (Pro-Tollxcer2- toll/Olbori/ (Cheongmac/Sikoku)/(Samheung/Olbori)]. The elite line has long culms and is high tillering. A promising line, SB941016-B-B-B-3, was selected in 1998. It performed well in the preliminary and advanced yield trials for two years (2000 and 2001). The line was designated Suwon389 through the regional yield trials (RYT) at 6 sites for 3 years (2004 to 2006).

Cv. Soman was developed from a cross made in 1994 between SB79124 [Bengei-Ginomeo-Buheung-Morsen-Hagane/Kangbori] and SB77189 [Samjug/Riso82//Olbori]. A promising line, SB941067-B-B-B-45, was selected in 1998. It performed well in the preliminary and advanced yield trials in 2002 and 2003. The line was designated Suwon404 through the RYT at 6 sites for 3 years (2004 to 2006).

Smooth awn and hooded type

Cv. Woocho was developed from a cross between F_1 [Olbori/ F_6 (SB77011)/ F_5 (Bengei//Hagane/Bunong)]/3/(Y7213/SD607//CM67/Millyang12) and (1012.2/IB65//Olbori)/ (Samheung//Suwon18/Kangbori) in 1994. Subsequent generations were handled with the bulk method and pedigree selection. SB94104-B-B-B-B-79 was selected as a smooth awn type and for agronomic appearance in 2001, and placed in preliminary yield trials. In 2003, it was

designated as Suwon396 and placed in the RYT.

Cv. Yuyeon was developed from a cross in 1996 between Suwon311 (CMB81A-1936; Kangbori//Miyuki-Millyang12) and the breeding line SB86648 [CMB81A-2149/Saeolbori//Buheung/Kangbori/5/Bera-olbori/Horisee-Buheung//Bengei/3/Olbori-Daechi-Hagane/4/*T. beared*]. SB961012-B-B-1, a promising line with hood gene from *T. beared* showed good character in potential forage yield in 2001. In 2004, it was designated Suwon406 and placed in the RYT at 5 locations of Korea for 3 years (2004 to 2006).

Auricleless type

The auricleless phenotype is a spontaneous mutant, controlled by a single recessive gene. The plant with this gene has erect leaf blades. A new barley cv., Dami, with the auricleless *lig* (other abbreviations: *al*, *li*, *aur-a*, etc.) gene, was developed for whole-crop forage. The cultivar derived from a cross between BGS60 and Kangbori. BGS60 (Frankowiak *et al.*, 2005) has the auricleless gene with good quality as whole-crop silage, while Kangbori has a high biomass with winter hardiness and resistance to Barley Yellow Mosaic Virus (BaYMV). Among the crosses made in 1996, a promising line with both high yield and lodging resistance in yield trials when tested at Iksan in 2003 and 2004 was designated Iksan414. The line was tested in the RYT at 7 locations for 3 years (2005 to 2007).

Results and discussion

Forage yield and quality of rough awn type forage barley cultivars

The characteristics of cvs. Youngyang, Sunwoo, Sangwon and Soman are shown in Table 1. The average heading and maturity dates of Youngyang were May 2 and June 6, respectively (Table 2). Plant height (83 cm), number of spikes per m² (637), ratio of leaf

sheath/whole length (culm+leaf) (18%) and the ratio of grain/whole plant weight (49%) in the RYT from 2000 to 2002 are shown in Table 3. It is resistant to BaYMV in Naju, Jinju, Millyang and Iksan regions of Korea. However, the response to other environmental stresses (cold and waterlogging tolerance) of Youngyang was similar to those of other cultivars (Table 4). The grain and dried whole crop forage yield potential of Youngyang in the RYT were 6.3 and 11.7 t/ha in paddy fields, respectively (Acosta *et al.*, 1991; Claudio and Adrian, 2000). Its silage quality was similar to that of other cultivars (Crovetto *et al.*, 1998; Filya, 2003).

Soman's heading and maturing dates were April 23 and May 26, respectively, which were 6 days earlier than those of Sunwoo (Table 2). Cv. Soman showed lower winter hardiness, but better resistance to lodging, shattering and BaYMV than those of check cv. Youngyang (Table 4). Its average forage yield in the RYT was 10.3 t/ha, which was 4% higher than that of the check cultivar (Table 5). Also, it showed 1.7% higher TDN content as whole-crop silage than did Sunwoo (Table 6).

Forage yield and quality of smooth awn and hooded barley cultivars

We have developed Wooho and Yuyeon with smooth awn and hood type in 2005 and 2006, respectively, as new ruminant-palatable barley cultivars (Table 7). The heading date of Yuyeon was April 27, which was 2 days earlier than that of check cv. Sunwoo, while Wooho was similar to that the check (Table 8). Yuyeon showed lower winter hardiness, but better resistance to lodging, shattering and BaYMV than the check cv. Sunwoo (Table 9). The average forage yields of Yuyeon and Wooho in the RYT were 10.5 and 11.1 t/ha, respectively (Table 10). These two cultivars would be suitable for the area where the daily minimum temperature is above -8°C in January in Korea.

Table 1. Inherent characteristics of forage barley cultivars with rough awns.

Cultivar	Growth habit	Leaf	Culm		Awn		Grain		Plant type
		Color	Width	Length	Density	Length	Color	Size	Color
Youngyang	Spring	Light green	Medium	Long	Compact	Long	Yellow	Medium	Yellow
Sunwoo	Spring	Green	Wide	Long	Compact	Long	Whitish yellow	Medium	Yellow
Sangwon	Facultative	Green	Wide	Long	Compact	Long	Yellow	Medium	Yellow
Soman	Facultative	Green	Wide	Long	Medium	Long	Whitish yellow	Medium	Yellow

Table 2. Heading and maturity dates of forage barley cultivars with rough awns in 3 regions of Korea.

Cv.	Area 1			Area 2			Area 3			Area 4		
	Heading	Maturity	Heading	Heading	Maturity	Heading	Heading	Maturity	Heading	Heading	Maturity	Maturity
Youngyang	May 7	June 10	May 1	May 1	June 2	Apr. 23	Apr. 23	June 5	May 1	May 1	June 6	June 6
Sunwoo	May 8	June 10	May 1	May 1	June 3	Apr. 26	Apr. 26	June 5	May 2	May 2	June 6	June 6
Sangwon	April 24	May 27	May 1	May 1	May 27	Apr. 26	Apr. 26	May 29	Apr. 28	Apr. 28	May 28	May 28
Soman	April 17	May 24	April 26	April 26	May 27	April 27	April 27	May 26	April 23	April 23	May 26	May 26

Table 3. Agronomic characteristics of forage barley cultivars with rough awn in 3 regions of Korea.

Cultivar	Culm (cm)		Number of spikelets per m ²		Ratio of sheath/ (culm+leaf) (%)		Ratio of grain/ whole plant (%)	
Youngyang	83		637		18		49	
Sunwoo	81		709		21		42	
Sangwon	94		682		22.5		43	
Soman	85		663		11.8		53.8	

Table 4. Resistance to disease and environmental stress of rough awned forage barley cultivars in 3 regions of Korea.

Cv.	Powdery mildew (0-9) ^a				BaYMV (0-9)				Lodging resistance (0-9)	Winter hardiness (0-9)	Waterlogging tolerance (0-9)
	AI	Field	Naju	Jinju	Millyang	Iksan					
Youngyang	7	1	0	0	1	1		1	6	5	5
Sunwoo	7	1	7	1	8	3		1	4	6	6
Sangwon	7	1	6	0	9	5		1	3	7	7
Soman	5	5	7	5	5	7		1	1	5	5

AI = artificial inoculation. Rating scales are: Powdery mildew: 0 = no plant infected, 9 = 100% infected; BaYMV: 0 = no plant lodging, 9 = 100% lodging; Winter hardiness: 0 = no plant killed, 9 = 100% killed; Waterlogging: 0 = no plant discolored, 9 = 100% discolored.

Table 5. Dry matter yield (t/ha) of forage barley cultivars with rough awns in 3 regions for 3 years

Cultivar	Year 1	Year 2	Year 3	Mean
Youngyang	10.3	11.2	13.5	11.7
Sunwoo	11.2	9.6	12.4	11.0
Sangwon	13.2	10.2	12.5	11.8
Soman	11.0	9.3	10.0	10.3

Table 6. Forage quality parameters of forage barley cultivars with rough awn tested at early dough stage.

Cultivar	Shattering rate (%)	Crude protein ^a (%)	ADF (%)	NDF (%)	TDN (%)	Silage grade (1–5)
Youngyang	42	9.8	30.7	50.9	60.2	3
Sunwoo	20	11.6	33.5	54.6	61.8	3
Sangwon	13	14.7	30.5	52.7	63.7	3
Soman	29	10.2	26.5	50.4	67.9	310.3

^a Analyzed at National Institute of Animal Science (NIAS) on material from HARI in 2005. Key to columns: ADF = Acid Detergent Fiber; NDF = Neutral Detergent Fiber; TDN = Total Digestible Nutrients; Flieg's silage evaluation: 1 (superior, >81), 2 (good, 61–80), 3 (common, 41–60), 4 (poor, 21–40), and 5 (very bad, <20).

Table 7. Inherent characteristics of cvs. Wooho and Yuyeon.

Cultivar	Growth habit	Plant type	Leaf Color	Leaf Width	Culm Length	Culm Diameter	Awn Length	Awn Type
Wooho	Facultative	Prostrate	Green	Wide	Long	Thick	Long	Smooth
Yuyeon	Facultative	Erect	Green	Wide	Long	Medium	Short	Hood

Table 8. Heading and dough stage dates of cvs. Wooho and Yuyeon (RYT 2003–2005).

Cultivar	Heading date	Dough stage date	Plant height (cm)	No. of tillers per m ²	Dry weight ^a (%)	
					Spike	Leaf blade
Wooho	April 29	May 27	95	662	40.3	15.7
Yuyeon	April 27	May 30	94	471	47.2	16.0

RYT = Regional yield trials; ^a Dry weight of spike was divided by whole plant and leaf blade divided by plant excluding spike.

Table 9. Resistance to disease and environmental stress in cvs. Wooho and Yuyeon.

Cultivar	Winter hardness				Lodging (0–9) ^b	Resistance to disease					
	Sheath's wilt (0–9)		Dead plants (%)			BaYMV (0–9)				Powdery mildew	
	High ridge	Level row	High ridge	Level row		Iksan	Naju	Infection Millyang	Security Jinju	Infection type ^a	Security (0–9)
Wooho	5	3	39.9	1.0	1	3	5	5	1	S	3
Yuyeon	5	3	41.6	1.9	0	3	5	3	1	S	5

^a Readings after artificial inoculation; ^b Average lodging in locations where it was recorded (0 = good, 9 = bad). Rating scales are: Powdery mildew: 0 = no plant infected, 9 = 100% infected; BaYMV: 0 = Resistance, 9 = Susceptible; Lodging: 0 = no plant lodging, 9 = 100% lodging;

Table 10. Dry matter yield (t/ha) of cvs. Wooho and Yuyeon evaluated at early yellow stage for 3 years.

Cultivar	Year 1	Year 2	Year 3	Mean
Wooho	10.2	10.8	12.3	11.1
Yuyeon	10.6	11.5	9.3	10.5

Table 11. Forage quality of cvs. Woooho and Yuyeon evaluated at early yellow stage for 3 years.

Cultivar	Crude protein (%)	ADF (%)	NDF (%)	TDN (%)	Silage grade (1–5)
Woooho	9.9	29.1	54.1	65.9	3
Yuyeon	10.0	28.5	52.8	66.4	2

Key to columns: ADF = Acid Detergent Fiber; NDF = Neutral Detergent Fiber; TDN = Total Digestible Nutrients; (c) Flieg's silage evaluation: 1 (superior, >81), 2 (good, 61–80), 3 (common, 41–60), 4 (poor, 21–40), and 5 (very bad, <20).

Table 12. Inherent characteristics of cv. Dami (HARI data, 2005–2007).

Cultivar	Growth	Plant	Leaf		Culm		Awn	
	habit	type	Color	Width	Length	Diameter	Length	Type
Dami	Facultative	Erect	Green	Broad	Long	Medium	Medium	Common
Sunwoo	Spring	Medium	Green	Medium	Long	Thick	Medium	Common

Table 13. Heading and maturing dates of cv. Dami (data from RYT 2005–2007).

Field type	Region	Heading date		Maturation date	
		Dami	Sunwoo	Dami	Sunwoo
Upland	Suwon	May 4	May 6	June 4	June 4
Paddy	Cheongwon	May 1	May 1	June 2	June 3
	Asan	May 3	May 4	June 2	June 1
	Yesan	May 3	May 2	May 30	May 29
	Gimje	April 30	April 30	May 29	May 28
	Daegu	April 26	April 22	May 25	May 22
	Jinju	April 26	April 25	May 30	May 27
	Mean	April 30	April 30	May 31	May 30

Table 14. Agronomic characteristics of cv. Dami (data from RYT 2005–2007).

Cultivar	Plant Height (cm)	No. of tillers per m ²	Lamina/Stem + Leaf (%)	Spike/whole plant (%)
Dami	97	643	16.0	50.0
Sunwoo	88	574	14.0	51.0

Table 15. Winter hardiness, lodging and disease resistance of cv. Dami (data from RYT 2005–2007)

Cultivar	Winter kill rate	Degree of lodging	Powdery mildew			BaYMV (0–9)		
		(0–9)	Infection type	Infection degree (0–9)	Iksan	Millyang	Infection Naju	Jinju
Dami	31.5	1	Susceptible	8	6	1	1	0
Sunwoo	37.8	3	Susceptible	8	6	7	2	2

Rating scales are: Powdery mildew: 0 = no plant infected, 9 = 100% infected; BaYMV: 0 = Resistance, 9 = Susceptible; Lodging: 0 = no plant lodging, 9 = 100% lodging.

Hooded and smooth awn types were derived from a cross. The two lines of smooth and rough awn showed no significant difference except in maturity date, while smooth awn lines had better silage qualities than those with rough awns. The cattle fed with silage from hood type cv. Yuyeon showed higher feed concentrate need (12.9 kg/day/body, 14% increase) and daily weight gain (1.46 kg/day, 35% increase) than those of the cattle fed with silage from rough awn cv. Olbori (no data). According to these

results, ruminant-palatable barley with smooth, hooded, awn-less type and fragile stem are good sources of breeding for whole crop forage.

Forage barley cv. Dami (auricleless type)

Cv. Dami has an erect plant habit, green leaf and stem similar to the check cv. Sunwoo (Table 12). Its heading date was April 30, maturing on May 31 in paddy field conditions, which is similar to those of cv. Sunwoo (Table

Table 16. DM yield of cv. Dami in regional yield trials in 7 locations of Korea (data from RYT 2005–2007).

Field type	Region	Dami (t/ha)				Sunwoo (t/ha)				Index (a/b×100)
		2005	2006	2007	Mean (a)	2005	2006	2007	Mean (b)	
Upland	Suwon	–	–	13.9	13.9	–	–	13.1	13.1	106
	Asan	10.6	11.4	15.3	12.4	8.6	8.5	12.0	9.7	128
	Cheongwon	15.8	10.5	9.8	12.0	12.1	10.9	9.8	10.9	110
	Yesan	16.5	11.2	11.0	12.9	15.7	10.3	10.6	12.2	106
	Gimje	13.0	10.2	9.9	11.0	13.1	9.8	7.5	10.1	109
	Daegu	–	–	18.4	18.4	–	–	20.4	20.4	90
	Jinju	8.1	10.4	7.8	8.8	6.4	8.8	5.9	7.0	124
	Mean	12.8	10.7	12.3	12.0	9.0	9.7	11.3	10.8	111
Paddy	Mean	12.8	10.7	12.3	12.0	9.0	9.7	11.3	10.8	111

Table 17. Forage quality of cv. Dami at early yellow ripe stage (data from RYT 2005–2007).

Cultivar	Crude protein (%)	ADF (%)	NDF (%)	TDN (%)	Digestibility	Silage grade (1–5)
Dami	7.5	28.5	50.1	66.4	56.6	2
Sunwoo	7.7	26.8	52.2	67.7	54.2	2

Digestibility analyzed at National Institute of Animal Science (NIAS) on material from Iksan in 2005, harvested 20 May. Key to columns: ADF = Acid Detergent Fiber; NDF = Neutral Detergent Fiber; TDN = Total Digestible Nutrients; (c) Flieg's silage evaluation: 1 (superior, >81), 2 (good, 61–80), 3 (common, 41–60), 4 (poor, 21–40), and 5 (very bad, <20).

13). Plant height (97 cm), number of spikes per m² (638), lamina/stem + leaf (%) and spike/whole plant are shown in Table 14. Dami has better winter hardiness and resistance to BaYMV than Sunwoo (Table 15). The average forage yield of cv. Dami was about 12 t/ha as dry matter (33 t/ha fresh matter) in paddy fields (Table 16). It also showed 7.5% crude protein content, 28.5% ADF, 50.1% NDF, and 66.4% TDN, including a higher grade of silage quality for-whole crop barley (Table 17). This cultivar would be suitable for the area where daily minimum temperature is above -8°C in January in Korea.

Conclusion

In Korea, barley cultivation for cereal food production has been being replaced by the production of forage barley. We have developed 7 forage barley cultivars with ruminant-palatable plant type, including smooth awn, hood and auricleless types, with high biomass as a whole crop, compared with barley cultivars for food. These cultivars have

advantages in forage or TDN yield per unit area. Moreover, it is very economic in land utilization because forage barley is produced in a cropping system following rice during the winter season. For commercial cultivation of the forage barley cultivars, the Ministry of Agriculture and Forestry of the Republic of Korea has had a seed supply program since 2007. The production of forage barley has recently caused great interest, with the rising fodder price and the reduction in Government purchasing of food barley. Forage barley cultivation requires heavy fertilization to obtain high yields, and it is a break crop that negatively affects grain quality for rice due to increasing protein content. Therefore, improved cultivation technologies are needed to overcome the problem of degradation of rice grain by residual fertilizer after forage barley cultivation in paddy fields. In the near future, we also plan to develop forage barley cultivars with awn-less, high biomass and lysine content, and with good silage fitness, such as high sugar content, for both solid and brittle stem types.

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Session 9

Biochemistry, cytogenetics and transformation

Genetic engineering in barley: current technologies and recent applications

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Introduction

Barley represents both a useful experimental model for a number of small-grain cereals as well as an agronomically important crop for feed, malt and food production. In recent years, a vast amount of different barley genetic resources has been generated and collected worldwide. Together with these resources, the development of reliable transformation technologies has stimulated a variety of approaches to functional gene analysis and genetically engineered breeding lines.

Methods of barley genetic transformation

The technical details of the gene transfer process are central to the establishment of powerful transformation technology. In contemporary methods, agrobacteria are employed as naturally evolved and artificially optimized vehicles to integrate recombinant DNA into the barley genome.

In our laboratory, an exceedingly powerful barley transformation method based on *Agrobacterium*-mediated gene transfer to immature embryos has been developed (Hensel *et al.*, 2008). Advancements were achieved through comparative experiments on the influence of various explant treatments and co-cultivation conditions. Beside the high transformation efficiency achieved in the highly amenable cultivar Golden Promise,

it was demonstrated that the established method is also suitable to generate transgenic plants from a range of barley cultivars and breeding lines. The experiments conducted to optimize the transformation method eventually resulted in as many as 80 stable transgenics per 100 immature embryos inoculated with *Agrobacterium tumefaciens*. However, this result is based on a group of only 270 embryos, and this experiment was confined to the transfer of reporter and selectable marker genes. Typically, average transformation efficiencies of some 20% are obtained.

A second method of barley transformation, which has been developed and thus far exclusively employed in our laboratory, is based upon infection of embryogenic pollen cultures with agrobacteria (Kumlehn *et al.*, 2006). In this method, we take advantage of the haploid nature of microspores giving rise to embryogenic microcalli, which are used as gene transfer target. However, since spontaneous genome doubling typically takes place very early in microspore cultures, haploid as well as diploid cells can be transformed. Although homozygosity of the transgene was shown for some of the primary transgenic lines produced by this method, these lines still required discrimination from the far more abundantly obtained hemizygous plants through copy number assessment along with time-consuming segregation analyses of the T_1 populations. Recently, however, following a more

advanced protocol, the haploid transgenic individuals are selected at the plantlet stage by flow-cytometry and then subjected to colchicine treatment. This procedure now routinely permits the exclusive formation of homozygous transgenic T_1 populations and thus substantially supports the retrieval of highly consistent data from experimental approaches to functional gene analysis, as well as the rapid identification of well-performing, true-breeding lines generated for barley improvement.

Pursuing a third approach to the stable transformation of barley, which was recently introduced by Holme *et al.* (2006), we have generated transgenic lines via infection of isolated ovules with *A. tumefaciens*. The frequencies of transgenic plant formation were fairly comparable with other methods of barley transformation routinely used. The major potential of this unique transformation method lies in the comparatively low genotype-dependence of the regeneration system employed, and in the fact that it is effective at still reasonable efficiency without the use of selective conditions during the *in vitro* culture procedure. As a consequence, transgenic barley instantly free of selectable marker can be produced. The analysis of representative numbers of transgenic lines obtained from all three gene transfer methods revealed that the T-DNA copy numbers are typically low, the generative transmission of the recombinant DNA is in accord with the Mendelian rules and the vast majority of the primary transgenics produce progeny that expresses the respective transgene product.

Each of the three transformation methods has specific advantages. While the use of immature zygotic embryos results in exceedingly high efficiency of transgenic plant formation, embryogenic pollen cultures enable us to produce instantly true-breeding transgenic lines, whereas isolated ovules potentially allow for the generation of transgenic plants from a broad range of genotypes, and without use of a selectable marker.

Transformation vectors

The binary vector systems that work well for many dicotyledonous species are, unfortunately, of only limited utility in the monocotyledons, largely because commonly used promoter sequences and/or selectable markers are ineffective in a monocotyledonous host. Recently, we have generated and functionally tested a novel set of modular binary vectors (the IPKb series), specifically tailored for cereal transformation and targeted to either over-expression or RNA-interference (RNAi)-mediated gene knock-down (Himmelbach *et al.*, 2007). In both vector types, the insertion of effector sequences is facilitated by the exploitation of GATEWAY destination cassettes, which permit the efficient, site-specific and reliable exchange of DNA-fragments between plasmids. Any DNA sequence can be readily transferred from an easily cloned entry vector to the binary destination vector via an LR reaction, a procedure that avoids the need for the digestion and ligation-based cloning of the typically rather large binary vectors. This is particularly advantageous in the context of RNAi vectors, in which two inverted DNA repeats need to be connected by a spacer or intron sequence. The IPKb vector set also includes derivatives both of the over-expression and RNAi types, in which various promoters, which are fully functional in monocotyledonous species, have been inserted to drive ubiquitous or epidermis-specific transgene expression. Furthermore, any other established or *de novo* isolated promoter sequence can be readily inserted upstream of the GATEWAY destination cassettes. Since specific plant selection markers may be preferred for some target hosts, or may be necessary for certain gene stacking strategies, the IPKb vectors have been constructed to allow for the ready introduction of further marker gene expression cassettes derived from a number of the compatible conventional binary vectors available.

Expression systems

The identification and employment of appropriate promoter sequences to drive expression of transgenes is vital to both the development of transformation technologies and its application in fundamental research and biotechnology. While promoters providing strong ubiquitous expression have been commonly used along with selectable marker and reporter genes in the scope of studies aiming to establish transformation methods, it became increasingly desirable to develop tissue- or cell-specific, or even inducible, expression systems. Unfortunately, the promoters originating from dicotyledonous plant species, which cover a wide range of specificities and expression strengths, normally do not work appropriately, if at all, in the genetic context of monocotyledons. Therefore, the choice of promoters available for barley is still fairly limited. Promoters that have been successfully used in our lab for transgene expression in barley drive ubiquitous, endosperm-, epidermis- and egg cell-specific as well as pathogen-inducible expression.

Generation of selectable marker-free transgenic plants

Through coupling genetic transformation with haploid technology, we have developed an efficient method to readily generate selectable marker-free transgenic lines instantly homozygous for the transgene. To this end, primary co-transgenic plants were produced via *Agrobacterium*-mediated gene transfer to immature embryos with two independent T-DNAs, carrying the selectable marker gene and the gene of interest, respectively. Fourteen different variants of embryo inoculation were tested, including mixtures of different *Agrobacterium* strains, clones with diverse binary vectors, and agrobacteria carrying two binary vectors or a vector with two T-DNAs. To simplify the segregation of unlinked integration loci, embryogenic pollen cultures were used

to produce populations of T_1 double haploids, among which individuals inheriting only the gene of interest were identified.

Application of transgenic barley in fundamental research and biotechnology

Based upon the enabling technology established, we have embarked on various projects aiming to functionally analyze candidate genes, and to improve barley by genetic engineering.

Taking advantage of the established transformation methods, thousands of transgenic barley plants were generated in recent years. We are especially interested in the analysis of genes putatively involved in interactions between cereals and fungi. A functional analysis of genes implicated in the complex molecular mechanisms of plant-pathogen interactions is often required to better understand these processes prior to directed approaches of genetic engineering towards improved resistance to pathogens. Some of the candidate genes used encode small RAC/ROP GTPases that serve as molecular switches in the signal transduction of many cellular processes, especially those initiated by environmental cues. For example, stable over-expression of the constitutively activated *racb* gene rendered barley more susceptible to powdery mildew, limited water retention in leaves and reduced responsiveness to ABA (Schultheiss *et al.*, 2005). Also, investigations have been conducted into the function of host genes putatively involved in barley-virus interactions. The barley eukaryotic translation initiation factor 4E gene (*Eif4E*) was identified to be putatively associated with resistance to Bymoviruses via a positional cloning approach. Through gene transfer to embryogenic pollen cultures of the resistant cv. Igri, which carries a naturally occurring mutant *eif4E* allele, with the wild-type *Eif4E* from a susceptible barley background, we

provided compelling evidence that this gene represents a host factor that is essential for the Bymovirus disease (Stein *et al.*, 2005).

Barley grains constitute a very useful system for the production of valuable recombinant protein. Our strategy to generate a vaccine against the H5N1 influenza A virus is based on the expression of haemagglutinin (HA), a major virus surface antigen, in plant tissue that may be used for massive oral immunization of birds. We have produced transgenic barley expressing codon-optimized HA1 antigen driven by the endosperm-specific α -gliadin promoter of wheat. Western blot analysis revealed a particularly high expression of HA1 antigen in the grains of two out of 84 transgenic lines.

Prospects

A better understanding of the cellular particularities of the transformation process and further technical improvements would be highly beneficial for future applications. Remarkable progress has been made in the elucidation of the general mechanisms of *Agrobacterium*-mediated gene transfer to plant cells including the recruitment of host factors. However, experimentally validated and detailed information about optimal conditions and particular requirements of agrobacteria for their efficient attachment and T-DNA transfer to target cells is still fairly limited. The current state of our understanding of these biological mechanisms may be sufficient for practical applications in most dicotyledonous plant species, yet the development and improvement of transformation technology for atypical *Agrobacterium* hosts, such as barley, will probably require a broader base of consolidated knowledge. In particular, comprehensive experiments examining the physical and chemical conditions and their implications in *Agrobacterium*-mediated gene transfer may result in exceedingly valuable information. The elucidation of particularities that limit the amenability

of cells from monocotyledonous plants as targets for *A. tumefaciens* would be of prime importance to facilitate the development of gene transfer protocols for genotypes or for regenerable cell types that are beyond the range of current barley transformation methods. For example, a routinely applicable and efficient transformation method based on the use of mature barley seeds or of target cells present shortly after their germination would render the tedious and time-consuming production of adult donor plants dispensable.

The transformation methods developed and the transgenic barley plants obtained are thought to represent a vital fundamental means to gain in-depth information on the cross-talk between plants and microbes as well as on physiological consequences. This increase of knowledge may substantially contribute to finding appropriate technical solutions for the agronomic challenges we are facing worldwide.

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Accessing the wild side of barley

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Abstract

Plant breeding relies heavily on accessing sufficient sources of genetic diversity. Barley (*Hordeum vulgare* L.) suffers from a narrow gene pool due to a long breeding history and an inbred, diploid nature. *Hordeum bulbosum* L. is the sole member of the secondary *Hordeum* gene pool and as an undomesticated, self-incompatible, out-breeding species, represents a major source of allelic diversity. In our research program we have used genomic *in situ* hybridization to demonstrate introgression of *H. bulbosum* chromatin into the barley genome. These introgression lines (ILs) were further characterized using interspecific molecular markers developed using public barley EST databases and cDNA-AFLP-based gene discovery. We have developed 175 ILs possessing mostly distal and some rare interstitial introgressions on 13 of the 14 chromosome arms. As *H. bulbosum* is a non-host for most barley pathogens, our research program has focused primarily on identifying disease resistance traits, although these lines are likely to contain valuable alleles for additional traits. Novel resistances derived from *H. bulbosum* have been successfully transferred into barley for leaf rust, *Septoria* speckled leaf blotch, powdery mildew, stem rust, BaMMV and scald, and also tolerance to BYDV. As with most wide hybridizations, introgressions of *H. bulbosum* chromatin in a barley background suffer from suppressed recombination, which can hamper further characterization without appropriate molecular marker resources.

Introduction

Access to genetic variation for barley improvement is critical to the ongoing success of the crop. *Hordeum bulbosum* is the sole member of the secondary gene pool of *Hordeum vulgare* and represents a valuable resource for barley genetic improvement. In the past, *H. bulbosum* has been used primarily in the production of barley doubled haploids. After crossing barley with *H. bulbosum*, the *H. bulbosum* genome is usually eliminated from the developing embryo, which can be rescued onto nutrient medium to regenerate haploid barley plants (Kasha and Kao, 1970). Fertility can be restored by treating the plantlets with colchicine to double the chromosome number. Interspecific hybrids are, however, occasionally produced, but complete chromosome retention is strongly influenced by parental genotype and temperature shortly after fertilization (Pickering, 1984). Occasionally these hybrids are partially fertile (triploid VBB or tetraploid VVBB) and can be used to produce introgression lines (ILs) through backcrossing to barley (triploids) or selfing (tetraploids). These ILs possess primarily single, distal introgressions of *H. bulbosum* in a barley genetic background and hence are effectively near-isogenic lines (NILs). Isolation of discrete portions of the *H. bulbosum* genome into barley allows identification and mapping of valuable *H. bulbosum* traits of use for barley improvement. The focus of this program has been primarily on breeding for disease resistance. However, these ILs represent a resource suitable for screening

allelic variation for a wide range of traits important for barley improvement including abiotic stress tolerance, malt and grain quality.

Determining the physical size and chromosomal location of the introgressions can be achieved using sequential genomic- and fluorescent *in situ* hybridization (Pickering *et al.*, 2000). However, in this paper we will describe the use and development of molecular markers to genetically characterize these ILs.

Materials and methods

Detection of ILs, from lines that are genetically identical to the barley parent, was initially performed using a single PCR assay. Based on a high copy number, retrotransposon-like sequence isolated by Bedbrook *et al.* (1980) from rye (*Secale cereale*), the pSc119.1 marker in combination with a rapid, crude DNA extraction (Johnston and Pickering, 2002) allowed the selection of putative ILs for further analysis.

To complement the pSc119.1 assay, 54 PCR markers have been developed which are fully informative across the barley cultivars and *H. bulbosum* genotypes used in our program. These markers are mostly co-dominant cleaved amplified polymorphic sequence (CAPS) markers generated from genomic re-sequencing of EST contigs derived from the HarvEST database (Wanamaker *et al.*, 2006). Briefly, allele sequences from four barley cultivars and four *H. bulbosum* genotypes were aligned using SeqScape (Applied Biosystems) and conserved species-specific SNPs and indels were targeted for CAPS development using BlastDigester (Ilic *et al.*, 2004) or for size separation. Species-diagnostic polymorphisms were confirmed by size separation or digestion of PCR products from a panel of 24 lines that included the original 8 parental genotypes, 8 interspecific hybrids and 8 ILs and/or chromosome substitution lines covering the appropriate chromosomal

location of the PCR marker (Figure 1).

Primer sequences and PCR product sequencing of one barley cultivar (Emir) and one *H. bulbosum* genotype (Hb2032) across 384 markers was kindly performed by Professor Kazuhiro Sato (Okayama University, Japan) allowing further marker development using the methods described above. The cDNA-AFLP technique (Bachem *et al.*, 1998) was also used to generate markers by extracting *H. bulbosum*-derived polymorphic bands directly from IL transcript profiles. This enabled markers to be generated from particular chromosomal regions for more saturated mapping experiments. Markers displaying polymorphisms between barley cultivars Steptoe and Morex were mapped using the doubled haploid population developed by Kleinhofs *et al.* (1993).

Progeny returning positive results from the pSc119.1 assay were screened using a subset of the diagnostic PCR markers spread across distal and proximal regions of all seven barley chromosomes. This enabled the chromosomal location and genetic size of each introgression to be determined.

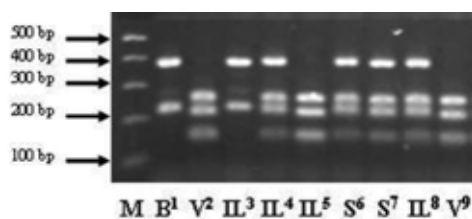


Figure 1. Cleaved amplified polymorphic sequence (CAPS) marker H31_5825 (2HL) digested with Sau96I (GGNCC) showing species-specific polymorphism between barley and *H. bulbosum*.

KEY: M = 1 kb plus ladder (Invitrogen); B = *H. bulbosum* genotype 2032; V = barley cultivars Emir (V²⁺) and Golden Promise (V⁹); IL = 2HL introgression lines – 182Q20 (IL³), 102C210/2/13/ (IL⁴), 38P18 (IL⁵), 102C211/1/15/PL1 (IL⁸); S = chromosome substitution lines 21R2 (2H^b (2H), 5H^b (5H)) (S⁶) and 21S1 (2H^b (2H), 3H^b (3H), 5H^b (5H)) (S⁷).

PCR markers were also developed to target the vernalization pathway loci *VrnH1* (Yan *et al.*, 2003) and *VrnH2* (Dubcovsky *et al.*, 2005) to allow analysis of ILs possessing winter-type alleles derived from *H. bulbosum*.

Results and discussion

The pSc119.1 diagnostic PCR assay detected all previously characterized ILs (Figure 2) and was used to screen uncharacterized progeny derived from both triploid and tetraploid interspecific hybrids.

A large degree of variation (5 to 44%, Table 1) was detected in the number of pSc119.1-positive progeny from each of these uncharacterized populations, but the proportions of pSc119.1-positive progeny derived from triploid or tetraploid interspecific hybrids were similar (Table 1).

Where possible, the co-dominant, locus-specific markers developed in the program were mapped using the Steptoe \times Morex population (Kleinhofs *et al.*, 1993) or located using previously characterized ILs. A total of 109 pSc119.1-positive but previously uncharacterized lines were screened using 38 PCR markers, allowing determination of the chromosomal location and genetic size of each introgression. A total of 93 introgression events were detected in this analysis. The

majority of lines (78) contained only a single introgression, with six lines possessing two introgressions and one line with three introgressions. Most of these *H. bulbosum* introgressions (97%) extended to the distal end of the chromosome arm, with only three interstitial or sub-distal introgressions identified. Introgressions were recorded on 13 of the 14 chromosome arms, with none on chromosome 3HL (Table 2), despite barley and *H. bulbosum* chromosome 3H homoeologues appearing to associate with the same frequency as other chromosomes (Pickering *et al.*, 2006).

Chromosome 2HL introgressions were by far the most frequently detected (29%; Table 2) and generally introgressions on long arms were found more frequently than those on the short arms of the same chromosome (Table 2). When the largest introgressions on each chromosome arm are taken into account, approximately 37% of the barley genetic map has been transferred from *H. bulbosum* (mostly the distal regions) into the barley genome. When combined with lines characterized prior to the use of pSc119.1, a total of 175 introgressions have been generated to date.

Of the original 109 pSc119.1-positive lines analyzed, 24 putative ILs still need to have

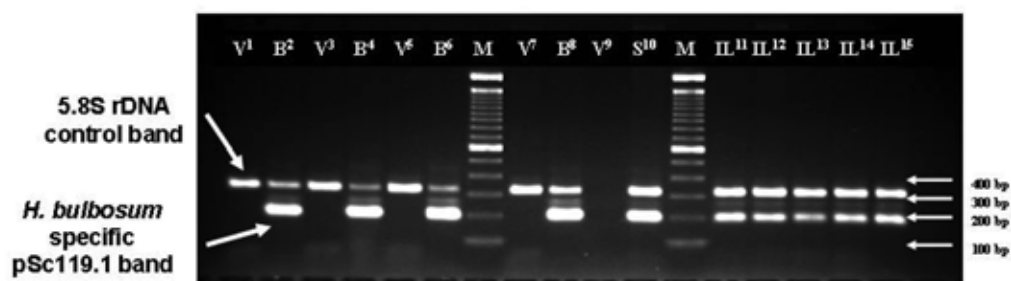


Figure 2. pSc119.1 PCR showing the presence of the *H. bulbosum*-specific 201 bp PCR product in lines that possess *H. bulbosum* chromatin.

KEY: M = 1 kb plus ladder (Invitrogen); V = barley cultivars Emir (V¹), Golden Promise (V³), Morex (V⁵), Steptoe (V⁷), Vada (V⁹) (failed PCR); B = *H. bulbosum* genotypes 2032 (B²), 2920/4 (B⁴), 2929/1 (B⁶), A17 (B⁸); S = monosomic chromosome substitution line 34L6 (4H^b (4H)); IL = introgression lines 102C2/16 (1H^bL) (IL¹¹), 38P18 (2H^bL) (IL¹²), 102C2/13 (3H^bS) (IL¹³), 53A8 (4H^bL) (IL¹⁴), 34L72 (4H^bL) (IL¹⁵).

Table 1. Frequency of pSc119.1-positive lines after PCR screening of unselected progeny from backcrossed triploid (VBB) and selfed tetraploid (VVBB) interspecific (*Hordeum vulgare* [Hv] × *H. bulbosum* [Hb]) hybrids.

Hybrid Code	Pedigree (Hv × Hb)	Positive Lines	% Positive Lines
Barl181P	Emir × A17/RP1	45/143	31.5
203S	Emir × A17	3/8	37.5
251V	Morex × 2032	4/74	5.4
VBB Hybrids		52/225	23.1
220A	Morex × 2920/4	7/27	25.9
228Q1	Morex × 2032	9/23	39.1
230H1	Morex × 2032	7/24	29.2
230H3	Morex × 2032	1/7	14.3
230H13	Morex × 2032	4/16	25.0
230H18	Morex × 2032	2/13	15.4
230H24	Morex × 2032	6/25	24.0
230H25	Morex × 2032	4/30	13.3
230S5	Morex × 2032	5/21	23.8
231D5	Golden Promise × 2032	1/5	20.0
231D8	Golden Promise × 2032	4/9	44.4
VVBB Hybrids		50/200	25.0
TOTAL		102/425	24.0

Table 2. Introgression locations detected using a selection of 38 single-locus markers spread across the barley genome (* denotes locations of the three interstitial introgressions).

Chromosomal Location (Arm)	Number of introgression events	Percentage
1HS	4	4.3
1HL	11	11.8
2HS	2	2.2
2HL	27*	29.0
3HS	3*	3.2
3HL	0	0.0
4HS	5	5.4
4HL	5	5.4
5HS	3	3.2
5HL	6*	6.5
6HS	10	10.8
6HL	12	12.9
7HS	2	2.2
7HL	3	3.2
Total	93	100

introgressions located. It is likely that these lines have either lost their introgressions in the generations subsequent to their original pSc119.1 screening, were false positives or have introgressions located in genomic regions not covered by the current markers. Another possibility is that during wide hybridization the pSc119.1 element, if active (unproven), may have transposed from *H.*

bulbosum into barley without actual transfer of chromatin between the species.

A series of rare interstitial introgressions located on chromosome 5HL was of particular interest as they resulted in the conversion of spring type barley cultivars into winter types due to the transfer of recessive winter *vrnH1* alleles from *H. bulbosum* into the barley cultivar Emir. However, one IL, 38U16, remained a spring type despite being homozygous for the *H. bulbosum* *vrnH1* allele, but located in the background barley cultivar Golden Promise. Analysis of the *VrnH2* locus (repressor of flowering) revealed that Emir was a rare spring type barley cultivar that still carried a functional repressor at *VrnH2*. Most spring type barley cultivars (including Golden Promise) possess deletions at the *VrnH2* locus (von Zitzewitz *et al.*, 2005) in addition to deletions in the first intron of *VrnH1* (Fu *et al.*, 2005) making it unresponsive to a functional *VrnH2* (Figure 3).

When 38U16 was crossed with Emir, winter-type plants were recovered in the F₂ population, but at a very high frequency, suggesting the presence of a segregation distorter locus. In fact from 88 F₂ plants, only 24 were spring type (72.5 expected)

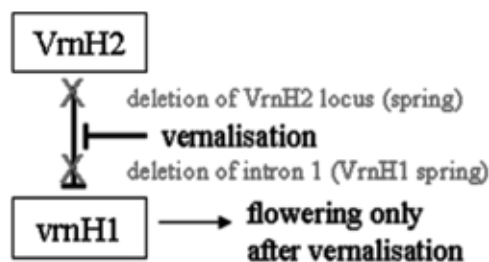


Figure 3. Proposed vernalization model (Szűcs *et al.*, 2007; Yan *et al.*, 2004)

despite the requirement for expression of the winter phenotype being at least a single functional (Emir) *VrnH2* allele (dominant) in combination with the homozygous *H. bulbosum vrnH1* allele (recessive). Further investigation revealed that the 5HL introgression (containing *vrnH1*) was exclusively retained in all 88 lines despite the backcross to Emir, with only two ILs having undergone some further recombination over a genetic distance of 70 cM (between markers *VrnH1* and H31_3326, Table 3). Analysis of the dominant *VrnH2* marker (present in cv. Emir but absent in cv. Golden Promise) on chromosome 4HL revealed normal 1:1 segregation (Table 3) and another co-dominant marker (H31_1079) located on 5HS and outside the introgression also segregated normally 1:2:1 for Emir: heterozygote: Golden Promise alleles (Table 3). This

indicated that the segregation distortion was restricted to the introgressed chromatin.

It is likely that this is the first example of a gametocidal or 'cuckoo' locus (Endo, 1979) to be found in cultivated barley resulting from interspecific hybridization. Such loci have been transferred from *Aegilops* spp. into wheat (*Triticum aestivum*) and have been used to develop cytological deletion stocks (Endo, 2007). Cytogenetic evaluation of an F₁ heterozygote revealed pycnotic nuclei in about 50% of the immature pollen grains (R. Pickering, unpublished data).

A degree of segregation distortion is common in many ILs, but, apart from the extreme example described above, this is exclusively at the expense of the introgressed alleles. In some ILs, homozygous BB genotypes are either completely absent (probably caused by embryo lethality resulting in semi-sterility) or produce very poor plants that fail to survive to maturity. Even in IL 38P18, which has a very small introgression and no evidence of yield penalty relative to its barley parent (Emir) (Pickering *et al.*, 2004), certation effects act to make gametes possessing introgressed *H. bulbosum* chromatin less competitive than wild type barley gametes. This results in fewer homozygous *H. bulbosum* genotypes in favor of heterozygous and barley-type progeny (Table 4) and is even more

Table 3. Marker analysis of the 333U (38U16 × Emir) population. Co-dominant markers *VrnH1*_Int1_3' and H31_2252 are located within the *VrnH1* gene (proximal 5HL) and H31_3326 (also co-dominant) is located sub-distally on chromosome 5HL. Markers *VrnH2* (dominant 4HL) and H31_1079 (co-dominant 5HS) are located outside the *H. bulbosum* introgression and both segregated as expected.

Genotypes	Observed (and expected) genotypes in markers located within the introgressed chromatin (5HL)			Genotypes	Observed (and expected) genotypes in markers located outside the introgressed chromatin	
	<i>VrnH1</i> _Int1_3'	H31_2252 (<i>VrnH1</i>)	H31_3326 (5HL)		<i>VrnH2</i> (4HL)	H31_1079 (5HS)
B	85 (22)	86 (22)	81 (22)	Emir	65 (66)	20 (22)
H	1 (44)	1 (44)	3 (44)	Heterozygous		42 (44)
V	0 (22)	0 (22)	0 (22)	Golden Promise	23 (22)	26 (22)
Fail	2	1	4	Fail		0
Total	88	88	88		88	88

V = homozygous barley genotype; H = heterozygous genotype; B = homozygous *H. bulbosum* genotype; Fail = failed PCR reaction (no observed genotype); numbers in parentheses represent expected genotype numbers for each marker.

pronounced in IL 182Q20, possessing a much larger introgression (Table 4).

Introgressions of *H. bulbosum* also tend to resist further recombination, with a high level of suppression previously measured in some chromosome locations relative to intraspecific barley populations (Korzun *et al.*, 1999; Ruge *et al.*, 2003). The size of introgressed chromatin seems to have a large effect on the extent of this suppression. Canady *et al.* (2006) working with interspecific hybrids between tomato (*Solanum lycopersicum*) and nightshade (*Solanum lycopersicoides*) reported that larger introgressions and whole substituted chromosomes experienced the highest recombination frequencies relative to intraspecific levels. In our own material this is also borne out in introgressions of different size located in the same chromosomal region. Marker analysis of interspecific recombination in F₂ progeny from IL 182Q20, which possesses an introgression covering >26% of the genetic length of chromosome 2HL, showed 2–3-fold suppression of recombination (measured in a cross with another IL 38P18, Figure 4) whilst analysis of a backcross population from IL 38P18 (covering ~2% of the genetic length of chromosome 2HL) revealed only two further recombination events detected from 715 F₂ progeny (data not shown).

Conclusions

The work detailed here represents a long-term effort to increase the accessibility of *H. bulbosum* genetic diversity for barley improvement. Efficient tools are now available to identify ILs from progeny derived from interspecific hybrids using the pSc119.1 assay. Further characterization of these ILs for introgression location and size is performed using locus-specific co-dominant PCR markers. Marker saturation also allows traits to be fine mapped within the introgressed *H. bulbosum* chromatin. However, this relies on further interspecific recombination to isolate the trait genetically and is influenced greatly by the size of the introgressed chromatin. Larger introgressions have been shown here and by others (Canady *et al.*, 2006) to achieve the highest recombination frequencies relative to intraspecific levels. Unfortunately, these larger introgressions are also more difficult to work with as they are more likely to suffer from poor vigor, reduced fertility and low seed set (strongly dependent on the chromosomal location and pedigree of the introgressed chromatin). Subtle traits may also be difficult to identify in larger introgressions, where there is a greater chance of being masked by undomesticated alleles. This collection of ILs represents a valuable resource for barley improvement and is

Table 4. Observed and expected (in parentheses) marker genotypes from the two recombinant F₂ populations 270R (Emir × 38P18) and 277A (38P18 × 182Q20). Both populations show significant (p < 0.001) deviation from the expected genotype frequency (1:2:1), favoring the detection of heterozygous (H) and homozygous barley (V) genotypes over homozygous *H. bulbosum* (B) genotypes. Chi-square test (χ²) was performed using GenStat (Lawes Agricultural Trust, version 9.2) with the maximum likelihood method (2 degrees of freedom (DF)).

Genotype	270R (4–5 markers × 715 lines)		277A (3 markers × 176 lines)	
	Observed (Expected 1:2:1)	(<i>VrnH1</i>)	H31_3326 (5HL)	Genotypes
B	85 (22)	86 (22)	81 (22)	Emir
H	1 (44)	1 (44)	3 (44)	Heterozygous
V	0 (22)	0 (22)	0 (22)	Golden Promise
Fail	2	1	4	Fail
Total	88	88	88	

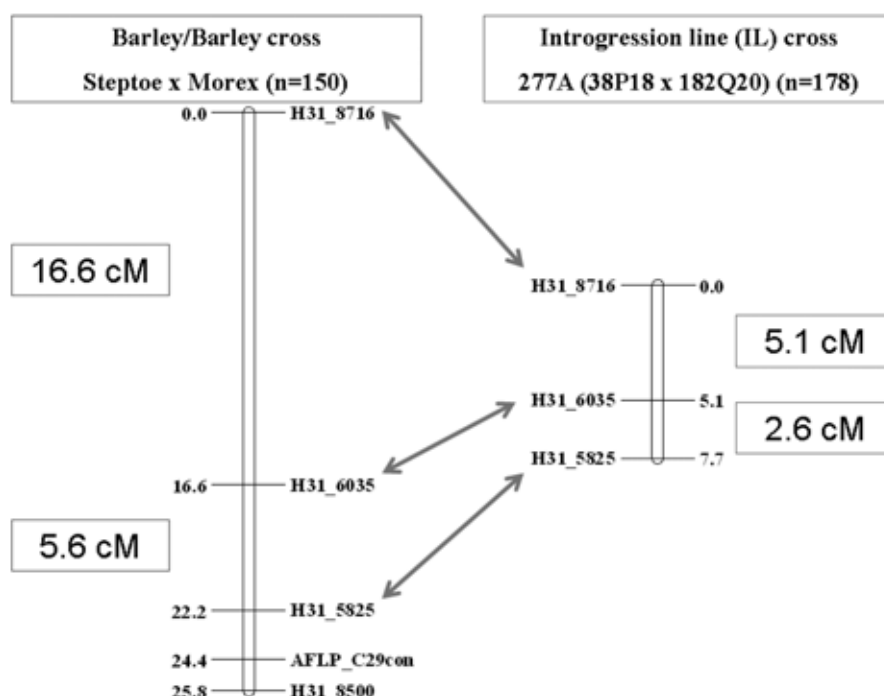


Figure 4. Comparative mapping of three markers (H31_8716, H31_6035 and H31_5825) across two populations: barley \times barley (Step toe \times Morex, $n = 150$), and interspecific region of a cross between two ILs (277A = 38P18 \times 182Q20, $n = 178$) showing reduced recombination frequency (2.2–3.3-fold) relative to the intraspecific frequency.

continuing to be distributed to researchers and breeders worldwide for targeting not only disease resistance, but also other traits of interest, such as tolerance to abiotic stress, forage potential and grain quality attributes.

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***Uzu*, a barley semi-dwarf gene, suppresses plant regeneration in calli derived from immature embryos**

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Abstract

Barley includes semi-dwarf varieties, called *uzu*, which are localized in parts of southwestern Japan, the southern Korean peninsula, and coastal areas of China. The *uzu* phenotype possesses dark green leaves and short coleoptiles, awns and spikes. It is controlled by a single recessive gene: *uzu*. *Uzu* results from the mutation in the brassinosteroid receptor kinase gene (*HvBR11*). Brassinosteroid synergistically acts with auxin on the plant morphology, which is an important plant hormone for tissue culture. For this study, tissue culture traits, including callus growth and shoot regeneration capability, are examined in F₂ populations derived from crosses between normal and *uzu* lines and in isogenic lines for the *uzu* gene. The *uzu* genotype shows a lower percentage of shoot regeneration than the normal genotype in F₂ populations and isogenic lines. The *uzu* gene negatively affects shoot regeneration. No significant differences were found in the callus growth capability between *uzu* and normal genotypes. *Uzu* isogenic lines show higher sensitivity to exogenous auxin for callus initiation than normal lines, when immature embryos were incubated on the media supplemented with several concentrations of 2,4-D in cultures at elevated temperature (25°C). Tissue culture traits of *uzu* might be regulated through cross-talk between brassinosteroid and auxin.

Introduction

Brassinosteroid, a kind of plant hormone, regulates a broad range of responses in plants: seed germination, stem and root elongation, vascular differentiation, leaf expansion, apical dominance, and others (Halliday, 2004). The signal transduction pathway of brassinosteroid has been analyzed using brassinosteroid-deficient and brassinosteroid-insensitive mutants in *Arabidopsis* (He *et al.*, 2000; Wang *et al.*, 2001). Several reports suggest that brassinosteroid acts synergistically with auxin on hypocotyl elongation and lateral root development, and that the signaling pathways of brassinosteroid and auxin converge the transcriptional regulation of common target genes (Bao *et al.*, 2004; Nemhauser *et al.*, 2004). At the same time, from other investigations, brassinosteroid independently regulates gene expressions from other hormones, such as auxin and cytokinin (Mussig *et al.*, 2002; Goda *et al.*, 2004). Interactions between brassinosteroid and other hormones remain unknown.

In addition to *Arabidopsis*, brassinosteroid-related mutants have been isolated from tomato and pea (Nomura *et al.*, 1997; Montoya *et al.*, 2002). In contrast to advanced research on molecular mechanisms of brassinosteroid biosynthesis and signaling in dicots, a limited number of brassinosteroid-deficient and brassinosteroid-insensitive

mutants were identified from rice (Hong *et al.*, 2005; Tanabe *et al.*, 2005); insufficiency of mutants yielded little information related to the molecular functions of brassinosteroid in monocots. *Uzu*, a barley semi-dwarf mutant, is controlled by a single recessive gene. These lines were cultivated in eastern Asian regions, which include parts of southwest Japan, the southern Korean peninsula and coastal areas of central China. *Uzu* lines show unique morphological characteristics, such as short awns, spikes, and coleoptiles, and dark green leaves. Recently, the *uzu* gene was cloned; it was a brassinosteroid receptor kinase gene, *HvBR11* (Chono *et al.*, 2003; Saisho *et al.*, 2004). *Uzu* is an important brassinosteroid-related mutant in monocots. The *uzu* phenotype was expressed by the natural mutation of *HvBR11* and such mutation showed insensitivity to brassinosteroid. Although the *uzu* gene acts in the signal transduction pathway of brassinosteroid, *uzu* lines showed higher accumulation of cytokinin in seedlings than normal lines (Honda *et al.*, 1995). *Uzu* lines also showed a lower level of endogenous indole acetic acid (IAA) in seedlings (Inouhe *et al.*, 1982). These results suggest that signals of brassinosteroid probably interact with the endogenous level of auxin and cytokinin.

Auxin and cytokinin control tissue culture traits, including callus formation and plant regeneration. Immature embryos and anthers were mainly used as explant sources in barley tissue culture. Endogenous hormonal levels of explants and calli affected embryogenesis and plant regeneration in maize and wheat (Jiménez and Bangerth, 2001a, b). Kraepiel *et al.* (1995) suggested that the ratio of auxin to cytokinin was important for callus proliferation and plant regeneration in tobacco cultured cells. For that reason, auxin and cytokinin are important factors for tissue culture traits. *HvBR11* mutation might affect callus formation and plant regeneration through modification of endogenous levels and/or signals of auxin and cytokinin.

To investigate the effects of brassinosteroid signaling on barley tissue culture traits, callus proliferation and shoot regeneration were examined in F_2 populations derived from crosses between normal and *uzu* lines, and in isogenic lines for *uzu* gene including different genetic backgrounds. The *uzu* gene suppressed shoot regeneration, suggesting that the signal transduction pathway of brassinosteroid acts on shoot regeneration in barley immature embryo cultures as a positive regulator similar to auxin and cytokinin. The *uzu* gene also modified the sensitivity to exogenous auxin for callus initiation and germination in immature embryos. We observed the effects of brassinosteroid signaling on tissue culture traits in barley.

Materials and methods

Plant materials

The F_2 populations of 82 individuals derived from a cross between Haruna Nijo (two-row and hulled normal barley) and Akashinriki (six-row and hullless *uzu* barley) and 92 individuals derived from a cross between H602 (*Hordeum vulgare* subsp. *spontaneum*, a two-row hulled normal barley) and Akashinriki, and parental varieties were grown in the field. Two independent isogenic lines of barley with the *uzu* gene were used. The *uzu* gene derived from Akashinriki was introduced into cvs. Ryohu and Hoshimasari (two-row hulled normal barleys) by 12-time and 10-time backcrossing with corresponding normal barley, respectively. Isogenic lines were grown in a growth chamber (15°C, 16-h photoperiod). These isogenic lines were provided by Dr K Sato of the Research Institute for Bioresources, Okayama University.

Uzu genotype determination

To detect the *uzu* genotype in F_2 individuals, the dCAPS marker was used as described by Saisho *et al.* (2004). Genomic DNA from F_2 individuals were used as a template for

mismatch-polymerase chain reaction (PCR). Restriction enzyme Hha I was used for detection of single nucleotide polymorphism (SNP) in *HvBR11*.

Tissue culture

Immature embryos (1–1.5 mm) were used as explants. In all, 42 immature embryos were cultured for callus induction of each parental variety and line. Of each F_2 individual, 23.4 F_3 immature embryos on average (Haruna Nijo \times Akashinriki) and 10.2 F_3 immature embryos on average (H602 \times Akashinriki) were cultured. Callus culture and shoot regeneration were performed as described in Rikiishi *et al.* (2003). Callus growth was evaluated using the mean number of calli per embryo transferred to the regeneration medium (MNC). Embryos with rudimentary calli were not included in the evaluation of callus growth. Shoot regeneration efficiency was evaluated according to the percentage of calli with shoots (PCS). The capabilities of callus growth and shoot regeneration in each F_2 individual were evaluated using the average values of F_3 immature embryos. In the experiments with isogenic lines, 14 immature embryos were cultured in a Petri dish and each line involved three dishes. Calli were induced under continuous darkness at 25°C for 4 weeks or 15°C for 6 weeks, and divided into about 5 mm diameter segments before they were transferred to the regeneration medium. Calli were incubated at 25°C for 4 weeks in 16-h photoperiods for shoot regeneration. The media for callus-induction and shoot-regeneration were identical to those used in the experiments with F_2 individuals.

Response to exogenous auxin

Immature embryos of isogenic lines were cultured on a modified MS medium (Rikiishi *et al.*, 2003) supplemented with different concentrations of 2,4-D (0, 0.01, 0.1 and 10 μ M). For each isogenic line, 14 immature embryos were cultured. Germination

percentages and fresh weight of calli were measured after culturing under continuous darkness at 25°C for 4 weeks or at 15°C for 6 weeks.

Results

Tissue culture traits in F_2 populations

The F_2 individuals from a cross between normal variety Haruna Nijo and *uzu* variety Akashinriki were examined for MNC and PCS, which respectively evaluate the capabilities for callus growth and shoot regeneration. Genotypes of 82 F_2 individuals were segregated into normal, hetero, and *uzu* genotypes; the numbers of F_2 individuals were 29, 40 and 13, respectively [$\chi^2(1:2:1) = 6.29$, $P = 0.04$]. The segregation ratio was distorted by lethality from disease attack in this F_2 population.

The respective MNCs of parental varieties, Akashinriki and Haruna Nijo, were 7.7 and 4.8. Akashinriki showed higher callus growth than Haruna Nijo. The respective MNCs of normal, hetero, and *uzu* genotypes were 6.5, 6.7 and 6.4 on average; these values were not significantly different (Table 1).

Although the calli showed no shoot regeneration in Akashinriki, the PCS of Haruna Nijo was 6.4% and the shoot regeneration capability was higher in Haruna Nijo. The respective PCS of normal and *uzu* genotypes were 8.4% and 1.7% on average, respectively; the normal genotype showed higher shoot regeneration capability than the *uzu* genotype (Table 1). Average PCS of the hetero genotypes (4.9%) were intermediate between those of normal and *uzu* genotypes.

In the F_2 population derived from a cross between normal lines, H602 (*H. v. subsp. spontaneum*) and Akashinriki, the numbers of normal, hetero, and *uzu* genotypes were 24, 42, and 26, respectively. These numbers were fitted to the expected ratio [$\chi^2(1:2:1) = 0.78$, $P = 0.68$]. In addition, MNC and PCS of H602 were respectively 4.4 and 12.6% on

Table 1. MNC and PCS of F₂ individuals derived from crosses between Haruna Nijo and Akashinriki, and between H602 and Akashinriki. Values are expressed as the mean ± SE.

Parent/Cross	Genotype	No. of F ₂ individuals	MNC	PCS
Haruna Nijo	+/+	-	4.8±0.2	6.4±1.0
H602	+/+	-	4.4±0.4	12.6±1.9
Akashinriki	<i>uzu/uzu</i>	-	7.7±0.4	0±0
Haruna Nijo × Akashinriki	+/+	29	6.5±0.4	8.4±1.1
	+/ <i>uzu</i>	40	6.7±0.2	4.9±0.5
	<i>uzu/uzu</i>	13	6.4±0.4	1.7±0.5
H602 × Akashinriki	+/+	24	4.5±0.3	12.1±1.8
	+/ <i>uzu</i>	42	4.7±0.2	8.5±1.2
	<i>uzu/uzu</i>	26	4.5±0.3	2.9±1.1

CD = Critical Difference, MNC = mean number of calli transferred to the regeneration medium; PCS = percentage of calli with shoots.

average (Table 1). Although MNC of H602 was lower than that of Akashinriki, H602 showed the highest PCS in parents. The MNC of F₂ individuals showed no significant difference in callus growth capability among the genotypes. The PCS of the normal genotype (12.1%) was higher than that of the *uzu* genotype (2.9%). The PCS of the hetero genotype was 8.5% on average. This value was intermediate between those of normal and *uzu* genotypes. In both F₂ populations, although callus growth was independent of the genotype, the shoot regeneration percentage of the normal genotype was higher than that of the *uzu* genotype.

Response of immature embryos to exogenous auxin

Immature embryos of two independent isogenic lines for the *uzu* gene were cultured on media supplemented with different concentrations of 2,4-D, and the effects of 2,4-D on embryo germination and callus formation observed. Under cultures at higher temperature (25°C), the germination percentage of normal isogenic line with Hoshimasari background (HN) decreased to 50% at 10 μM of 2,4-D, although immature embryos of HN germinated completely at less than 1 μM of 2,4-D (Figure 1A). Consequently, 2,4-D inhibited the germination of immature embryos at higher concentration in HN

under higher temperature conditions. The germination percentage of the *uzu* isogenic line with Hoshimasari background (HU) was lower than that of HN without 2,4-D at 25°C.

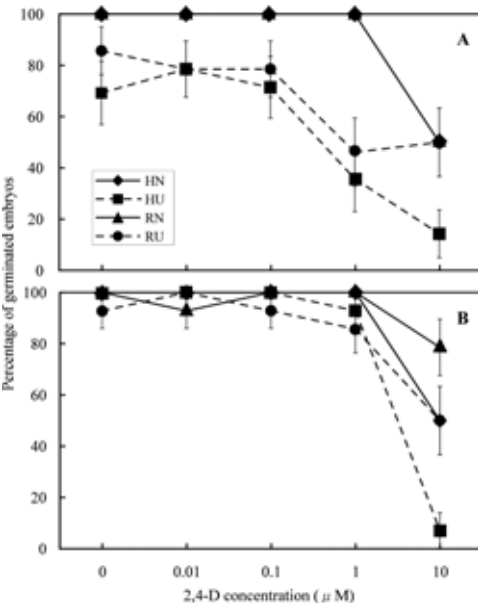


Figure 1. Percentage of germinated embryos on the medium with different concentrations of 2,4-D after 4 weeks (A: 25°C) and 6 weeks (B: 15°C) in isogenic lines. HN and HU respectively indicate the normal and *uzu* lines with Hoshimasari background. RN and RU indicate isogenic lines with the Ryohu background.

Germination percentages of HU showed no differences at less than 0.1 μM of 2,4-D, although 2,4-D inhibited the germination of immature embryos at 1 μM (35.7%) and 10 μM (14.3%). Although the germination percentage of normal isogenic line with Ryohu background (RN) only decreased at 10 μM of 2,4-D, the *uzu* isogenic line with Ryohu background (RU) showed lower germination percentages at more than 1 μM of 2,4-D at 25°C. At the lower temperature (15°C), all lines showed high germination percentages with no differences at less than 1 μM of 2,4-D and inhibition was observed on the germination in 10 μM concentration (Figure 1B). Normal and *uzu* lines showed no differences on the reduction of germination percentage for cultures at lower temperatures.

Regarding cultures at the higher temperature (25°C), immature embryos of

HN formed calli with more than 1 μM of 2,4-D. HU showed callus formation in 0.1 μM of 2,4-D; vigorous growth of calli was observed in 1 μM of 2,4-D (Figure 2A). The *uzu* line initiated callus formation at lower concentration of 2,4-D than the normal line. In the Ryohu isogenic lines, the fresh weights of calli were higher in the *uzu* line than those in normal line, except at 10 μM of 2,4-D. At lower temperatures (15°C), all lines showed less callus formation at less than 0.1 μM of 2,4-D. Marked callus growth was observed at greater than 1 μM of 2,4-D; the fresh weight increased depending on the 2,4-D concentration (Figure 2B). Similarly to the germination percentage, normal and *uzu* lines showed no significant differences in callus formation at lower temperatures.

Discussion

Shoot regeneration efficiency of isogenic lines

Shoot regeneration efficiency was examined in calli derived from immature embryos of isogenic lines under cultures at different temperatures (15°C and 25°C) during callus induction. The PCS of HN and HU were, respectively, 37.3% and 17.8% in calli induced at lower temperature (15°C) (Figure 3). Actually, HN showed higher percentage of shoot regeneration than HU. The PCS of HN and HU were 22.9% and 8.4% in calli induced at higher temperature (25°C), respectively; in addition, the shoot regeneration percentage was much higher in calli of normal lines than those of *uzu* lines. Lower shoot regeneration capability of *uzu* lines was shown to be independent of the culture temperature during callus induction. In the isogenic lines with Ryohu background, RN also showed a considerably higher percentage of shoot regeneration than RU at both temperature conditions.

In the F_2 populations from crosses between normal and *uzu* lines, the percentage of shoot regeneration in calli derived from

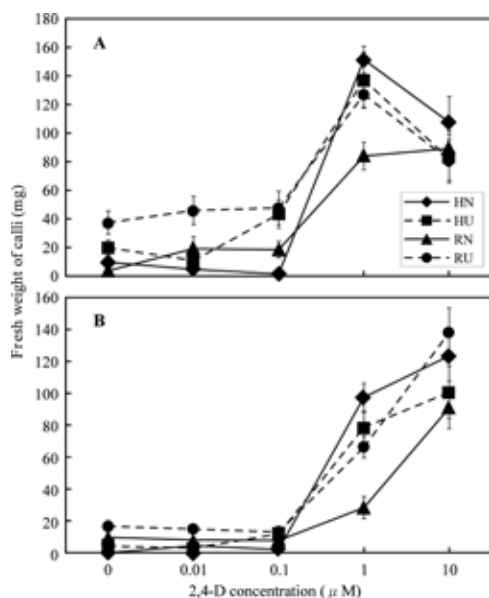


Figure 2. Fresh weight of calli on the medium with different concentrations of 2,4-D after 4 weeks (A: 25°C) and 6 weeks (B: 15°C) in isogenic lines. HN and HU respectively indicate normal and *uzu* line with Hoshimasari background. RN and RU indicate isogenic lines with the Ryohu background.

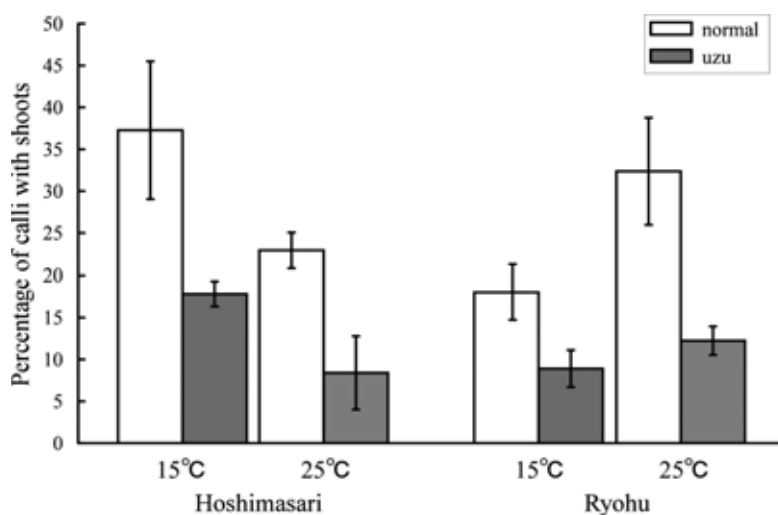


Figure 3. Percentage of calli with shoots (PCS) of the calli induced at different temperatures in isogenic lines.

immature embryos was lower in the *uzu* genotype. Shoot regeneration percentages of the hetero genotype were lower than that of normal genotype. In addition, the PCS of F_2 individuals was determined on the average of calli derived from F_3 immature embryos. Due to the hetero genotype segregates of normal and *uzu* genotypes in the F_3 generation, the values of the hetero genotype involve those of normal, hetero and *uzu* genotypes. Estimation of shoot regeneration efficiencies affected the lower values of the hetero genotype in F_2 individuals. In this study, the shoot regeneration capability was determined as dependent on the *uzu* genotype. Lower shoot regeneration percentages of *uzu* genotypes were also observed in isogenic lines, just as for F_2 populations. Ohkoshi *et al.* (1991) indicated that no *uzu* varieties showed a high frequency of shoot regeneration in a screening for regeneration capability in barley immature embryo culture. A QTL for shoot regeneration with a large effect was found on chromosome 3H in recombinant inbred lines derived from a cross between Azumamugi (*uzu* barley) and Kanto Nakate Gold (normal barley); the locus was identical

with or very close to the *uzu* locus (Mano and Komatsuda, 2002). These findings suggest that the *uzu* gene negatively regulates shoot regeneration in calli derived from barley immature embryos.

Isogenic *uzu* lines had different responses to exogenous auxin from normal lines on the inhibition of germination and callus initiation. *Uzu* lines showed higher sensitivity to exogenous auxin at higher temperature (25°C), but sensitivity to auxin was similar in normal and *uzu* lines at lower temperature (15°C). The auxin sensitivity of *uzu* lines is dependent on temperature during incubation. The *uzu* gene has pleiotropic effects on the elongation of the second internode from the top in a temperature-dependent manner (Saisho *et al.*, 2004). The elongation of the second internode in *uzu* lines is specifically inhibited under high temperature conditions. Sensitivity to exogenous auxin of immature embryos increased at higher temperature, similar to the elongation pattern of the internode. These results support that higher sensitivity to exogenous auxin in *uzu* lines is also caused by the pleiotropic effects of the *uzu* gene.

The *uzu* phenotype is attributed to the natural mutation on the brassinosteroid receptor kinase gene, *HvBR11* (Chono *et al.*, 2003; Saisho *et al.*, 2004). Signal transduction of brassinosteroid and auxin showed interdependency: both hormones act synergistically on the hypocotyl elongation and in promoting lateral root development (Bao *et al.*, 2004; Nemhauser *et al.*, 2004). Distorting signals of brassinosteroid affect auxin signals. Brassinosteroid might have positive regulation on the shoot regeneration of calli derived from barley immature embryos through synergistic interaction with auxin signals. At the same time, *uzu* lines accumulated excess cytokinin and showed lower levels of endogenous IAA in seedlings (Inouhe *et al.*, 1982; Honda *et al.*, 1995). Calli can regenerate into whole plants in response to auxin and cytokinin. Kraepiel *et al.* (1995) suggested that the auxin/cytokinin ratio is important for plant regeneration. The *uzu* gene might affect shoot regeneration as a result of the inadequate balance of endogenous levels.

Uzu lines showed higher sensitivity to auxin in terms of germination and callus initiation at higher temperature (25°C). Clouse *et al.* (1996) indicated that brassinosteroid-insensitive mutants, *br11*, of *Arabidopsis* enhanced the inhibition of root growth by exogenous auxin. Signals of brassinosteroid modified the sensitivity to auxin in barley. Although brassinosteroid and auxin synergistically act on shoot regeneration in barley immature embryo culture, the *uzu* gene that showed insensitivity to brassinosteroid enhanced sensitivity to auxin in immature embryos. Halliday (2004) suggested that the mode of interaction between brassinosteroid and auxin is different dependent on tissues. Effects of the *uzu* gene on auxin sensitivity and shoot regeneration showed different responses to temperature conditions. Therefore, the independent signaling pathways of brassinosteroid might control these phenotypes.

The *uzu* variety is an important brassinosteroid-insensitive mutant for investigating the molecular mechanism of signal transduction in monocots. At the same time, *uzu* has been known as a major semi-dwarf gene in classical barley genetics: several isogenic lines for the *uzu* gene were developed. Consequently, *uzu* mutants are inferred to be useful tools for research into brassinosteroid functions. This study found novel effects of the *uzu* gene on barley tissue culture traits in isogenic lines. The *uzu* gene suppresses shoot regeneration of calli derived from barley immature embryos. This result indicates that signal transduction of brassinosteroid takes part in the regulation system of shoot regeneration in a similar way to auxin and cytokinin. The relationship between the *uzu* gene and shoot regeneration should be investigated further for elucidating cross-talk among plant hormones, including brassinosteroid, auxin, and cytokinin.

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Session 10

Functional genomics

Functional genomics approaches to tackling abiotic stress tolerance

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Abstract

Abiotic stresses such as extreme temperatures, low water availability, high salt and mineral deficiencies or toxicities severely diminish productivity of cereal crops. These stresses are becoming increasingly important because of the declining availability of good quality water, land degradation and community pressures to move away from chemical intervention in agriculture. Barley is frequently grown in hostile and consequently very low yielding environments. Extensive genetic studies and surveys of landrace and wild germplasm have indicated extensive variation for abiotic stress tolerance, but this has been difficult to exploit due to the relatively poor background knowledge of the molecular basis for stress in this species. Interconnected signal transduction pathways that lead to multiple responses to abiotic stresses have been difficult to study using traditional approaches because of their complexity and the large number of genes and gene products involved in the various defensive and developmental responses of the plant. Functional genomics is now widely seen as providing tools for dissecting abiotic stress responses in barley, through which networks of stress perception, signal transduction and defensive responses can be examined from gene transcription, through protein complements of cells, to the metabolite profiles of stressed tissues.

Introduction

Abiotic stresses such as extreme temperatures, low water availability, high salt levels and mineral deficiency and toxicity are frequently encountered by plants in both natural and agricultural systems. In many cases, several classes of abiotic stress challenge plants in combination. For example, high temperatures and scarcity of water are commonly encountered in periods of drought, and can be exacerbated by mineral toxicities that constrain root growth.

Higher plants have evolved multiple, interconnected strategies that enable them to survive abiotic stress. However, these strategies are not well developed in most agricultural crops. Across a range of cropping systems around the world, abiotic stresses are estimated to reduce yields to less than half of those possible under ideal growing conditions (Boyer, 1982).

Traditional approaches to breeding crop plants with improved stress tolerance have thus far met with limited success – in part because of the difficulty of breeding for tolerance traits in traditional breeding programs. Desired traits can be crossed into crop species from wild relatives and for barley, extensive abiotic stress tolerance has been identified in screens of landraces and related wild species. It is estimated that only around 30% of the wild variation has been used in modern barley varieties. A similar

situation appears to apply to rice and wheat. There is considerable interest at present in using the emerging technologies of genomics as a means to identify key loci controlling stress tolerance and as a tool for screening for allelic variation in the wild and landrace gene pools. Delivery of the outcomes of genomics can be through conventional breeding although genetic transformation will offer a more rapid and efficient option.

Will genomics provide the tools for understanding stress tolerance in these species and lead to increased rates of genetic gain for tolerance?

Molecular biology of abiotic stress tolerance

Drought stress

Crop plants grown under drought conditions are exposed to a combination of stresses that are attributable to high temperatures, excessive irradiance, soil resistance to root penetration, and low water potential. Loss of leaf water causes some passive loss of turgor in guard cells. Absciscic acid production is also induced and leads to a further loss of stomatal turgor. The resulting stomatal closure causes a concomitant decrease in CO₂ availability in the leaves, and hence in assimilate availability to the plant. Although the photosynthetic machinery has a range of photoprotective mechanisms to dissipate excess light energy, the continued exposure of leaves to excessive excitation energy can lead to photoreduction of oxygen and the generation of highly toxic reactive oxygen species (ROS), such as superoxides and peroxides (Niyogi, 1999). These dangerous compounds cause chemical damage to DNA and proteins, and can therefore have serious or even lethal effects on cellular metabolism. Plants have evolved several strategies to deal with ROS, including the production of chemical antioxidants such as ascorbic acid, glutathione and α -tocopherol that directly remove potentially damaging electrons

from the ROS, and enzymic systems such as peroxidases and superoxide dismutases that scavenge the electrons enzymically (Alscher *et al.*, 1997; Foyer *et al.*, 1997; Holmberg *et al.*, 1998). The enzymes often use metals such as iron, zinc, copper or manganese as electron acceptors, so the metal ions must be available if enzymic detoxification of ROS is to proceed. In this way, oxidative stress may be linked to mineral deficiencies. In related responses, reactive aldehydes produced through perturbations in redox balances can be removed by the action of aldehyde dehydrogenases and aldose/aldehyde reductases (Kirch *et al.*, 2001), and superoxide production in mitochondria can be limited by the alternative oxidase (Purvis, 1997).

Another adaptive mechanism for protection against drought is the maintenance of turgor during periods of drought by adjusting the osmotic pressure of cells. There are two main routes whereby this can be achieved. Firstly, the cell can sequester ions into cellular compartments. Secondly, specialized osmolytes such as proline, glycine betaine, mannitol, fructans, trehalose, ononitol and ectoine can be synthesized to re-adjust cellular osmotic potential. These osmolytes are also active in scavenging ROS, especially if they are targeted to the chloroplast (Shen *et al.*, 1997). Other specialized organic molecules can be used to protect cellular membranes against physical damage, and proteins against unfolding. Dehydration induces the partitioning of amphiphilic molecules such as glycosylated flavonols and hydroquinones into membranes; these compounds increase membrane fluidity and depress phase transition temperatures (Hoekstra *et al.*, 2001).

Proline and sugars can coat protein molecules, exclude solute from their surfaces and thereby reduce the rate of unfolding (Hoekstra *et al.*, 2001). During extreme desiccation, tolerant plants synthesize large amounts of non-reducing disaccharides, such

as trehalose, which can substitute for water by satisfying hydrogen bonding requirements of polar amino acid residues at protein surfaces, and maintain the folded, active states of the proteins (Crowe *et al.*, 1997). Some late embryogenesis abundant (LEA) proteins and dehydrins might act in a similar fashion (Close, 1996; Dure *et al.*, 1989). Transgenic rice expressing wheat LEA genes PMA80 and PMA1959 showed enhanced drought and salt tolerance in glasshouse tests (Cheng *et al.*, 2002) and the barley gene *HVIA* led to enhanced yield in field grown transgenic wheat under drought stress (Bahieldin *et al.*, 2005). Small heat shock proteins (HSPs) may also contribute a general protective function in desiccation tolerance (Wehmeyer and Vierling, 2000), presumably through the maintenance of proteins in a folded state.

During extreme desiccation in the desert resurrection plants *Selaginella lepidophylla* and *Myrothamnus flabellifolius*, high trehalose concentrations replace essentially all the water in cells and convert the cytoplasm into a stable, intracellular glass (Hoekstra *et al.*, 2001; Leyman *et al.*, 2001). Trehalose is only found in high concentrations in a few species that are adapted to extreme drought stress, although lower levels have been detected in *Arabidopsis*, where it is believed to play a regulatory role in carbon metabolism (Leyman *et al.*, 2001) or stress tolerance (Zhu, 2001).

The genetics of drought tolerance has been based around the use of a range of screens. Table 1 shows some recent studies investigating the genetic control of abiotic stress tolerance in barley. A series of studies has identified around 70 QTL loci for different components of drought tolerance

including leaf relative water content, leaf osmotic potential, osmotic potential at full turgor, osmotic adjustment, water-soluble carbohydrate concentration, osmotic adjustment and carbon isotope discrimination (Diab *et al.*, 2004; Teulat *et al.*, 2001, 2002, 2003). These results imply an extremely complex control of drought tolerance.

In summary, adapted plants have evolved a range of strategies to enable them to survive the multiple elements of drought stress, and this provides opportunities to transfer to and optimize key protective strategies in barley, where drought tolerance mechanisms are not always well developed.

Cold and frost stress

When ice crystals form in plant tissues, severe osmotic and mechanical stresses result. Ice generally forms first in the extracellular space, water moves out of the cell along the osmotic gradient so created, and the osmotic stress is thereby imposed. Mechanical damage includes expansion-induced lysis, phase transitions and fracture lesions in membranes, and physical damage can be caused simply by the formation of large ice crystals. In addition, freezing can induce the production of ROS, which damage membrane components, and can cause protein denaturation (Thomashow, 1999).

The similarities between the consequences of drought and cold stress are clearly evident and, as one might expect, the plant responses during cold acclimation or in species adapted to cold climates are often similar to those observed in drought stress. Antioxidant defence mechanisms are invoked, together with the production of

Table 1. Stress tolerance loci mapped in barley.

Stress	Chromosome	Reference
Boron tolerance	2H, 3H, 4H, 6H	Jefferies <i>et al.</i> , 1999
Manganese efficiency	4HS	Pallotta <i>et al.</i> , 2000
Frost at anthesis	2H, 5H	Reinheimer <i>et al.</i> , 2004
Drought	over 70 QTL	Diab <i>et al.</i> , 2004; Teulat <i>et al.</i> , 2001, 2002, 2003
Seedling desiccation at germination	7 QTL	Zhang <i>et al.</i> , 2005

HSPs, the synthesis of high concentrations of intracellular proline and sugars, and the secretion of increased levels of sugars into the apoplastic space. The phase transition temperatures of membranes can be lowered through the action of fatty acid desaturases and the incorporation of amphiphilic proteins into membranes (Thomashow, 1999). Small, highly stable proteins characterized by the presence of multiple amphipathic α -helices, similar to the LEA proteins, are also synthesized at high levels during acclimation or in adapted species. These are likely to stabilize membranes, protect proteins from unfolding and, in some cases, inhibit the recrystallization of ice through surface patches of hydrophilic amino acid residues. There are additional suggestions that antifreeze proteins in winter rye (*Secale cereale*) might have evolved specialized roles in protection against cold stress from (1,3)-beta-glucanases, chitinases and thaumatin-like proteins of the pathogenesis-related protein families (Yu and Griffith, 2001).

The genetic basis for cold and frost tolerance has been extensively studied in wheat and barley. Not surprisingly, the timing and nature of exposure requires different mechanisms, and different genes appear to be involved. However, in all cases there is a close relationship between photoperiod control, vernalization and cold tolerance. For example, Reinheimer *et al.* (2004) identified two key loci involved in frost tolerance at anthesis in barley (Figure 1). One of these loci co-segregated with an earliness (*Eps*) and a vernalization (*Vrn H1*) gene on chromosome 5H. Although there may be a single locus on 5H involving earliness, vernalization and frost tolerance, the locus on 2H appears to be specific for tolerance to frost at anthesis and is a clear target for positional cloning.

Salt stress

Osmotic and ionic components of salt stress can be identified and plants subjected to salt stress invoke two response pathways (Zhu, 2001). The osmotic component has been

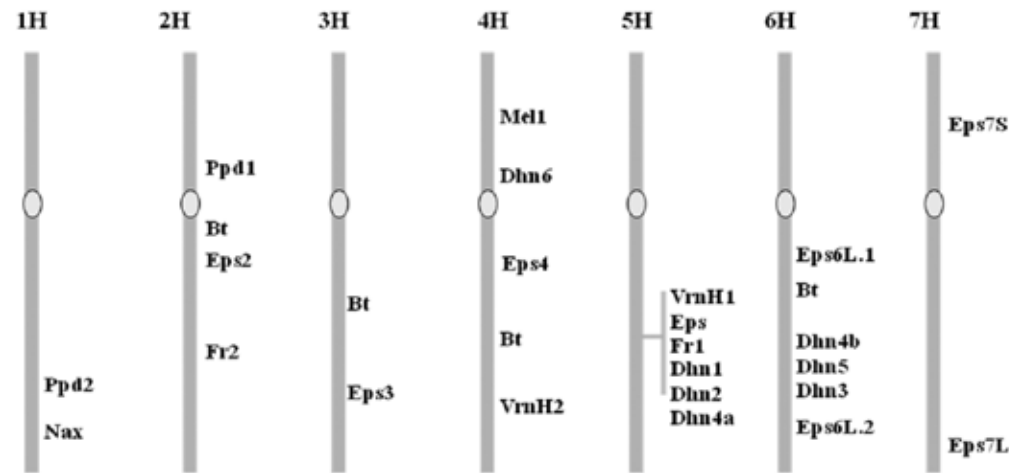


Figure 1. Location of several abiotic stress tolerance loci in barley.

KEY: Bt = boron tolerance (Jefferies *et al.*, 1999); Nax = Sodium exclusion (unpublished); Mel1 = manganese efficiency (Pallotta *et al.*, 2000); Eps = earliness per se (Laurie *et al.*, 1995); Ppd = photoperiod (Laurie *et al.*, 1995); Vrn = vernalization (Snape *et al.*, 2001); Fr = tolerance to frost at anthesis (Reinheimer *et al.*, 2004); Dhn = dehydrin (Campbell and Close, 1997). Although over 70 QTLs have been identified for tolerance to aspects of drought, these have not been included in the map.

discussed above under drought and cold stress. The ionic response is essentially an attempt by the plant to detoxify cells, because high concentrations of cellular salt interfere with membrane integrity, enzyme activity and nutrient acquisition. In the last-named case, ROS may be generated and elicit the usual plant reactions. Ionic homeostasis is normally maintained through the action of various ion transporters and non-selective ion channels. The sequestering of Na^+ ions from the cytoplasm into vacuoles appears to be a particularly important strategy in salt stress management by the plant, as suggested by the observation that many naturally salt-tolerant halophytes rely on this strategy. Furthermore, transgenic tomato plants over-expressing an *Arabidopsis* vacuolar $\text{Na}^+\text{-H}^+$ antiport protein can grow and produce fruit in the presence of 200 mM NaCl (Zhang and Blumwald, 2001). This raises the exciting possibility that salt tolerance could be engineered into important crop plants such as wheat and barley through the transfer and appropriate expression of a single gene.

Loci related to sodium exclusion have been identified in barley (Table 1 and Figure 1) and, again, these are good targets for positional cloning.

Mineral toxicity and deficiency

Micro- and macronutrients in soils are key determinants of plant growth and development. In most cropping systems, at least some of the soil nutrients will be present at sub-optimal concentrations or will be bound to soil in such a way as to limit their availability for plant uptake. For example, most cereals cropping soils in Australia are Zn-deficient. Others are deficient in Mn, Cu and, when other stresses are present, Fe. In other cases, toxic levels of minerals such as B and Al can limit yield potential. Boron is an essential micronutrient that is phytotoxic at high concentrations, and is of particular relevance in Australia. Thus, both mineral deficiency and toxicity represent important

abiotic stresses commonly encountered by crop species. The stresses can often, but usually in part only, be managed through fertilizer application. Mineral stresses can intensify other stresses, especially water stress, when plants are exposed simultaneously to both.

Multiple and varied functions for micronutrient minerals have been identified in plants. These functions include co-factors for many different enzymes (e.g. oxidoreductases), light harvesting and carbon assimilation processes in photosynthesis, and a role in pectin structure in cell walls. Plant responses to stress imposed by mineral deficiencies or toxicity are not always well defined, but are likely to involve, at least in part, changes in specific plasma membrane ion transporting pumps, carriers and channels (Barbier-Brygoo *et al.*, 2001). For example, toxic levels of Al slow root growth through processes that involve the inhibition of plasma membrane $\text{H}^+\text{-ATPases}$, the blockage of plasmodesmata, and oxidative damage (Sivaguru *et al.*, 2000; Ezaki *et al.*, 2001). Al impairs plant growth on nearly 1 billion of the world's 3 billion hectare of cropland, including about 35 million hectare in the USA.

The identification of adapted barley lines (Table 1), coupled with the molecular mapping of characteristics such as Mn efficiency, Zn efficiency, grain Fe and Zn density, and B toxicity tolerance (Graham *et al.*, 2001; Huang *et al.*, 2000; Jefferies *et al.*, 1999; Pallotta *et al.*, 2000), indicates that significant advances can be made in the management of abiotic stresses associated with mineral availability in cereal crops, through a functional genomics approach. The poor Mn efficiency of released cereal cultivars exemplifies the difficulty of achieving good adaptation by empirical breeding methods, even though a major locus is involved (Pallotta *et al.*, 2000). In the first instance, genes and gene systems controlling B tolerance, Mn efficiency, Zn efficiency and Cu efficiency will be identified from adapted

varieties, wild relatives and native grasses, with the expressed purpose of integrating them into elite varieties of barley.

Stress networks

A striking feature of plant adaptation to abiotic stresses is that multiple responses, involving complex networks that are interconnected at many levels, are activated when abiotic stresses are encountered. Plants increase their tolerance to 'environmental insults' through both physical and interactive molecular and cellular changes that are triggered by the stress. It is not always possible, therefore, to attribute a particular response to a specific abiotic stress. For instance, freezing temperatures, low water availability and high salt concentrations can all cause a lowering of cellular osmotic potential and thereby activate osmotic stress responses. These osmotic stress responses can operate via both an ABA-dependent and an ABA-independent signaling pathway (Ishitani *et al.*, 1997). In addition to the induction of osmotic response pathways, salt stress simultaneously activates a second, ionic response, through which ion transporters shuttle ions between various cellular compartments in attempts to maintain ionic homeostasis (Zhu, 2001). Drought and cold stresses will similarly activate additional, more specific response pathways. In another example, drought tolerance and tolerance to boron toxicity are closely related in cereals, where boron-toxic soils restrict root development. Thus, stresses induced by soil drying might incorporate stress attributable to water shortage, osmotic stress and nutrient deficiency.

Plant responses to abiotic stress are affected at several levels, and these eventually result in slowing or cessation of growth. Following perception of the stress conditions, signal transduction pathways are activated and lead to alterations in gene expression, as measured by the abundance of mRNA species, in the protein profiles of cells, in the activities of key enzymes, and in the relative flux through

and between different metabolic pathways. In turn, the alterations in cellular activity result in molecular and cellular changes that constitute the network of abiotic stress responses invoked to protect the plant against the unfavorable environmental conditions.

Signaling pathways

Perception of abiotic stress conditions by higher plants leads to the transduction of a signal that relays information within and between cells. An early event in many stress responses is the elevation of cytoplasmic Ca^{2+} levels, which leads to the activation of signal transduction pathways involving Ca^{2+} -dependent protein kinases and Ca^{2+} -regulated protein phosphatases (Knight and Knight, 2001). In the case of osmotic stress, perception is believed to occur through a plasma membrane histidine kinase (Urao *et al.*, 1999). Genes encoding many of the enzymes have been identified in *Arabidopsis* and their functions have been confirmed in loss-of-function mutants. The presence of C-repeat-binding factors and drought responsive elements (CBF/DREB) and ABA-responsive elements (ABF/ABRE) in the promoters of genes for both drought, salt and cold signal transduction probably represents a point of pathway convergence that enables responses of the two stimuli to be coordinated, although there are clearly specific Ca^{2+} -dependent pathways for different stresses (Knight and Knight, 2001). Oxidative stress pathways also appear to interconnect with the Ca^{2+} -mediated response pathways. Many, but not all, abiotic stress responses can be induced by ABA treatment.

The nature of general stress responses has opened the option of engineering tolerance through up-regulation of transcription factors and other regulatory components of the stress response pathway. The use of HSPs, chaperones and LEA proteins has been described above. Transcription control has also been used to enhance stress tolerance in plants (Vinocur and Altman, 2005). Expression of the *Arabidopsis*

DREB1A transcription factor under the control of a stress-inducible promoter (RD29A) led to significantly increased drought tolerance in greenhouse-grown wheat (Pellegrineschi *et al.*, 2004).

Functional genomics approaches

With the above background, how can we launch effective studies to transfer information from model species to the cereals, and what information do the cereals have to offer other crops in understanding and manipulating stress tolerance? On the surface barley appears to have little to offer. The genomes are large and poorly characterized relative to the model species. However, the level of abiotic tolerance shown by barley is greater than most other crop species and the diversity in the landrace and wild gene pool for barley tells us that still greater tolerance is achievable.

Role of model species

Many of the recent advances in genomics research have been founded around developments in model species. *Arabidopsis* has led the way, but, more recently, resources and information on rice and maize have gained in importance. The near-complete sequencing of the *Arabidopsis* and rice genomes has been crucial to the success of these models. For *Arabidopsis*, the mutant and tagged populations and extensive micro-array databases have provided powerful resources for gene discovery and functional analysis. The micro-array information covers a diverse series of stresses, including cold, salt, heat, water deficit and UV treatments. Through these and related studies in *Arabidopsis*, many genes involved in the control of abiotic stress tolerance have been elucidated, and, in some cases, the information has been transferable to crop plants. A clear example can be seen with the DREB transcription factors. These were first identified in

Arabidopsis as being involved in the control of drought and cold responses (Qin *et al.*, 2004). Subsequent experiments identified orthologous genes in the cereals maize (Dubouzet *et al.*, 2003), rice (Bahrman *et al.*, 2004) and wheat (unpublished), in addition to several other species. In the case of wheat, the DREB-1A transcription factor has been used to generate transgenic lines that show increased drought tolerance in preliminary trials (unpublished). A further example is the ornithine amino transferase gene that was found to confer salt tolerance when over-expressed in *Arabidopsis* (unpublished). This gene has been used to transform wheat and the transgenic lines have been evaluated in field trials in Australia.

Although these two examples indicate that genes discovered in *Arabidopsis* can be used directly or via orthologues to engineer abiotic stress tolerance in cereals, the key validation of this approach will only be revealed when the field trials, currently under way, have been completed. Many of the crucial stress responses in crop plants relate to their behavior as a crop, when growing as part of a plant community and when faced with multiple stresses. Therefore, the preliminary results with the *Arabidopsis* genes are still well distant from real application.

Mutant populations

There has been a long history of mutation breeding in barley, with several important varieties resulting from selection of mutant phenotypes (Ahloowalia and Maluszynski, 2001). However, systematic development of mutant populations as a genomics resource has only commenced recently. A large barley mutant population constructed for mutation screening based around detection of single-base mismatches was described recently (Caldwell *et al.*, 2004) and a similar populations have been developed by groups in Australia, Germany, Italy and USA.

Work has also been underway to develop transposon-tagged populations of barley

using the maize Ac/Ds system (Cooper *et al.*, 2004; Zhao *et al.*, 2006; Ayliffe *et al.*, 2007). This will also provide an important resource for functional analysis of cloned genes.

Mapping and map-based cloning

The importance of barley as a crop species and the long history of systematic breeding have resulted in extensive information of the genetic control of a wide range of traits, and also in the identification of broad diversity for stress and disease tolerance. The advent of molecular marker techniques and the links to breeding programs has resulted in a large expansion of mapping studies. Importantly, many of the studies have involved field evaluation of abiotic stress tolerance. The information shown in Table 1 and Figure 1 covers only recent studies, but indicates both the diversity and complexity of abiotic stress mapping programs.

Molecular markers have also proved important in surveys of cultivated, landrace and wild relatives of barley (Table 2). From these studies extensive variation has been identified and not only is this being used directly in several wheat and barley breeding programs, but also allows extension of the mapping work. The diversity found in abiotic stress tolerance will provide an important resource for validation of candidate genes and will also provide a mechanism for rapid delivery of genomics outcomes to pragmatic breeding programs. The concept here is to use candidate genes identified from genomics studies to assess stress tolerance mechanisms used in the landrace or wild lines that show elevated tolerance. Where variation is identified in candidates, either in the structural

gene or in expression levels, the locus can be transferred and tested in non-adapted or cultivated germplasm for assessment. In this way the variation is not only used to validate candidates for stress tolerance but also to provide a tool for allele discovery. Desirable alleles can be transferred by conventional breeding and selection.

Positional cloning pre-supposes the availability of high resolution linkage maps, and the development of these has involved large segregating populations. Association mapping may provide an alternative, but this is not sufficiently developed in wheat and barley for application at present (Powell and Langridge, 2004; Caldwell *et al.*, 2006). The availability of the rice genome sequence and the generally strong synteny between rice and barley means that rice can be used to generate potential markers close to the target locus. Since most known abiotic stress loci have been identified as QTLs, high resolution mapping is complicated by difficulties in clear definition of phenotypes. Breaking the trait down to well defined components, or eliminating confounding loci from the populations, may help in dealing with this difficulty.

The extensive mapping of particular traits, such as drought tolerance, in multiple cereals and using different populations may provide a means for locating common loci. For example, components of drought tolerance have been mapped in almost all major cereals by several groups around the world. However, the merging of populations between the cereals has not yet been attempted. Similarly, could one look for syntenous loci controlling salt tolerance in wheat, barley, rice and maize and use the comparison to generate a large ‘virtual’ mapping population? Computational tools for these types of analysis are in the process of development and this approach may be feasible in the not too distant future.

The key to map-based or positional cloning in barley is the ability to combine genetic data with information coming from a range of genomics-type approaches. This was

Table 2. Recent studies using molecular markers to survey germplasm for drought tolerance.

Germplasm pool	Reference
Drought in cultivated	Rizza <i>et al.</i> , 2004
Drought in cultivated, landraces and wild	Forster <i>et al.</i> , 2004
Drought in wild barley	Nevo <i>et al.</i> , 2005

recently demonstrated through the cloning of the 4H locus controlling boron tolerance in barley (Sutton *et al.*, 2007). Here, to identify the gene, high resolution mapping was combined with transcript profiling and sophisticated bioinformatics search using data from *Arabidopsis*, in situ localization of transcripts in barley tissues and expression screening of cDNAs in yeast.

Positional cloning work in barley is now well advanced for several boron tolerance QTLs (Sutton, pers. comm.) for loci determining salinity tolerance (Shavrukov, pers. comm.) and frost tolerance (Chen, pers. comm.).

Transcript profiling

Estimates of gene number in the cereals are very similar to other complex organisms; for example, in barley, gene number estimates range from around 30 000 to 50 000 (Zhang *et al.*, 2004).

There are now well over 1 million cereal EST sequences in the public databases, with wheat and barley dominating. The large number of ESTs and the diversity of cDNA libraries that have been used to generate the sequences, has made 'electronic Northern' a useful method for assessing gene expression and this provides a good first measure of transcript abundance.

Several micro-array and macro-array platforms have been generated for the cereals. Affymetrix arrays have been developed for barley (Close *et al.*, 2004). In a reference experiment using a series of well defined developmental stages, the 22K Barley 1 Gene Chip identified 18 481 transcripts showing expression above background (Druka *et al.*, 2006).

Transcript databases are now being accumulated for barley germplasm exposed to a range of stresses (unpublished data). In combination with the information now being generated from protein and metabolite profiling, these databases will provide a valuable resource to support a range of gene discovery projects.

Proteomics and metabolomics

There has only been one reported study of the application of proteomics to the study of abiotic stress tolerance in barley (Patterson *et al.*, 2005, 2007). This study focused on assessing protein differences between boron-tolerant and -intolerant germplasm. Importantly, the study revealed an association between boron and iron nutrition that now forms the basis for more detailed studies.

A proteomics study of drought- and salt-stressed rice plants found that around 3000 proteins could be detected in a single gel, and over 1000 could be quantified (Salekdeh *et al.*, 2002). This study found 42 proteins that changed in abundance or position in response to the stresses. Several of the key proteins were identified and are the subject of further studies.

The importance of metabolite changes during plant responses to abiotic stress suggests that detailed metabolite profiling may provide valuable insights into stress response mechanisms. Metabolomics is a relatively new area of research and the first report on the use of this technique in assessing the impact of abiotic stress in barley has only recently appeared (Roessner *et al.*, 2006). Again the published study reported on difference in metabolite in boron-tolerant versus -intolerant germplasm. Importantly, metabolite profiles are now being accumulated for barley germplasm grown under stressed and non-stressed conditions. These databases are showing which metabolites are involved in general stress responses, which are stress or tissue specific, or both, and which metabolites are associated with tolerant versus intolerant germplasm (Roessner, pers. comm.).

Metabolite profiling is also expected to be particularly useful in assessing the impact of transgene expression on plant performance. This was recently demonstrated in a study of metabolites in barley plants expressing a sodium pump derived from the moss *Physcomitrella patens* (Jacobs *et al.*, 2007).

Future directions

The outline above indicates the complexity of abiotic stress responses, but also shows that much of the information generated in model species is likely to have application in the cereals. Key processes of stress tolerance, including the signaling pathway components such as transcription factors, HSPs, chaperones and LEA proteins, ROS scavenging and synthesis of osmoprotectants, ion and water transporters, and a range of related processes appear to be common across plant species—indeed, many are also found in animals. These provide targets for research in the less well studied cereals. However, barley will also provide insights that have not been revealed in some of the model species that have formed the basis for much recent study.

The main advantage of barley is the strong genetic information on abiotic stress tolerance. While some of the QTL studies appear frighteningly complex, such as the drought mapping work (Diab *et al.*, 2004), there are clear target regions where QTLs are clustering. Barley 2H and 5H seem to play key roles. The possibility of combining QTL data from across the cereals is particularly attractive, and several projects have been initiated to attempt this.

The rapid advances in genotyping technology has meant that high quality maps can be rapidly generated. This has exposed the weaknesses of poor phenotyping in many studies, and the limitations in the sizes and structures of some mapping populations. Addressing these problems will be an important undertaking over the next few years. Improvements in techniques for evaluation of stress responses, including the automation of some measurements, will improve the throughput and, hopefully, the accuracy of phenotyping.

A second advantage offered by barley is the extensive collection of landrace and wild germplasm. It is estimated that less than 40% of the available variation has been captured. Many of the diversity screens of

these species have identified levels of stress tolerance well beyond that seen in current varieties. It is also important to remember that these species already show a far higher degree of tolerance to many abiotic stresses than other crop plants. This is the key reason why barley is so important for cropping in low yielding environments. The availability of this additional variation opens up the opportunity for exploring stress tolerance mechanisms that may not be fully developed in other species, and also provides scope for allele discovery and the use of allelic diversity for functional analysis.

The complexities of abiotic stress responses essentially preclude the precise experimental dissection of individual abiotic stresses, and suggest that further studies of individual stresses might not be the best approach. Genomics, proteomics and metabolomics, coupled with a strong bioinformatics capability, now enable a 'systems' approach to be taken in the study of plant responses to abiotic stresses. Thus, the entire system of networks of signaling pathways and key interconnecting processes that lead to the multiple defensive responses can be described in detail. New technologies, including improvements to positional cloning, mean that an understanding of plant responses to abiotic stresses and the basis for diversity may well be achievable for the first time. This understanding can be used for the manipulation of the responses, or their transfer to important cereal crop species through either conventional, marker assisted or transgenic approaches.

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Transcriptome study of storage protein genes of field-grown barley in response to inorganic nitrogen fertilizers

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Abstract

The storage proteins of barley, in terms of both amino acid profile and quantity, are traits strongly influenced by the amount of nitrogen applied. Given this, we performed a developmental expression analysis of the genes from barley grains grown under field conditions to further our understanding of the molecular and biochemical mechanisms underpinning nitrogen utilization.

A barley grain specific micro-array, where a comprehensive set of genes involved in nitrogen mobilization, storage protein synthesis and amino acid metabolism were assembled, was used to obtain a global but focused gene expression profile under different N regimes. Reviewing the expression of the storage protein homologues within the families revealed markedly different temporal profiles; for example, some alleles were expressed very early in development. Furthermore, the differential temporal expression of the homologues suggested that the genes of the different storage protein families were subject to different transcriptional regulation and responded differently to environmental stimuli. This finding may open the intriguing possibility of breeding selectively for specific alleles or homologues to confer an enhanced amino acid profile of the barley storage proteins.

Introduction

Substantial increases in agricultural production have been realized over many decades. Higher yields of crops plants have been achieved through the development of superior varieties, improved disease management, and application of inorganic nitrogen fertilizers (Evenson and Gollin, 2003). However, while production has increased, the changes in agricultural practices have led to undesired effects on the environment. As an example, in Denmark, groundwater reservoirs have been shown to accumulate potentially toxic nitrogenous compounds well above the permitted limits. Therefore, in recent years substantial efforts have been directed towards reducing the environmental load through restrictions on the use of nitrogen fertilizers. To meet the demands for low-nitrogen fertilizer regimes in Danish and European agriculture, the nitrogen utilization efficiency of crop plants has to be improved. Therefore, it is crucial to obtain more information about dry matter production, nutrient uptake and carbon partitioning, especially during grain filling as this will provide crucial information to empower future breeding initiatives targeted at grain yield and quality.

Barley (*Hordeum vulgare* L.) is the most widely grown cereal in Denmark and is used in compound feeds for monogastric animals

and for malt production. However, Danish agriculture has since the mid-1990s been subject to reduced levels of nitrogen fertilizer inputs, which have affected the barley grain quality (Madsen, 2006). Therefore, knowledge of the genetic basis of barley grain quality under low N input agricultural practice could facilitate reduced environmental load while maintaining productivity and quality.

Nitrogen is an important macronutrient, which can limit plant growth and performance (for reviews, see Crawford, 1995, and Stitt, 1999). Both nitrogen and carbon have been shown to act as signals regulating gene expression, which is in turn modulates cellular processes (reviewed in: Gutiérrez *et al.*, 2007; Palenchar *et al.*, 2004; Rolland *et al.*, 2001; Stitt *et al.*, 2002). A number of studies have addressed the emerging central role of nitrogen orchestrating the response of plants at the transcriptomic level. For example seedlings, shoots and roots of *Arabidopsis* (Wang *et al.*, 2000, 2003, 2004; Scheible *et al.*, 2004), the roots of tomato wheat (Lu *et al.*, 2005) and rice (Lian *et al.*, 2006) have been investigated with respect to the impact of nitrogen on plant development at the molecular level. However, knowledge on the biochemical and molecular mechanisms controlling N uptake, assimilation, and recycling is still fragmentary (Mickelson *et al.*, 2003).

Barley (*Hordeum vulgare* L.), besides its importance as a crop, is an established model plant for agronomic, genetic and physiological studies (Raun and Johnsson, 1998). The influence of N fertilizer levels and timing of application on grain yield and grain protein content has been investigated (Penny *et al.*, 1986; Bulman and Smith, 1993; Hirel *et al.*, 2005), and recently reviewed by Hirel *et al.* (2007). In order to assess the changes in grain quality in relation to reduced N input, we have chosen to study the developmental expression profile of genes from field-grown barley grains under different nitrogen regimes. To facilitate this, we constructed a

cDNA micro-array with 1035 genes primarily derived from the Clemson University cDNA library generated from developing barley grains. A custom-made cDNA micro-array was implemented to elucidate key candidate genes essential for grain filling and regulation of amino acid biosynthesis and storage protein accumulation, as this tool allows the analysis of complex interconnecting metabolic pathways. The output of the study will provide a powerful resource for future breeding initiatives directed at improving the nutritional quality of barley under low nitrogen inputs.

Materials and methods

Plant material

Spring barley (*Hordeum vulgare* L.) cv. Barke was grown in three field plots of 19.8 m² (12 m × 1.65 m) during the summer of 2005, in Flakkebjerg, Denmark. Immediately after sowing, the plots were fertilized with NS24-7 (DLA Agro), which contains 12% ammonium, 12% nitrate and 7% sulphur, at a rate of 50 kg/ha N (low N dosage, LN), 120 kg/ha N (normal N dosage, NN) and 150 kg/ha N (high N dosage, HN). After one week the plots were fertilized again with PK 0-4-21 (DLA Agro) at a rate of 25 kg/ha P and 60 kg/ha K. The plots were sprayed one month after sowing with a broad-spectrum herbicide mixture containing Express ST (Tribenuron-methyl 50%; E.L. du Pont de Nemours & Co), Oxitril CM (loxynil 17.32%; Bayer Crop Science) and Starane 180s (Fluroxypyr 180 g/L; Dow Agrosiences) herbicides. Individual spikes were tagged and harvested in the morning (08:00 to 09:00) at 10, 15, 18 and 25 days after pollination (DAP) (see individual grains at different time-points and treatments in Figure 1). Samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis. The mature grains of each nitrogen-level plot were also harvested and used for the yield calculations (16% water content of dry weight) at 51 DAP.

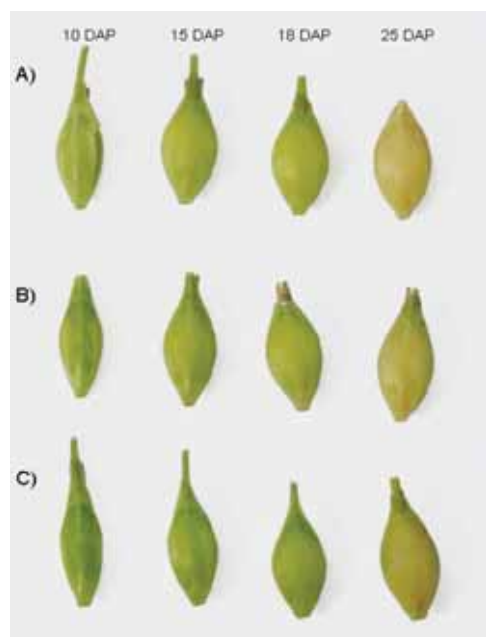


Figure 1. A phenotypic presentation of harvested grains under different nitrogen regimes during grain development. Grains grown at (A) 50 kg/ha N; (B) 120 kg/ha N; and (C) 150 kg/ha N, at 10 days after pollination (DAP), 15 DAP, 18 DAP and 25 DAP.

Near-infrared spectrophotometer

The mature grains were also harvested and analyzed for water (%), starch (%) and protein content (%) in our laboratory using a near-infrared spectroscopy analyzer (Foss Tecator, Infratec 1241, Grain Analyzer v.3.40). The near-infrared spectroscopy (NIRS) analyzer was calibrated and linked to the Danish Near-Infrared Transmission (NIT) network (Buchmann *et al.*, 2001).

Pre-processing of micro-array data

The pre-processing was carried out according to Hansen *et al.* (2007), and annotations of the probes can be found at URL: <http://www.genome.clemson.edu/projects/barley/>

Micro-array design, data pre-processing, and identification of differential expression

The array contained 1035 genes. Each cDNA probe was spotted in triplicate in three sub-grids across the slide to control for potential sources of variation in hybridization across the area of the slide (technical replicates). The micro-array experiments were performed using samples collected from field-grown barley subject to three different nitrogen regimes (50, 120, and 150 kg/ha) at four time points (15, 18 20 and 25 DAP). An interwoven loop experimental design was chosen (Altman and Hua, 2006) in combination with three biological replicates per treatment, resulting in 18 hybridizations, while each hybridization contained two different factors (time and treatment) for each slide (Figure 2). This strengthens the statistical test of the two-way ANOVA identifying systematic error. Moreover, the ANOVA is based on well-developed statistical theories, which ensures that changes in gene expression as small as 1.2-fold can be detected as highly significant (Jin *et al.*, 2001). The hybridization protocol was performed according to Eisen and Brown (1999) with modifications according to Hansen *et al.* (2007). Data acquisition and analysis was performed on an arrayWoRx micro-array scanner (BioChipReader, Applied Precision, USA) using the arrayWoRx 2.0 software Suite.

Clustering using partitioning around medoids (PAM)

Co-regulated genes were identified by generating a distance matrix using a Pearson correlation between the expression values with the highest confidence limits. The statistical package used was R (Becker *et al.*, 1988; <http://www.r-project.org/>). The distance matrix was subsequently clustered by the Partitioning Around Medoids method (PAM) (Kaufman and Rousseeuw, 1990) using the cluster package in R. The PAM algorithm is a robust version of k-means, and

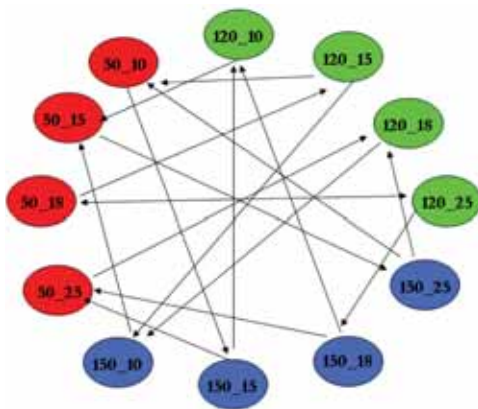


Figure 2. An interwoven loop experimental design was used to assess the impact of applied nitrogen regimes on gene expression, during barley grain development, (Altman and Hua, 2006). The colored circles represent the nitrogen regime: Red=50, Green=120, Blue=150 kg N/ha. The first number in the circle corresponds to the nitrogen treatment and the second to the days after pollination (DAP). The RNA was prepared from three independent biological samples, and 36 independent RNA samples were hybridized in total. Within the interwoven loop figure the arrows indicate the specific pairwise combinations used for each hybridization. The RNA sample at the head of the arrow was labeled with Cy5, while the RNA sample at the tail of the arrow was labeled with Cy3.

it searches for a specified number of medoids (representatives), k , around which clusters are constructed. The clusters were generated by minimizing the sum of the dissimilarities of all observations and assigning them to their closest medoid.

Real-time RT-PCR expression analysis

Total RNA was isolated from a pool of three individual grains from the middle of three independent barley spikes using FastRNA Pro Green Kit (Bio101, Systems, France) and re-suspended in 50 μ l DEPC-treated water according to manufacturer's manual. The diluted RNA was quantified using a GeneQuant II DNA/RNA calculator (Pharmacia Biotech, Piscataway NJ, USA). First strand cDNA synthesis and real time RT-PCR were carried out as described by Hansen *et al.* (2007). Primer Express software (Applied Biosystems, Foster City, CA, USA) was used to design the primers. Primers for the hordein family were designed towards homologous regions identified using sequence alignments generated from accessions recovered from the genbank database.

To investigate the specificity of each primer set (Table 1) a dissociation curve analysis was implemented. Expression level units of each gene of interest were calculated relatively to a calibrator and normalized to the housekeeping gene *actin* in samples (Livak and Schmittgen, 2001). For the individual candidate genes, the expression at 18 DAP and 25 DAP was calibrated to the expression at 10 DAP (calibrator). The Ct value was obtained for each specific gene in the samples, followed by a quality check of linear regression (R^2) and relative expression calculation for each gene using parameters of the software REST© according to Pfaffl *et al.* (2002).

Table 1. Stress tolerance loci mapped in barley.

Gene product	Forward primer (5' \rightarrow 3')	Reverse primer (5' \rightarrow 3')
C-hordein	ATAATTCCTCCAGCAACCTCA	GATGGTGCACATCATTTATTCA
B-hordein	GCAAGGTATTCTCCAGCAGC	TAAGTTGTGGCATTTCGCACG
D-hordein	GACAGTCCACCGAGATGGCTA	CGATTACCGCCACAAAGAGG
γ -hordein	TCCACCTAACTGCTCCACC	CGGAGACTACCAACAATATGGT
Glutelin	ACCATGGTTCGAAATGTGTTCAAT	TTAATAGTTGCCCCGGGGCTA

Results and discussion

Storage products of the harvested barley grain grown under different nitrogen regimes

Yield was 41.6 kg/ha for the low-nitrogen treatment, 55.5 kg/ha for the normal nitrogen and 56.5 kg/ha for the high-nitrogen applications. We observed a relatively higher proportion of developed tillers at normal and high nitrogen levels compared with low nitrogen level. In the LN treatment, the yield decrease was 25% when the fertilizer level was decreased 42%, while in the HN treatment 25% fertilizer increase corresponded to 2% yield increase only.

The harvested seeds from the three different nitrogen treatments were analyzed for protein and starch contents. NN grains contained 10.4% protein compared with LN and HN, containing 8.3% and 11.1% protein, respectively. The starch content was highest in LN grains (64.8%) whereas NN and HN grains had 61.6% and 60.2%, respectively.

Phenotypic profiling

LN grains matured earlier (18 DAP) compared to NN and HN grains as measured by the color of the spikes and the hardness of the endosperm according to Zadoks code 87 (Figure 1, Zadoks *et al.*, 1974). The flag leaves of LN barley showed increased rate of senescence at 15 DAP compared to the green and broad flag leaves of NN and HN barley. The senescence started in the NN flag leaf at 18 DAP while HN stayed green until 22 DAP (data not shown).

Gene expression profiles affected by time, nitrogen and interaction

The two-way ANOVA highlighted genes affected by time, nitrogen treatment and the interaction of the two factors, namely time \times nitrogen (T \times N). The 145 most significantly affected by time were clustered

into three clusters, which correlated with an early, middle and late developmental stage (Figure 3). The value of $k = 3$ was identified by manual inspection as the optimal number of clusters and it divided the data into three categories, one having the highest expression at day 10, another at 15–18 days, while the last cluster showed the highest expression at 25 days (Figure 3).

The development transcriptional profile of storage proteins

It has long been recognized that there must be intricate and complex cross talk between the primary metabolism and storage product pathways. It is widely suggested that storage product accumulation occurs in the later phases of grain or seed development in preparation for a period of dormancy before germinating, and this would be fuelled by the utilization of the storage products (for review see Shewry and Halford, 2002). However in our studies we observed storage product gene expression not only in the latter period of development but also the early stages. This is in line with a recent study, which reported early expression of hordeins in microspore-derived embryogenic development (Pulido *et al.*, 2006). Pulido and colleagues (2006) suggested that these proteins might be synthesized and consumed according to the requirements of the embryogenic microspores and early embryos.

Barley storage protein is made up of glutelin, albumins, globulin and hordeins that are encoded by multi-gene families. To assess the expression of these families we have included homologues for barley storage proteins in the micro-array and observed considerable variation in the temporal gene expression profile of the members of the family. For example, of the five B hordeins genes represented on the array, the expression profile of two falls within cluster 1, a further two exhibit an expression characteristic of cluster 2, while one gene appears to

be expressed late in development and corresponds to cluster 3. Similar differences were observed for the expression patterns of the five B-hordein genes, with two present in cluster 1 and three belonging to cluster 2, while the two D hordein genes were represented in cluster 2. Among the seven

significantly expressed globulin genes, two belonged to cluster 2 and five to cluster 3. The expression patterns of genes coding for a hordein C homologue, the albumins and the lysine-rich glutelin genes were all associated with cluster 3, where the respective mRNA levels increased late in development.

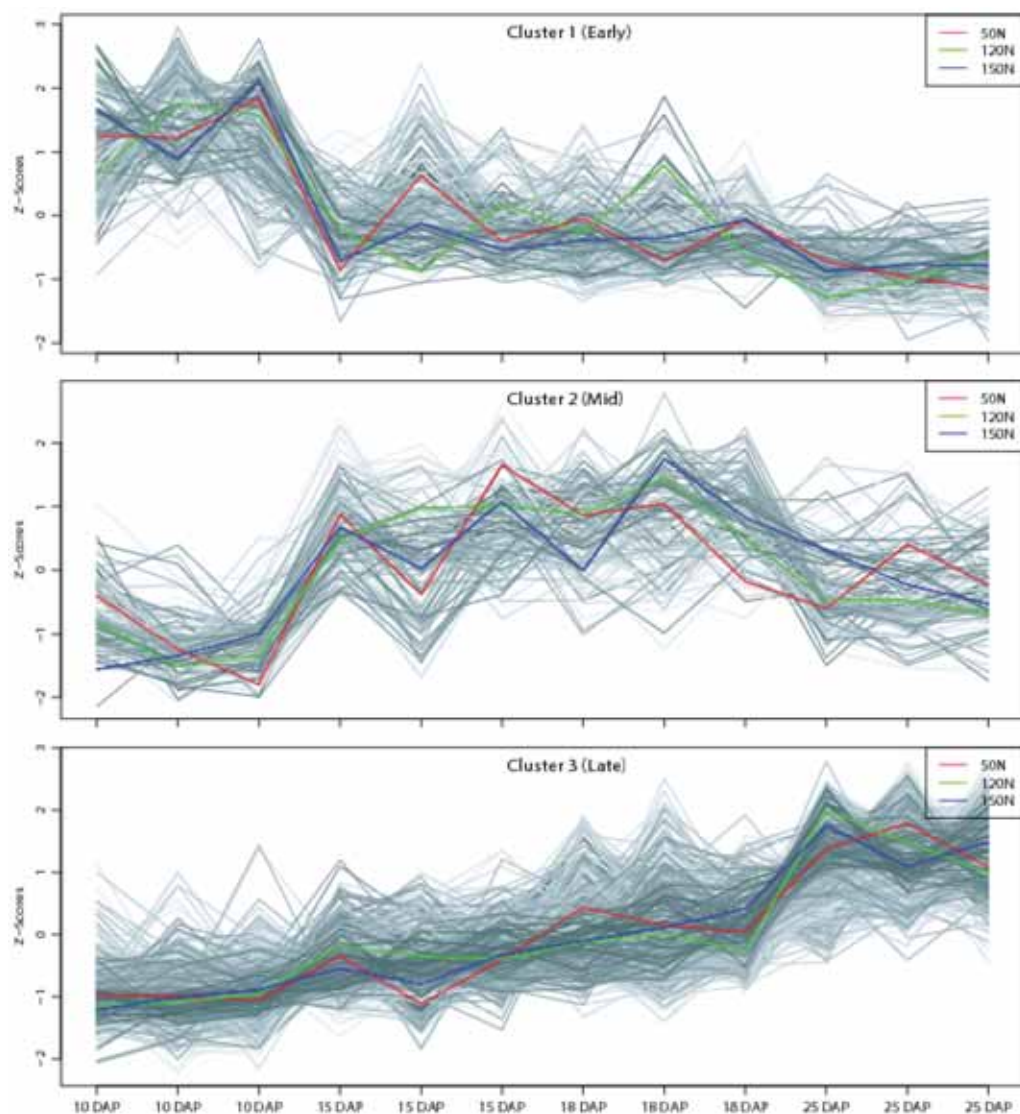


Figure 3. Cluster analysis. The gene expression profile of the 146 most significantly regulated genes representing early, middle and late phases of the field-grown barley grain. The relative expression is depicted by the Z-score (transformed standard deviation) separated by the sampling time points. The colored lines indicate the average gene expression during development.

Reviewing the temporal expression profiles of the homologues within the storage protein families, it is apparent and very striking that storage protein genes are expressed very early in development. Similar evidence has been reported by Rahman *et al.* (1984) using *in vitro* biochemical techniques, which demonstrated protein synthesis, thus the gene expression we observed seems to coincide with protein production.

Given this, the interplay as adjudged by the differential temporal expression of the homologues, suggests that the genes of each family of proteins are subject to different transcriptional regulation. This implies that the regulatory units of the genes respond to different developmental or environmental stimuli, opening the intriguing possibility of breeding selectively for specific alleles or homologues to confer an enhanced amino acid profile of the barley storage proteins.

Validation by RT-PCR

The gene expression profiles obtained from the micro-array experiments were validated by real-time RT-PCR for a selection of genes (Figure 4). We used primers homologous to all members of the appropriate gene families present on the micro-array so the RT-PCR results represented an average expression level among the family members.

This was confirmed when we created, from the micro-array absolute expression values, an average profile for the different hordein homologues (Figure 5 shows examples of gamma-hordeins and glutelins). The profile of the C-hordein (data not shown) and the three glutelin homologues (Figure 5) indicated that transcription of the respective genes continued to increase up to 25 DAP, which correlated with the results of the cDNA micro-array and confirmed that the genes belong to cluster 3. Similarly, the

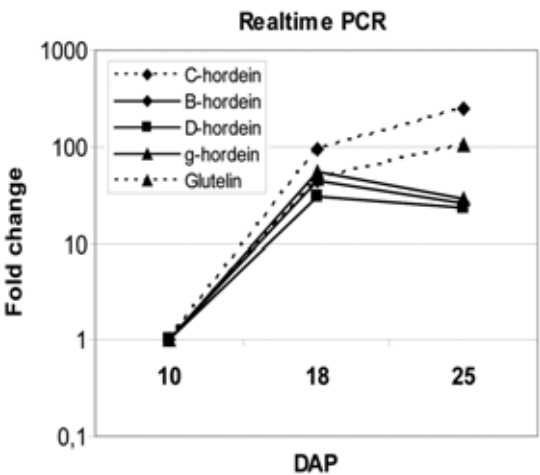


Figure 4. Genes of interest validated by real-time RT-PCR. Values of fold changes calculated relative to 10 DAP are presented on a logarithmic scale.

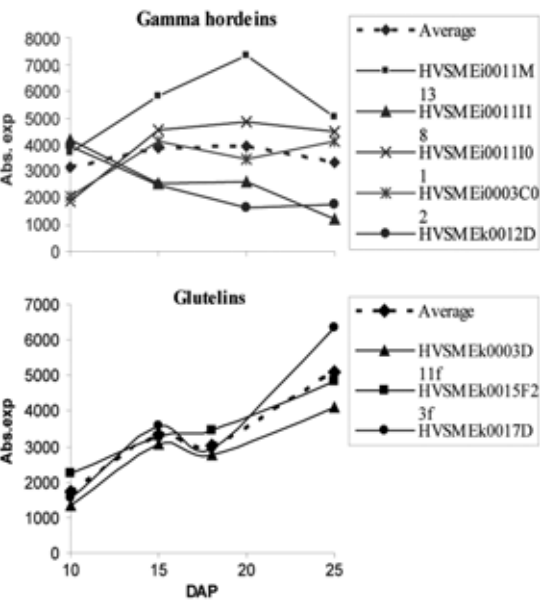


Figure 5. The absolute expression profiles of the gamma-hordein and glutelin genes mentioned in the RT-PCR experiments: absolute expression values and the created average profile for the different homologues of the genes present on the micro-array chips.

RT-PCR results matched the average profile pattern for the B-, D- (data not shown) and gamma-hordeins (Figure 5).

Sulphur-rich and sulphur-poor storage proteins were highly affected by N

It has been widely demonstrated that N nutrition increases the total protein fraction of the grain, which correlates with the increase in the prolamin content, e.g. gliadins, glutelins in wheat (Wieser and Seilmeier, 1998; Triboui *et al.*, 2000, 2003) and hordeins in barley (Shewry *et al.*, 2001). However, the metabolic or structural storage protein albumins, globulins and glutelin are not significantly affected by increasing of the level N (Pechanek *et al.*, 1997; Wieser and Seilmeier, 1998).

In our experiments, genes encoding both metabolic and structural storage proteins were affected by nitrogen or $T \times N$ interaction, or both. Four genes coding for metabolic or structural storage proteins, two glutelins and two globulins, were up-regulated in the presence of elevated levels of N and the developmental expression patterns was apportioned to Cluster 3, while genes coding for three γ -hordeins exhibited developmental profiles belonging to Cluster 2. Interestingly, different homologues of the γ -hordein family were affected by $T \times N$, as two $\gamma 1$ -hordeins and a single $\gamma 3$ -hordein were affected out of four present on the array. Figure 6 shows the example one of the γ -hordein genes affected by the different N regimes. Overall, the expression of the storage protein genes was higher in NN and HN grains than in LN grains.

Conclusions

The data we present provides transcriptomic analysis of cereal grain development of field-grown material. It is based on a set of genes chosen from cDNA libraries of developing barley seeds. Although the available micro-array data set deposited in the BarleyBase (<http://www.plexdb.org>) is very comprehensive, it is limited to

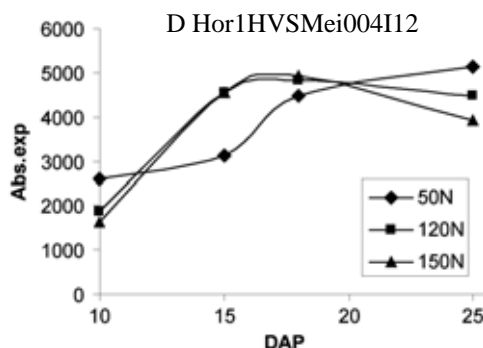


Figure 6. The absolute expression profiles of the γ -hordein affected by the different N regimes: absolute expression values of the homologue (HVSMEi004I101).

greenhouse material, with 20 DAP being the oldest developmental stage reported. Our study identified a large number of genes responding to nitrogen and $T \times N$ interaction from a range of different biological processes: genes of storage protein accumulation; genes involved in cell wall metabolism and starch biosynthesis; and cell cycle coding genes. Abiotic and biotic stress-related genes were affected as well. We described and discussed in this paper the temporal expression profiles of a range of genes involved in storage protein accumulation.

We conclude that the grain-specific micro-array coupled pathway-specific analysis is a fast, reliable and cost-effective tool for monitoring temporal changes in the transcriptome of the gene families. The most intriguing aspect of this study was the observed differences in the expression patterns of the alleles or homologues of the different gene families coding genes influenced by time, nitrogen and $T \times N$ interaction. Therefore, micro-array analysis could provide the knowledge required for rational design of breeding selectively for specific alleles or homologues to achieve better amino acid composition and increase the utility of barley grain as food and feed.

Acknowledgement

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The barley plastome mutator genotype

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Abstract

The barley chloroplast mutator genotype is the only one so far known to induce a wide spectrum of cytoplasmically inherited mutants in monocots. Initially it was described as inducing a high rate of various chlorophyll-deficient types. Later it was also possible to isolate several families displaying other characteristics, such as dwarfism, slow growth and modified responses to environmental conditions and herbicides. Major plastid-DNA changes were not detected in mutants derived from this mutator. Interestingly, the mutants so far molecularly characterized correspond to single nucleotide mutations. Three of them were transitions in the *infA* gene, affecting different conserved regions of the corresponding protein that is homologous to the bacterial translation initiation factor IF1. This allowed us to describe an *infA* gene mutant in higher plants for the first time. Besides, in a mutant very sensitive to high temperature, one transition and one base insertion were observed in the *ycf3* locus that encodes for a PSI-assembly chaperone. A fifth transition, conferring tolerance to herbicides acting on PSII (atrazine, diuron and bromacil), was detected in the *psbA* gene. The wide spectrum of mutants and the subtle DNA changes induced by this mutator genotype show it to be a unique source of variability that could be exceptionally valuable to explore the potential functionality of the otherwise highly conserved plastid genome.

Introduction

The first report about this barley mutator genotype was presented at the 6th IBGS, held in Helsingborg (Prina, 1991). It corresponds to a single nuclear mutant gene that, acting as recessive, induces several different chlorophyll deficient types, it being the first time that a mutator inducing such a wide spectrum of cytoplasmically inherited mutants was observed in a monocot species (Prina, 1991, 1992). It is also interesting that several viable mutant types, easy to multiply and stabilize by backcrossing with normal genotypes, were observed (Prina, 1996). Major plastid-DNA changes were not detected in those mutants (Colombo *et al.*, 2006). However, sequencing of some candidate genes that were proposed based on the peculiar characteristics of some of those phenotypes allowed us to identify several point mutations. A compilation of published and unpublished results on this field is presented here.

Materials and methods

Cloning and sequencing of *infA* gene were performed as described by Landau *et al.* (2007). Primers for *ycf3* (2 kb) PCR amplification were:

P1: 5'-TTATTCAAATTCAAAGCGCTTCGTA-3' and
P6: 5'-ATGCCTAGATCCCGTGTAATGGAA-3'.

Amplification was performed in a final volume of 50 µL using 10 µL 10× buffer, 1

μL 50 mM MgSO₄, 1.6 μL 10 μM primer P1, 1.6 μL 10 μM primer P6, 1.6 μL 10 mM each nucleotide dNTPs mix, 1 μL 5 U/μL Taq Pfx (Invitrogen), and 20 ng DNA. Internal primers for PCR sequencing of *ycf3* were designed:

P0: 5'-CTAAGTTTCAAACCCCTAATTTTAT-3'
 P2: 5'-TTTTTTTAGTTGTATCGACCCAGTCGC-3'
 P3: 5'-GCCCGGAAATAATATTCCAAAGCCT-3'
 P4: 5'-CACCAATGAATCTATTAATGCTA-3'

PCR amplification of the *psbA* gene was done as described by Rios *et al.* (2003).

Results

An overview of the results obtained by sequencing different candidate genes in several mutator-induced mutants and a wild type control are presented. We successfully identified several different point mutations in the plastome.

Three of the point mutations were identified in the plastid gene *infA*, which was postulated as responsible for the CL2 phenotype, a curious chlorophyll-deficient phenotype that shows a delayed greening mainly localized on the top of the first leaf blade (Prina, 1996; Prina *et al.*, 2003). This gene encodes for a protein that is homologous to the bacterial translation initiation factor IF1, whose functions in higher plants are, so far, unknown (*cf* Landau *et al.*, 2007). The three mutants mentioned above were independently originated in different pools of mutator plants. Two were T→C transitions, affecting nucleotide 157 (CL2, GenBank AY488513) and nucleotide 97 (CL2-like 1, GenBank AY743911), and the third one was an A→G transition at position 185 (Landau and Prina, pers. comm.). The three mutations found in the *infA* gene corresponded to highly conserved residues of the IF1 protein (Landau *et al.*, 2007; Landau and Prina, pers. comm.).

Another successful postulation was made in relation to CL3 (Prina, 1996), a homogeneous light green mutant that is very sensitive to high temperature

(Martinez *et al.*, 2005). Several results coming from experiments with different growing temperatures and light intensities (Landau, pers. comm.) suggested that one of the chaperones involved in photosystem I (PSI) assembly could be responsible for that phenotype. They are encoded by the plastid loci *ycf3* and *ycf4*. Interestingly, one transition (T→C) at position 528 and one base insertion (T) at position 150 were observed, corresponding to one of the two introns of the *ycf3* locus, i.e. intron 1, that resulted in a defective splicing (Landau, pers. comm.).

In an effort to test the possibilities of generating changes in traits other than chlorophyll deficiencies, we chose atrazine-tolerance as a model for selection experiments. Two atrazine-tolerant families were obtained after two generations of selection by applying atrazine solutions on a pool of mutator plants (Rios *et al.*, 2003). Sequence analysis of the *psbA* gene showed a transition (A→G) at the 790 position, corresponding to an amino acidic change of serine-to-glycine in the D1 protein of PSII (Rios *et al.*, 2003). This result agrees with identical molecular changes conferring strong atrazine-tolerance previously observed in several other species (*cf* Rios *et al.*, 2003). Both tolerant families were isolated from the same pool of plants carrying the mutator genotype and therefore it is not possible to judge the independence of the mutational events from which they originated.

Discussion and conclusions

On the basis of the wide spectrum of induced mutants and the observation of several viable and normal-vigor mutant phenotypes, the barley mutator genotype here presented has been proposed as a potentially important source of genetic variability (Prina, 1991, 1992, 1996; Prina *et al.*, 1996, 2000). It was postulated that the nuclear genotype responsible for such recurrent occurrence

of plastome mutants is probably related to failures in a DNA-repair mechanism (Prina, 1992). Judging from the results of the molecular analyses so far obtained (Rios *et al.*, 2003; Colombo *et al.*, 2006; Landau *et al.*, 2007), this mutator genotype is inducing subtle DNA changes to the plastid genome. To our knowledge, such a mutator constitutes—at least for monocot species—a unique source of variability for the otherwise highly conserved plastid genome. Targeting Induced Local Lesions in Genomes (TILLING), which consists of applying a powerful screening methodology in populations carrying high frequencies of point mutations induced by chemical mutagenesis (McCallum *et al.*, 2000; Slade and Knauf, 2005; Till *et al.*, 2007), recently became a key strategy for making nuclear gene allelic series available, and elucidating nuclear gene functions in higher plants. However, plastome mutagenesis is so far a subject not so easy to deal with, in this context, and the barley mutator genotype here presented appears to be suitable material for use as a source of plastome point mutations to meet the challenge of developing a TILLING strategy targeting the plastid genome.

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Session 11

Barley development

Ethiopian barley research and development

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Abstract

The government of the Federal Democratic Republic of Ethiopia has launched Agricultural Development Led Industrialization (ADLI) as a main strategy to bring economic growth. This is primarily because agriculture accounts for about 50% of GDP, 85% of employment, 90% of foreign currency earning and 70% of the supply of raw materials for heavy and medium-scale industries. Moreover, the Plan for Accelerated and Sustainable Development to End Poverty (PASDEP) program has been launched to ensure food security and reduce poverty by half by 2015, and is in its second phase of implementation. The focus is to transform small-scale agriculture to commercial farming systems. Attempts were made to align the national agricultural research efforts to the governmental development strategies and programs. In this regard, indigenous crops will receive high emphasis for their food security role. These include cereals (tef, maize, sorghum, wheat, barley), roots and tubers (enset, Irish potato, sweet potato), and vegetables (garlic, onions and tomato). Barley can be considered as a dependable food security crop for the majority of highland farmers. In the Ethiopian context, research on barley is broadly classified into food and malting barley to meet specific requirements. In the past ten years, encouraging research results have been registered in variety development and promotion; optimum agronomic practices have been recommended; and information on genetic variability further enriched. The

food barley variety development efforts emphasized evaluation and utilization of landraces, while malting barley research focused primarily on evaluation of introductions and crosses. Region-specific optimum fertilizer recommendations were identified. Currently, the demand for malting barley is increasing due to the emerging breweries. Genetic variability studies on barley landraces from the central highlands, using SDS-PAGE, illustrated a range of variation within accessions. Evaluation of barley landraces for waterlogging tolerance showed apparent differences in P concentration and uptake between the susceptible and tolerant landraces. A national barley research and development workshop was held in collaboration with ICARDA in 2007 to re-visit the achievements and constraints in barley research and development of the previous decade. Future barley research and development directions were identified during the workshop.

Introduction

In Ethiopia, agriculture accounts for about 50% of GDP, 85% of employment, 90% of foreign currency earning and 70% of the supply of raw materials for heavy and medium-scale industries. The Government of the Federal Democratic Republic of Ethiopia has launched Agricultural Development Led Industrialization (ADLI) as a main strategy to bring economic growth. Moreover, the Plan for Accelerated and Sustainable Development to End Poverty (PASDEP) program was launched to ensure food security

and reduce poverty by half by 2015, and is in its second phase of implementation. The focus is to transform small-scale agriculture into commercial farming systems. Increased extension and research services that respond to farmers needs, taking into account the different conditions in different parts of the country and likely market demand, has been and will continue to be the focus of this program. Several-fold increases in productivity and production and export volume of crops and livestock are envisaged. Attempts were made align the national agricultural research efforts to governmental development strategies and programs. In this regard, indigenous crops will get high emphasis, reflecting their role in food security. These include cereals (tef, maize, sorghum, wheat, barley), roots and tubers (enset, Irish potato, sweet potato), and vegetables (garlic, onions and tomato). Barley can be considered as a dependable food security crop for the majority of highland farmers.

Encouraging research results have been seen in variety development and promotion; optimum agronomic practices have been recommended; and information on genetic variability further enriched. In this paper, an overview is provided of barley research and development efforts in Ethiopia in the past ten years.

Importance of barley

Barley is considered as a dependable food security crop for the majority of highland farmers because it is grown in marginal areas where the choice of other cereals is limited. In comparison with other cereals, it is harvested earlier and provides relief from food shortages during the long rainy season; has better stability of production, with wide adaptation to different production systems. Moreover, it is the sole raw material for commercial malting and brewing plants, with no alternatives available. Hence, in the Ethiopian context, research on barley is

broadly classified into either food or malting barley, to satisfy the specific requirements of each sector. There is an increasing trend in barley production, with the area expanding from 0.92 million hectare to nearly 1 million in 2007/08, except in the 2006/07 production year. Productivity has also increased, from 1.2 to 1.4 t/ha for the same period, but it is still much below the world average (CSA, 2008).

Variety development efforts and achievements

A decentralized research system exists, where federal and regional research centers are able to release varieties for their own local circumstances. In the past ten years, encouraging research results have been seen in variety development and promotion. Variety development efforts emphasized the evaluation and utilization of landraces in the case of food barley, and introductions and crosses in the case of malting barley. Over 40 food barley varieties have been released by national and regional research programs since the start of barley research, of which 22 were released since 1993 by national and regional research centers. Except three (EMBSN 5th 2/95-3-3-3 named as Bentu; EMBSN 5th 46/95-9-9-5 or Desta; and BI 95-198 or Yedogit, all released for low-moisture environments) and EH 1700/F7 B1.63 from hybridization, they were all selections from landraces for either specific or wide adaptation.

Cvs. Beka and Holker are the old malting barley varieties still in production. In an effort to find new alternative varieties, 34 malt barley varieties were introduced from ICARDA, Canada and Kenya during 2003 to 2005, and three varieties (cv. Haruna-Nijo, CDC select and Miscal-21) were registered in 2006. However, two proved to be inferior in disease resistance and yield compared with Miscal-21. Industrial-scale malting and brewing quality tests showed that Miscal-21

qualified as a malting barley and was recommended for further extension. HB 52 and HB 120 were also promising varieties but quality tests demonstrated low extract yield, slow fermentation, slow beer filtration and low alcohol content.

Fertilizer recommendations

Although 60:60 kg/ha N and P is the blanket recommendation, area-specific optimum fertilizer recommendations were identified. In northwest Ethiopia, a drastic increase in response to N was achieved in contrast to P. Depending on the location, 64 to 96 kg/ha N and 23 to 69 kg/ha P was found economical for food barley production. For the low-rainfall barley growing environments (northeastern Ethiopia) 46 kg/ha N was found optimum, but different levels of P (0 to 69 kg/ha) were recommended to give options for poor farmers. For central Ethiopia, 41 kg/ha N and 46 kg/ha P_2O_5 is still the recommendation. Generally, although fertilizer recommendation depends on locations, it is generally advised to use

60 kg/ha N and 60 kg/ha P for red soils, and 25 kg/ha N and 30 kg/ha P for brown soils (Liben *et al.*, 2006).

Area-specific fertilizer trials for quality malt barley production are ongoing for the northwest and central Ethiopia (Bekoji, Kofele, Shashemene and Digelu-Tijo). Results from Bekoji indicated that effects of N, P and $N \times P$ for grain yield and N and P effects for 1000-kernel weight were significant ($P < 0.01$). Rates of 36–46 kg/ha of $N-P_2O_5$ applied as 100 kg/ha diammonium phosphate (DAP) and 40 kg/ha urea, 54–46 kg/ha of $N-P_2O_5$, applied as 150 kg/ha DAP and 19.6 kg/ha urea and 54–69 kg/ha of $N-P_2O_5$ applied as 150 kg/ha DAP and 58.7 kg/ha urea, yielded better with yield advantage (YA) of 39%, 42% and 44%, respectively (Table 1). They were also acceptable with respect to protein content and 1000-kernel weight, and they experienced low levels of lodging. The highest marginal rate of return (589.3%) was obtained from 36 kg/ha N and 46 kg/ha P_2O_5 (100 kg/ha of DAP and 40 kg/ha of urea) and can be considered the economically optimum rate for quality malt barley production at Bekoji (Table 2). This rate will be verified for malt barley producing farmers.

Table 1. Mean values of P_2O_5 , N and their interaction for different characters at Bekoji.

$N-P_2O_5$	DH	DM	PLH	LOD (%)	TKW	PC (%)	GYH	% YA
0-0	107	164	95	0	41.1	10.5	1.4	0
18-46	94	158	102	3	43.1	11.0	2.0	30
36-46	94	157	106	6	43.0	10.7	2.3	39
36-69	92	157	106	3	42.9	10.9	2.4	42
54-69	92	155	110	6	42.6	11.3	2.5	44

DH = Days to heading; DM = Days to maturity; PLH = Plant height; LOD = Lodging; TKW = 1000-kernel weight; PC = Protein content GYH = Grain yield (t/ha); YA = Yield advantage.

Table 2. Partial budgeting and marginal rate of returns for fertilizer trials at Bekoji.

$N-P_2O_5$	DAP-Urea (kg/ha)	GY (t/ha)	Adj. GY (5%)	GB (birr)	TVC (birr)	NB (birr/ha)	MRR (%)
0-0	0-0	1.4	1.3	7150	0	7150	-
18-46	100-0	2.0	1.9	10450	760	9690	434.2
36-46	100-39.1	2.3	2.2	12100	1040	11060	589.3
36-69	150-19.6	2.4	2.3	12650	1262.2	11387.8	247.5
54-69	150-58.7	2.5	2.4	13200	1535.9	11664.1	200.9

DAP = diammonium phosphate; GY = Grain yield; GB = Gross Benefit; TVC = Total Variable Cost; NB = Net Benefit; MRR = Marginal rate of return.

PARAMETERS: Price of malting barley = 5500 birr/tonne; Price of Fertilizer: DAP = 7.50 birr/kg, Urea = 7.00 birr/kg. Cost of fertilizer application: 10 birr/qt.

Barley genetic variability studies

Genetic variability studies on barley landraces using SDS-PAGE and AFLP illustrated varying ranges of variation within accessions that enriched knowledge on landraces and their utilization. Landraces from specific locations were compared that showed landraces within some locations are genetically close (0.45) while in other locations genetic dissimilarity it is as high as 0.61. Moreover, AFLP analysis of the genetic relationship between farmers' cultivars from central Ethiopia illustrated genetic dissimilarity of 0.372 to 0.728 (Assefa, 2003).

Estimates of genetic parameters from crosses of selected barley landrace lines highlighted the importance of additive gene actions for yield components under optimum growing conditions, but both additive and non-additive gene actions were important for grain yield. At the same time, evaluation of barley landraces for variation in response to waterlogging showed apparent differences in P concentration and uptake between the susceptible (e.g. Feres Gama) and the tolerant landrace Mage (Assefa and Labuschagne, 2007). Differences for N concentration were also noticed, although the magnitude was not comparable to that for P.

Promotion of released varieties

Promotion of some of the released varieties with their production package has shown promising results, but the scaling-up efforts are not to the extent required. In northeast Ethiopia, for instance, food barley varieties Shedho and Estayish were the preferred varieties, with a marginal rate of return (MRR) of 17 to 51 and a yield advantage of 3–5 qt over the local check. Participatory

seed production of these varieties, involving 144 farmers, was implemented to ensure their wide-scale promotion. Misrach was found well adapted to north, northeast and the central highlands of Ethiopia, with good acceptance by farmers. HB 1307 is also under promotion in the central highlands of Ethiopia.

Malting barley is becoming a good source of income and it can be made a dependable income generating commodity for farmers if they receive regular training, a high quality seed supply system is ensured, and breweries offer an attractive price to the producers. There are six breweries currently operating, but only a few use locally produced malt barley. The Asela malt factory (AMF) is the sole receiver of malt barley produced by farmers. The annual malt requirement of the factory is 45 700 t/yr (i.e. 64 000 t raw malt barley) but supply is only 15 000 t. Almost 94% of the supply is from small-scale producers and the remainder is from commercial farms. Encouraging efforts were made to facilitate domestic malt barley supply to AMF and in the course of intervention farmers in the Arsi zone responded positively and increased land under malt barley production (Table 3). As a follow-up to that effort, a three-year project on scaling-up of HB-1533 and Miscal-21 production was developed by Kulumsa Agricultural Research Center (KARC) and implemented jointly by pertinent malt barley development actors, including KARC and AMF. In 2006, 176 farmers were involved in 7 Weredas [districts] of Arsi and West Arsi. Excluding those farmers who entered the system through farmer-to-farmer seed exchange schemes, 200 farmers also benefited from the second year effort. Farmers developed great interest, especially in the production of Miscal-21, which was good in grain yield, grain weight, protein content and germination. The project was expected to finish this cropping season.

Table 3. Land under malt barley in Arsi and West Arsi Districts and harvest supplied to Asela malt factory (AMF).

Year	Land under malt barley (ha)	Supply of malting barley to AMF (t)
1997	28 721	16 645
1998	43 409	17 423
1999	26 594	3 867
2000	16 800	6 520
2001	25 857	18 436
2002	31 153	21 924
2003	32 545	18 171
2004	43 651	23 249
2005	43 655	20 526
2006	38 660	13 833
Mean	33 104	16 060

SOURCE: Assefa Malt Factory. Based on paper presented at the 2nd Barley Research and Development Review Workshop, November 2006, Holetta, Ethiopia (unpublished).

Future research and development directions

- The focus given to landraces and efforts to release varieties is encouraging. However, evaluation and selection for low input conditions to address the needs of resource-poor farmer was not emphasized.
- Varieties for low and erratic rainfall environments are lacking and this lack needs addressing, together with the need for strong promotion of the varieties already released.
- There had been an aggressive germplasm evaluation for scald and net blotch resistance, and useful materials have been identified; but the effort was wasted because the information was not compiled in a useable way. Moreover, there is lack of coherence among breeders and pathologists, with very little commitment to joint efforts.
- The price of fertilizer is escalating while soil fertility is declining. Hence, looking for alternative sources of nutrients along with proper soil and water management practices is not only a priority but also a survival issue, especially in the highlands.
- Value addition for food barley to increase

its economic significance beyond its role as a food security crop is an area to look into.

- The demand for malt barley cannot be met with current levels of production, and it will require continued efforts to involve small-scale farmers in a wider scale of production. Moreover, a strong crossing program is required to find alternative varieties to Holker and Beka.

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Peruvian barley program

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Abstract

The genetic and agronomic improvement of barley in Peru is mainly in the hands of the Cereal Research Program of Universidad Nacional Agraria de La Molina, since 1968 with the support of the Backus Foundation and the International Atomic Energy Agency (IAEA). The main results have been the release of 9 improved cultivars for food that are grown in 90% of the barley area; the productivity at national level increased from 0.85 t/ha in 1978 to 1.2 t/ha in 2006. The introduction of improved agronomic technology to the central highland has contributed to increase the yield in farmers' fields to 4000 kg/ha. In the past, barley production was mainly for family subsistence, while today there is a larger quantity of better quality barley grains in the local market and for the food industry.

Introduction

Barley was introduced into Peru by Spaniards during the conquest period. In Peru, barley is the fourth most important food crop in terms of area. Barley is popular primarily because it is one of the few crops suitable for the extensive and marginal highlands, where frost and drought are common. Farmers claim that barley grows well in poor soils with little or no fertilizer. Barley is produced by indigenous subsistence-level farmers as a basic food staple for their families and as a feed. It is served in a variety of ways and contributes substantially to the caloric and nutrient content of the peasant diet. A food consumption survey indicated that barley

contributes on average 20% of the caloric intake of families.

Barley yields in the highlands are low. In 2007, above 3000 m elevation, 150 000 ha were cultivated with a harvest of 190 000 t of grain, equivalent to a mean yield of 1200 kg/ha, of which some 70% is used as food and 30% as feed, seed and other uses. Imported barley and malt are used for beer production.

The main diseases are stripe rust (*Puccinia striiformis* f. sp. *hordei*), leaf rust (*Puccinia hordei*), net blotch (*Pyrenophora teres*), barley stripe (*Pyrenophora graminea*) and leaf blotch (*Cochliobolus sativus*).

The objectives of the Peruvian barley program are:

- To develop improved varieties with high yield, good disease resistance, wide adaptation and good quality for the commercial food industry and as traditional food.
- To determine suitable production technologies for the various barley zones in the country.
- To transfer the knowledge and technology generated to agronomy students, agronomists and farmers.

Methodology

The national germplasm collection and ICARDA material are used to develop new cultivars using hybridization, mutation induction and doubled haploids. Head \times row planting is used from F_2 to F_6 or from M_2 to M_4 . The promising rows are harvested as spikes (10–20). The homogenous barley lines are evaluated for yield using experimental

trials in different locations and years. The test to select for grain quality is made from F_4 or M_4 to later generations. The genetic material is evaluated in two growing seasons per year.

Multiple disease resistance is a major objective because of the large number of barley pathogens present in the highlands. The genotypes selected could have either vertical or horizontal resistance.

Agronomic trials are performed to develop adapted cropping technologies for the different barley growing areas.

Results and discussions

Germplasm collection

Over a period of 39 years a large collection of 12 000 barley genotypes from various sources have been evaluated in the coastal and highland regions. Different sources of resistance and tolerance to biotic and abiotic stresses, suitable maturity, adaptation, morphological characters associated with good yielding ability and quality have been identified.

Development of improved cultivars

Nine cultivars were released during the 39 years. Of these, seven—Zapata, UNA 80, UNA 8270, Yanamucló, Buenavista, UNALM 94, UNALM 96—were obtained through hybridization, and two, UNALM95 and Centenario, by mutation induction. Eight cultivars are covered barley and one, UNALM 95, is hulless barley. All cultivars are used mainly as food.

Yield increased from 1500 kg/ha in Zapata to 4000 kg/ha in UNALM96 and Centenario under farmers' field conditions. Increase in the grain yield average depends on the genotypes and can vary from 33 to 75% (Wych and Rasmusson, 1983; Sylvey, 1986). This performance could be improved more due to their better adaptation to the modern cultural practices. Ortiz *et al.* (2002) found increased yield due to genetic improvement of 13% for

two-row and 34% for six-row Nordic spring barley cultivars. Žaková and Benková (2006) noted significant progress in the performance of barley cultivars developed from 1900 to 2003 in Slovenia.

Tolerance to lodging was improved due to the reduction of plant height from 180 cm to 100–120 cm. Ortiz *et al.* (2002) found a reduction of 1 cm in height in Nordic spring barley cultivars over a period of 5 to 6 years, which partially contributed to increase their performance. Žaková and Benková (2006) found that barley cultivars in Slovenia between 1900 and 2003 became generally shorter, which contributed to resistance to lodging.

Resistance to disease, mainly stripe rust and leaf rust, has improved. In the highlands this is the main criterion for release of cultivars, reflecting the poverty of farmers and respect for the environment. Cultivars exhibit varying grades of resistance, and some of them, such as UNA 80, is still resistant since release in 1980 (Sandoval-Islas *et al.*, 1998).

Other characters improved were the life cycle, and morphological characters of the spikes to provide tolerance to hail.

Quality characters such as grain plumpness, 1000-kernel weight, test weight and protein content improved. Madic *et al.* (2005) indicated that 1000-kernel weight is a good indicator of the quality of malting barley, because the amount of malt extract is positively correlated with grain size. Ferrio *et al.* (2004) reported increase in grain size of barley by genetic improvement. Žaková and Benková (2006), in their study of barley cultivars released over a period of 100 years, found a slight increase in 1000-kernel weight. Madic *et al.* (2006) reported the effect of growing conditions on 1000-kernel weight. Globally, there is evidence of improvement in the quality of food and malting barley (Swanston and Ellis, 2002; Tamm, 2003).

The Peruvian improved cultivars are now cultivated on almost 90% of the area used for barley production and they have contributed

significantly to the total increased production and average yield of barley in Peru. As the use of fertilizer and other technologies in barley production is rather exceptional, the increase of yield from 0.85 t/ha in 1978 to 1.2 t/ha in 2003 was mainly achieved through genetic improvement.

Development of crop technologies

New cultivars require appropriate agronomy technology to express fully their yield and quality potential. Experiments were conducted to find the right fertilizer dose, the seasonal seeding time, seeding methodology and weed control. The application of the resulting recommendations permitted yields close to 4000 kg/ha in farmers' fields.

Economic impact of new barley cultivars

The research work provides valuable tools (improved cultivars and technology) for the agricultural communities of the Peruvian Andes. Their use in some areas resulted in doubling or tripling of yield compared with traditional cultivars.

This program has contributed to the development of a growing food industry that produces barley flakes, barley pearl grains, flour and high nutritional value foods for the consumers through small- and medium-scale industries located in the highlands.

These achievements were obtained with the collaboration of NGOs such as CARITAS PERU and ADRA PERU, and of local governments.

These contributions are very valuable because they are in the field of agriculture, resulting in greater food provision and increased work sources. National statistics

indicate that approximately 49.6% of the Peru population lives in poverty or extreme poverty; three million of those people live in rural highland areas, and about 100% of national barley production is harvested in this area, primarily for human consumption.

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Participatory varietal selection of barley in the highlands of Tigray (northern Ethiopia) with implications for breeding in low input conditions

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Abstract

Barley is one of the most important cereals grown in Tigray (northern Ethiopia), a region with low and erratic annual rainfall. Officially released varieties have not been adopted by farmers as they do not perform well under the prevailing conditions. Therefore farmers in northern Ethiopia either continue growing their old landraces or develop their own varieties by selection. To compare the performance of improved and farmer-developed varieties, a participatory varietal selection (PVS) trial was initiated on-farm and on-station.

Four improved varieties (IV), two farmer-developed (FDV) varieties, and three rare farmer varieties (RFV) were tested and trials were conducted for two years (2004–2005) in a number of locations representing the highland areas of barley cultivation. Strong effects of years, locations, genotypes and genotype \times environment interactions were found. For the low input conditions of Tigray, the FDV Himblil was more preferred to others. The RFVs did not perform well. The IVs HB-42 and Shege were rejected, while Mistrach and Dimtu were accepted, though their expected yield advantage was not realized under low input conditions. It was also shown that environments in the breeding stations diverge significantly in rainfall and

fertility from the conditions in farmers' fields. However, the experimental data corresponded well with farmers' ratings, and subsequent spread of the varieties in 2005 demonstrated that PVS involving farmers and their innovations in trials can lead to a participatory breeding program in barley.

Introduction

Barley is one of the most important cereal crops grown for their livelihood by the resource-poor farmers of the Tigray region in northern Ethiopia. Its productivity is low, ranging from 0.8 to 1.8 t/ha, depending on the season and applied inputs (Fekadu and Skjelvag, 2002). Officially recommended released varieties have not been adopted by farmers. HB-42 and Shege are recommended varieties but not adopted in Tigray. We wished to investigate the causes for this poor adoption and the traits required for adaptation to the low input conditions of this part of Ethiopia.

Low input areas have benefited far less from the yield increases achieved by formal modern breeding. The frequent failure of varieties developed through this system has been ascribed to the fact that the varietal development and testing is done in conditions not representative of those of resource-poor farmers, and that breeding materials

evaluated were only partly relevant to such conditions. This has led Ceccarelli *et al.* (1998) to propose that breeding for drought tolerance has to be based on direct selection for grain yield in the target environment, as one possible way to improve the efficiency of selection of improved varieties. Selection for specific adaptation is particularly important in breeding crops predominantly grown in unfavorable conditions, because these environments tend to be more different from each other than favorable environments (Ceccarelli and Grando, 2007) and tend to produce repeatable cross-over interactions with favorable environments (Ceccarelli, 1996).

A complementary trend in recent years has shown that technology development can greatly benefit by involving farmers in a participatory manner. Participatory Varietal Selection (PVS) and Participatory Plant Breeding (PPB) (Witcombe *et al.*, 1996) are the terms used to indicate farmers making their choices among the final (or nearly final) and segregating generations, respectively. The success of such programs in identifying preferred varieties and fostering improved

adoption of varieties are well documented (Joshi and Witcombe, 1996; Abay *et al.*, 2000; Sperling *et al.*, 2001; Ceccarelli *et al.*, 2001; Mekbib, 2006; Ceccarelli and Grando, 2007) and thereby enhance productivity levels.

To develop and provide varieties adapted to the fragile environments of Tigray region and to meet farmers' criteria, increased farmer participation in development and experimentation of new varieties is an unused potential. A PVS program was therefore initiated with different varietal types of barley to understand why improved varieties have not been adopted by farmers, and to assess the merit of PVS in the Tigray region. More detailed analyses of the present work have been published by Abay *et al.* (2008) and Abay and Bjørnstad (2009).

Materials and methods

First, a search process for barley varieties led to the selection of nine varieties (Table 1). The varieties are grouped as Farmers Developed Varieties (FDV), Improved Varieties (IV) and Rare Farmers' Varieties

Table 1. Characteristics of the test varieties used in the experiment

Variety	Land under malt barley (ha)	Row type	Caryopsis type	Maturity type	Year of release or status
HB-42	Exotic × indigenous cross IAR/H/81/compound 29// compound 1420/coast	Six	Covered	Late	1984
Shege	Pure line selection from accession 1622-05	Six	Covered	Late	1996
Misrach	Pure-line selection from Kulmsa 1/88	Six	Covered	Late	1998
Dimtu	Pure-line selection from accession 3369-19	Irregular	Covered	Late	2001
Himblil	Farmers' Developed Variety through pure line selection	Six	Covered	Late	Preferred locally since 2000
Demhay	Farmers' Developed Variety through pure line selection	Six	Naked	Late	Preferred locally since 2000
Rie	Farmers' variety	Six	Covered	Late	Rare farmers' variety
Sihumay	Farmers' variety	Six	Covered	Late	Rare farmers' variety
Atona	Farmers' variety	Irregular	Covered	Late	Rare farmers' variety

(RFV). “Rare” types are defined by farmers as those grown either in mixed cultures or only by a few households in neighboring villages. Varieties were either identified as suitable or deliberately included because of their adoption constraints. The IVs Dimtu and Misrach are intended for both early and late sowings and for both high and low input conditions. HB-42 and Shege are released for high input, but deliberately included because of their official recommendation for cultivation in Tigray. Himblil and Demhay are FDVs in Tigray and the three RFVs were Rie, Sihumay and Atona.

Because of the nature of the test varieties, the research addresses only the highland barley growing areas of Tigray. The experiments were conducted for two production main seasons in 2004 (7 locations) and 2005 (14 locations). The trial was unbalanced in terms of locations \times years,

but balanced for varieties \times year and varieties \times locations. The location of the test sites are presented in Figure 1. Two of the test sites in the second year were Ethiopian Institute of Agricultural Research (EIAR) station sites Holleta and Debrebrhan, from whence the IVs were released. These research sites are located in the central and north Shewa regions, respectively.

Decentralized participatory trials

The trials lasted for two years in the four villages of Bolenta, Habes, Buket and Mugulat. Participating farmers were selected during the visits, with deliberate inclusion of seed selectors where feasible. The seed selectors from Bolenta (from where two FDVs were procured), Buket and Habes villages were involved. Criteria for selection

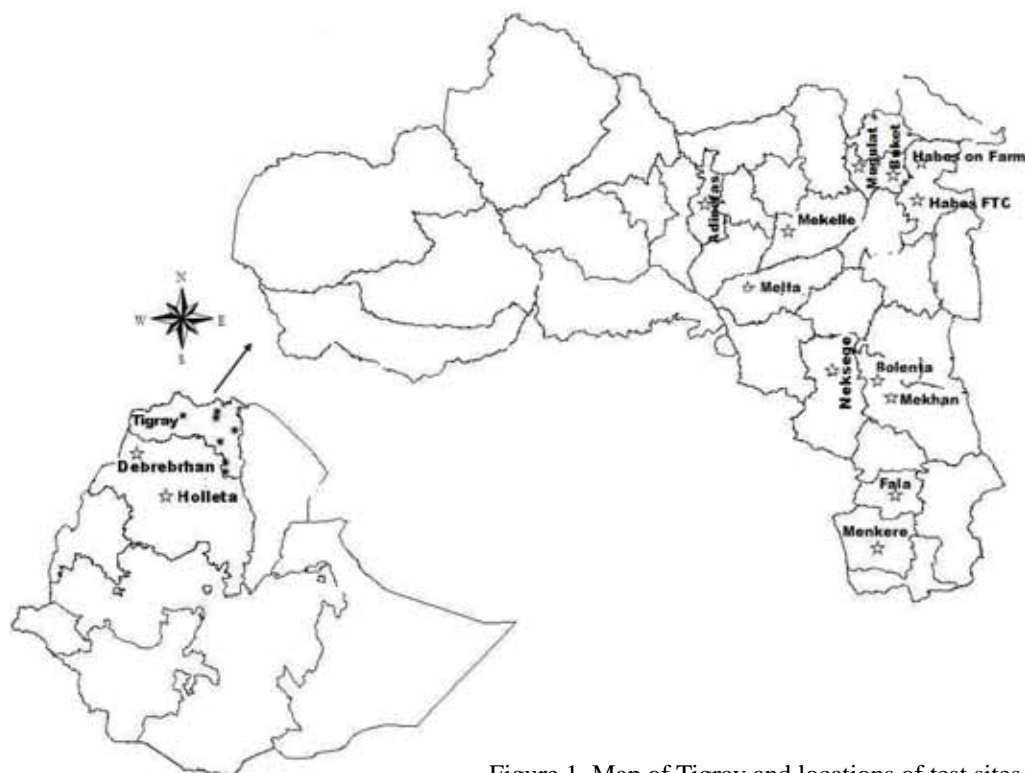


Figure 1. Map of Tigray and locations of test sites.

of farmers were different across villages. In Habes and Buket farmers were selected from different clusters of villages, assumed to represent existing diversity and constraints of the area such as soil fertility, waterlogging and disease pressure. In Mugulat and Bolenta, most experimenter farmers were from 1 or 2 clusters in order to facilitate follow-up and monitoring by the development agent (DA). In Tigray, nearly 30% of the households are female headed. Due representation was given to such households, and, on average, 20% of the experimenter farmers were women.

The trials followed the “mother and baby” design (Snapp, 1999; Witcombe, 2002). A flow chart of the trials is shown in Figure 2. In the mother trial, two farmers were involved in each village as replications, and each of them received the complete set of varieties. The trials were managed as per farmers’ practice; including soil preparation, weeding and no fertilizer was applied. The baby trials are based on paired plots, where a farmer received only one test variety in order to grow it alongside to their local variety under their management. In each village, 1 kg seed of each test variety was randomly given to five farmers. A total of 180

farmers, 45 from each village, were involved in baby trials. The objective of these trials was to test varieties under the varied farmer managements and environmental conditions. Quantitative and qualitative data were recorded from both trials. Mutual visits within villages were organized for participatory evaluation at three stages of crop growth (vegetative, flowering and grain filling). Household preferences were assessed based on household level questionnaires (HLQ) providing their matrix ranking level of the introduced variety. Following the methods explained by Christinck *et al.* (2005), the criteria and varieties were listed in a matrix to understand the choices between the varieties.

On-station and FTC trials

On station trials were conducted at Mekele (in the capital of Tigray) and on Debrebrhan and Holleta stations. Ten trials in Tigray were conducted at Farmers Training Centers (FTC). A randomized complete block was used with two replications, each containing the same 9 varieties plus the local check. In all locations the soil was ploughed 2-3 times and seeds were planted in six rows 2.5 m long and 20 cm apart. A seed rate of 100 kg/

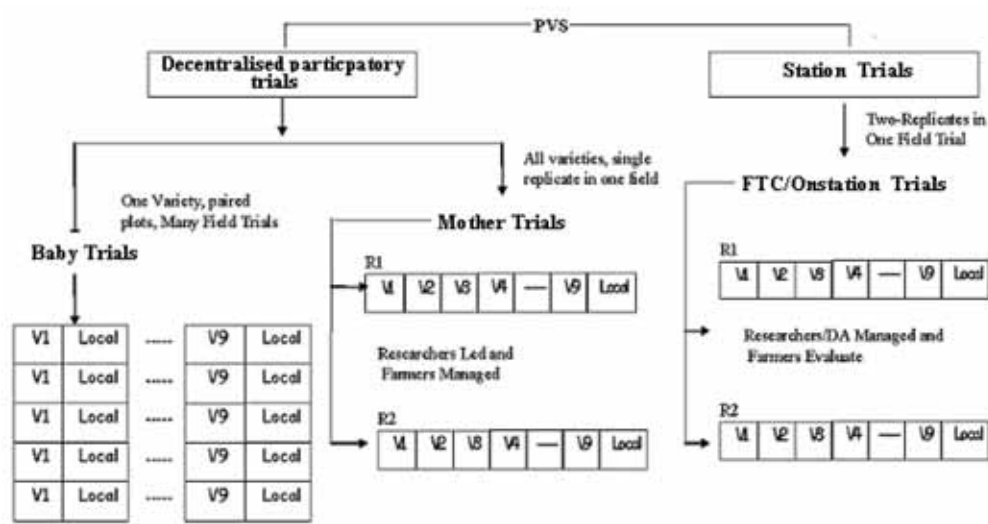


Figure 2. Chart of experimental design for station and on-farm (mother and baby) trials

ha was used uniformly. In the trials planted on-station, the experiments received DAP fertilizer before planting at a rate of 100 kg/ha and 50 kg/ha urea. The planting dates ranged from 27 June to 6 July, in both years. Local checks used were different across locations: two-row barley varieties were used at Adinefas, Melfa, Habes FTC, Habes on-farm, Mugulat, Buket, Neksege and Mekele, while six-row varieties were sown at Menkere, Mekhan, Bolenta, Fala, Holleta and Debrebrhan. The trials in farmers' fields were managed by farmers while the FTC and on-station trials were managed by the researchers, Development Agents (DAs) and technical staff of the research sites.

Data collection and statistical analysis

Days to heading (DH) and days to maturity (DM) were recorded as the number of days from date of sowing to the 50% appearance of ear heads and 75% discoloration of the spike in a plot, respectively. Plant height (PH) was measured in cm from ground level to the base of the main spike at maturity. The central four rows (2 m²) were hand harvested and hand threshed, in order to record the grain yield (in grams, later converted into kg/ha). Thousand-grain weight was recorded in grams as the average of three samples of 1000 grains from a plot after harvest. Rainfall and minimum and maximum temperatures were recorded from meteorological stations. For Neksege, Menkere, Mekele, Holleta and Debrebrhan, data were obtained at the site, but for other sites data were obtained from meteorological stations located in the closest district headquarters.

In this paper, only the data for grain yield, total rainfall, distribution and its coefficient of variation were analyzed across locations and years. Analysis of variance, using Proc GLM of SAS version 9.1, (SAS, 2001) was performed for grain yield of year \times location combinations (environments) in the Multi-Year, Multi-Location Trials (MYMLT).

Coefficients of variation (CV) were calculated to measure the precision of experiments and the mean standard error differences to compare the differences between varieties.

After confirming a significant presence of Genotype \times Environment Interactions (GEI), GGL (Genotype and Genotype \times Location) bi-plot analysis was performed. The bi-plot analysis is a multivariate analytical technique that graphically displays a two way table and allows visualization of the relation among genotypes, environments and their interactions (Yan, 2001). The underlying influence of the year variation was explored further by visualizing the 'which won where' feature in the GGE bi-plots (Yan *et al.*, 2000). Finally, the qualitative data collected from household questionnaires was subjected to a Chi-square test (Virk *et al.*, 2003), where the number of farmers responding 'better' and 'not better' in relation to the local variety were arranged in a two way table.

Results

Rainfall data

The annual total and temporal distribution of the rain was highly variable between years and locations, and was higher in 2005 than 2004. The annual rainfall distributions varied. Rainfall in the growing seasons ranged from 330 mm in Mugulat to 1509 mm in Debrebrhan, as the extremes (Figure 3). The average grain yield ranged from a low of 528 kg/ha in Neksege to a high of 2428 kg/ha in Holleta.

As shown in Figure 3, yield was not determined by rainfall alone. Although the rainfall at Debrebrhan was greater than Holleta, the latter gave 63% higher yields. In both years, rainfall at Menkere—the wettest site in Tigray—was about twice as much as at Neksege (the driest site). However, the yield at Menkere was only 53% of that at Bolenta and 65% of that at Mugulat. This implies that there are other factors that determine yield apart from rainfall, as shown by Ceccarelli

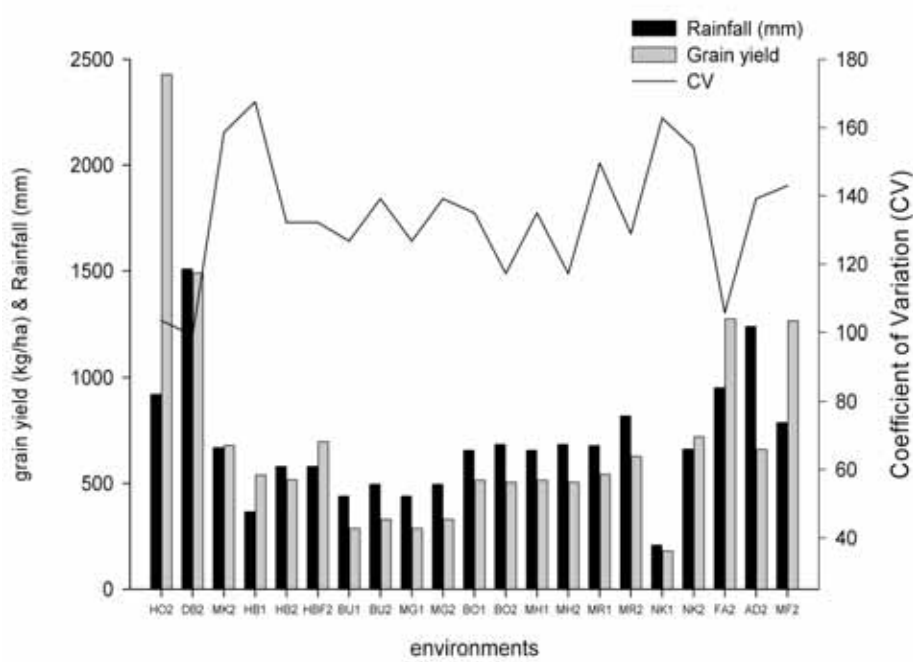


Figure 3. Total rainfall, average grain yield and coefficient of variation by test environments.
HO = Holleta; DB = Debrebrhan; HB = Habes; HBF = Habes FTC; BU = Buket; MG = Mugulat; BO = Bolenta; MH = Mekhan; MR = Menkere; NK = Neksege; FA = FALA; Ad = Adinefas; MF = Melfa.

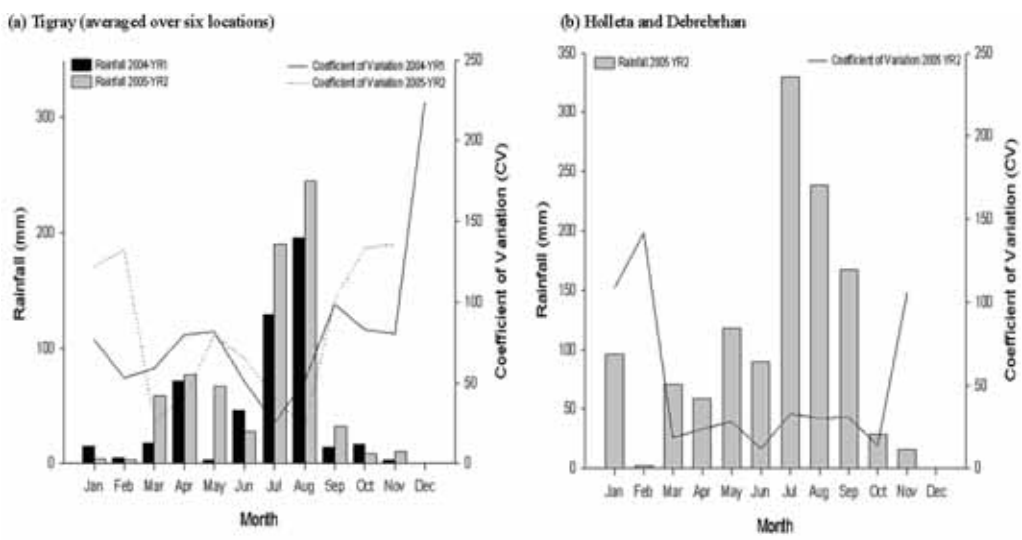


Figure 4. Mean monthly rainfall and CV in rainfall of experimental sites

Table 2. Combined analysis of variance of grain yield (kg/ha) for 2004 and 2005 barley variety trials in Ethiopia.

Multi-Environments		Trial (MET)
Source	DF	MS
Genotype (G)	9	1 812 224.42**
Environment (E)	20	3 013 931.26**
G x E	180	136 924.71**
Error	21	57 291.56

Multi-Environments		Trial (MET)
Source	DF	MS
Genotype (G)	9	199.53 **
Location (L)	6	96.99**
Year (Y)	1	516.34**
L x Y	6	58.23***
G x Y	9	11.80 **
G x L	54	6.95 **
G x L x Y	54	4.22 **
Error	7	7.08

DF = degrees of freedom; MS = Mean of the squares; ** = $P < 0.01$; *** = $P < 0.001$; Environment = year \times location.

et al. (2007) in Mediterranean environments. The effective rainfall in the main season started in May in Holleta and in July in the eastern zone of Tigray, but the rain in May is crucial for timely sowing of six-row varieties. The locations in Tigray had highest rainfall (54–70% of the total) in July and August, which corresponded to the sowing and vegetative stages of the crop, while the rainfall was less or absent in September, which subjected the crop to drought stress during the grain filling stage (Figure 4).

Variety performance and interaction with locations and years (The MYMLT analysis)

All sources of variation in the ANOVA were significant (Table 2). The coefficient of variation (CV) ranged from 6 to 16%, indicating the consistency of the experiments over the two years and locations (Table 3). The overall mean yield (Table 3) indicated highly significant differences between the genotypes. The FDV Himblil was the best, and significantly different from other varieties, not only in the high-yielding environments of Bolenta and Habes, but also at the driest site (Neksege) and in the waterlogged soils of Menkere. Misrach was superior to the two-row local checks of Buket, Habes and Mugulat, but not at the driest site (Neksege). It did not differ significantly from the six-row local checks at Bolenta and Mekan, and it was inferior to the local check at Menkere. In the relatively fertile soils of Bolenta and under waterlogged locations, the yield advantage of Himblil over the next best, Misrach was 330 kg/ha at Bolenta, and 378 kg/ha at Menkere. The superior performance of Himblil in waterlogged soils indicates a potential and genotypic variability for waterlogging tolerance, which is being further investigated. The recommended varieties, HB-42 and Shege, were consistently the lowest yielding at all sites (Table 3).

Table 3. Mean grain yield (GY in kg/ha) of barley varieties grown at seven locations over two years.

Variety	Trial site							Mean GY
	Bolenta	Habes	Mugulat	Buket	Menkere	Mekhan	Neksege	
Atona	960e	648e	782d	723c	787e	732e	522e	729g
Demhay	1195cd	880d	782d	778c	738f	781d	656d	815e
Dimtu	1149d	939c	1330a	917b	728f	828c	764c	950d
HB-42	455g	105f	156f	203e	219h	297g	100h	219i
Himblil	1620a	1283a	1089b	1161a	1239a	952a	1010a	1193a
Local	1262c	970c	950c	951b	958b	933ab	900b	989c
Misrach	1250c	1042b	1056b	1182a	861c	916b	948b	1036b
Rie	1180cd	889c	726d	725c	852c	789d	282g	778f
Shege	819f	706d	800d	558d	820d	681f	483f	695h
Sihumay	1413b	875d	612e	523d	490g	658f	386g	708h
Location mean	1130	834	828	772	769	757	605	811
s.e.d	78	57	44	63	31	30	19	16
CV	10	14	11	16	8	8	6	11

s.e.d.= standard error of the data; data entries followed by the same letter are not significantly different; CV= coefficient of variation.

Comparing high- and low-input trials

Considering the amount of rainfall (Figure 3) and differences in the mean yield of test sites (Figure 4), Debrebrhan and Holleta were classed as high yield potential, and Tigray sites as low yield potential, sites. The high-input sites received total rainfall of 1509 mm (Debrebrhan) and 609 mm (Holleta), with relatively good distribution (Figures 3 and 4). At these sites, during the experimental seasons, 46% (Debrebrhan) and 16% (Holleta) of the rain fell in July, August and September, compared with the low-input sites of Tigray, where 54–70% fell in July–August (Figure 4). In Tigray, it was not only the low total rainfall but also its uneven distribution that caused the lower yields.

The mean yields in the two groups of environments are shown per variety in Figure 5. The average correlation in yield of varieties between groups was negative (-0.213). Particularly for Shege the contrast is sharp. The respective yield advantages of Shege and HB-42 over Himblil were 1016 and 560 kg/ha on the high yielding sites, but the reverse was true for low yielding sites (Figure 5). If the experiments had been conducted only at high input sites, Shege and Misrach would be recommended. However Shege is one of the lowest yielding varieties in the low-input conditions of Tigray, and Misrach is

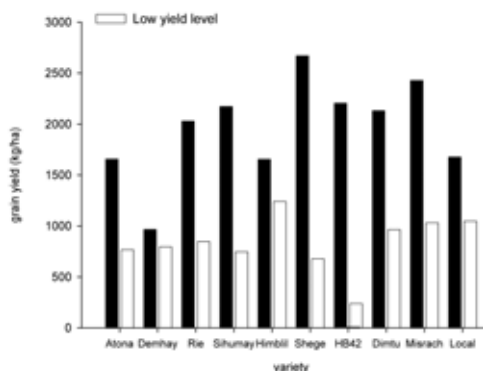


Figure 5. Grain yields of high and low input trials by variety

not significantly better than the local variety, though much better than Shege and HB-42. This is an indication of the need to select in the target growing environments.

GGL bi-plot analysis

The ‘which won where’ feature of the GGL bi-plot, based on the 2004 and 2005 yield data, is presented in Figures 6a and 6b. The two years were highly informative because of their contrasting rainfall distributions. A further grouping of environments is also illustrated. The bi-plots explained 86.1% and 77.4% of the total variance for the years 2004 and 2005, respectively. In 2004, all Tigray sites fell in same sector and shared the same ‘winner genotype’, Himblil. In 2005, Himblil was also the winner at all Tigray sites. In contrast, Misrach and Shege were the winners at Debrebrhan and Holleta and the slightly obtuse angle between the two groups of environments corroborates the weakly negative correlation mentioned above. Therefore, despite the presence of $G \times L$ interaction, Himblil yielded the best or close to the best in both years. This suggests that, within Tigray locations, the genotype main effects dominated the $G \times E$ interactions effect, and that it is possible to select for widely adapted varieties like Himblil throughout the highland Tigray region. HB-42 performed consistently poorly in both years, while the other varieties reacted differently. This variety can be considered as stable because of low GEI. However, as described by Yan and Tinker (2006), stability is meaningful only if it is associated with high mean yield performance.

Farmer preferences and perceptions

Chi-square test analysis was performed for the matrix ranking of all experimental entries, including local varieties (Table 4). The overall farmers’ preferences across the 7 sites are presented in Figure 7. HB-42 was rejected by 98% of experimenter farmers ($P < 0.05$).

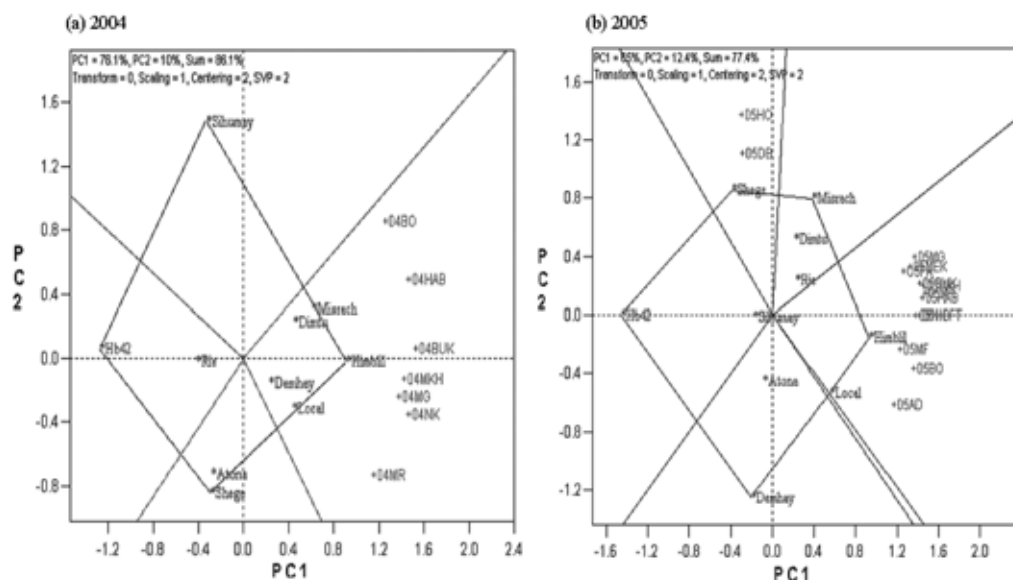


Figure 6. Yearly Polygon view for yield data from 2004-2005 in Tigray only (a) and all locations (b). The names of the test sites are abbreviated in upper case (BO = Bolenta; HAB = Habes; BUK = Buket; MKH = Mekan; MG = Mugulat; NK = Neksege; MR = Menkere; HO = Holleta; DB = Debrebrhan; AD = Adinefas; MF = Melfa; HBFT = Habes FTC; MK = Mekelle; FA = FALA).

Dimtu was preferred by farmers in Mugulat because they observed its “stay green” trait during late season drought. However, it was not preferred at Bolenta because of its less compact and thin spike. The RFVs ‘Rie’

and Sihumay’ were not preferred since their extended vegetative period made them prone to late season drought, but preferred for their grain quality (Table 4). The preference for high grain yield was only significant for Himblil. Overall, the preference was only significant for Himblil and Misrach, the latter not for yield but for its overall preference, indicating the multiple criteria and flexibility of farmers in maintaining varieties (Table 4). The experiments also allowed information exchange among farmers and increased the familiarity with new varieties of barley. As shown in Figure 8, the area expansion of varieties indicates the acceptance by farmers. The variation between villages for area allocation of each variety associated with the adaptation of varieties. HB-42 was not preferred as it was not planted in the second year, except at Buket and Mugulat by one and two farmers, respectively (Figure 8). In other words, the PVS showed tangible results already in its second year.

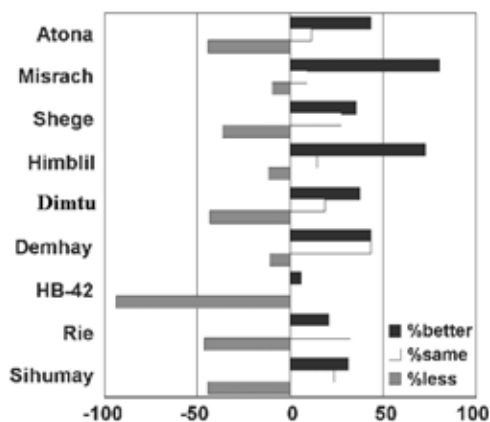


Figure 7. Overall preferences ranking of barley varieties in three districts of Tigray.

Table 4. Chi-square test on preference ranking of test varieties by traits in 2005

Variety	Emergence			Days to heading			Yield			Grain quality†			Overall preference		
	B	NB	χ ²	B	NB	χ ²	B	NB	χ ²	B	NB	χ ²	B	NB	χ ²
Misrach1	8	12	0.8	5	15	5.0**	12	8	0.8	13	7	1.8	16	4	7.2**
Atona	7	18	4.84**	4	21	11.56**	7	18	4.84*	7	18	4.84*	11	14	0.36
Shegel	2	23	17.64**	2	23	17.64**	6	19	6.76**	11	14	0.36	9	16	1.96
Himblil2	16	10	1.38	6	19	6.76**	18	7	4.84*	11	13	0.17	19	7	5.53**
Dimtu1	5	16	5.76**	4	17	8.04**	8	13	1.19	5	16	5.76**	8	13	1.19
Demhay2	4	15	6.37**	0	19	19**	5	13	3.56	6	10	1.0	9	10	0.1
HB-421	1	14	11.27**	0	15	15**	0	15	15**	2	13	8.1**	1	14	11.26**
Rie	10	14	0.67	0	24	24**	3	21	13.5**	17	7	4.1**	8	16	2.67
Sihumay	3	22	14.44**	0	25	25**	4	21	11.56**	10	15	1.0	8	17	3.24

Variety types: 1 = Improved varieties; 2 = Farmer developed varieties; * = Significant at 5% level; ** = highly significant at 1% level; Farmer rankings: B = Better; NB = Not better; † = ranking for grain quality was related to seed color and perception of product quality.

Discussion

The merit of PVS for barley improvement in Tigray

The experiments have shown that PVS is a viable method for identifying preferences, constraints and the potential of varieties. The PRA methods corresponded well with the data from the mother and baby trials, and the analysis showed that the level of accuracy was acceptable. Also, the preliminary experiences with the dissemination of varieties are promising. For further work, strong collaborative networks have been established between the farming communities and regional extension systems. A group of farmers experimenting with introduced and local varieties were spontaneously organized in an “Association for Barley”, established to share information, ideas and seeds that they found to be more productive than other varieties (both introduced and local). This study contributes a methodology for breeders and agricultural experts on how to perform varietal selections. Much work was done to identify farmers who are interested in improving varieties. The combined efforts of farmers, breeders and development agents may lead to more acceptable varieties being tested, to the benefit of farmers.

Choice of breeding strategies and genetic diversity

The decentralization effect of varietal testing combined with on-farm, FTC and on-station trials showed the importance of conducting selection in the target environment. The correlations between performance under stress and under favorable growing conditions were poor, indicating high G × E interactions. This is similar to that found by Ceccarelli (1994, 1996), who observed that the largest gains for barley improvement for drought stress are expected from direct selection under stress conditions. In this study, we found that the FDV Himblil out-yielded the recommended varieties, which also indicates the importance of specific adaptation.

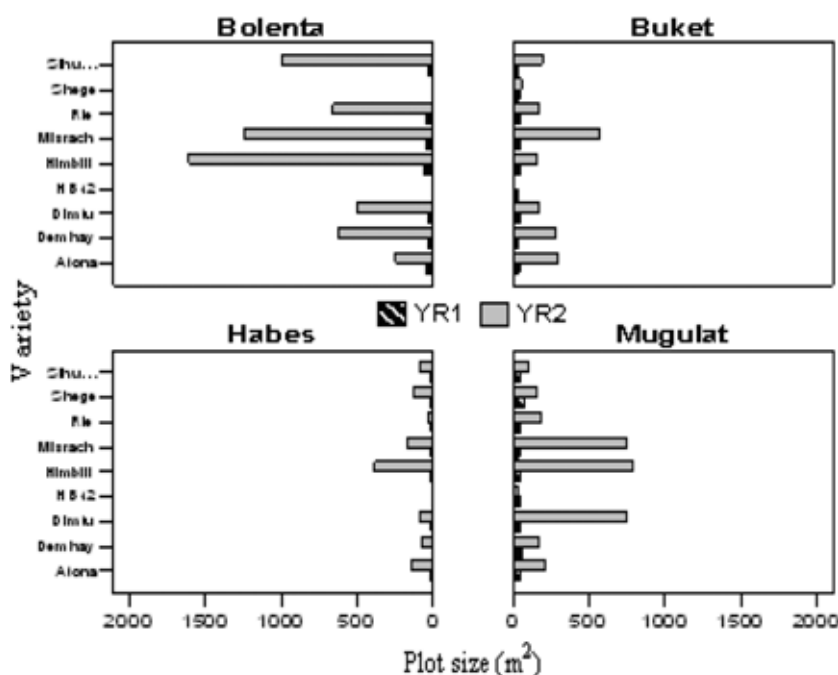


Figure 8. Diffusion of preferred varieties by year and variety.

The expected yield advantages of the IVs Misrach and Dimtu based on previous information were not met. With the exception of under high-input conditions, the two varieties did not show any yield increase and were not significantly different from the local, even though their performance and the farmers' preference was much more positive than for Shege and HB-42. The relatively better preference of these two IVs can be associated with the progress in improving barley breeding in Ethiopia by using relevant germplasm and testing methods (Sinebo, 2002). Their performance in the highlands of Tigray approximates to that of Himblil. Misrach comes from a landrace in Arsi and was promoted in Debrebrhan due to its superior waterlogging tolerance. In view of the extensive testing of landraces performed by the Ethiopian Institute of Agricultural Research (EIAR) since 1988 (Birhanu *et al.*, 2005), it was surprising that Himblil—selected by a farmer in Bolenta—performed

as well as or better than Misrach. This might indicate that if a wider genetic diversity base was used by including local germplasm from Tigray, even better cultivars could be produced.

The value of local varieties as a source of drought resistance was shown by Grando *et al.* (2001). Himblil can be considered as a good source of abiotic stress tolerance, both to drought and waterlogging stresses. It is more stable than the improved varieties under the conditions of low input and low rainfall typical of Tigray. The superiority of this variety is not associated with its escape mechanism as was explained for variety Saesa by Sinebo (2004). Saesa is a two-row, extra early variety, while Himblil is a six-row and medium-late maturing variety selected by a farmer under low-input conditions. The superior performance of Himblil, in contrasting seasons and low-input conditions, confirms the importance of specific adaptation, as negative results were

obtained when it was tested in high-input, high-yielding conditions. At the same time, improved varieties, like HB-42 and Shege, selected under these conditions responded positively only there.

An interesting further hypothesis is whether the waterlogging tolerance also confers a tolerance to later drought stress when rainfall ceases in September, due to deeper root systems.

Choice of testing sites for recommendation of varieties

When looking at various testing environments, the differences between high and low input conditions were more important than differences between farmers' fields. The differential response of varieties has indicated the relevance of conducting selection in the target environment. The negative relation between high and low input trials is consistent with the findings of Ceccarelli *et al.* (1992).

It can be concluded that there are good reasons for farmers not to adopt HB-42 and Shege delivered through the HIEP (High Input Extension Program). The recommendation of inappropriate IVs also makes farmers lose confidence in IVs for future technology adoption. It is therefore important that the Bureau of Agriculture (BoA) and Ethiopian Seed Enterprise (ESE) officers should revise the methodology used to recommend varieties. FDVs should also be formally recognized and promoted in the target environment. Himblil could be a potential candidate for formal release. The results of this study indicate that it is possible to improve grain yield of crops in low-input, abiotic-stressed target environments. The successes obtained can be summarized as (1) improved varieties are either inferior or no better than the local varieties; and (2) joint evaluation of varieties with farmers as research partners helps in rapid dissemination of varieties and information.

Acknowledgements

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Effect of decomposed coffee husk and nitrogen fertilizer on growth and yield of barley (*Hordeum vulgare* L.) on nitisols of Kokate, Southern Ethiopia

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Abstract

A field experiment was conducted during the 2003 crop season at Kokate, southern Ethiopia, to study the combined effects of decomposed coffee husk and N fertilizers on growth and yield of barley. There were two factors in this experiment, namely coffee husk at five levels (0, 0.675, 1.350, 2.025, and 2.7 t/ha) and nitrogen fertilizer at five levels (0, 15, 30, 45 and 60 kg/ha N). The recommended rate of phosphorus (26.4 kg/ha) in the form of triple super phosphate was applied uniformly to all plots at time of sowing. The results from this study indicated that coffee husk alone or combined with nitrogen showed no significant effect on either growth or yield of barley. However, the main effect of nitrogen was significant ($P < 0.05$) on days to heading, plant height, spike length, biomass and grain yield. In general, grain yield tended to be higher under nitrogen treatment than under coffee husk. The highest soil organic matter content of 4.6% was obtained from a treatment combination of 2.025 t/ha coffee husk and 60 kg/ha N. The highest grain yield (2979 kg/ha) was obtained from a treatment combination of the highest rate of coffee husk (2.7 t/ha) and 45 kg/ha N. In contrast, the lowest grain yield (1854 kg/ha) was obtained from 0.675 t of coffee husk per ha alone. Although the effect of organic residue is more long-term,

the present study showed that barley treated with a highest rate of coffee husk and 75% of the recommended N fertilizer rate (45 kg/ha) yielded better than other treatments.

Introduction

Globally, barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in production terms after wheat, maize and rice. The crop is indigenous to the highlands of Ethiopia, where many cultivars and a great variety of forms exist (Onweume and Sinaha, 1991). Barley is preferred to other cereals for its early maturity, relieving hunger; amenability for belg (short rainy season) growing, and hence double cropping; and stability of grain yield compared with other cereals under low and erratic rainfall, poor soil fertility and other stress conditions (Hailu Gebre and van Leur, 1996; Yirga *et al.*, 1998).

In Ethiopia, barley is grown on about 1.47 million hectare. The total production is about 1.55 million tonne with an average yield of 1.1 t/ha. The area under barley cultivation in the Southern Nations Nationalities and Peoples' Regional State (SNNPR), Ethiopia, is about 123 000 ha and production is estimated to be about 150 000 t, with a mean yield of 1200 kg/ha in the main cropping season (MOA, 2000). In the Wolayita zone, where the experiment was conducted, barley

is the fourth most important cereal after maize, sorghum and tef in area coverage (BOA, 1998).

Despite its importance and cultivation on extensive areas of land, the yield and productivity of barley in the country in general, and in the Wolayita zone in particular, is low compared with its potential. Among the major constraints on increased production of barley are poor soil fertility, limited supply of production inputs (fertilizer and improved seed), low prices for the produce, and undeveloped markets (Hailu Gebre, 1978).

Soil fertility is one of the major production constraints in the southern region of Ethiopia (Getahun and Tenaw, 1990). The various factors accounting for the poor soil fertility include topography, soil erosion, deforestation, population pressure, and continuous cultivation without proper soil fertility maintenance (Tenaw, 1996). Undulating topography, particularly in the mid- and high-altitude areas, has led to erosion problems, yet, because of the high population pressure, even steep slopes are put under cultivation. Small land holdings (<0.5 ha) per household are also the result of population density. Not only small landholdings, but also continuous and intensive cropping without use of fertilizers is a consequence of population pressure. All these factors have hastened a decline in soil fertility that affects crop production, resulting in low crop yields. The crop market price also fluctuates. Farmers with many expenses (tax, clothing, education, social activities, etc.) and yet with declining income cannot afford to buy chemical fertilizers.

Poor soil fertility is also due to nitrogen and phosphorus nutrient deficiencies (Bekele *et al.*, 1996). Due to the high cost of chemical fertilizers, increasing from year to year, and the low income of the farmers, it has become unaffordable to use mineral fertilizers for soil fertility maintenance and to ensure crop productivity.

The effect of applying available organic resources and agricultural wastes on improving crop productivity remains poorly understood from the perspective of organic matter dynamics. The use of underutilized organic resources as a means of providing nutrients to the crop and improving the efficiency of fertilizer use warrants greater attention by agricultural scientists (Woomer and Muchena, 1993).

In the absence of application of inorganic fertilizers, use of locally available organic materials alone may frequently be inadequate, both in quantity or diversity, to supply the nutrient demand. Strategies for improving nutrient management require assessment of these needs and evaluation of the organic and inorganic resources required for supplementation. Inorganic fertilizers coupled with effective management of organic matter are critical for the intensification of traditional farming systems to meet the immediate demand for high food production.

Ethiopia produces 215 000 t/yr of clean coffee for export and domestic use, both in almost equal proportion (FAO, 1997). This yield is associated with an estimated 242 000 t of by-products, which is being wasted annually. The southern region of Ethiopia has favorable weather condition for coffee and more 160 000 ha of coffee plantations. In the region, there are 324 coffee processing plants, of which 229 are wet processing plants, and 95 are dry processing plants, together producing a total of 33 750 t of husk (Tsige-Yohanes and Steinbach, 1996).

Currently, coffee pulp from wet processing is simply dumped into rivers, which are also the water source for domestic use by the population in close proximity to the processing sites. The pollution arising from a factory producing 1 t/day of parchment coffee is 20 000 L of effluent (Finney, 1990), which produces an equivalent biological oxygen demand comparable to that caused by 2000 people. The coffee husk from dry processing, in contrast, is either burnt deliberately or by

spontaneous combustion (Tsige-Yohanes, 1989). All these result in environmental pollution.

Coffee by-product (pulp or husk) as an organic fertilizer can be an alternative for the resource-poor farmer. However, the nutrients in the husk may be poor for plant growth because of low nutrient availability, thus large quantities of husk are required, which becomes too bulky to transport to the point of application. At the same time, the use of chemical fertilizers alone is costly. This situation calls for combined application of coffee husk and chemical fertilizers to make the production process more sustainable.

The aim of this project was to study the combined effects of decomposed coffee husk and N fertilizers on growth and yield of barley on Nitisols of Kokate (Wolayita zone). The specific objectives of the project were (1) to evaluate the response of barley (growth and yield) to decomposed coffee husk and N fertilizer, and (2) to investigate the combined effects of decomposed coffee husk and N fertilizer on some soil characteristics.

Materials and methods

The experiment was conducted at the Kokate research site of the Awassa Agricultural Research Center during the 2002 main crop season. Kokate (6°52' 25.4" N and 37°48' 26.9"E) is located 375 km south of Addis Ababa on the Addis Ababa–Arbaminch road, in the Wolayita zone of the Southern Nations, Nationalities and Peoples Regional State. The site is located at an elevation of 2100 masl. The soils of Kokate are Nitisols.

The experimental area had been under wheat without fertilizer before it was used for the present study. The straw of wheat had also been removed for animal feed from the field after harvest. The field was oxen ploughed four times before incorporation of the coffee husk.

There were two factors at five levels each in this experiment. The factors were coffee

husk and N fertilizer. The five levels of coffee husk were 0, 0.675, 1.35, 2.025, and 2.7 t/ha (based on 2.2% N in coffee husk (Assefa, 1996) and the five N levels were 0, 15, 30, 45 and 60 kg/ha N, corresponding to 0%, 25%, 50%, 75% and 100% of the recommended nitrogen fertilizer rate (60 kg/ha N). The recommended rate of phosphorus (26.4 kg/ha P) in the form of triple super-phosphate (TSP) was applied uniformly to all plots at the time of sowing of barley on 25 July 2002.

The coffee husk was collected from the processing plant six months before sowing and allowed to decompose in a pit 2 m deep and 1.5 m wide. The decomposed coffee husk was incorporated into the experimental plots a week before sowing the barley. Urea (46% N) was drilled within rows of barley as nitrogen source at the time of sowing.

A plot size of 6 m² (2 m × 3 m) with 20 cm spacing between rows was used. The improved food barley variety HB-42 was sown at a rate of 100 kg/ha. The two-factor experiment was conducted in a five by five factorial arrangement using a randomized complete block design with four replications.

The agronomic and yield data collected on a plot basis included date of emergence, date of heading, tillers with spike, plant height, spike length, seeds per spike, 1000-seed weight, above-ground biomass weight, grain yield, and disease and pest scores.

Date of emergence was recorded when 50% of the plants in the plot emerged, while date of heading was recorded when 50% of the plant in a plot produced heads. The number of fertile tillers was counted on a 50 cm row length in the middle two rows after heading. Plant height (in cm) was measured on 10 randomly selected plants per plot from the soil surface to the tip of the spike excluding the awn, and the spike length was measured on the same plants at physiological maturity. Days to maturity was taken when 50% of plants in a plot showed light white to yellowish plant color. The crop was harvested manually with a sickle close

to the soil surface from 1.6 m \times 3 m (4.8 m²) for measurement of the agronomic and yield data. The total above-ground biomass was weighed after sun drying at harvest. Ten random spikes from each plot were sampled and threshed separately to determine the number of kernels per spike. The yields of these spikes were later added to the plot yield to determine the final plot yield. Grain yield and 1000-seed weight were adjusted to 12.5% moisture level. Straw yield was determined as the difference between total aboveground biomass and grain yield.

Before the application of coffee husk, 50 random soil samples (0–15 cm) were collected from the experimental area using an auger, and two composites were made for determination of total N, available P, organic matter content and soil pH.

Soil samples (0–15 cm) were also collected from each treatment (four samples from each plot and composition per treatment) after the crop was harvested and analyzed for total N, organic matter and pH. Loose surface crop residue was brushed aside before taking the soil sample. The samples were dried and crushed to pass through a 2 mm sieve.

Soil pH was determined by a glass electrode pH meter in a 1:2.5 soil/water suspension. Soil texture was expressed by using Bouyoucos hydrometer method (Dey, 1965). Soil organic matter was determined according to Walkely and Black wet oxidation method (Allison, 1965) while total nitrogen was determined by Kjeldhal method. The Olsen method was used for available phosphorus determination.

The coffee husk before and after decomposition was sampled to determine its total nitrogen (%) and organic matter (%) content. The procedure applied for plant tissue analysis was applied to analyze coffee husk.

At harvest, plant samples were collected from the fourth row of every plot. The straw and seed were separated manually and weighed to add to plot grain and straw yields.

The samples were dried at 70°C for 24 hrs, ground to pass a 1 mm sieve, and analyzed for total nitrogen (%).

Statistical analysis was carried out following the procedure described by Gomez and Gomez (1984) using MSTAT C computer software (Freed *et al.*, 1989).

Results and discussion

The soil analysis before application of the treatments and sowing barley revealed that the soil was clay-textured, with a pH (H₂O) of 5.62, total N of 0.19%, available P content of 33 g/kg, and organic matter content of 4.05%.

Addition of coffee husk did not produce marked variation in soil organic matter content (Table 1). However, there was a tendency for soil organic matter to increase with increasing rates of applied coffee husk. In the control plots, the organic matter content was 4.19% whereas in coffee husk-treated plots (even in the case of 2.7 t/ha) organic matter content was 4.41%, implying an addition of only 0.22% organic matter over control. The organic matter content of decomposed coffee husk was 31%, i.e. 31 kg per 100 kg of decomposed coffee husk. The organic matter content of 2.7 t equates to circa 810 kg/ha. The contribution to the soil organic matter due to the application of coffee husk is only about 0.04%. The application of N fertilizer also showed a similar effect on soil organic matter content. The highest soil organic matter content (4.6%) was observed from a treatment combination of 2.025 t/ha coffee husk and 60 kg N/ha, followed by the treatment combination of 2.7 t/ha coffee husk and 15 kg N/ha, and 45 kg N/ha without coffee husk, having similar soil organic matter content of 4.58% (Table 1).

Similar to that of organic matter, application of coffee husk did not increase the total N content of the soil (Table 2). The possible reason for the decrease in total N in soil could be due to denitrification in the presence of

Table 1. The influence of coffee husk (CH) and N fertilizer on soil organic matter (%).

CH (t/ha)	Nitrogen (kg/ha)					CH Mean
	0	15	30	45	60	
0.000	4.19	4.44	4.22	4.58	4.47	4.38
0.675	4.47	4.45	4.47	4.18	4.46	4.41
1.350	4.41	4.53	4.52	4.53	4.19	4.44
2.025	4.27	4.37	4.45	4.34	4.60	4.41
2.700	4.41	4.58	4.32	4.32	4.41	4.41
N mean	4.35	4.47	4.4	4.38	4.43	

Table 2. The influence of coffee husk (CH) and N fertilizer on soil total nitrogen (%).

CH (t/ha)	Nitrogen (kg/ha)					CH Mean
	0	15	30	45	60	
0.000	0.26	0.24	0.24	0.26	0.28	0.26
0.675	0.24	0.26	0.27	0.29	0.26	0.26
1.350	0.24	0.26	0.24	0.24	0.30	0.26
2.025	0.24	0.25	0.21	0.27	0.26	0.25
2.700	0.24	0.24	0.21	0.27	0.27	0.25
N mean	0.24	0.25	0.24	0.27	0.27	

decomposable coffee husk ($\text{NO}_3^- \rightarrow \text{N}_2$) and the low N content of decomposed coffee husk (1.01%). However, the combination of 0.675 t/ha coffee husk with 15, 30 or 45 kg N/ha showed increased total soil nitrogen content after barley harvest.

In line with this result, Selvakumarie *et al.* (2000) reported that absence of variation in the availability of soil N with addition of fly ash (FA), a by-product of thermal power stations where electrical energy is obtained by firing finely powdered coal, at graded levels. They attributed that the result was due to very little total nitrogen content (0.6%) of FA, which might not have been sufficient enough to cause any increase in the level of available nitrogen and soil organic matter in the post-harvest soil.

The clay textured soil of the experimental site might have also protected the organic matter by adsorbing otherwise readily available substrates, making them less available to the soil population, by stabilizing the newly formed metabolite, and by increasing the longevity of soil organisms, as described by Russell (1988). Several investigations have also revealed that burying crop residue with low N content in soils

immobilizes N, whereas high-N-containing crop residues improve N mineralization (Ladd, 1981; Azam *et al.*, 1985; Smith and Sharpley, 1990).

Several factors, such as soil moisture, temperature, oxygen, soil pH, also affect the rate of mineralization, and various critical nitrogen concentration values below which immobilization occurs have been reported: 1.1 to 1.9% N (Janzen and Kucey, 1988); 1.5 to 1.7% N (Allison, 1973); 1.2 to 1.3% N (Jenkinson, 1984), while residues with nitrogen greater than 1.8 to 2% release the element (Jenkinson, 1984). In order to supply the needs of soil microbes during the decomposition of most crop residues, a minimum residual N content of 1.5–1.7% (C/N ratio of 25–30) has been suggested (Allison, 1973; Smith and Elliott, 1990). The nitrogen content of the coffee husk used in this study was 1.01%, which is below the minimum residual N content for decomposition; hence immobilization might have taken place. This is because the micro-organisms take up mineral N and decrease its concentration during decomposition of plant material with wide C/N ratios. However, neither the N content of the material nor the

C/N ratio is a safe guide to the effect on the mineral nitrogen content of the soil, although there is usually a loss of mineral nitrogen, at least temporarily, if the material contains less than about 1.8% N, corresponding to a C/N ratio of about 30 (Russell, 1988).

The concentration of N (1.01%) in decomposed coffee husk in this study (Table 3) might not have been sufficient enough to cause any increase in the level of total nitrogen in the post-harvest soil. Tolessa (1999) reported that about 33% of the N from farm-yard manure (FYM) is likely to be used by the maize crop in the first year (direct effect), the remaining may become available to the second and to a smaller extent to subsequent crops grown on the same land (residual effect). Thus, the choice between FYM and inorganic fertilizer is a matter of nutrient content, economics, transportation and accessibility.

The C/N ratio of about 18 of decomposed coffee husk was also too high to release the element to the crop (Table 3). The effect may last for several weeks, during which period microbial respiration results in the loss of carbon as carbon dioxide and a narrowing of the C/N ratio. As the microbial cells die, or act as the food source for predators, some of the nitrogen within them is mineralized. According to Russell (1988), net immobilization would take place during the first period, whilst there will be

mineralization during the second, which explains the results of our study.

Application of coffee husk resulted in decrease in the soil pH (Table 4), as was also the case during its decomposition. The probable reason for the decline in soil pH and that of coffee husk during decomposition might be due to the release of hydrogen ions (Brady and Weil, 2002).

Grain N increased with the application of 0.675 t/ha of coffee husk, but decreased thereafter. At the same time, increased rates of applied N increased grain N content. On average, the highest grain N content of 2.0% was obtained from the highest rate (60 kg N/ha) whereas the lowest grain N content of 1.61% was obtained from the control (0 kg N/ha) (Table 5).

Straw N decreased with increasing rates of coffee husk up to 1.35 t/ha (Table 6). Possible reasons for this reduction might be either a dilution effect or the transfer of N to grain. At the same time, straw N increased with application of 45 and 60 kg N/ha.

Phenology and growth of barley

Results of the analysis of variance for the phenology and growth parameters considered in the study are presented in Table 7. There were no significant effects of coffee husk on days to emergence, days to heading, days to maturity, spike length, and plant

Table 3. Chemical analysis of coffee husk.

Coffee husk state	pH	Organic carbon (%)	Organic matter (%)	Total N (%)	C/N ratio
Un-decomposed	7.50	43.20	74.47	1.36	30
Decomposed	6.51	17.92	30.89	1.01	18

Table 4. The influence of coffee husk (CH) and nitrogen fertilizer on soil pH.

CH (t/ha)	pH at various levels of applied N (kg/ha)					CH Mean
	0	15	30	45	60	
0.000	5.57	6.74	6.29	6.00	5.75	6.07
0.675	5.61	5.61	5.55	5.49	5.31	5.51
1.350	5.90	5.74	5.69	5.70	5.66	5.74
2.025	5.64	5.60	5.66	5.65	5.52	5.61
2.700	5.51	5.54	5.53	5.42	5.42	5.48
N mean	5.65	5.85	5.74	5.65	5.53	

Table 5. The effect of coffee husk (CH) and N fertilizer on total nitrogen content (%) of barley grain.

CH (t/ha)	Total N at different applied N (kg/ha) levels					CH Mean
	0	15	30	45	60	
0	1.45	1.60	1.50	1.65	2.39	1.72
0.675	1.66	2.51	1.80	1.73	1.96	1.93
1.35	1.71	1.56	1.71	2.25	1.77	1.80
2.025	1.65	1.55	1.72	1.73	1.96	1.72
2.7	1.60	1.45	1.65	1.85	1.91	1.69
N mean	1.61	1.73	1.68	1.84	2.00	

Table 6. The effect of coffee husk (CH) and nitrogen on total nitrogen content (%) of barley straw.

CH (t/ha)	Total N content (%) of barley straw at different applied N (kg/ha) levels					CH Mean
	0	15	30	45	60	
0	0.58	0.43	0.53	0.53	0.55	0.52
0.675	0.43	0.37	0.53	0.43	0.53	0.46
1.35	0.52	0.48	0.43	0.47	0.48	0.48
2.025	0.42	0.43	0.48	0.53	0.83	0.54
2.7	0.48	0.53	0.43	0.63	0.48	0.51
N mean	0.49	0.45	0.48	0.52	0.57	

Table 7. Effect of coffee husk (CH) and N fertilizer on phenology and growth of barley.

Treatment	DTE	DTH	DTM	PHT (cm)	SPL	BMS (kg/ha)
CH (t/ha)						
0.000	8.8	78.9	113.7	76.6	5.9	7806
0.675	8.8	78.8	113.7	73.8	5.9	7506
1.350	8.8	78.4	113.6	75.2	5.8	7488
2.025	8.6	78.4	113.4	77.7	6.0	7923
2.700	8.7	78.6	113.9	77.1	6.0	7952
Ni (kg/ha)						
0	8.9	79.6 a	113.7	73.0 c	5.6 c	6923 c
15	8.6	78.5 b	113.5	74.2 bc	5.7 bc	7360 bc
30	8.7	78.1 b	113.4	77.4 ab	5.9 bc	7893 ab
45	8.7	78.0 b	113.6	76.8 ab	6.0 ab	8177 a
60	8.8	78.9 ab	114.1	79.1 a	6.3 a	8321 a
CV%	5.12	2.21	1.03	7.33	7.99	12.16
LSD 5%						
CH	NS	NS	NS	NS	NS	NS
N	NS	1.1	NS	3.52	0.35	593
CH × N	NS	NS	NS	NS	NS	NS

DTE = days to emergence; DTH = days to heading; DTM = days to maturity; PHT = plant height; SPL = spike length; BMS = biomass. NS = not significant. Values in the same column followed by the same letter are not significantly different at 0.05% probability.

height. Similarly, days to emergence was not significantly influenced by N fertilizer, although N fertilization had a significant ($P < 0.05$) effect on days to heading and a highly significant ($P < 0.01$) effect on plant height and spike length. Neither CH nor the interactions with N had significant effect on plant height and spike length.

Plant height increment due to the application of the highest rate of N (60 kg/ha) over the check was about 6 cm, whereas for the highest rate of coffee husk (2.7 t/ha), the increase over the check was only about 0.49 cm (Table 7). An increase of 2.6% (0.16 cm) of spike length was also observed due to the application of 2.025 t CH/ha compared

with the control. At the same time, growth and phenology of barley were not affected by factor interaction (CH \times N).

Yield and yield components of barley

The analysis of variance showed non-significant effects of CH on yield and yield components of barley (Table 8). The yield components (number of fertile tillers, seeds per spike, seed weight, and harvest index) were not significantly affected by application of coffee husk. Similarly, N fertilizer had no significant effect on number of fertile tillers, seeds per spike and seed weight. The effect of nitrogen fertilizer on grain yield of barley was, however, highly significant ($P < 0.01$). Although the effect of CH on yield and yield components was not significant, a slight increase of about 3.1% in seeds per spike and 1.7% (42 kg/ha) increase in grain yield were observed due to the application of 2.025 t CH/ha compared with the control. The yield advantage due to the application of 2.7 t CH/ha over the check was 5% (121 kg/ha) (Table 8). Barley grain yield obtained with an application of 2.7 t N/ha was 2517 kg/ha, which is similar to the amount obtained from the application of 30 kg N/ha (2513

kg/ha) and marginally lower than the yield obtained from the highest nitrogen rate (60 kg N/ha) (Table 8). Similar to this, Ghosh and Sharma (1999) reported that the yield produced with FYM alone was comparable to yield following the application of 20 and 40 kg N/ha. The authors indicated no significant difference in grain yield due to the application of 20 and 40 kg N/ha and those treated with FYM, whereas the maximum yield was produced when FYM application was supplemented with 40 kg N/ha.

Previous experiments conducted at Awassa and Areka proved that coffee husk served as a means of conserving moisture at times of moisture stress and resulted in maize grain yield increases of more than 230 kg/ha under both fertilized and unfertilized conditions (Tenaw and Kelsa, 1998). However, the moisture conservation aspect of coffee husk application was not studied in this experiment.

Russell (1988) reported that in the year of application most of the phosphate and potassium are as effective as phosphate and potassium, whereas only about one quarter to one-third of the nitrogen is as effective as that present in fertilizers. He stated that the incorporation of low quality residue of maize

Table 8. Effect of CH and N on yield (kg/ha) and yield components of barley.

Treatments	TWS	SPS	TGW (g)	GY (kg/ha)	Harvest Index
			CH (t/ha)		
0.000	56	31	49.2	2396	0.306
0.675	53	32	48.9	2354	0.311
1.350	54	32	48.2	2388	0.313
2.025	54	32	48.0	2438	0.307
2.700	56	32	47.7	2517	0.314
			Ni (kg/ha)		
0	51	31	48.6	2154 c	0.307
15	53	32	49.1	2296 bc	0.308
30	55	30	48.6	2513 ab	0.317
45	56	33	48.8	2598 a	0.316
60	57	33	46.9	2531 ab	0.302
CV (%)	22.64	14.46	6.01	16.74	9.15
			LSD 5%		
CH 5%	NS	NS	NS	NS	NS
N 5%	NS	NS	NS	255.2	NS
CH \times N 5%	NS	NS	NS	NS	NS

TWS = tillers with spike, SPS = seeds per spike, TGW = Thousand Grain Weight, GYD = grain yield. NS = not significant. Values in the same column followed by the same letter are not significantly different at 0.05% probability.

stover resulted in the reduction of maize grain yield, which was due to N immobilization of about 4 kg N/ha. Rodell *et al.* (1980) obtained similar result using cattle manure and maize stover in Zimbabwe.

Grain yield showed an increment of 15% (377 kg/ha) due to the application of 60 kg N/ha over the check (Table 8). Amsale *et al.* (1997) also reported significant effect of N fertilizer on grain yield, straw yield, plant height and days to heading of barley. Application of N and P fertilizer improved grain yield by 199% and 62% and 1000-grain weight by 4% and 2.6% in 1987 and 1988, respectively. Straw yield was improved by 72% in 1988 (Amsale *et al.*, 1997). An increase in straw yield with N application on cereals, particularly wheat and barley, is well documented (Jedel and Helm, 1992; Zebarth and Sheard, 1992). Nitrogen increases vegetative biomass, which results in increased straw yields. This is in agreement with the present result.

The application of N did not result in significant difference of seeds per spike and 1000-grain weight. The result of this study agrees with that of Zebarth and Sheard (1992) and Jedel and Helm (1992), who found that increased N rates resulted in increased production of spikes per unit area, which were balanced by reduced kernel weight.

In general, barley growth, yield and yield components were not significantly influenced by factor interaction (CH \times N). However, a biomass and grain yield advantage of 30% (2396 kg/ha) and 31% (916 kg/ha) were obtained over the check due to the combined application of 2.7 t CH/ha and 45 kg N/ha, respectively. The highest grain yield of 2979 kg/ha was obtained from a treatment combination of the highest rate of CH (2.7 tons/ha) and 45 kg N/ha followed by no CH but only 45 kg N/ha application, which gave a grain yield of 2681 kg/ha. At the same time, grain yield of 1848 kg/ha was obtained from the application of 0.675 t CH/ha alone. Neither the main effects of CH and N fertilizer

nor their interaction effect was significant on harvest index. This is in contrast to the result of Jedel and Helm (1992), who reported a decrease in harvest index of barley with increased soil fertility.

Hegde (1997) reported that use of FYM to substitute 50% of N needs increased total production by 10.5% compared with the treatment receiving all the recommended nutrients as inorganic fertilizer alone. The differential response to N substitution from organic sources could probably be related to variations in soil characteristics and temperature regimes (Prasad and Power, 1991; Nambiar *et al.*, 1992).

Association of characters

Spike length, harvest index and biomass were correlated strongly and positively with grain yield ($r = 0.66, 0.57$ and 0.75 , respectively). Plant height was also significantly and positively correlated with spike length, biomass and grain yield. Similarly, spike length showed significant and positive correlation with biomass and grain yield ($r = 0.77$ and 0.67 , respectively) (Table 9). The linear correlation between biomass and grain yield was highly significant ($P < 0.01$) while total soil N was correlated significantly with biomass ($P < 0.05$).

This result is in line with the report of Sinebo (2002) on his study of yield relationships of barley grown in a tropical highland environment. He reported consistent and positive correlation of grain yield with straw yield, plant height and kernels per spike.

Conclusion

Production constraints on barley in Ethiopia are diverse, but poor soil fertility, particularly that of nitrogen, and phosphorus nutrient deficiencies are common. The effect of applying available organic resources and agricultural wastes in improving crop productivity remain poorly understood from the perspective of organic matter dynamics.

Table 9. Linear correlations of growth, yield, yield components and some soil characteristics.

	DTM	TWS	PHT	SPL	SPS	BMS	THW	GY	HI	TSN	SOM
DTM											
TWS	-0.059										
PHT	0.149	0.469*									
SPL	0.031	0.370	0.777*								
SPS	-0.047	0.461*	0.074	0.132							
BMS	0.227	0.427*	0.847*	0.768**	0.306						
THW	0.030	-0.205	-0.248	-0.385	-0.098	-0.13					
GY	0.065	0.268	0.587*	0.665**	0.254	0.751**	-0.042				
HI	-0.144	-0.122	-0.157	0.042	0.010	-0.106	0.095	0.574**			
TSN	0.279	0.099	0.304	0.384	0.309	0.433*	-0.264	0.179	-0.257		
SOM	0.175	0.165	-0.138	0.042	-0.015	0.008	0.168	0.020	0.022	-0.251	
pH	-0.272	-0.090	-0.202	-0.209	-0.360	-0.327	0.185	-0.346	-0.118	-0.118	-0.263

DTM = days to maturity; TWS = fertile tillers; PHT = plant height; SPL = spike length; SPS = seeds per spike; BMS = biomass weight; THW = Thousand Grain Weight; GY = grain yield; HI = Harvest Index; TSN = total soil nitrogen; SOM = soil organic matter. *, ** = significant at 5% and 1%, respectively.

Strategies for improving nutrient management require the assessment of these needs and the evaluation of the organic and inorganic resources required for supplementation. This study was therefore, aimed to study the combined effects of decomposed CH and inorganic N fertilizer on growth and yield of barley on nitisols of Kokate (Wolayita zone, Ethiopia).

Five levels of CH: 0, 0.675, 1.350, 2.025, and 2.7 t/ha (based on 2.2% N in CH) and five levels of N: 0, 15, 30, 45 and 60 kg N/ha, corresponding to 0%, 25%, 50%, 75% and 100% of the recommended nitrogen fertilizer rate (60 kg N/ha) were used in factorial combination. The recommended rate of phosphorus (26.4 kg P/ha) in the form of TSP was applied uniformly to all plots at the time of sowing of barley. The amount of N present in decomposed CH was only 1.01%, and not as reported in the literature (2.2%).

The results indicated that coffee husk alone or combined with nitrogen has no significant effect on either growth or yield of barley. However, the main effect of N was significant ($P < 0.05$) on days to heading, plant height, spike length, biomass and grain yield. In general, grain yield tended to be higher under N treatment than under CH. Although the effect of CH was not significant, a slight increase of about 2.6% (0.16 cm) in spike length and 3.1% seeds per spike,

a biomass increase of 146 kg/ha and a 121 kg/ha increase in grain yield were observed from the highest rate (2.7 t/ha) compared with the control (no fertilizer). Despite non-significant interaction effects of CH \times N, the highest grain yield (2979 kg/ha) was obtained from a treatment combination of the highest rate of coffee husk (2.7 t/ha) and 45 kg N/ha, whereas the lowest grain yield (1854 kg/ha) was obtained from 0.675 t CH/ha alone. Application of coffee husk did not result in any marked difference in soil organic matter content and total soil nitrogen after barley harvest, although the highest soil organic matter content (4.6%) was obtained from a treatment combination of 2.025 t CH/ha and 60 kg N/ha. Although any organic residue effects are more long term, the present study showed that a high rate of CH and 75% of the recommended N fertilizer rate (45 kg/ha) yielded better than other treatments.

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Productivity selection methods for efficiency in barley breeding in Tunisia

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Abstract

The Tunisian barley breeding program was initiated late in 1893. Barley is mostly cultivated by sheep owners, but it is also grown for grain production in the intermediate zones and for hay. The aim of this study was to compare three selection methods (bulk, pedigree and single seed descent) applied in the early generations of barley segregating populations in two different environments, namely a sub-humid environment at Beja and a semi-arid one at Kef. Selection was carried out on the F_1 , F_2 and F_3 generations in three consecutive growing seasons (2000–2003) in Beja and Kef. Biological yield (BY), grain yield (GY), straw yield (SY) and 1000-kernel weight (TKW) were evaluated. Data were subjected to an Additive Main Effects and Multiplicative Interaction (AMMI) model to determine the efficiency of each method of selection for each environment. The results showed that the bulk method was very effective for the selection of TKW in semi-arid environments, while pedigree selection was more efficient in high-input environments. In sub-humid and semi-dry areas, the cross Martin \times Rihane-03 was selected using the pedigree selection methodology as the more efficient selection for biological yield, grain yield and straw yield.

Introduction

Barley is the second major cereal crop in Tunisia. More than 80% of the barley produced in Tunisia is used for feed, with the rest is used for food and malt.

The first crossing program contributed to select varieties adapted to local conditions, in addition to pre-existing landraces such as Souihli, Ardhaoui, Frigui, and Beldi. The introduction from Algeria in 1931 of the six-row cv. Martin was successful (INRAT, 1965). Indeed, sixty years later, cv. Martin still covers around 20% of the area sown to barley (Daaloul, 1996).

Attempts to diffuse two-row barley cultivars date back 40 years, with the introduction of line Barley 552 from Australia and cv. Prior, and later, in 1962, cv. Ceres. These varieties were essentially cultivated for malt, but because Tunisian farmers are used to growing six-row barleys, they soon disappeared from production and seed markets. Rejection of two-row barley by farmers was again confirmed more recently when newer ICARDA varieties were registered in 1985: cvs. Faïz, Roho and Taj (El Faleh *et al.*, 1985). The registration and the marketing in 1987 of Rihane-03, originating from ICARDA, contributed to the achievement of record production, estimated at 8 million quintal, during 1990/91 and 1995/96. Indeed, Rihane-03 maintained the same yield (31 to 32 q/ha) during the two growing seasons compared to other varieties. These results accelerated its adoption by farmers, and in the next 10 years, it extended to 40% of the barley area, compared with 40% cultivated with the local barley landraces and 20% sown to Martin (Daaloul, 1996). In the drought of 1996–97, Rihane-03 achieved a good yield; it is resistant to lodging, but is susceptible to

powdery mildew and net blotch. Improved cultivars have contributed effectively to grain yield and production records in the 1990s.

Our study investigated the merits of three breeding methods: pedigree (Ped), bulk (Bk) and single seed descent (SSD). A complete diallel cross (6×6) was sown in April 2000 at Beja. The F_3 generations were planted in Beja and Kef in 2002/03 using a split design using the three selections methods. Biological yield (BY), grain yield (GY), straw yield (SY) and 1000-kernel weight (TKW) were evaluated. AMMI analysis was used to estimate the most efficient selection and breeding method across locations and genotypes to be adopted for the relevant environment.

Materials and methods

Plant material and experimental design

A complete diallel cross (6×6) was sown in 1999/2000 using six local or improved, two- and six-row barley cultivars (Table 1). Parents were chosen on the basis of long history grown variety, a new improved successful grown variety, local landrace and introduced material. These cultivars characteristics are presented in Table 1.

The F_1 s were planted in Beja in 2000/01. The F_1 trial used a randomized complete block design (RCBD) with 3 replications, from which 19 derived crosses were selected within 30 bulked F_1 hybrids rows. These were replicated as F_2 offspring in a RCBD, adding the six parents in two locations at Beja and Kef in 2001/02. Selection was done in the F_2 crosses as Single Seed

Descent (SSD), Pedigree (Ped) and Bulk (Bk), generating three sets of material: 19 F_3 Pedigree inbred lines; 19 F_3 SSD inbred lines and 19 F_3 Bulk inbred lines. The three sets of material were grown in a split plot design with three replicates to explore the genotype \times environment interaction within the three breeding methods. F_3 inbred lines from a comparative screening trial are represented in Table 2. The seeding rate was a mixture of one spike kernels per genotype for the pedigree method, a two-gram mixture of kernels per genotype for the bulk method and 40 kernels of 40 spikes per genotype for SSD method viable seeds per each two lines of 2.5 m. The F_3 trial was conducted in 2002/03 at the INRAT experimental stations of Beja (37° N, 9° E, 165 masl) and Kef ($36^\circ 14'$ N, $08^\circ 27'$ E, 518 masl).

Field evaluation

Parameters evaluated included: biological yield (BY), grain yield (GY), straw yield (SY) and 1000-kernel weight (TKW).

Statistical analysis

Statistical analyses were carried out using GenStat (Genstat 4 Committee, 2004). Genotype \times Selection method interaction was partitioned according to the additive main effects and multiplicative interaction (AMMI) model, as proposed by Zobel *et al.* (1988). The magnitude of the selection response was also determined using AMMI model. AMMI analysis combines analysis of variance and principal component analysis into a single model with additive and multiplicative parameters (Zobel *et al.*,

Table 1. Origin, code and nature of parents

Cultivar or landrace	Code	Nature	Origin	Spike type	Use
Martin	A	Algerian local cultivar (introduced in 1931)	Tunisia	six-row	Food/Feed
Taj	B	New improved cultivar	Tunisia	two-row	Food/Malt
Ardhaoui	C	Local Tunisian barley landrace	Tunisia	six-row	Food/Feed
Sahli	D	Local Tunisian barley landrace	Tunisia	six-row	Food/Feed
Rihane-03	E	New improved cultivar	ICARDA	six-row	Food/Feed
Salmas	F	Introduced improved cultivar	ICARDA	two-row	Malt

Table 2. F₃ inbred lines from the comparative screening trials at Beja and Kef, Tunisia.

No.	Code	Cross
1	AB	Martin (6-row) × Taj (2-row)
2	AC	Martin (6-row) × Ardhaoui (6-row)
3	AD	Martin (6-row) × Sahli (6-row)
4	AE	Martin (6-row) × Rihane-03 (6-row)
5	AF	Martin (6-row) × Salmas (2-row)
6	BA	Taj (2-row) × Martin (6-row)
7	BD	Taj (2-row) × Sahli (6-row)
8	BE	Taj (2-row) × Rihane-03 (6-row)
9	BF	Taj (2-row) × Salmas (2-row)
10	CA	Ardhaoui (6-row) × Martin (6-row)
11	DA	Sahli (6-row) × Martin (6-row)
12	DB	Sahli (6-row) × Taj (2-row)
13	DE	Sahli (6-row) × Rihane-03 (6-row)
14	EA	Rihane-03 (6-row) × Martin (6-row)
15	EB	Rihane-03 (6-row) × Taj (2-row)
16	ED	Rihane-03 (6-row) × Sahli (6-row)
17	EF	Rihane-03 (6-row) × Salmas (2-row)
18	FA	Salmas (2-row) × Martin (6-row)
19	FB	Salmas (2-row) × Taj (2-row)
20	A	Martin (6-row)
21	B	Taj (2-row)
22	C	Ardhaoui (6-row)
23	D	Sahli (6-row)
24	E	Rihane-03 (6-row)
25	F	Salmas (2-row)

1988). AMMI first computes the additive main effects for genotypes and environments, and then analyzes the interaction by principal components analysis (Gauch, 1992). A principal components model, fitted to the residuals from the ANOVA, and the resulting scores, called the IPCA (I for interaction) are calculated for both the genotypes and the environments (Gauch, 1992).

Table 3. Scores of the first four AMMI selections with selection methods at Beja, Tunisia, for biological yield (BY), grain yield (GY) and straw yield (SY).

Location	Selection method	Rank	Score	First four AMMI selections**			
BY	Bk	3	-14.644	B	EB	EF	BD
	Ped	1	23.534	AD	DB	BE	AE
	SSD	2	-8.889	BA	AE	E	BF
GY	Bk	2	-3.208	EB	DB	DE	B
	Ped	1	16.020	AD	AE	EA	BE
	SSD	3	-12.811	E	DA	BA	FB
SY	Bk	1	17.253	AF	BD	C	B
	Ped	3	-15.031	DB	EF	AD	BE
	SSD	2	-2.223	BA	AE	AF	BF

Selection methods are Bk = Bulk; Ped = Pedigree; SSD = single seed descent. Lines are A = Martin; B = Taj; C = Ardhaoui; D = Sahli; E = Rihane-03; F = Salmas.

Results and discussion

Advanced derived lines showed better adaptation to stressed environments within improved selection methodologies. The AMMI model generated predictive optimizing selection methods for agronomic characters such as GY, BY, SY and TKW.

Tables 3 and 4 show the selection method scores and the first four AMMI selections per method for agronomic characters at Beja and Kef, respectively.

The results showed the significant efficiency of pedigree selection methodology applied in both sub-humid (Beja) and semi-dry (Kef) locations. These results have been clearly demonstrated, especially in Beja, where IPCA-AMMI scores showed high grain yield (16.02) and biological yield (23.5), while in Kef, IPCA-AMMI scores showed that the first four AMMI selections were at least the parents. Indeed, these results indicate that pedigree selection is more efficient in a high-input environment where predicted genotype offspring should be developed toward greater yield and also included high product quality. In fact, the first cross-selection in the sub-humid area is already AD (Martin × Sahli). Martin, as female parent, showed all the characteristics of a good combiner by transferring to its offspring its adaptive sub-humid traits, inherited from its selection history, as the result of mass selection from an Algerian population in Lakhuat, Tunisia, in 1931.

Table 4. Scores of the first four AMMI selections within selection methods at Kef, Tunisia, for biological yield (BY), grain yield (GY) and straw yield (SY).

Location	Selection method	Rank	Score	First four AMMI selections**			
BY	Bk	3	-30.718	AB	BF	FB	DB
	Ped	1	36.541	F	AE	B	FB
	SSD	2	-5.823	BF	AB	FB	C
GY	Bk	3	-20.962	AB	FB	BF	E
	Ped	1	21.495	A	B	C	AE
	SSD	2	-0.533	AE	FB	C	AD
SY	Bk	3	-22.283	AB	BF	C	DB
	Ped	1	31.080	F	AE	ED	FB
	SSD	2	-8.796	BF	AB	F	FB

Selection methods are Bk = Bulk; Ped = Pedigree; SSD = single seed descent. Lines are A = Martin; B = Taj; C = Ardhauoi; D = Sahli; E = Rihane-03; F = Salmas.

In the sub-humid and semi-dry areas, the cross AE (Martin \times Rihane-03) has been selected following the pedigree selection methodology as the more efficient AMMI selection for biological yield, grain yield and straw yield (Tables 3 and 4). It had been demonstrated that cv. Martin as a female parent could confer the productivity traits for non-stressed environments, whereas Rihane-03 as a male parent confers its wide adaptability to derived lines.

TKW as a main yield component has been AMMI selected as a bulk parameter yield determinant in semi-arid areas and as pedigree parameter yield determinant in sub-humid areas. We note that genotype AB, derived from Martin \times Taj has been selected in the pedigree method as a promising genotype, but its reciprocal BA (Taj \times Martin) has been selected in all the three selection procedures. This can be explained by the row type (Taj is a two-row cultivar) that can develop homogenous plump grains, especially in sub-humid areas.

Martin has been selected as female parent for semi-dry areas following the bulk selection methodology (Table 5).

Finally, the best selection criteria depend on selection methodologies suitable for the target environment. The best bulks were significantly superior for TKW in an unpredictable semi-dry location. Pedigree procedure should identify the best inbred lines for grain yield and biological yield in high-input environments. Plant selection and head row selection within the best families were efficient procedures to achieve significant genetic gain in non-stressed locations. The poor performance of the SSD method is explained by its inefficiency before the F₅ generation. Single seed procedures did not exploit all the environment variation, in contrast to pedigree and bulk methods.

Conclusion

Our results show that multivariate analysis is an effective tool in identifying the best barley genotypes for crossing and selection.

Table 5. Scores of the first four AMMI selections within selection methods at Beja and Kef for TKW.

Location	Selection method	Rank	Score	First four AMMI selections**			
Beja	Bk	3	-1.595	EB	BA	BE	BD
	Ped	1	2.363	BA	DB	B	AB
	SSD	2	-0.768	BA	EB	BE	DB
Kef	Bk	1	2.170	AB	EF	AF	BA
	Ped	2	0.372	AF	EF	EB	FB
	SSD	3	-2.542	AB	EB	AF	BE

Selection methods are Bk = Bulk; Ped = Pedigree; SSD = single seed descent. Lines are A = Martin; B = Taj; C = Ardhauoi; D = Sahli; E = Rihane-03; F = Salmas.

The AMMI model has generated a predictive optimizing selection method for agronomic characters. In comparison with bulk and SSD, the pedigree method of breeding was more efficient in favorable areas. A complete diallel crossing strategy enables the breeder to decide on male versus female parents in the crossing program.

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Session 12

Success stories

Cloning of barley genes known only by their phenotype

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Introduction

One of the great success stories of the barley molecular era is that we have developed the tools, technology and knowledge to enable the cloning from the large barley genome of genes that are known only by their phenotype. Since most, or perhaps all, of the genes relevant to the unique functions of barley and agriculture are known only by their phenotype, this is an extremely important accomplishment. The story, of course, is not complete, since we still have a long way to go to make gene cloning easy and affordable; nevertheless, what we have accomplished is a great success story. Instead of dwelling on the technology or tools, I will take a look back at the history of this development, discuss research from my laboratory relevant to the topic, and comment on future prospects.

There are only a few ways that genes known only by their phenotype can be cloned. These are: (1) map-based cloning; (2) transcript-based cloning; and (3) transposon insertion mutant cloning. We have made progress on all of these fronts, but only map- and transcript-based cloning have tangible results so far.

A bit of history

Map-based cloning depends first and perhaps foremost on the availability of maps. Barley molecular map development started with our publication in 1988 (Kleinhofs *et al.*, 1988) of the molecular mapping of the barley nitrate reductase genes, which we had previously cloned by a simpler technique based on the

availability of protein sequences or antibodies. This was followed by a publications from Tom Blake's group in 1990 (Shin *et al.*, 1990) and from the Schulze-Lefert group in 1991 (Hinze *et al.*, 1991). However large-scale efforts came later (Graner *et al.*, 1991; Heun *et al.*, 1991; Kleinhofs *et al.*, 1993). The maps developed were primitive by today's standards, but they, particularly the Steptoe \times Morex map produced by the North American Barley Genome Mapping Project, serve as the foundation on which most maps are built today.

These maps, although limited, were the starting point for development of high-resolution maps for specific traits and their utilization for map-based cloning. The first map-based cloning success reported was of the *Mlo* gene (Buschges *et al.*, 1997). This was not only a great technical achievement, but also the *Mlo* gene has turned out to be very interesting and unique in the plant disease resistance gene world. Cloning of the *Mlo* gene also demonstrated a unique solution to the problem of confirming that the candidate gene cloned is actually the one you wanted to clone. The availability of large numbers of *mlo* mutants was very important in this case. The mutants were used for allele sequencing and also for a unique experiment demonstrating recombination within the gene and the isolation of a wild-type recombinant.

At this early stage in barley molecular genetics development, a mere 10 years ago, most barley scientists did not have access to a yeast artificial chromosome (YAC)

or bacterial artificial chromosome (BAC) libraries, and thus no means of developing BAC or YAC clone physical contigs that would lead to a physical map and an opportunity to identify potential candidate genes. The Keygene barley YAC library was not available to the general scientific public.

When we started our quest to clone the stem rust resistance gene *Rpg1*, we expected that we would be able to exploit rice-barley synteny to facilitate the cloning. This expectation was reasonable, given that the *Rpg1* gene was located near the telomere of barley chromosome 1(7H) and that this chromosome had synteny with rice chromosome 6, which was the first chromosome targeted for sequencing by the Japanese Rice Genomics group. Diligent synteny work and sequence analysis, however, soon made us realize that this was not going to happen and we set about developing the first and still the foremost barley BAC library freely available in the public domain (Yu *et al.*, 2000). Although it fell upon me to acquire the funding from various sources (not including any genome grants – which did not exist at the time), several people, in particular due to their diligent efforts and cooperation, made it possible. For the funding, I want to particularly single out the Tritici Mapping Initiative, which donated the first US\$10 000, followed by the North American Barley Genome Mapping Project, Anheuser Busch and many other smaller donations. Among the people who made this possible, I particularly want to single out my technician, Dave Kudrna, who prepared the plant material and traveled to Clemson University to assist in the arraying of the library, and Rod Wing's laboratory, who did most of the work at cost

Barley-rice synteny

Barley (wheat)-rice synteny has been studied at the macro- and micro-levels in numerous laboratories. It was expected to play a major role in barley gene cloning, and it has, to the

extent that it provides multiple molecular markers useful for defining the region of interest in high-resolution genetic maps. However, there are only a few examples of finding rice gene homologues of the barley gene of interest in the syntenic region. The cloning of the *Ror2* gene is one example of direct exploitation of barley-rice synteny (Collins *et al.*, 2003). An excellent example of exploiting barley-rice synteny was provided by the cloning of a major quantitative trait locus (QTL) for (1,3;1,4)-beta-D-glucan content in barley (Burton *et al.*, 2006). This is the one and only example, so far, of cloning of a barley QTL. This QTL, mapped earlier (Han *et al.*, 1995), appears to encode a family of cellulose synthase-like genes. Synteny with wheat has provided a much easier route to cloning barley genes, but there are not that many wheat genes that have been cloned to date.

Unique barley genes

Roger Wise's laboratory succeeded in cloning the powdery mildew resistant *Mla* gene cluster, exploiting the Keygene YAC and the public cv. Morex BAC large insert libraries (Wei *et al.*, 1999). The *Mla* gene cluster consists of typical NBS-LRR genes. A clever single-cell transient assay (Shirasu *et al.*, 1999a) was used to identify and clone specific genes from the *Mla* cluster (Haltermann *et al.*, 2001).

An interesting barley gene, required for resistance to powdery mildew, was initially identified as a mutant (Jorgensen, 1996). The gene, designated *Rar1*, was cloned and shown to contain two tandem Cysteine and Histidine-Rich Domains (CHORDs) defining a novel eukaryotic Zn²⁺-binding protein (Shirasu *et al.*, 1999b). This gene has turned out to be an important regulator of several disease resistance genes and is conserved not only in plants, but also in protozoa and metazoa. The cloning of the *Rar1* gene led to the identification of another important gene, *Sgt1*, which functions in SCF-mediated protein ubiquitylation (Austin *et al.*, 2002).

Map-based cloning in barley has led to the identification of genes that are unique and not previously identified, even in highly cloned organisms such as *Arabidopsis*. Besides *Rar1*, that list includes *mlo* and the stem rust resistance genes *Rpg1*, *Rpg5* and *rpg4* that we have cloned, and the barley gene *HvHox1* that determines the tow- vs six-row barley spike phenotype (Komatsuda *et al.*, 2007).

The barley stem rust resistance genes

The first barley gene cloned entirely in the public sector using the cv. Morex BAC library was our stem rust resistance gene *Rpg1* (Brueggeman *et al.*, 2002). *Rpg1* turned out to be a unique protein kinase with two tandem kinase domains, one a functional kinase and the other a pseudokinase (Nirmala *et al.*, 2006). The pseudokinase does not function in autophosphorylation, but is required for *Rpg1*-mediated stem rust resistance. This requirement was demonstrated by mutating the two adjacent lysines (K) at position 152

and 153 to asparagines (N) and glutamine (Q), respectively, in the kinase 1 domain and mutating two lysines KK-461,462-NQ in the kinase 2 domain. The kinase 2 domain mutation abolished autophosphorylation, indicating that it is a functional kinase, but the kinase 1 mutation did not alter the RPG1 autophosphorylation activity, indicating that it is a pseudokinase. Mutations in both or either kinase domain were not able to support *Rpg1*-mediated stem rust resistance, indicating that both are required for disease resistance (Figures 1 and 2).

The function of the kinase 1 (pseudokinase) domain is not clear, but we speculate that it is involved in protein-protein interactions essential for the disease resistance signaling pathway initiated by the *Rpg1* gene. Such interactions are demonstrated by the yeast two-hybrid assay, which showed that the KK-152,153-NQ mutant failed to interact with the *HvPTI1* and other proteins (Figure 3). The wild type RPG1 protein interacts with *HvPTI1* in the yeast two-hybrid assays and presumably

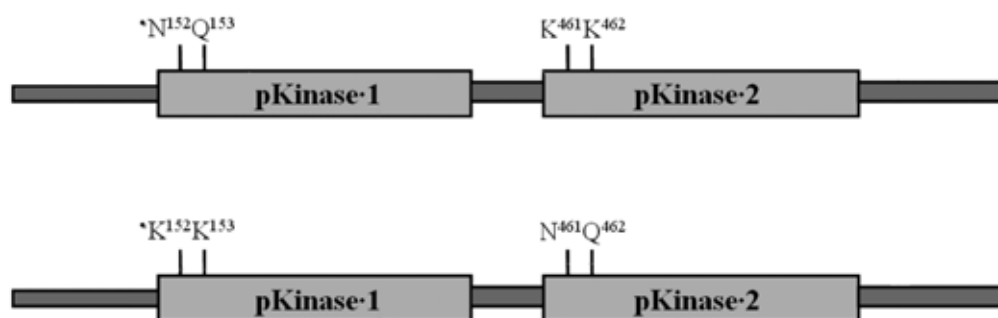


Figure 1. The wild-type RPG1 protein consists of two tandem protein kinase-line domains. It has conserved lysine (K) residues at positions 152, 153, 461 and 462. In order to investigate the function of the two kinase domains, we mutated the conserved lysines to asparagines (N) and glutamine (Q) in each domain. These mutants were then used to study autophosphorylation. They were also transformed into the susceptible cultivar Golden Promise and assayed for RPG1 degradation and ability to confer *Rpg1*-mediated resistance to stem rust (*Puccinia graminis* f.sp. *tritici*) pathotype MCC. Results showed that only protein kinase 2 domain was functional in autophosphorylation and protein kinase 1 was a pseudokinase. However, both mutants failed to degrade the RPG1 protein as is observed with the wild-type RPG1 (data not shown) and both failed to confer resistance to pathotype MCC (Figure 2).

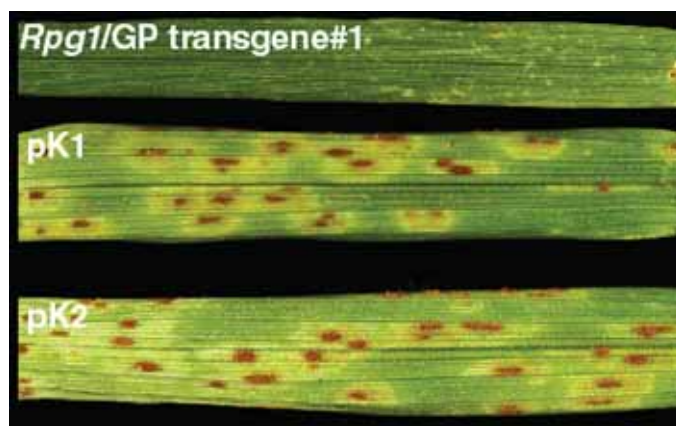


Figure 2. *Rpg1*-mediated stem rust resistance. Wild-type and mutant *Rpg1* genes were transformed into cv. Golden Promise and tested for resistance or susceptibility to *Puccinia graminis* f.sp. *tritici* pathotype MCC. The wild-type cv. Golden Promise is highly susceptible (not shown), but when transformed with the wild-type *Rpg1* gene it becomes highly resistant (left leaf). Golden Promise transformed with pK1 and pK2 mutants (described in Figure 1) are highly susceptible, showing that both kinase domains are essential for disease resistance.

indicates that this interaction is essential for the disease resistance function.

We also demonstrated that the RPG1 protein is rapidly degraded upon infection of the barley plant with the stem rust fungus (Nirmala *et al.*, 2007). The RPG1 protein degradation was blocked by mutations in both kinase 1 or kinase 2 domains (Figure 1). The KK-461,462-NQ mutation in the kinase 2 domain blocks autophosphorylation and therefore presumably phosphorylation of critical interacting proteins, resulting in loss of function of the *Rpg1*-mediated disease resistance signaling pathway. Why the KK-152,153-NQ mutation in the pseudokinase 1 domain blocks the RPG1 protein degradation is not clear. The role of the RPG1 protein degradation in the disease resistance is also not clear. We speculate that the RPG1 protein acts as a negative regulator of a complex of proteins involved in the disease resistance-signaling pathway. Degradation of RPG1 releases this complex from the negative

regulation and allows it to function. However, the lack of the RPG1 protein *per se* is not sufficient to induce disease resistance signaling, even when all the other components are present. This is illustrated by the cultivar Golden Promise, which is completely lacking the *Rpg1* gene, but is highly susceptible to stem rust. However, when Golden Promise is transformed with an intact *Rpg1* gene driven by its own promoter, it is rendered highly resistant to stem rust (Horvath *et al.*, 2003). This clearly indicates that all the components needed for functional stem rust resistance are present in Golden Promise except the *Rpg1* gene. Thus, the RPG1 protein is a negative and a positive regulator of the stem

rust disease resistance-signaling pathway.

We are just beginning to learn what other proteins are essential for the *Rpg1*-mediated stem rust resistance function. The yeast two-hybrid assay identified the Pto interactor Pti1 as potentially involved with *Rpg1*-mediated resistance. The barley homologue, designated HvPTI1, is a strong interactor with RPG1 in the yeast two-hybrid assay, but we have not been able to demonstrate its requirement for disease resistance in the virus-induced gene silencing (VIGS) assay. There are several reasons possible for this, the most straightforward interpretation being that HvPTI1 is not involved. This seems unlikely because, in addition to the interaction observed in the yeast two-hybrid assay, we have also shown that HvPTI1 is trans-phosphorylated by RPG1 *in vitro*. Other explanations may be that the HvPTI1 protein may be required in small quantities and the VIGS assay simply does not knock

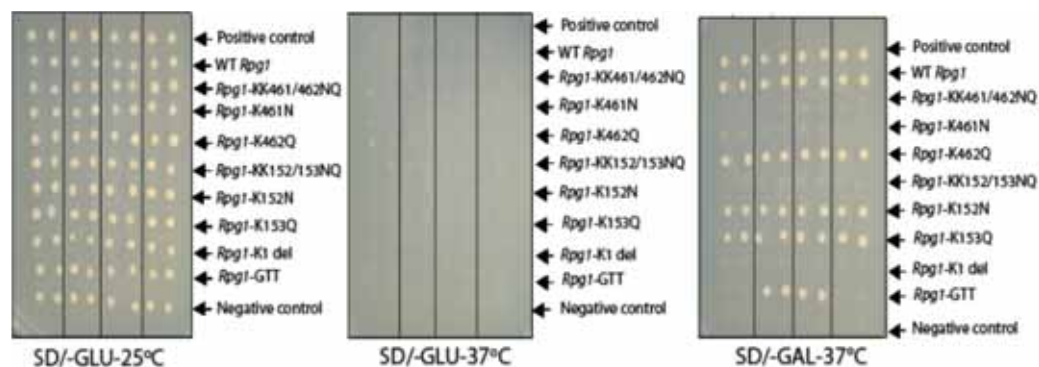


Figure 3. Yeast two-hybrid assay for protein-protein interaction. A full-length *Rpg1* cDNA (Horvath *et al.*, 2003) was cloned into the pSos vector (Stratagene, CA) at the *NotI* site of the multiple cloning site so as to express RPG1 as a fusion protein with hSos. A barley cDNA library was constructed in the pMyr vector and screened according to the manufacturer's recommendations. Left panel shows yeast cells grown in glucose at 25°C (everything should grow), middle panel are yeast cells grown in glucose at 37°C (nothing should grow), right panel are yeast cells grown in galactose at 37°C (only cells containing physically interacting proteins should grow). Data shows that wild-type RPG1 and mutants K462Q, K152N, K153Q (do not inactivate autophosphorylation) do not disturb RPG1 interaction with the protein listed above the panel (PTI1, Myb factor, RIN4, and HSP17). Mutants that disrupt autophosphorylation (KK461,462NQ and K461N) and mutants with unknown function (KK152,153NQ, RPG1-pK1 deletion) do not interact with the shown proteins. An interesting case is RPG1-GTT mutant. This mutant has a GTT insertion in the pK1 domain resulting in a serine (S) to arginine (R) conversion plus insertion of a phenylalanine (F). The resulting mutant is susceptible to pathotype MCC infection and fails to interact with PTI1 and HSP17 in the yeast two-hybrid assay. These data indicate that the PTI1 and HSP17 interactions are important for Rpg1-mediated resistance, but do not exclude the importance of the other interactions as well.

down the mRNA sufficiently for the plant to show a phenotype.

Other potential players in the *Rpg1*-mediated stem rust resistance pathway have been discovered by mutagenesis. A mutation resulting in susceptibility to stem rust was discovered in a fast-neutron irradiated M_2 field of *Rpg1*-containing, stem rust resistant, cv. Morex. The gene responsible was designated *Rpr1*, for Required for *Puccinia graminis* Resistance. *Rpr1* was cloned by transcript-based cloning, the first demonstrated case of this methodology in barley (Zhang *et al.*, 2006). The *Rpr1* gene encodes a serine/threonine-specific protein kinase. It apparently functions downstream of the *Rpg1* action because it does not interact with RPG1 in the yeast two-hybrid assay.

A map-based cloning approach succeeded in isolating two other stem rust resistance genes in my laboratory. The stem rust resistance genes *Rpg5/rpg4* conferring resistance to *Puccinia graminis* f.sp. *secale* isolate 92-MN-90 and *P.g.* f.sp. *tritici* pathotype QCC, respectively, are very closely linked on chromosome 7(5H) long arm. Based on recombinants selected from >5000 gametes, we identified two BAC clones that span the region and define the *Rpg5/rpg4* locus to a 70 kbp DNA segment. The region was sequenced from the susceptible cv. Morex BAC clones identifying 5 possible candidate genes. Sequencing of the same genes from the resistant cv. Q21861 identified that one of the RGA-like genes was truncated in Morex by an insertion/deletion (indel) event. This

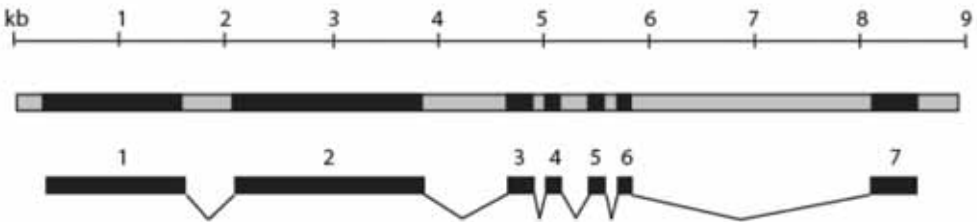
gene turned out to be the correct candidate gene, based on elimination of all the other candidate genes by allele sequencing and high-resolution genetic mapping. Allele sequencing of susceptible alleles and VIGS analysis further supported the conclusion that this is the correct *Rpg5* gene.

The *Rpg5* gene encodes a resistance (R) protein with a unique structure consisting of a nucleotide binding site (NBS), leucine rich region (LRR) and serine/threonine protein kinase (S/TPK) all in one very large gene (Figure 4). The gene spans approximately 9 kbp of genomic DNA and encodes a 4.8 kbp mRNA. The mRNA encodes a predicted 1378-amino-acid protein. The protein kinase domain is essential for the gene to function in stem rust resistance, since cvs. with intact NBS-LRR domains, but lacking the S/TPK domain, are susceptible to the rye stem rust isolate 92-MN-90. The combination of NBS-LRR and protein kinase domains for functional disease resistance was

not unexpected, since two other previously characterized protein kinase R-genes, *Pto* and *PBS1*, require interaction with NBS-LRR genes, *Prf* and *RPS5*, for functional disease resistance. However, this is the first reported case of all three domains being found on the same disease resistance gene. In fact, a search of the *Arabidopsis* and rice databases failed to identify a single annotated gene with a similar structure. It is interesting how frequently the cloning of barley genes have resulted in the identification of gene structures that have not previously been reported.

High-resolution mapping and numerous recombinants identified the actin depolymerizing factor gene *HvAdf2* as the probable *rpg4* gene (Brueggeman *et al.*, 2008). The *rpg4* gene encodes a 147-amino-acid protein. The role of *Adf* genes in cytoskeleton re-arrangements is well documented (Remedios *et al.*, 2003; Wasteneys and Galway, 2003) and have been

A. gene structure



B. protein structure

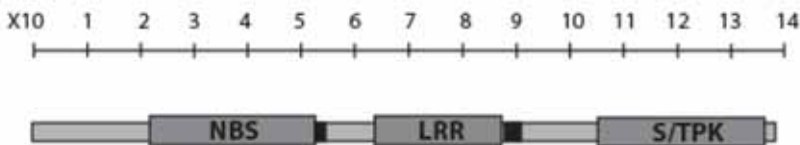


Figure 4. *Rpg5* predicted gene and protein structure. The *Rpg5* gene, conferring resistance to rye stem rust isolate 92-MN-90 is predicted to have 7 exons and encode a protein with the three disease resistance gene domains, i.e. nucleotide binding site (NBS), leucine rich repeats (LRR) and serine/threonine protein kinase (S/TPK). It is also predicted to encode two transmembrane domains identified as black bars in Figure 4B.

reported to also function in non-host disease resistance (Kobayashi *et al.*, 1997; Yun *et al.*, 2003). However, this is the first case where an *Adf* gene is implicated in gene-for-gene resistance. It is possible that the *rpg4* gene functions in cooperation with the *Rpg5* gene and it is the latter that provides the pathotype-specific recognition.

Besides the *Rpg5* and *rpg4* genes, the locus encodes one more *R*-like NBS-LRR gene and another *Adf* gene. Their function is unknown. The *Rpg5/rpg4* locus consists of an approximately 70 kbp genomic sequence and does not undergo recombination. The lack of recombination is probably due to the presence of the insertion/deletion event at the distal end of the cluster. In addition to the identified resistances to wheat stem rust pathotype QCC (*rpg4*) and rye stem rust isolate 92-MN-90 (*Rpg5*), this locus also confers resistance to the wheat stem rust pathotype MCC and race TTKS (Ug99) (Figure 5) (Steffenson *et al.*, unpublished). The Ug99 resistance is particularly interesting since this is a new race that is spreading rapidly and is highly virulent on most of the cultivated barley and wheat. Thus the *Rpg5/rpg4* locus is unique and highly interesting and needs to be understood and exploited for multiple stem rust resistance breeding.

Transcript-based gene cloning

Transcript-based cloning promises to provide a quicker and less expensive way than map-based cloning to isolate genes known only by their phenotype. Transcript-based cloning takes advantage of micro-array technology and mutants using the Barley1 micro-array that has been developed to facilitate barley molecular genetics work. Ability to use transcript-based cloning depends on the availability of mutants, preferably induced by fast neutron or gamma irradiation, or some other treatment that induces deletions. Deletions are not absolutely required, since

some stop codon mutants will also result in much reduced transcript abundance, but deletions are almost guaranteed to do so. Besides mutants, one needs a micro-array containing most or all, if possible, of the genes expressed by the wild-type organism. Comparison of the wild-type transcript profile with that of the mutant identifies genes whose

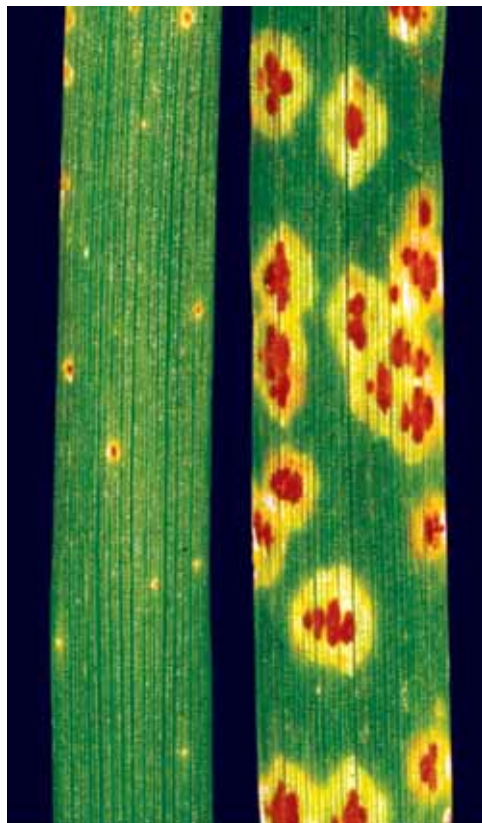


Figure 5. Resistance in barley to the stem rust race TTKS (Ug99). The Ug99 stem rust race was first discovered in Uganda and is virulent on most of the commercial wheat and barley cultivars grown worldwide. The parents Q21861 (left) and SM89010 (right) and their doubled-haploid progeny were evaluated against Ug99. Q21861 is highly resistant and the Ug99 resistance mapped to the *Rpg5/rpg4* genomic interval. The *Rpg5* gene is probably involved in this resistance, but further work is required to verify it.

expression is highly down- or up-regulated in the mutant. For transcript-based cloning we are interested in the highly down-regulated genes, which become the candidate genes for the phenotype in question. The candidate genes identified on the micro-array need to be further characterized by DNA and RNA PCR, Southern blotting, allele sequencing of wild-type and mutant alleles, VIGS analysis and stable transformation, if possible. One or more of these techniques will allow a highly probable conclusion as to which candidate gene is the correct one.

Summary and future prospects

In summary, many tools and technologies needed to be developed for barley to allow us to progress to the stage where cloning of genes known only by their phenotype becomes possible. Perhaps most important among these has been the development of a BAC library, EST sequences, a micro-array, and, of course, the ability to sequence large genome regions for a reasonable cost. Although barley workers were not involved in the improvement of sequencing technology, they stand to benefit tremendously from the recently developed and rapidly becoming available 454 sequencing technology. This is particularly important for barley workers since they work with a very large genome and mostly with very limited funds. The development of tools and technologies for gene cloning allowed us to isolate interesting plant disease resistance genes and to study their function. These studies will eventually result in the development of improved barley cultivars.

Although we have come a long way, the way ahead is also long, and may be difficult. Today we can theoretically clone any gene that we can identify by phenotype. However, it requires the ability to carry out very precise mapping and phenotyping. This is still very difficult for the quantitative traits (QTLs) that

represent by far the greatest majority of traits of economic and agricultural importance. What will it take to facilitate QTL cloning? From my point of view, it will primarily be the ability to quickly and inexpensively sequence large regions of the genome. Such sequencing may become more approachable by the completion of the barley genome sequence (or at least the gene-rich regions). One sequence by itself does not completely solve the problem, as we have experienced. Often insertion/deletion regions scramble the adjoining sequences and make it impossible to sequence through by PCR based on primers designed from the lone sequence. Additional lambda or BAC libraries may need to be made from the cv. carrying the desired gene in order to fully sequence the allelic region. These are, however, minor obstacles in comparison with the lack of at least one complete barley genome sequence. Hopefully we will hear about progress toward a barley genome sequence at this conference. If it is forthcoming, it will provide yet another milestone in our quest for the ability to clone barley genes that are known only by their phenotype.

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Breeding for resistance to barley leaf rust in a highly favorable environment for diseases

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Abstract

Barley leaf rust, caused by *Puccinia hordei*, has been present generally at low incidence for over 20 years in Uruguay. During 2005 and 2006, the disease reached epidemic levels, causing heavy losses in grain yield and grain size in susceptible cultivars if chemical control was not used. The increase in importance of the disease was associated with the appearance of a new race of *P. hordei*, virulent to widely grown barley cultivars. The *P. hordei* population is relatively stable in the Southern Cone of America, only three races having been identified since 1995. The third race acquired virulence on *Rph9.z*, which is probably present in Defra-derived cultivars, widely used in Uruguay and Brazil. Sources of resistance, mostly adult plant resistance identified since 1999, have been routinely used in crosses in the barley breeding program of the Uruguayan National Institute for Agronomical Research (INIA) for the last 10 years. Combining information from field nurseries and seedling infection type data allowed identification of highly effective resistance, expressed as low levels of disease under heavy leaf rust epidemic challenge. The resistance of these materials derives from European germplasm. The number of resistant advanced lines increased significantly after three cycles of selection for resistance under heavy disease pressure during 2004–2006, which indicates that an important genetic advance in breeding for leaf rust resistance was achieved.

Introduction

Barley is planted on 140 000 ha in Uruguay. Spring types are sown in late autumn and early winter (June–July) and harvested in late spring (mid-November–December). Only malting types are used, since over 90% of production is exported as malt. Average yield is 2.4 t/ha, limited mostly by elevated temperature during grain filling and excess precipitation (1000 mm/yr), which also favors the development of many pathogens. The most prevalent diseases are spot blotch (*Bipolaris sorokiniana*) and net type net blotch (*Pyrenophora teres* f. sp. *teres*). However, scald (*Rhynchosporium secalis*), *Fusarium* head blight (*Fusarium* spp.), spot type net blotch (*Pyrenophora teres* f. sp. *maculata*), powdery mildew (*Blumeria graminis* f. sp. *hordei*) and leaf rust (*Puccinia hordei*) are sporadically very important.

Leaf rust has been present with low incidence and severity in commercial fields since the early 1970s, when a severe leaf rust epidemic occurred. After more than 30 years, leaf rust was again observed at early tillering during 2005, heralding a severe leaf rust epidemic (Germán, 2006). A similar epidemic occurred during 2006 (Germán, 2007). Both epidemics were associated with the appearance of a new race of *P. hordei*, virulent on most commercial cultivars used in Uruguay (Germán *et al.*, 2005) and the widely grown cultivar BRS195 used in Brazil. The new race was first detected in 2004 in samples from the Uruguayan National

Institute for Agronomical Research (INIA), La Estanzuela Experimental Station, where unusually high infections were observed.

Losses in susceptible cultivars estimated during 2006 were as high as 66% in yield, 89% in yield of plump grains (grains >2.5 mm) and 73% in the percentage of plump grains (Castro *et al.*, 2009). In severely affected variety trials, the coefficient of infection (CI) of leaf rust had a negative association with yield, yield of plump grains, percentage of grains >2.5 mm and a positive association with lodging and straw breakage. Leaf rust CI explained from 12 to 54% of the variation of these traits when the correlation was significant (Germán *et al.*, 2007). Due to the high losses caused by the disease, the use of fungicides increased from an average of 0.7 treatments per ha prior to 2005, to 1.3 treatments per ha in 2005. Even when leaf rust was efficiently controlled by fungicides when these were applied according to recommendations, crops of susceptible cultivars required two, and in some cases three, fungicide treatments.

During the epidemic years 2005 and 2006, it was clearly demonstrated that leaf rust has the potential to cause extremely severe economic losses under Uruguayan conditions, and research efforts related to the control of the disease were significantly increased.

The objective of this paper is to provide information on virulence in the pathogen population, resistance in commercial cultivars, sources of resistance, and incorporation of resistance into elite germplasm.

Materials and methods

The *Puccinia hordei* population

To study the different virulence combinations present in the pathogen population, leaf rust samples were collected from commercial and experimental fields annually. Inoculum from each sample was increased on a susceptible

genotype and single-pustule isolates tested on a differential set with 16 near isogenic lines with single genes in a common Bowman background, developed by Dr J. Franckowiak (NDSU), which carry resistance genes *Rph1.a*, *Rph2.b*, *Rph2.t*, *Rph3.c*, *Rph4.d*, *Rph5.e*, *Rph6.f+Rph5.e*, *Rph7.g*, *Rph8.h*, *Rph9.i*, *Rph9.z*, *Rph10.o*, *Rph11.p*, *Rph13.x*, *Rph14ab* and *Rph15ad*. The races were also checked on the old differentials to confirm results.

Evaluation of barley lines and cultivars for leaf rust reaction

Adult plant reaction was assessed in two types of field trials: 5 m² plots from one replication of the Official Variety Trials (INIA-INASE), planted at four locations, and 0.6 m² plots in a leaf rust nursery, artificially inoculated with a mixture of local races when the disease was not naturally present. Leaf rust severity was estimated as percentage of infection according to the modified Cobb scale (Peterson *et al.*, 1948) and reaction was scored according to Stackman *et al.* (1962), as R resistant, MR moderately resistant, MS moderately susceptible and S susceptible. To summarize information, the leaf rust CI was calculated, multiplying severity by a coefficient assigned to different reactions (R = 0.2; MR = 0.4; MRMS = 0.6; MS = 0.8; and S = 1.0).

Seedling reaction to single pathogen races was evaluated in a greenhouse. Different barley cultivars and lines were planted as hill plots (6 to 8 seeds per material) on plastic trays filled with a mixture of soil, sand and substrate. Eight-day-old seedlings were inoculated with urediniospores suspended in mineral oil, kept in a dew chamber overnight and then transferred to a greenhouse bench for 12 days at 20–22°C. Seedling reaction was scored with a 0–4 scale representing different infection types (IT) (Stackman *et al.*, 1962). IT 0–2 were considered a resistant reaction and 3–4 a susceptible reaction.

Breeding for resistance to leaf rust

Sources of resistance were first identified in the field tests. These were also tested with single isolates in greenhouse tests. Field-resistant materials that were susceptible at the seedling stage were considered to carry adult plant resistance (APR) and preferentially used for crossing. However, some materials carrying effective seedling resistance genes not known to be present in the local germplasm have also been used.

A modified pedigree selection scheme was used to advance generations, incorporating off-season nurseries to advance the F_3 and the F_5 generations. Phenotypic selection for low leaf rust infection started in F_4 families. F_8 and more advanced elite lines were tested for leaf reaction to single races in the leaf rust nursery and in the greenhouse.

Results and discussion

The *Puccinia hordei* population

Only three races of the pathogen have been identified since 1995 (Germán, 2007), indicating that the *P. hordei* population is relatively stable. The three races can be differentiated by their reaction to leaf rust resistance genes *Rph3.c*, *Rph9.z* and *Rph8.h*. Only race UPh1 (Uruguay *P. hordei* 1), avirulent on *Rph3.c*, *Rph9.z* and virulent on *Rph8.h*, was isolated during 1993–1998. UPh2, first isolated in 1999, acquired virulence on *Rph3.c*. UPh3, first detected in samples from 2004, is virulent on *Rph3.c*, *Rph9.z* and avirulent on *Rph8.h*. Genes *Rph7.g*, *Rph13.x* and *Rph15.ad* are effective and genes *Rph1.a*, *Rph2.b*, *Rph2.t*, *Rph4.d*, *Rph5.e*, *Rph6.f*, *Rph9.i*, *Rph10.o* and *Rph11.p* are ineffective to the *P. hordei* races identified in Uruguay (Germán, 2007). Virulence on *Rph9.z* is the most relevant characteristic of race UPh3. This resistance gene is probably present in the German cultivar Defra, which was widely used in crosses in Uruguay and Brazil. Defra and derived cultivars with

Rph9.z were resistant to leaf rust in Uruguay until 2003, but showed susceptible field reaction since 2004 (Germán *et al.*, 2007). The new race predominated during 2004 and 2006, and was present in similar frequency to UPh1 during 2005 (Germán, 2007). Race UPh3 probably originated in Paraná, Brazil, where virulence on *Rph9.z* was observed in 2003 (G. Arias, pers. comm.).

The present situation: genetic resistance in current cultivars

Widely grown cultivars were susceptible (S) or had intermediate (I) field reaction to leaf rust during 2004–2006 (Table 1); during 2007, the area of cultivars with I, moderately susceptible (MS) or S field reaction was still high (77.6%). New resistant cultivars have been released but are still sown only a small area.

Norteña (N.) Daymán (ND, USA), MUSA 936 (Uruguay), N. Carumbé (ND, USA), and AMBEV 488 (Uruguay) had S, MS, I and moderately resistant (MR) field reaction to leaf rust, respectively, and do not carry effective seedling resistance. This information indicates that N. Carumbé and AMBEV 488 have intermediate and good levels of APR to leaf rust, respectively. The German cultivar Perún is MS under field conditions and seedling resistant (R) only to UPh1, suggesting it probably carries *Rph3.c*. Uruguayan cultivars INIA Ceibo and INIA Arrayán, and the German cultivar Ackermann (Ac.) Laisa had S, I and R field reaction, respectively, low seedling IT to races UPh1 and UPh2 and high IT to UPh3 in the seedling stage. This information suggests that these cultivars probably have *Rph9.z* and that INIA Arrayán and Ac. Laisa have intermediate and high levels of APR to leaf rust, respectively.

Sources of resistance

Effective sources of resistance have been identified since 1999 (Table 2). Many of these materials were susceptible in the seedling stage and resistant in the adult plant stage in

Table 1. Area, average and maximum field infection, field and seedling reaction and characterization of the resistance to leaf rust of commercial barley cultivars.

Cultivar	Area		Average CI		Max. value	Year	Field rxn. ^a	<i>Puccinia hordei</i> races			Resistance	
	2005	2006	2005	2006				UPh1	UPh2	UPh3	Seedling	APR ^b
Norteña Daymán	16.9 ^c	16.9	46.2 ^d	35.7	90 SMS	2006	S	3+	3	4	—	—
INIA Ceibo	24.5	20.2	34.4	32.7	90SMS	2006	S	;	1	3	<i>Rph9.z</i>	
Perún	5.4	7.5	35.3	25.5	80MS	2006	MS	0	3	33+	<i>Rph3.c</i>	
MUSA 936	13.8	14.3	26.4	23.3	80MS	2006	MS	3	3	3		
Norteña Carumbé	16.1	15.7	24.7	23.6	85MS	2005	I	3+	3	3+c		+
INIA Arrayán	—	0.1	23.8	18.5	70MS	2006	I	0;	0:	33+	<i>Rph9.z</i>	+
AMBEV 488	7.7	7.0	12.3	12.1	35MSS	2006	MR	3	3	3+		++
Quilmes Ainara	3.8	2.4	20.0*	10.0*	50MR	2005	MR	2	2	2	?	
Ackermann Madi	1.5	4.0	7.5*	3.0*	25RMR*	2005	R	1=;	1	2	?	
Danuta	—	6.7	—	2.9	40RMR*	2004	R	0	0	21	?	
Ackermann Laísa	1.1	1.9	12.0*	2.8*	20MSMR*	2005	R	0	0	3	<i>Rph9.z</i>	++

^a Rxn = reaction; ^b APR = adult plant resistance; ^c as percentage of the total area; ^d coefficient of infection (CI) in the Official Variety Tests or * Leaf rust nurseries (La Estanzuela and Young) (Germán *et al.*, 2007); (e) severity, using modified Cobb scale (Peterson *et al.*, 1948), and reaction (Stakman *et al.*, 1962); (f) R = resistant, MR = moderately resistant, I = intermediate, MS = moderately susceptible, S = susceptible; (g) seedling infection type: IT = infection type based on a 0-4 scale (Stakman *et al.*, 1962), where 0-2 = resistant, and 3-4 = susceptible; (h) + and ++ = intermediate and high APR levels, respectively; 33 = between 3 and 4; c = extra Chlorosis.

the field, indicating they possess APR to leaf rust. This resistance traces back to European germplasm, and is probably of the same nature as the partial resistance described by Parlevliet and Van Ommeren (1975) and Parlevliet *et al.* (1980). The genetic control of leaf rust using APR has been given priority in the breeding program since it is presumed to be durable.

The first sources of APR to leaf rust used in INIA breeding program (CLE 209, CLE 210 and GP 313) were selected in 1999, under heavy epidemic conditions at La Estanzuela experimental field. These

materials had similar leaf rust reaction to cv. Vada, a reference material for APR to leaf rust. GP 313 was introduced from Germany and sib lines CLE 209 and CLE 210 derive their resistance from the German cultivar Aphrodite. These materials have been widely used in crosses and are the basis of the most effective resistance present in recent elite INIA lines. Since 2004, other materials with APR to leaf rust have been identified and used as sources of resistance.

Due to the relatively low variability of the pathogen and to increase the genetic variability of the basis of resistance, some

effective major genes have also been used in crosses (*Rph9.g*, *Rph13.x*, *Rph15.ad*), as well as other seedling resistant materials with unknown resistance (Table 2).

Breeding for resistance to leaf rust and genetic progress

Severe localized leaf rust infections occurred at La Estanzuela in 2004 and the widespread epidemics of 2005–2006, as well as the presence of variability in resistance to the disease, allowed a rapid improvement in the level of resistance of INIA's germplasm (Germán *et al.*, 2007). Both direct phenotypic selection for low leaf rust reaction and indirect selection for yield and grain size were performed. As a result, the proportion of resistant lines increased, as indicated by the number of resistant INIA lines evaluated in the Official Variety Trials during 2006 (Table 3). Ten out of 11 INIA lines tested in trials during 2006 and 2007 were R or MR to leaf rust, six of which derive their resistance from GP 313, CLE 209 and CLE 210.

The frequency of F_8 resistant lines also increased markedly from 2004 (without

previous selection for leaf rust resistance) to 2006, after two years of selection (Figure 1). These F_8 lines were derived from different crosses routinely managed by the breeding program. The number of F_8 lines tested in the leaf rust nurseries during 2004, 2005 and 2006 were 79, 66 and 62, respectively. The average leaf rust CI of the susceptible checks INIA Ceibo and Norteña Daymán was high and similar during 2004, 2005 and 2006 (67, 54 and 63, respectively), which demonstrates that a high and uniform disease development occurred in the nurseries.

Prospects and challenges

The situation for barley leaf rust during the last 30 years indicates that it is important to maintain breeding for resistance as a long-term objective, even after a long period when the disease has been of minor economic importance. The pathogen has the potential to build up severe epidemics and cause very severe damage when large areas are planted with susceptible cultivars.

Genetic resistance is considered the best control measure against barley leaf rust, since

Table 2. Origin, cross, resistance genes, first year used in crosses, seedling reaction to *P. hordei* race UPh3 and field reaction at two locations in Uruguay of sources of resistance to leaf rust.

Material	Origin	Cross	R gene	First Cross	IT UPh3	Leaf rust 2006	
						LE	Young
Checks							
Bowman (S)					3+ ^a	99S ^b	40MS
VADA (R)					3	TR	0
Adult plant resistance							
CLE 209	INIA (Uru)	Aphrodite/E. Quebracho		1999	3	2MR	0
CLE 210	INIA (Uru)	Aphrodite/E. Quebracho		1999	3+	5M	2MRMS
GP 313	Germany	(Camelot-W15122-W17267)Libelle		2000	3	5R	0
F0425-	INIA (Uru)	N.Daymán/CLE177		2005	23	5MR	2MR
F0429-	INIA (Uru)	N.Carumbé/CLE175		2005	3-	15MS	20MS
F0449-	INIA (Uru)	CLE178/CLE185		2005	3	10MRMS	5MS
NCL 9970	MUSA (Uru)	AmBev 488/Diamalta.		2006	3	5R	2MS
Seedling resistance							
I931-21-	ND, USA	Bowman*84//ND7771//CI6193/MT81195	<i>Rph7.g</i>	2003	:	70R	5MRMS
I981-4-383-	ND, USA	Bowman*6/PI 531849	<i>Rph13.x</i>	2003	0;	60R	10MRMS
I952-282-	ND, USA	PI355447*5/Bowman	<i>new</i>	2003	0;	70R	10R
KE 5	Germany	(Aura-Estanzuela-Rika-MG43733)*Alexis	<i>Rph7.g</i>	2004	:	30R	5MRMS
ND 23250	ND, USA	ND19922/ND19974//ND19119	<i>Rph15.ad</i>	2006			
F0412-	INIA (Uru)	PI 3554475/*Bowman//N Carumbé	<i>Rph15.ad</i>	2004	0;	50R	0

^a Based on Stakman *et al.* (1962), where 0–2 = resistant, and 3–4 = susceptible; ^b severity using a modified Cobb scale (Peterson *et al.*, 1948) where T = trace; reaction (Stakman *et al.*, 1962), R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible.

Table 3. Cross, first year of evaluation in the Official Variety Trials, field and seedling reaction and presence of adult plant resistance in all INIA cultivars and elite lines tested in 2006.

Cross	First Evaluation	Field Reaction	Seedling IT Uph3	APR
Norteña Daymán (susceptible check)		S ^a	3+4 ^b	
INIA.Ceibo FNCI 22/Defra	1998	S	3	
INIA.Aromo CLE 150/LBP(14376/2646)	1999	S	3	
INIA I.Viraró Defra/CLE 166	2002	MR	1	
INIA.Arrayán Defra/CI 5791	2002	I	33+	+
CLE 232 Defra/CLE 169	2002	MS	23-	
CLE 240 ND 16680/N.Carumbé	2004	MS	3+4	
CLE 246 CLE 169/RH 94 2954	2005	S	3	
CLE 247 N.Carumbé/CLE 175	2006	MR	3+	++
Emir/3/Api/CM67//Bus/4/Shyri/5/Sen's				
CLE 248 '6/E.Quebracho/7/C.Cangüe	2006	R	3	++
CLE 249 Clipper/3/FM 519/Alexis//PFC 85185	2006	R	22+	
(Camelot-W15122-				
CLE 250 W17267)Libelle/CLE 194//I. Ceibo	2006	R	2+3	++
CLE 251 N. Daymán/CLE 209	2006	R	2+3	++
CLE 252 N. Daymán/CLE 209	2006	R	2+3	++
CLE 253 Perún/I. Aromo	2007	R	33+	++
CLE 254 N.Carumbé/I. Aromo	2007	MS	3+	
CLE 255 N. Carumbé/CLE 210	2007	MR	3+	++
CLE 256 I. Ceibo/CLE 210	2007	R	2+3	++
CLE 257 CLE 210/I. Ceibo	2007	R	--	

^a R = resistant, MR = moderately resistant, I = intermediate, MS = moderately susceptible, S = susceptible; ^b based on Stakman *et al.* (1962), where 0–2 = resistant, 3–4 = susceptible, 33+ = more than 3 but less than 4; 23- = between 2 and 3-, 2+3 = between 2+ and 3; ^c + and ++ = intermediate and high APR levels, respectively.

it does not represent an increased cost of production to farmers and is environmentally safe. New resistant elite lines combining adequate resistance to leaf rust, agronomic traits and generally acceptable malting quality have been developed. However, new races of the pathogen virulent against widely grown, previously resistant, cultivars have developed in the Southern Cone of America and the pathogen may continue to evolve. The identification and utilization of APR was one strategy used to increase the duration of the resistance. APR to leaf rust could be efficiently transferred to elite lines through phenotypic selection.

To minimize the risk represented by the appearance of new virulent races it is necessary to maintain diversity in the basis of resistance. There is little information

regarding the identity of the genes or gene combinations conferring effective seedling resistance in Uruguayan barley germplasm; some major genes, including newly identified genes, are being incorporated into adapted backgrounds to increase diversity.

Diversity in APR is also desirable, since virulence to this type of resistance might also arise. Previous studies showed that partial resistance is quantitatively inherited (Parlevliet, 1976, 1978; Qi *et al.*, 1998, 1999). Modern molecular tools will be very valuable to study the diversity of the genetic basis of this resistance and to identify APR sources carrying different resistance genes.

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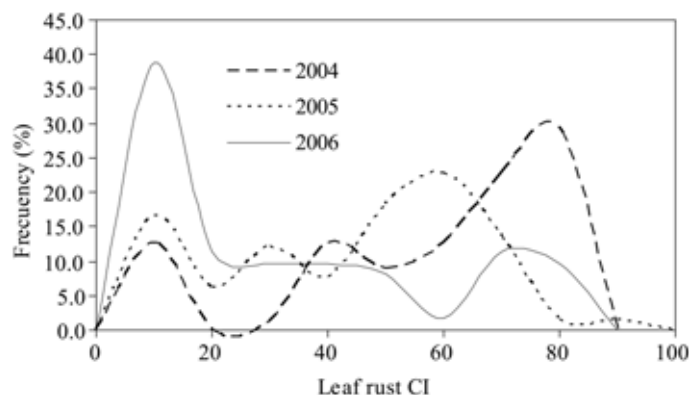


Figure 1. Frequency of F_8 breeding lines according to their leaf rust coefficient of infection (CI) in the 2004, 2005 and 2006 leaf rust nurseries.

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The evolution of barley in Brazil: contributions of Embrapa in 30 years of research and development

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Abstract

Increased beer consumption since 1920 has challenged scientists and farmers to establish malting barley production in Brazil. The major challenge has been adapting barley to give an economic yield under the natural acid soil and wet and warm growing conditions of Brazil. Currently, malt consumption is 1.1×10^6 t/yr while production is 0.3×10^6 t/yr. Barley production increased substantially from 20 to 70% of the supply needs of the malt industry. The increasing trend is attributed to yield and quality improvements that have established barley as a competitive crop. This competitiveness is mainly due to the results of 30 years of research and extension at Embrapa (Brazil Agricultural Research System). The widespread use of Embrapa's technologies, including varieties, the "No-Till" Production System (NTPS), crop agronomy and protection since the early 1990s have increased yield from 1500 kg/ha in the 1980s to 2500 kg/ha today. Since the release of BR 2, Embrapa varieties have gained grower preference, taking up 70% of the acreage. Varieties resistant to net blotch, a very limiting factor, have reduced fungicide use. Current modern short, lodging-resistant, varieties (e.g. BRS 195, BRS Greta) are more competitive in the NTPS, where improved soil properties and production technologies boost the yield of barley to 6000 kg/ha in favorable seasons. Varieties Embrapa 127 and BRS Borema match closely the quality of known elite malting cultivars under Brazilian environmental conditions.

Introduction

Barley was firstly introduced into Brazil in 1586, but became a crop only in the 20th Century, when commercial production started in 1930, although only production for malting has been competitive in economic terms. The only barley used for feed is the amount produced annually that does not meet malting quality. Production has evolved but is still concentrated in southern Brazil in regions where cool springs favor malting quality. Major growing areas are between latitudes 24° and 31° S, in the highland plateaus (400 to 1100 masl) of Rio Grande do Sul, Santa Catarina and Parana states. The regional climate is classified as a Koppen's cfa (humid subtropical), with rainfall well distributed throughout the year and a mean temperature in the warmest month of $>22^\circ\text{C}$. Barley is sown in late autumn to early winter, following a summer crop of soybean or maize, in the traditional double cropping system area. The crop cycle is completed in 110–140 days, depending on the variety, location, planting date and seasonal temperature and rainfall regimes. Harvesting takes place from late October to early December. Subsoil acidity causing aluminium toxicity is a recurrent problem; the originally acid soils restrict root growth beyond the limed layer, making barley more vulnerable to moisture stress than the more acid-soil tolerant crops such as wheat and oats. Rainfall during the growing season averages 700 mm. High seasonal and regional variability in the amount and distribution of rainfall are the major limiting

factors affecting yield and quality. Excessive rainfall during the reproductive stage, particularly in El Niño Southern Oscillation (ENSO) years, is the most unfavorable environment for malting quality. Seasonal drought or temperature stresses (heat, frost) at critical stages, and fungal and virus diseases, are other major limiting factors. Since 2000, a small amount of malting barley has been produced under irrigation in tropical Brazil in the states of São Paulo, Goiás and Minas Gerais.

The objective of this paper is to present 30 years of effort by and research results of Embrapa in attempting to tailor barley to be a competitive crop in the acid soil and high rainfall growing conditions of southern Brazil.

Barley production

Since the beginning of commercial barley production in Brazil it has been produced under contract between farmers and the malting-brewing industry. The malting industry provides seed of varieties they want to malt and buy the production that meets the quality standards established in the contract. In the beginning, and for some time, the seed distributed by the industry was imported, mainly coming from Argentina. Local production was small and confined to the Rio Grande do Sul, Santa Catarina and Paraná states, in areas where forest had been cleared for agricultural production by settlers of European origin, mainly German, Italian and Russian. Until the end of the 1950s, barley was produced manually or using animal power and only on high-pH, fertile soils, while wheat and rye were grown on poor acid soil lands suitable for mechanization. Locally selected varieties more tolerant to soil acidity/aluminum toxicity allowed barley to grow in the mechanized areas, boosting production during the 1960s, when acreages of up to 40 000 ha were recorded. Average grain yield on-farm was then close to 1 t/ha. Production

almost disappeared early in the 1970s due to frequent crop failures and competition from the international market.

The Federal Government barley and malt self-sufficiency plan launched in 1976 boosted both barley production and the capacity of the malting industry. As part of the plan, Embrapa, the Ministry of Agriculture Agricultural and Livestock Research System founded in 1973, was assigned the task of providing the requisite scientific and technical support. Embrapa, through its National Wheat Research Unit, started working with barley in 1976, with the mission of expanding, diversifying and coordinating all the country's research and development efforts on the crop.

The public support for research and production finally established malting barley as a commercial crop in southern Brazil, giving the farmers an alternative winter crop to grow on the stubble of a summer soybean or maize harvest. Rio Grande do Sul and Paraná have since become the major barley and malt producing states, with Rio Grande do Sul leading with an average of 60% of total production. Since 2000, barley has also been produced in small amounts, under irrigation, in Goiás and DF in central Brazil and São Paulo in the southeast.

Publicly-funded support to barley production ended by the late 1980s, leaving the fate of local production to the whim of the malting industry. Higher production costs than for other winter crops, mainly due the susceptibility of varieties to net blotch, then the major disease, and the lower price of imported barley, reduced local acreage to the levels of late 1970s. Domestic production started recovering in 1995, with the release the variety BR 2 by Embrapa, the first Brazilian variety resistant to net blotch. It was widely adapted and highly competitive in grain yield, and suitable for production under the NTPS production system then beginning to be adopted in the region. The increased competitiveness of barley under NTPS, as

well as price increases in the international market, made domestic production interesting again to the brewing industry. Malting barley production in the last decade averaged 310 000 t/yr, supplying 60 to 70% of current malting capacity, which produces around 30% of the country's brewing malt requirement. Brazil is a major malt importer and domestic brewing consumption has steadily increased in recent years, indicating an increased demand for both malt and barley. Current trends indicate a significant increase in local malting capacity in a few years, and a consequent increased demand for malting barley. Considering the current agronomic and quality competitiveness of barley, it can be postulated that there will be a steady growth in both acreage and production of malting barley in Brazil.

Barley research

Public barley research was initiated together with that on wheat in 1920. During the 1930 to 1975 period, research was carried out by the brewing industry, interested in malting barley production, with little or no support from government institutions. Major efforts then were directed to breeding, soil improvement and crop agronomy. Under the leadership of Embrapa, research efforts significantly increased after 1976, with inputs from state research systems, universities and farmer organizations. Besides breeding, during the official support to domestic production, barley benefitted from a substantial amount of research on crop production and crop protection. Unfortunately, cuts in official incentives to the crop negatively affected the resources allocated to barley research from public sources.

Since 1990, Embrapa is practically the sole public institution with significant research inputs in barley. In the private sector, AmBev and Cooperativa Agraria run individual research programs, with major efforts in variety development and crop

agronomy. Academic studies on barley are almost inexistent today. Embrapa Trigo runs a malting barley breeding program with formal technical and financial support from the malting industry (currently AmBev, Cooperativa Agraria and Malteria do Vale). Since its onset, Embrapa's breeding program work has integrated with the research and quality analysis capabilities of the malting industry. The varieties obtained from the program are incorporated into commercial production through the partners' seed production and farmer technical assistance apparatus, after intensive agronomic and malting quality evaluation.

Embrapa's barley improvement program was implemented in 1976, at the National Wheat Research Center, located in Passo Fundo, RS (28°15' S, 52°24' W, 687 masl), one of the country's most important barley production regions. Since its beginning, the major goals of the program have been the assembling of an enhanced and broad germplasm pool and developing new barley cultivars adapted to local soil and weather conditions and showing competitive yield, malting quality, and disease resistance. Collecting viable seeds from the locally developed germplasm and from exotic genetic material was the first task of the program. Cultivars and breeding lines made available by the brewing companies Brahma and Antarctica and by the state institutions IAPAR (Paraná) and IAC (São Paulo) became the germplasm base. Crossing work started in 1978, aiming to develop breeding lines by combining the locally-adapted superior malting lines with known sources of disease resistance from the USDA world collection, Australia, Canada, Europe, South America, and USA.

The program has been pursuing the idea that to be competitive under local conditions a phenotype (ideotype) should combine the largest possible number of the following traits: acceptable malting quality; high yield potential (over 3000 kg/ha for rainfed and

5000 kg/ha for irrigated conditions); kernel plumpness over 85%; early maturity (<140 days for rainfed and <120 days for irrigated conditions); short straw (<80 cm); resistance to lodging and to pre-harvest sprouting (PHS); and disease resistance (net blotch, powdery mildew, leaf rust, BYDV, spot blotch and head scab). The methodology applied has evolved from strictly conventional breeding to a combination of the available conventional and modern methods. Nowadays, 30% of crosses made are advanced through Single Seed Descent (SSD) and Doubled-Haploid (DH) methods, and 70% through selected bulks up to the F_4 . DHs are produced through anther culture. Using SSD, the materials are advanced by three generations per year. Selection for adaptation to soil and environmental conditions and for disease resistance is made under field conditions, under natural and artificial pathogen infection. Selection for malting quality is delayed until the line selection stage, based on the industry's quality analysis output.

Results

The Embrapa research program has been underway for more than 30 years now, and both the germplasm developed and the knowledge generated have made substantial contributions to the consolidation of a malting barley industry in Brazil, and also, in a wider context, to the knowledge on the performance of the crop in acid soil subtropical/tropical areas (Minella and Sorrells, 1992, 1997, 2002; Peruzzo and Arias, 1996). During this period local barley production experienced significant progress. Compared with the 1970s, when Embrapa started researching barley, average acreage has tripled (304%), production is six-fold (603%) and yield has doubled (207%) (Table 1).

During the 1980s the average farm grain yield increased almost 30% due mainly to improved agronomy and plant protection practices developed by Embrapa and

partners. Significant improvements were obtained in the geographical distribution of production (production zoning); seeding time and density; soil fertility increases through liming and P and K amendments; N fertilization; and disease (mainly net blotch) and insects (mainly aphids) control methods. The aphid population that was causing severe cereal crop losses was brought under control through a massive release of introduced natural enemies.

During the 1990s the observed yield increase in farmers field was even greater than during the 1980s, one major factor being the difference resulting from the wide use of Embrapa cultivars, which became available to farmers after 1992 (Tables 1 and 2), and the adoption by most of the producers by the end of the decade of NTPS practices (Silva and Minella, 1996; Minella, 2000).

The release of BR 2, Embrapa's first true malting barley, in 1989 has been credited as a cornerstone in both barley breeding and production in Brazil (Minella *et al.*, 1999). Due to its earliness, short straw, wide adaptation, high yield potential and resistance to net blotch, BR 2 was the leading variety for 12 years (1994 to 2003). It was sown on over 80% of the acreage, peaking at 91% in 1997. Its wide use allowed a reduction of at least one fungicide spray against net blotch, thus lowering both production cost and impact on the environment. The net blotch resistance of BR 2 and derivatives continues to be as effective as it was 20 years ago. It is believed that the use of BR 2 represented a 40% yield increase, bringing back the competitiveness of the local crop in comparison with home grown wheat or imported barley.

Barley production was resumed with the release of the Embrapa cultivars in 1995 and is currently stabilized at around 310 000 t/yr, satisfying on average 65% of the malting capacity. Before the advent of the Embrapa varieties and the introduction of NTPS technology, the country was importing on average 70% of the barley used for malting.

Table 1. Current average acreage, production and yield as absolutes and percentages relative to the 1970s for malting barley in Brazil

	Acreage		Production		Yield	
	(ha)	% of 1970s	(t)	% of 1970s	(kg/ha)	% of 1970s
1970s	42 962	100	50 018	100	1 124	100
1980s	102 947	239	146 884	293	1 459	130
1990s	93 812	218	197 230	394	2 036	181
2000s	138 425	322	311 233	622	2 250	200
Last Decade ¹	130 638	304	301 851	603	2 328	207

¹ Data for 1997–2006.

Table 2. Evolution of the malting barley crop in Brazil in the last 15 years.

Year	Acreage (ha)	Production (t)	Yield (kg/ha)	Embrapa (%) ¹
1992	57 018	117 835	2 067	5.0
1993	62 184	105 702	1 700	30.4
1994	53 269	98 176	1 843	45.6
1995	73 462	117 811	1 654	81.5
1996	83 575	223 981	2 680	89.5
1997	124 909	277 604	2 222	91.1
1998	137 720	310 383	2 253	90.2
1999	123 894	314 895	2 542	86.3
2000	136 664	307 303	2 249	65.0
2001	135 640	274 888	2 027	60.0
2002	145 156	224 403	1 546	51.0
2003	136 971	381 220	2 783	63.0
2004	146 803	395 277	2 692	73.0
2005	127 961	282 245	2 207	72.7
2006	90 661	250 291	2 761	57.6
Last 10 years	130 638	301 851	2 328	71.0

¹ Proportion of the acreage seeded with Embrapa varieties.

In this century, the average yield is 10% higher of that of the 1990s, and this increase has been attributed to the release and wide use since 2003 of the high yielding, short straw and lodging resistant variety BRS 195 (Minella, 2005) and to greater use of nitrogen when growing this cultivar. Because of its superior yield potential and acceptable lodging resistance in areas for several years under NTPS (chemical, physical and biologically improved soils), BRS 195, released in 2002, has been the leading variety in both rainfed and irrigated areas. In general, it yields 1000 kg/ha more than the regular (non-dwarf) varieties. Lodging has become a major problem for regular type varieties in soils with a long history under the NTPS production system.

The average malting quality of Brazilian produced barley has also improved substantially (Table 3), and varieties Embrapa 127 and BRS Borema (Minella *et al.*, 2006) have a malting quality profile competitive with those of world top varieties, such as cv. Scarlett.

Table 3. Progress in malting barley traits in Brazil in the last 30 years

Trait	1970	2000	Change (%) 1970–2000
Grain yield (kg/ha)	1 124	2 250	200
Kernel plumpness (%)	65.0	90.0	138
Barley protein (%)	12.5	9.5	76
Malt extract (%)	79.0	82.5	104

The release of BRS 180 six-row barley in 1999 (Silva *et al.*, 2000), was the starting point for the production of malting barley in the cerrado region (Goiás and Minas Gerais states) under pivot irrigation. Since 2005, there has been limited production for malting in irrigated areas of São Paulo, where BRS 195 has been competitive in yield and also malting quality. Due to the long distance from the irrigated areas of Goiás and Minas Gerais to the nearest malting plant (São Paulo), production in those regions is not yet competitive with imported barley and currently is not happening. Major yield limiting factors in the cerrado have been lodging and occasional occurrence of rice blast caused by *Pyricularia grisea*.

The soil improved production capacity under NTPS has doubled the yield potential of barley and nowadays productivities >6000 kg/ha in the farmer's fields are already common in favorable seasons. The yield potential of BRS 195 under irrigation is >8000 kg/ha. In areas under NTPS for more than a decade, barley has been also much less affected by acidity and/or aluminum toxicity of the subsoil than when soil was tilled. A negative effect associated with the NTPS production system consolidated in southern Brazil, has been the increased occurrence of head scab (*Fusarium graminearum*) and spot blotch (*Bipolaris sorokiniana*) diseases. Reducing losses due to these fungi to acceptable levels is currently the major challenge for barley researchers, mainly plant pathologists, breeders and agronomists. The level of genetic resistance to these pathogens in current cultivars is far below the desired one, and the variability in the germplasm being used is not promising for a genetic solution of the problem.

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The Western Australian Barley Industry Development Strategy

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Abstract

Over the last few years a number of changes have taken place within the Western Australian barley industry. The formation of national bodies such as Barley Australia (BA) and Barley Breeding Australia (BBA), along with the implementation of the National Variety Testing (NVT) program, has helped to bring a coordinated approach to the barley industry in Australia. However, at the state level, the beginnings of deregulation in marketing, reduced state government funding for industry communication, and an increased rate of new variety releases alongside those from private seed development companies has the potential to bring confusion and fracture the lines of industry communication.

A Barley Industry Development Officer (BIDO) is employed to ensure that effective communication flows through the industry. The role of the BIDO is to remain impartial of competing interests and utilize information from a wide range of sources to deliver concise messages through the industry networks at a state level, yet with links that span the nation.

Background

In the past, communication and industry extension had been generally aligned to the state public breeding programs, with little national focus. Most information transfer was through state government departments. Mainly this was with an agronomic and production message. There was a low

turnover of varieties and a generally slow rate of change. The regulated market place facilitated a very simple value chain from producers to customers, whether domestic or export.

During the last decade many changes have occurred in the barley breeding industry, most notably in the field of plant breeding for new varieties. The introduction of plant breeders' rights (PBR) and end-point royalties (EPR) or crop improvement royalties (CIR) to help fund breeding programs, and the introduction of private breeding companies, have been major factors in the development of a greater number of varieties and their release into the market place. There is also the development of international plant breeding companies forming alliances with agribusiness in an endeavor to develop new varieties. In addition to this, much of these variety breeding programs are being pitched to have an impact over a greater area of the nation.

In 2006, Australia's six state-based, public barley breeding programs agreed to form Barley Breeding Australia (BBA), a joint-venture national breeding program. BBA is an unincorporated joint venture between the:

- Grains Research and Development Corporation;
- Queensland Department of Primary Industries and Fisheries;
- NSW Department of Primary Industries;
- Department of Primary Industries, Victoria;
- The University of Adelaide; and
- Department of Agriculture and Food, Western Australia.

BBA is the coordinator of Australia's national public barley breeding investment, servicing Australia's barley growers, marketers and end users. The parties committed to jointly service the needs of all Australian barley growers and end users through a national breeding effort conducted at three nodes (Warwick for the north; Adelaide for the east; and Perth for the west). Each node, whilst regionally based, has a slightly different focus, with the western node focusing on breeding barley for soils with an acidic to neutral profile, the southern node focusing on breeding barley for soils with a neutral to alkaline profile, and the northern node specializing in feed grain quality.

Despite this coordinated approach, there is still competition between the nodes, which leads to nodes putting their varieties in the best light for growers to maximize adoption. This can lead to confusion among growers and conflicting messages. Thrown in with this is the national adoption of varieties developed from programs from elsewhere. This requires information to be cross-regionalized to verify the success or otherwise of varieties, and to also enhance the commercial supply of seed to growers.

To assist in varietal evaluation, the peak funding body for grains crop research, the Grains Research and Development Council (GRDC), has coordinated the development of the National Variety Testing (NVT) program in recent years. This project is complemented in Western Australia by its own Crop Variety Testing (CVT) program run by the Department of Agriculture and Food, Western Australia (DAFWA), a program with at least 30 to 40 years of continuity behind it.

Within research and development, rationalization of state department and industry funding has meant a greater level of discrete research project development, with the resultant effect of fewer researchers necessarily spending extended career paths within the industry. Extension of research has also been outsourced to private agronomists

and agribusiness. Yet these key industry players in crop production need a clear and unified source of information.

Deregulation of the market place, more advanced in the eastern states, is beginning to occur in Western Australia. The value chain has become more complex, while at the same time experiencing a degree of rationalization. Mergers have occurred between grain receival companies and marketers or end users, such as between the Australian Barley Board (ABB) and Ausbult and Joe White Maltings (JWM) in South Australia. In Western Australia this has also occurred between Co-operative Bulk Handling (CBH) and the Grain Pool. This has had the resultant impact of having fewer people in the state's barley industry having to do the same work, while with in Western Australia's case, an expanding area of barley production occurring.

The outcome of this has meant that communication is now more complex. Competition in the market place generates a degree of secrecy or "commercial in confidence" information. Information outputs need to be rated for value and importance and in places obtained from multiple sources to develop the "Big Picture". To this end a well defined network is essential that promotes confidence and a willingness to share in order to collect information while at the same time to deliver it to the various users along the industry value chain and be seen as not being compromised by industry players.

To facilitate the information exchange there has developed the formation of Barley Advisory Councils to act as industry reference boards. These Councils are made up from representatives of growers, farm agronomists, marketers, maltsters and brewers, the livestock feed industry, breeders, and researchers. Within their region of influence, each Council monitors the progress of breeding lines from the time their potential is identified until release as a malting or feed variety. The Councils integrate all information on a breeding

line—quality, agronomy, disease resistance, market potential—and can make a holistic assessment as to the merit of a variety both on-farm and in the market place. Within Western Australia, the Western Region Barley Council (WRBC) is the state's peak body. The council is heavily relied upon by the BBA's western node for variety evaluation and has a close relationship with Barley Australia (BA), a national peak body to represent the barley industry partners.

BA is the peak body representing the Australian barley logistics, marketing and malting industry and is “an independent, nonprofit industry organization established to enhance communication and co-ordination between industry and customers, and promote the competitive appeal of Australian barley through national accreditation and quality assurance.”

BBA also works with BA where “BBA is committed to developing and maintaining a strong consultative relationship with BA and the broader Australian barley and malt/brewing industries.”

Within this very dynamic industry there exists the Barley Industry Development Officers or BIDO's, whose role is to filter and facilitate the exchange of information in a timely and concise way. Nationally, there are three BIDO's, each aligned to a broad

cropping region of Australia: Northern, Southern and Western (Figure 1). While working independently of BA, the BIDO's are fundamental to nurturing the industry relationships that have developed, as discussed above, and are closely aligned to the respective state councils and so linked to BBA.

This paper outlines the status of the Western Australian barley industry and discusses industry development. It presents the process we are undertaking to advance the production in Western Australia of high yielding barley varieties that meet the quality requirements of the domestic and international markets. It outlines the methods used to develop a communication plan, the results of a scoping study, and outlines the schematic plan for collection and dissemination of information within the barley industry.

The Western Australian industry

Western Australia is well suited to barley production, particularly malting barley, having good climatic conditions. The generally dry harvest period found in the main cropping areas of the state lessens the risk of weather damage and downgrading of malt barley varieties. Western Australia is



Figure 1. The Australian BIDO network. Working within the whole value chain to identify weakness, improve efficiency and communication.

also located in close proximity to many key importing countries, especially those of SE Asia.

Variety adoption

Although the malting variety Stirling has been a popular variety grown for many years in Western Australia, it is now no longer the number one variety. Malt varieties Baudin and Gairdner now share the mantle of being the most popular varieties (Table 1). The area sown to Gairdner, though, has now declined from 2003, when it occupied 31% of the total area sown, while the area sown to Baudin (a variety of similar maturity, but higher malting quality, plumper grain, though more susceptible to leaf disease) has grown markedly since 2003.

While there has been rapid adoption of Baudin, to the point that Baudin is now the dominant variety in the state, a new malt variety, Vlamingh, released to growers in 2006 and bulked up in 2007, looks like being a keen competitor to both Baudin and Gairdner in the next few years. Grower

interest in the variety Buloke also increased in 2007. Buloke originated from the state of Victoria some 4 years prior and as a result of performance in the NVT and CVT programs looks likely to be a contender in the medium-to-lower rainfall areas in the state, especially if it becomes an accredited malt variety. The accreditation process is managed by BA and any news on the status of Buloke was expected to be known by March 2008. Should this occur, then seven varieties could be received as malting quality in Western Australia at the 2008/09 harvest, putting pressure on the capacity of grain handlers to segregate each variety.

While malting varieties represent 75% of the varieties sown, malt barley receivals are only about 42% of barley production on average. Half the production is downgraded to feed barley as it does not meet quality specifications. This is due to a combination of a run of poor seasonal conditions; growers sowing high yielding malt varieties like Gairdner and Baudin to chase yield rather than quality; the inherently smaller grain

Table 1. Percentage of the area sown to barley varieties in Western Australia 2001-2007 (Source: Cooperative Bulk Handling).

Variety	Percentage (%) of barley area sown to each variety by season						
	2001	2002	2003	2004	2005	2006	2007
Baudin	—	0	1.2	13.4	20.7	23.1	24.4
Gairdner	16.6	24.8	31.1	25.0	23.0	23.4	21.4
Stirling	63.7	54.7	45.7	33.0	26.1	20.6	16.6
Mundah	4.0	5.3	6.8	9.7	11.0	12.8	15.4
Hamelin	—	0	0.3	4.1	7.4	9.9	11.5
Yagan	0.9	2.4	3.4	5.5	5.0	3.1	4.5
Dash	—	—	—	—	0	0.6	1.0
Molloy	0.6	0.4	0.4	0.9	0.9	1.0	0.8
Fitzgerald	1.1	0.8	0.7	0.9	0.8	1.0	0.8
Barque	0.2	0.1	0.3	0.6	0.6	0.8	0.6
Schooner	6.5	6.4	5.3	3.4	2.5	1.7	0.5
Other feed	6.3	5.2	4.9	3.5	2.2	2.0	2.4
Malt	86.9	85.9	83.6	78.9	79.6	78.7	74.5
Feed	13.1	14.1	16.4	21.1	20.4	21.3	25.5
Top 5 varieties	84.3	84.8	85.1	85.2	88.2	89.8	89.4

size of varieties like Gairdner and Baudin compared with Stirling; and inappropriate management in some situations.

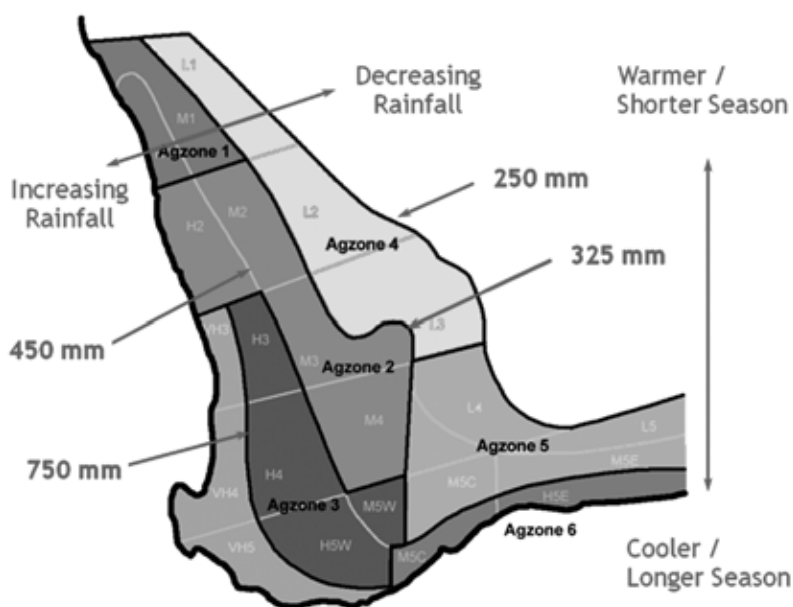
The percentage of area sown to feed-only barley varieties has increased significantly in recent years, with feed varieties making up 25% of the total area sown to barley in 2007. Mundah and Yagan were the most popular feed-only varieties, making up 15 and 4.5%, respectively, of the total area sown to barley. There has been a strong swing to feed barley varieties in some districts of the state (Agzone 1) associated with a run of poor seasons and a strong presence in Agzone 4 (Figure 2). The introduction of high-yielding feed-only varieties from BBA (i.e. cvs. Fleet, Hindmarsh, Lockyer, Roe and Hannan) will only heighten grower interest.

The barley industry development project has cultivated a close relationship with the WRBC, the peak barley industry body in Western Australia, to foster the development of the barley industry. Agronomic and

extension packages resulting from this collaboration have resulted in the widespread adoption of three new general malting varieties in Western Australia: cvs. Gairdner, Baudin and Hamelin. With cv. Vlamingh being bulked up by growers in 2007, widespread sowings were expected in 2008, despite unknown domestic and international market demand.

Industry growth

The Western Australia barley industry has been increasing in value over the last decade. This reflects a combination of factors: a slight increase in the area planted; steady yield improvements; increases in the nominal price for barley; as well as additional domestic malting capacity at the JWM malting plant in Perth. Barley now commands an area of around 1 million ha and production is averaging 2 million tonne/yr. This is about double the production seen in the mid-1990s. Depending on the season, the main areas



of production are within the central, great southern and southern shires of the state. The area sown to barley has increased significantly in the central region but has remained static in southern zones. Barley is a less important crop in the more northern areas. The biggest barley growing areas are the predominantly medium rainfall Agzones 2 and 5. Between them, they constitute some 60% of the barley area. The high rainfall Agzone 3 constitutes around 20% of the barley area.

Industry development

The development of an extension or industry development plan is a complex process. The framework presented in this paper is based

on the work of Jenny Crisp and Colin Holt (2002) titled *Framework for planning an extension strategy with rigour*. Jenny Crisp is a Development Officer with DAFWA and Colin Holt is a Consultant with the ARID group. The framework used is a forward planning tool and comprises 12 sequential steps, which are outlined in Table 2.

Aims of industry development strategy

Using the abovementioned framework, an industry development plan was developed and presented to industry. The industry development strategy thus developed aims to:

1. Guide an industry development program to

Table 2. 12 steps to planning an extension strategy, and the reasoning behind each step (Crisp and Holt, 2002).

Step	Title	Reasoning
Step 1	Gather project background	Investigating the project background sets the scene, and provides a strong starting point for developing an extension strategy.
Step 2	Identify stakeholders	Who the stakeholders are will influence development of the extension strategy, from setting objectives early on through to detailed activity planning.
Step 3	Develop extension purpose, objectives and outcomes	Developing the extension purpose, objectives and outcomes provides direction and focus for planning and implementing an extension strategy.
Step 4	Check for congruency	Checking whether extension purpose, outcomes and project needs are aligned or congruent will pick up any gaps and inconsistencies in logic. This adds rigor to an extension strategy.
Step 5	Test assumptions	Identifying and testing assumptions underpinning extension success is critical, as the implications of these assumptions can then be assessed and planned for in the extension strategy.
Step 6	Analyze situation in more detail	Four analysis tools provide a means to understand the extension situation, and to generate more ideas for activity planning. (The tools are SWOT, Forcefield analysis, Six thinking hats, and Change model.)
Step 7	Think about broad extension models	If your extension situation is similar to an existing extension model, some of their thinking about assumptions, processes, tools, etc., may be useful to you.
Step 8	Brainstorm possible activities	A brainstorming session will gather as many ideas and thoughts as possible about potential extension activities. This step throws the net wide before making the final choice of activities. It incorporates data from all previous steps.
Step 9	Choose activities to best achieve outcomes	Choosing activities is about focusing in on which of the many activities listed in Step 8 will best deliver extension outcomes and purpose. Critical thinking and reasoning are important for this step.
Step 10	Prioritize	Prioritizing activities is deciding which outcomes are more important to the overall project, and how easy they would be to deliver in terms of resources needed.
Step 11	Putting it all together	Finally, all the information generated and decisions made need to be put together in a working document, with action plans and timelines for activities.
Step 12	Evaluation and review	Introduces key concepts of project and program evaluation and review. Highlights the cyclical nature of planning.

assist barley breeders through a pathway to market for new barley varieties suited to Western Australia, involving:

- (a) delivering high-level information on agronomic issues in barley production to breeders, marketers, and end users; and
 - (b) assisting the facilitation of uptake of new varieties by growers to match their requirements and the demand from marketers and end-users.
2. Provide guidelines to growers and industry on the production and marketing of new barley varieties, including importance of varietal purity and segregation, target quality parameters (protein, screenings), and correct use of crop husbandry.
 3. Act as a conduit for reciprocal information flow between growers, agribusiness, researchers, breeders and industry (marketers and end-users) to support a viable barley industry.

Methods

A scoping study of the barley industry was conducted in May 2007, based on a review of the activities of previous BIDO's and discussion with key industry members, including Barley Australia, BBA and marketers. Areas of opportunity and threat to the industry were identified and investigated, a set of extension priorities identified, and purposes defined for the industry development strategy.

Results

Three main priorities have been identified for the successful development of the barley industry in the Western Region.

- Improvement in the quality of barley to meet the needs of markets.
- Adoption of new barley varieties suited to market requirements.
- Promotion of the Western Australian barley industry and improve communication and cooperation for the Australian barley industry.

These three main priorities have the overall aim of advancing the production in Western Australia of high yielding barley varieties that meet the quality requirements of the domestic and international market.

There are 5 main purposes for this industry development strategy.

1. Enable the rapid adoption of the latest research results and market information to increase grower income through improved grain quality and yield of barley, implying
 - extending current research and market information on barley to growers and industry, and
 - improving decision-making on marketing barley.
2. Provide growers and agribusiness with information to support the appropriate selection of malting and/or feed barley varieties, and the potential impact of management on yield and quality.
 - Improve the selection of varieties suited to each region.
 - Manage barley to minimize risk and meet the quality requirements of the market being targeted.
 - Collate data on barley production in Western Australia.
3. Coordination and communication between the barley industry partners to foster industry development within Western Australia.
 - Improve links between members to strengthen the barley industry.
 - Involve industry in decision-making with the Western Region Barley Council for the benefit of growers.
 - Cultivate networks of communication at regional and national levels to enhance the barley industry.
4. Promote biosecurity in the Western Australia barley industry.
 - Management of biosecurity through the Grain Guard program.
5. Maintenance of the resource base.
 - Ensure long-term sustainability of the barley industry with respect to the resource base.

Collection and delivery of information

Many sources of information are used to progress the strategic development of the barley industry in Western Australia. The state government's DAFWA Barley Agronomy project provides information on variety performance in regional areas using data collected from its own research trials and that collected by the CVT and NVT projects. Variety-specific agronomic projects help to develop specific management packages to maximize production and quality. The BBA and plant pathologists help to embellish this. Commercial agribusiness and agronomists help to provide valuable feedback to the BIDO and researchers along with key growers and grower groups. Information on quality and

acceptance for commercial requirements are also fed back from the malting and brewing industry, grains quality researchers, marketers, feed grains industry and BA.

The BIDO collects, analyzes and synthesizes the outcomes from all the research and evaluation programs and external sources to tailor information to key audiences. Figure 3 provides a schematic plan for the collection and delivery process of information within the barley industry in Western Australia.

The sorts of information delivered include:

- Variety guides, which are increasingly more important as they reduce confusion within the industry and can be localized for agricultural districts within the state. Recent examples include the variety recommendation guide published as

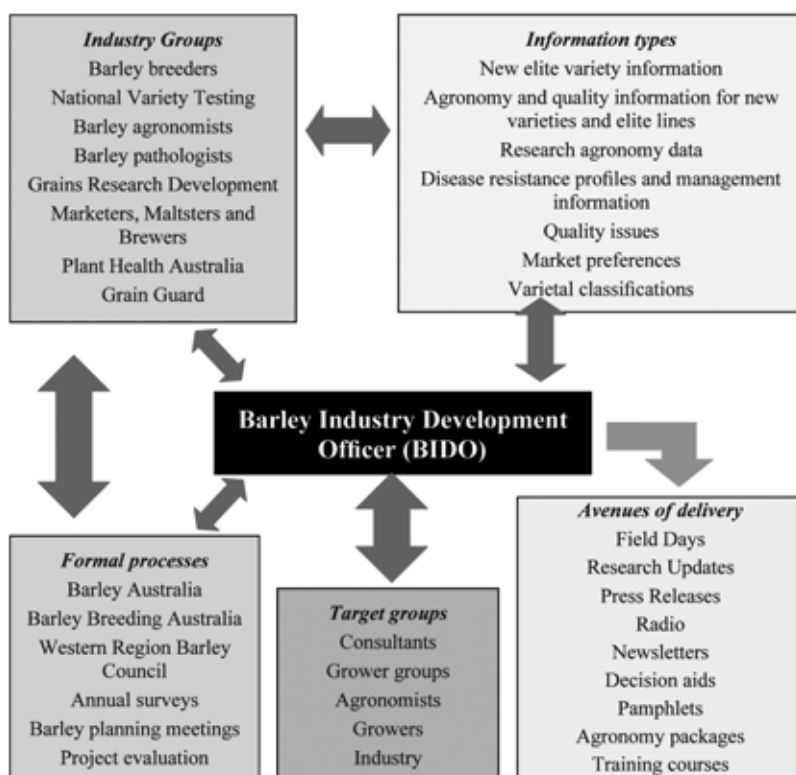


Figure 2. Schematic plan of the collection and delivery process of information within the barley industry, facilitated by the BIDO.

Farmnote 257 (Russell *et al.*, 2007a) and the comparison of new feed barley varieties published as Farmnote 261 (Russell *et al.*, 2007b).

- Management packages in the form of Farmnotes and Bulletins to growers. These are a vital part of ensuring the adoption of new varieties and management practices to assist in the maintenance of a quality product for the market place. The most recent management advice is as guidelines for Vlamingh barley (Farmnote 289; Russell *et al.*, 2008a) and an update for Baudin barley as Farmnote 290 (Russell *et al.*, 2008b). Segregation of varieties is of increasing importance with the development of many new malting varieties. Close liaison with producers, grain handlers and end users is essential to develop an understanding of the key issues requiring communication among all parts of the industry.
- Market and production information to track trends in the industry and to use for strategic planning.
- Promotion of the crop using a variety of media techniques and the attendance at grower, council and industry meetings. Techniques of communications depend on the audience being addressed. Brochures, pamphlets and leaflets, along with e-mail, Web sites, newspaper, radio and TV, all have a place in information delivery. The DAFWA Web site (www.agric.gov.au) has a section under 'Crops' >> 'Barley' titled: *The Barley Site: a guide to barley production in Western Australia* that encompasses general information regarding growing barley in Western Australia. Topics include seed selection, crop establishment, crop nutrition, integrated weed management, diseases and their management, harvesting and storage. Presentations at key industry meetings and less formal, but nevertheless no less important, field days with growers and agribusiness are valuable networking events.

Evaluation

The implementation of this strategy should result in:

- Agribusiness and growers selecting appropriate barley varieties that the market place wants and that are suited to their environment and can be delivered into the appropriate segregation.
- Agribusiness and growers identifying risk management strategies for optimum barley production.
- Agribusiness and growers being able to access management information for barley production on-line through the DAFWA Web site (www.agric.wa.gov.au) barley page.
- Rapid adoption of new barley varieties and management practices by growers.

The provision of a report documenting a numerical inventory of extension products, activities and the quantified impact of these priorities and purposes on the barley industry of Western Australia was planned for 2009. Key DAFWA and external clients were to be sought to gauge their reactions and assess the extent to which the project had raised awareness of Barley Industry information.

Conclusions

The vision of the industry development plan is an improvement in the skills of Western Australian barley growers in their ability to grow barley of a quality that meets market requirements.

The outcomes of the industry development plan are:

- An industry development program that assists barley breeders with a pathway to market for new barley varieties suited to Western Australia.
- Unbiased guidelines on the production and marketing of new barley varieties, how to achieve quality targets through crop management, and the role of barley in the farming system.
- A conduit for reciprocal information flow

among growers, agribusiness, researchers, breeders and industry to support a viable barley industry.

The communication delivered aims to involve growers themselves, their agronomists and advisers, maltsters, brewers, grain handlers, marketers—in essence, all parts of the supply chain in the growing, marketing and delivery of a high quality product.

Our goal is to maximize returns from barley by applying specific management strategies during production, harvest, storage and when selling the grain. We aim to work with industry to improve:

- Understanding of how grain quality affects malt and beer quality.
- Ability to alter grain quality by modifying paddock management practices.
- Marketing techniques to optimize returns for barley.
- On-farm storage of barley to maintain quality for processors.
- Ability to reduce the level of quality defects before, during and following harvest.

At the time of writing, the current *The Western Region Barley Industry Extension Strategy and Communication Plan* covered the period from July 2006 to June 2009. Evaluation of the current plan should be undertaken from January 2009, and then lead into the development of a new plan from July 2009.

As the Western Australian barley industry moves forward in a deregulated market it is critical that a whole-industry communication ethos is implemented to minimize issues within the supply chain and ensure that Western Australian barley is of the highest quality for sale into domestic and export markets.

The role of the BIDO in Western Australia is not only to focus on the needs of Western Australian growers, also but to work with the northern and southern BIDO's and BA to grow Australian barley. The role is to remain impartial of competing interests and utilize information from a wide range of sources to deliver precise messages through the industry networks at state and national levels.

Acknowledgements

Fellow Barley Industry Development Officers Kym McIntyre (North) and Mary Raynes (South) who have been working on similar barley development projects. Financial support from the Grains Research and Development Corporation is acknowledged, along with the support from staff from the Department of Agriculture and Food (Western Australia), and industry members of the Western Region Barley Council.

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Relevant Web sites

- ARID group (ARID): www.arid.com.au
- Australian Barley Board (ABB): www.abb.com.au
- Barley Australia (BA): www.barleyaustralia.com.au
- Barley Breeding Australia (BBA): www.barleybreedingaustralia.com.au
- Co-operative Bulk Handling (CBH): www.cbh.com.au
- Department of Agriculture and Food, Western Australia (DAFWA): www.agric.wa.gov.au
- Grains Research and Development Corporation (GRDC): www.grdc.com.au
- Joe White Maltings (JWM): www.joewhitemaltins.com.au
- National Variety Testing (NVT): www.acasnvt.com.au
- Plant Breeders Rights (PBR): www.pbr.ipaaustralia.optus.com.au

WORKSHOPS

Generation Challenge Program (GCP) Workshop

The workshop was held on the first day of IBGS10 and was attended by 70 scientists in the Bibliotheca Alexandrina, though it was competing with another workshop at the same time. Numerous scientists involved in GCP attended the workshop, as well as scientists currently not involved in GCP. Michael Baum [ICARDA] gave an overview of current status, objectives, ongoing projects in the Generation Challenge Program (the GCP Introduction PowerPoint™ presentation from the Web page was used).

The introductory presentation was followed by a presentation on a competitive grant “Allele Mining Based on Non-Coding Regulatory SNPs in barley germplasm” given by Maria von Korff [MPIZ, Cologne, Germany; former ICARDA Post-doctoral Fellow]. It is becoming increasingly clear that the study of changes in DNA coding for proteins is only one aspect of genetic variation, and for many traits, notably tolerance to environmental stresses, the amount of gene expression is also likely to be of great importance. A key problem in genetics is how to identify this type of variation. In the project funded by GCP, a robust approach to identify plant genes that harbor such regulatory variants is proposed and elaborated. The approach is novel, and particularly amenable to plants since it is based on monitoring gene expression in experimentally created hybrids. The principle of the approach is based on the hypothesis that the relative abundance of allelic transcripts when estimated for individuals in the heterozygous condition will be devoid of trans-acting influences and environmental factors, which can confound micro-array-based experiments. The approach is robust, scalable and particularly well suited to

crop plants, where the ability to produce sexually derived heterozygous hybrids is not rate limiting. A successful outcome will provide a new mechanism to connect genotype to phenotype based on changes in gene expression rather than on changes in the structure of an encoded protein. This approach will be used to characterize a series of genes identified, to reveal potential candidates for tolerance to drought, frost, boron and salinity stresses. The approach is generic and widely applicable. The project will also involve training researchers in Developing Countries and create a high quality collaborative network of researchers delivering new knowledge on genetic diversity and translatable outputs for the Developing World.

Dominique This [Montpellier SupAgro, Agropolis-France] gave a presentation on the project “A dataset on allele diversity at orthologous candidate genes in GCP crops (ADOC)”. The ADOC project aims to characterize allelic diversity at orthologous loci of candidate genes for drought tolerance in seven GCP crops (rice, barley, sorghum, bean, chickpea, cassava and potato), working with reference collections of around 300 accessions for each crop, selected by crop partners. Six gene families (ERECTA, DREB, SS, SPS, ASR and INV) were selected as the initial subset of target genes during an initial scientific exchange with gene specialists and advisers. Apart from the DREB gene family, for which a specific focus has been given to DREB2A, they represent a set of relatively small gene families acting at different levels of the drought stress response (transcriptional regulation, carbohydrate metabolism, etc.) for which a comparative analysis of the whole gene family was decided.

Joanne Russell [SCRI, UK], gave an overview of the newly initiated competitive project “Genomic dissection of tolerance to drought stress in wild barley”. *Hordeum spontaneum* introgressions into *H. vulgare* cv. Harrington and Syrian-Jordanian landraces are genotyped with the Illumina SNP chip (1536 SNPs) to identify by linkage and association mapping most important introgressions for drought tolerance. Marker assays will be derived for MAS applications for the barley breeding programs and steps undertaken in future to isolate the genes responsible.

Wafa Choumane [Tishreen University, Syria] gave a presentation on a commissioned project “Developing and genotyping a composite germplasm set of barley”. Within the Generation Challenge Program Subprogram 1, ICARDA was assigned responsibility developing a composite set

of 3000 barley accessions, representing the range of diversity of the crop and its wild progenitor, *Hordeum vulgare* subsp. *spontaneum*. The collection was characterized with EST-derived and genomic SSR markers, and a reference collection harboring about 90% of the genetic diversity of the composite collection was extracted. This reference set, after seed multiplication, would be available to other barley researchers on demand for association studies.

All the presentations raised considerable interest; numerous technical details were discussed following each presentation. In the general discussion, a number of attendees requested more information on the next dates for commissioned and competitive funding applications, showing an increased interest in participating in the Generation Challenge Program.

Barley Genetic Linkage Groups, Barley Genome, Genes and Genetic Stocks Workshop

Udda Lundqvist, Overall Coordinator (e-mail: udda@nordgen.org) and Agnese Kolodinska Brantestam (e-mail: agnese.kolodinska@nordgen.org).
Box P.O. 41, Nordic Genetic Resource Centre, SE-230 53 Alnarp, Sweden.

The following topics were brought up for discussion during the workshop:

1. The current coordination system of today and its function in the future.
2. Whole-genome coordination.
3. Integration of molecular and morphological marker maps.
4. Nomination of coordinators.
5. Barley Genetics Newsletter.
6. The International Database for Barley Genes and Barley Genetic Stocks, and GrainGenes.
7. Symbolization and nomenclature problems of barley genes.
8. Maintenance of barley genetic stocks.
9. International Overall Coordinator.

1. Coordination system

Discussions focused on its activities, both current and in the near future. There are many possible technologies for identifying genes today, and progress is being made in integration of gene-based maps, especially at the Scottish Crop Research Institute (SCRI). Bill Thomas, Scotland, presented a summary of past activities and how the coordination group was started, initially on NIL, SNP and AFLP marker integration. When now working on Illumina SNPs marker high throughout genotyping, 15 000 markers, QTLs and other technologies can give us much more information about each chromosome. He also gave an overview of the progress in integration of gene-based maps: (a) 4500 Illumina SNP markers; (b) 1000 genes on the IPK Gene Map; (c) 2500 genes in the Japanese EST map; (d) 1500 SFP markers; (e) 1000 Bowman lines mapped with BOPA1; and (f) 11 000 QTLs. It was suggested to have not each chromosome coordinated but store and coordinate all 7 chromosomes together.

2. Whole-genome coordination

Several participants stressed that we also need whole-genome coordination. This should be a team task, since the biology of mutants should be evaluated, and this is a huge effort and a full-time task. For the time being, the time is not ready for one person to handle this. Therefore the workshop recommended continuing with the current system. For publications of general QTL locations, the workshop recommended that the estimated Bin map position of the gene/QTL must be included.

3. Integration of molecular and morphological maps

Andy Kleinhofs [USA] has been trying to integrate molecular and morphological maps for many years, with notable success. This is increasingly of great importance. There exist many good maps that could be used as references, including SSR maps and DART maps. The quality of

data of these maps is very high and linkage groups are easily detected. We need all the basic morphological genes with associated information and to have them in good shape. In the San Diego, USA, meetings in 2008, only very few groups were represented that are working on molecular genotyping. David Marshall [SCRI, Dundee, Scotland] agreed to take over this responsibility.

4. Nomination of coordinators

Below is a list of the Chromosome/Linkage Groups and Genetic Stocks Collections, together with the names of the individuals who have agreed to be responsible for each.

a. Overall Chair and chromosomes:

Overall chair	U. Lundqvist, Sweden. assisted by: A. Kolodinska Brantestam, Sweden.
Chromosome 1H (5)	G. Backes, Denmark. Chromosome 2H J.D. Franckowiak, Australia.
Chromosome 3H	L. Ramsey, UK.
Chromosome 4H	A. Druka, UK. (replaces Brian Forster, who resigned)
Chromosome 5H (7)	G. Fedak, Canada.
Chromosome 6H	V Blake, USA.
Chromosome 7H (1)	L .Dahleen. USA.

b. Integration of molecular and morphological maps:

D. Marshall, UK. (replaces A. Kleinhofs, USA, who wished to step down).

c. Genetic Stocks and Collections:

Barley Genetic Stock Center:	H. Bockelman, USA. (replaces An Hang, who retired in 2007)
Trisomics and aneuploids:	H. Bockelman, USA. (replaces An Hang, who retired in 2007)
Translocations and BTT:	A. Houben, Germany.
Desynaptic Genes:	A. Houben, Germany.
Autotetraploids:	W. Friedt, Germany.
Disease and pest resistance genes:	M. Sutherland, Australia. (replaces B. Steffenson, USA, who wished to step down)
Eceriferum genes:	U. Lundqvist, Sweden.
Chloroplast genes:	M. Hanssom, Denmark.
Male-sterile genes:	M. Therrien, Canada.
Spike morphology genes:	U. Lundqvist, Sweden, and M. Stanca, Italy.
Semi-dwarf genes:	J.D. Franckowiak, Australia.
Early maturity genes:	U. Lundqvist, Sweden.
Barley-wheat genetic stocks:	A.K.M.R. Islam, Australia.

Coordinators are expected to conduct current literature searches and such research in their area of responsibility. Updated information should be published annually in Barley Genetics Newsletter.

5. Barley genetics newsletter

Barley Genetics Newsletter (BGN) was established in 1970 after decisions made at the 2nd International Barley Genetics Symposium (IBGS) in Pullman, USA, with its first volume appearing in 1971. The original idea was to report short preliminary barley research notes,

descriptions of barley genes and stocks, chromosome locations, barley maps and literature references.

One of the initiators of the Newsletter and the IBGS, Bob Nilan, USA, was attending the workshop and became acknowledged. After some discussions and opinions, the workshop decided strongly to continue the BGN in electronic format as it is the only forum for the barley community to publish updated and revised gene descriptions and short notes. Phil Bregitzer, USA, is continuing to act as main editor. Its availability should be more widely advertised and reminders for submissions will be sent several times a year.

6. International Database for Barley Genes and Barley Genetic Stocks, and GrainGenes.

Udda Lundqvist gave a short demonstration of this database with its own special address www.untamo.net.bgs. Most parts are linked to GrainGenes. Several participants in the workshop stressed that both databases are not easy and simple to use and rather time consuming. If you are familiar with barley nomenclature, genetics and Bin maps you get the information you need. Victoria Blake, USA, the coordinator for GrainGenes, promised to solicit suggestions to improve its usability and usefulness.

7. Symbolization and nomenclature problems of barley genes.

Udda Lundqvist informed the meeting of germplasm problems in connection with the Untamo database that Morten Huldén (the former head of the information department at the Nordic Genetic Resource Center and now responsible for this database) ran into when he was including revised descriptions.

- (a). Germplasm stocks should be assigned a GSHO number and seed samples should have been submitted to the Stock Center before descriptions are published.
- (b). References such as 'unpublished' and 'personal communication' should be avoided.
- (c). There are no clear definitions of disease and pest resistance genes.
- (d). When revising a gene and moving it from one locus to another, both descriptions should be revised and published.

In the discussions, several participants agreed that in many cases the rules for assigning genes, alleles and germplasm stock numbers have been ignored. In particular, the nomenclature rules for pest and disease resistance genes failed to conform. The former coordinator stressed that this nomenclature had existed for a long time, but nobody was willing to do the allelic tests; too often the literature has not been checked thoroughly, and temporary names simply assigned in order not to miss the allele. The new coordinator asked as a matter of urgency that new resistance names, genes and symbols should first be accepted by the coordinator before publishing.

The workshop also recommended strongly that the relevant rules be published annually in Barley Genetics Newsletter.

8. Maintenance of barley genetic stocks

The workshop acknowledged the existence of different barley collections worldwide, the necessity to maintain and keep them in good condition, and to update all information continuously.

9. International overall coordinator

The workshop recommended that the current chairs for the barley linkage groups and collections should continue.



International Center for Agricultural Research in the Dry Areas