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RESEARCH PAPER

Mapping of QTL associated with nitrogen storage and remobilization in barley (*Hordeum vulgare* L.) leaves

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Abstract

Nitrogen uptake and metabolism are central for vegetative and reproductive plant growth. This is reflected by the fact that nitrogen can be remobilized and reused within a plant, and this process is crucial for yield in most annual crops. A population of 146 recombinant inbred barley lines (F_8 and F_9 plants, grown in 2000 and 2001), derived from a cross between two varieties differing markedly in grain protein concentration, was used to compare the location of QTL associated with nitrogen uptake, storage and remobilization in flag leaves relative to QTL controlling developmental parameters and grain protein accumulation. Overlaps of support intervals for such QTL were found on several chromosomes, with chromosomes 3 and 6 being especially important. For QTL on these chromosomes, alleles associated with inefficient N remobilization were associated with depressed yield and higher levels of total or soluble organic nitrogen during grain filling and vice versa; therefore, genes directly involved in N recycling or genes regulating N recycling may be located on these chromosomes. Interestingly, the most prominent QTL for grain protein concentration (on chromosome 6) did not co-localize with QTL for nitrogen remobilization. However, QTL peaks for nitrate and soluble organic nitrogen were detected at this locus for plants grown in 2001 (but not in 2000). For these, alleles associated with low grain protein concentration were associated with higher soluble nitrogen levels in leaves during grain filling; therefore, gene(s) found at this locus might influence the nitrogen sink strength of developing barley grains.

Key words: Barley, grain protein concentration, *Hordeum vulgare* L., nitrogen remobilization, nitrogen storage, QTL, yield.

Introduction

Nitrogen is quantitatively the most important mineral nutrient taken up from the soil by plants (Marschner, 1995). A thorough understanding of its assimilation, transport and metabolism is central to modern plant biology, both with respect to understanding basic plant function and with respect to breeding or engineering crop plants for desirable traits.

The most important source of nitrogen for many crops (with the remarkable exception of legumes capable of symbiotic nitrogen fixation) is nitrate. Depending on the plant species and its physiological status, nitrate can be stored or reduced and assimilated in both roots and leaves (Marschner, 1995). In cereals, nitrate assimilation is predominantly localized in the shoots (Cooper and Clarkson, 1989; Larsson *et al*., 1991). In leaf vacuoles, nitrate can accumulate to high levels, which is undesirable if vegetative plant parts are used for food or animal feed (Marschner, 1995). The largest fraction of assimilated nitrogen is used for protein synthesis, while smaller fractions are present in other macromolecules (nucleic acids) and in a large number of different primary and secondary metabolites (Peoples and Dalling, 1988). Among these, amino acids, and especially the amides (glutamine, asparagine), occupy a central position, both in terms of the role they play in metabolism (Ireland and Lea, 1999) and because of their importance for long-distance

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transport of reduced nitrogen in the phloem (Bush, 1999). In mesophyll cells of fully developed cereal leaves, over 50% of the total nitrogen is present in the photosynthetic apparatus in chloroplasts (Peoples and Dalling, 1988). In many annual crops including cereals, nitrogen recycled from senescing vegetative plant parts during grain-filling is important for nitrogen yield (Feller and Fischer, 1994). Because of its importance for crop physiology, senescence has been intensively studied using biochemical and molecular approaches (Feller and Fischer, 1994; Quirino *et al*., 2000), but a comprehensive understanding of the regulation of this complex developmental process has not yet been achieved.

Barley (*Hordeum vulgare* L.), besides its importance as a crop, is an established model plant for both genetic and physiological studies (Koornneef *et al*., 1997; Forster *et al*., 2000). Some aspects of plant nitrogen metabolism have been studied in detail in this species (Warner and Kleinhofs, 1992; Sueyoshi *et al*., 1995; Botrel and Kaiser, 1997; Kronzucker *et al*., 1999; Vidmar *et al*., 2000), and well-defined genetic maps are available (Forster *et al*., 2000). The focus of this study was the analysis of causal relationships between leaf nitrogen metabolism and grain protein accumulation, using a combination of quantitative genetic and biochemical tools. A population of 146 recombinant inbred lines (RILs), derived from a cross between two varieties with marked, highly heritable differences in grain protein concentration, was used for this investigation. A 110 point linkage map has previously been established for these lines and used to map QTL controlling grain protein concentration (See *et al*., 2002). Here, total nitrogen was assayed in flag leaves at three developmental stages from anthesis through maturity to monitor total nitrogen accumulation and remobilization. These data were complemented with the analysis of nitrates and amino nitrogen (amino acids, small peptides) to follow continued uptake of inorganic nitrogen into the leaves as well as levels of transportable N compounds after anthesis. QTL and correlative analyses on these parameters were used and compared to QTL obtained for grain protein concentration to identify loci important for cereal nitrogen recycling.

Materials and methods

Plant material

Two barley (*Hordeum vulgare* L.) varieties, 'Lewis' (CI15856) and 'Karl'(CI15487), were chosen as parents for this study based on marked differences in grain protein concentration at maturity. Karl is a six-rowed variety which produces grain of consistently lower protein concentration than most other varieties. Lewis is a commonly grown two-rowed barley. A 146-member recombinant inbred population was developed by single seed descent from a cross and used to map the genes responsible for barley grain protein concentration (See *et al*., 2002). For the present study, F_8 and F_9 plants were grown in two independent replicates in a randomized block design near Bozeman, MT in summer 2000 and 2001 using standard farming practice. Flag leaves (10 leaves per sample) from all 146 lines as well as the parental lines, Lewis and Karl, were collected around anthesis, mid-grain fill and plant maturity, immediately frozen in liquid nitrogen, transported to the laboratory in liquid nitrogen and stored at –80 °C. Leaves were weighed, ground to a fine powder in liquid nitrogen using a mortar and pestle, and stored again at –80 °C until analysis.

The fact that heading date is one of the parameters which segregated in this population (see Results) made it impossible to collect samples for all lines at exactly the same developmental stages. Therefore, the dates were chosen in such a way that the first sampling time point was at anthesis ±3 d for most lines, before any potential changes of leaf nitrogen metabolism associated with the main phase of grain-filling. The last harvest date was characterized by mature plants, and leaves were fully senesced for all lines. Some influence of the developmental stage on the assayed parameters is to be expected for the middle harvest date.

Total nitrogen quantification

Total leaf nitrogen was analysed using a combustion method in a Leco FP 528 protein analyser (Leco Corporation, St Joseph, MI). Approximately 100 mg leaf powder was used, the exact weight determined using an analytical balance, and the sample was processed according to the manufacturer's instructions. The determined nitrogen concentrations (percentage of total sample weight) were used for correlative and QTL analysis (see below). Additionally, as leaf weights change during development and senescence, measured leaf weights were used to calculate total nitrogen on a per-leaf basis (mg $leaf^{-1}$) and to determine changes in total leaf nitrogen between anthesis and maturity (ΔN, mg $leaf^{-1}$).

Quantification of nitrates and soluble α-NH_2 nitrogen

For the extraction of soluble nitrogenous compounds, 50–100 mg of leaf powder was heated at 80 °C for 15 min in 500 µl (<75 mg) or 1000 µl H_2O (⩾75 mg) in a 1.5 ml tube, extracted with a small pestle (fitting the conical bottom of the micro tube) using a motor unit for 30 s, again heated to 80 °C for 15 min, centrifuged at full speed in an Eppendorf centrifuge, and supernatants were used for nitrate and amino nitrogen quantification, either directly or after storage at –80 °C.

For nitrate analysis, NO_3^- was reduced to NO_2^- enzymatically, using immunoaffinity-purified corn leaf nitrate reductase (The Nitrate Elimination Co. [NECi], Lake Linden, MI) as outlined by the manufacturer's protocol, and NO_2^- was determined colorimetrically using the sulphanilamide–*N*-naphthylethylenediamine method (NECi, Lake Linden, MI). Optical densities were determined after 10 min at 540 nm in a SPECTRAmax PLUS^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA). The method was calibrated using 0–10 nmol KNO_3.

A solution of 150 ppm TNBS (2,4,6-trinitrobenzene sulphonic acid) in 50 mM Na-borate buffer pH 9.5 (150 µl per well; total assay volume including sample: 200 µl) was used to assay soluble α-NH_2 nitrogen. The method was calibrated using 0–50 nmol glycine. Optical densities were measured at 405 nm 60 min after the addition of TNBS reagent.

Statistical and QTL analysis

Data were collected for both independent replications for all nitrogen uptake, storage, and remobilization traits from both years (2000 and 2001) and analysed using the General Linear Model procedure of SAS (SAS Institute Inc., 1990). Phenotypic correlations were calculated among traits using least square mean values for each genotype combined across replications and environments. Narrow

sense heritability on an entry-mean basis for the recombinant inbred lines was determined for these traits using the following equation:

$$\acute{o}^2_A/(\acute{o}^2_G+\acute{o}^2_{GE/E}+\acute{o}^2_{e/RE})$$

where $\acute{o}^2_A$ represents additive genetic variance, $\acute{o}^2_G$ represents genotypic variance, $\acute{o}^2_{GE/E}$ represents genotype by environmental variance divided by the number of environments, and $\acute{o}^2_{e/RE}$ represents error variance divided by the number of replications multiplied by the number of environments.

The 110 point linkage map developed by See *et al*. (2002) for the population of barley lines used in this study was used for genetic analyses. This map is based mostly on AFLP markers anchored to linkage maps with previously mapped morphological, storage protein and SNP markers (Kleinhofs *et al*., 1993; Liu *et al*., 1996; Kuenzel *et al*., 2000). QTL analysis was conducted using the PlabQTL Version 1.1 mapping program (Utz and Melchinger, 1996). Composite interval mapping employing the covariate SELECT option of PlabQTL was performed for detection of QTL. This option uses step-wise multiple regression to select cofactors. For QTL model building and detection of QTL×environment interactions, a LoD threshold of 2.5 was used. The additive effect of a marker was calculated by PlabQTL as ((mean of the homozygous Karl class–mean of the homozygous Lewis class)/2). QTL support intervals are calculated as the point along the significance peak at which the LoD score is 1.0 unit less than the peak LoD score. The phenotypic variance (σ^2_p) explained by a single QTL was estimated by the square of the partial correlation coefficient (R^2). The phenotypic variance (σ^2_p) explained by the QTL model was estimated by the adjusted correlation coefficient (R^2_{adj}), which accounts for the number of predictors in the QTL model.

QTL analyses of grain yield, heading date and grain protein concentrations have been published previously for this population (See *et al*., 2002). These data have been adapted and re-analysed using composite interval mapping to demonstrate relationships between leaf and grain nitrogen composition, plant development and metabolism.

Results

Genotypic variation and heritability

A wide range of values was observed in the recombinant inbred lines (RILs) for all traits measured in this study (Figs 1, 2), although differences between the parental lines Lewis and Karl, which were originally selected for their highly heritable difference in grain protein concentration, were smaller for most parameters, demonstrating transgressive segregation in both directions.

Specifically, both mean values and distribution for total flag leaf nitrogen were comparable for the first (around anthesis) and second (mid-grain fill) harvests, with a mean value of ~1.6% total leaf nitrogen (based on fresh weight) at anthesis and 1.45% at mid-grain fill (Fig. 1A, B). By contrast, much higher variation, from 1.25 to >3%, was found at maturity (Fig. 1C). As the leaves were fully senesced and partially desiccated at this point, this reflects a lower average per-leaf nitrogen content than on the previous dates (data not shown). The high variation among lines for nitrogen remaining in leaves at maturity reflects differences in nitrogen remobilization (which may be partially explained by differences in structural N content), suggesting that this population can be used for the genetic analysis of this parameter.

Figure 1D shows differences (mg leaf^{-1}) in total leaf nitrogen content between the first (anthesis) and last (plant maturity) harvest dates. Although leaf nitrogen metabolism is a dynamic process, characterized by both import and export of nitrogen compounds, this parameter gives an indication of the quantity of nitrogen exported from flag leaves to developing grains. Again, a wide range of variation, from <1 to 2.5 mg leaf^{-1}, was observed, with a mean of ~1.5 mg leaf^{-1}.

Soluble inorganic (nitrate; Fig. 1E, F) and organic (amino nitrogen; Fig. 1G, H) nitrogen compounds were quantified to gain a better understanding of overall nitrogen metabolism in the leaves. Freshly acquired nitrogen is primarily imported through the xylem in the form of nitrate in cereal leaves (Marschner, 1995), whereas amino acids are the main transport forms used to export nitrogen from both mature and senescing leaves (Bush, 1999). Nitrates varied from ~5 to 65 ppm (based on fresh weight) around anthesis (Fig. 1E) and continued to accumulate in leaves during grain fill, reaching concentrations of 300 ppm in some lines by mid-grain fill (Fig. 1F). As for the other nitrogen-related parameters, considerable variation in leaf nitrate was obvious in the population used for this study. The distribution for nitrate at anthesis appears slightly skewed on the linear scale used; this skewness can be eliminated and the data appear normally distributed when a logarithmic scale is used (not shown). Similar concentrations and distributions of amino nitrogen were found at anthesis and mid-grain fill (Fig. 1G, H), but overall concentrations and means were slightly lower at the later harvest date.

Leaf nitrogen parameters were compared with a number of developmental (leaf weight, heading date, yield) parameters and with grain protein concentration (Fig. 2A–D). Leaf fresh weights at mid-grain fill (Fig. 2A) were considered representative of leaf size, as they correlated well with leaf weights at anthesis (not shown). Correlations between leaf weights at mid-grain fill and maturity were less significant (not shown), as different parameters (e.g. remobilization efficiency) become more important at the later time point. Leaf size, grain protein percentage, grain yield, and flowering date all varied widely among lines in this population. Leaf weights ranged from 80 to >200 mg leaf^{-1} (Fig. 2A), while heading dates ranged from 2 July to 12 July (between 182 and 192 Julian days, Fig. 2B), and grain protein concentration ranged from 11% to 17% (Fig. 2C). Yield ranged from 1.5 t ha^{-1} to 5.5 t ha^{-1} (Fig. 2D).

Narrow-sense heritability was calculated for leaf nitrogen parameters and leaf weight. For leaf nitrogen concentration (% of FW) it was found to be 34% at anthesis, 57% at mid-grain fill and 48% at maturity. For difference in leaf nitrogen content between anthesis and maturity

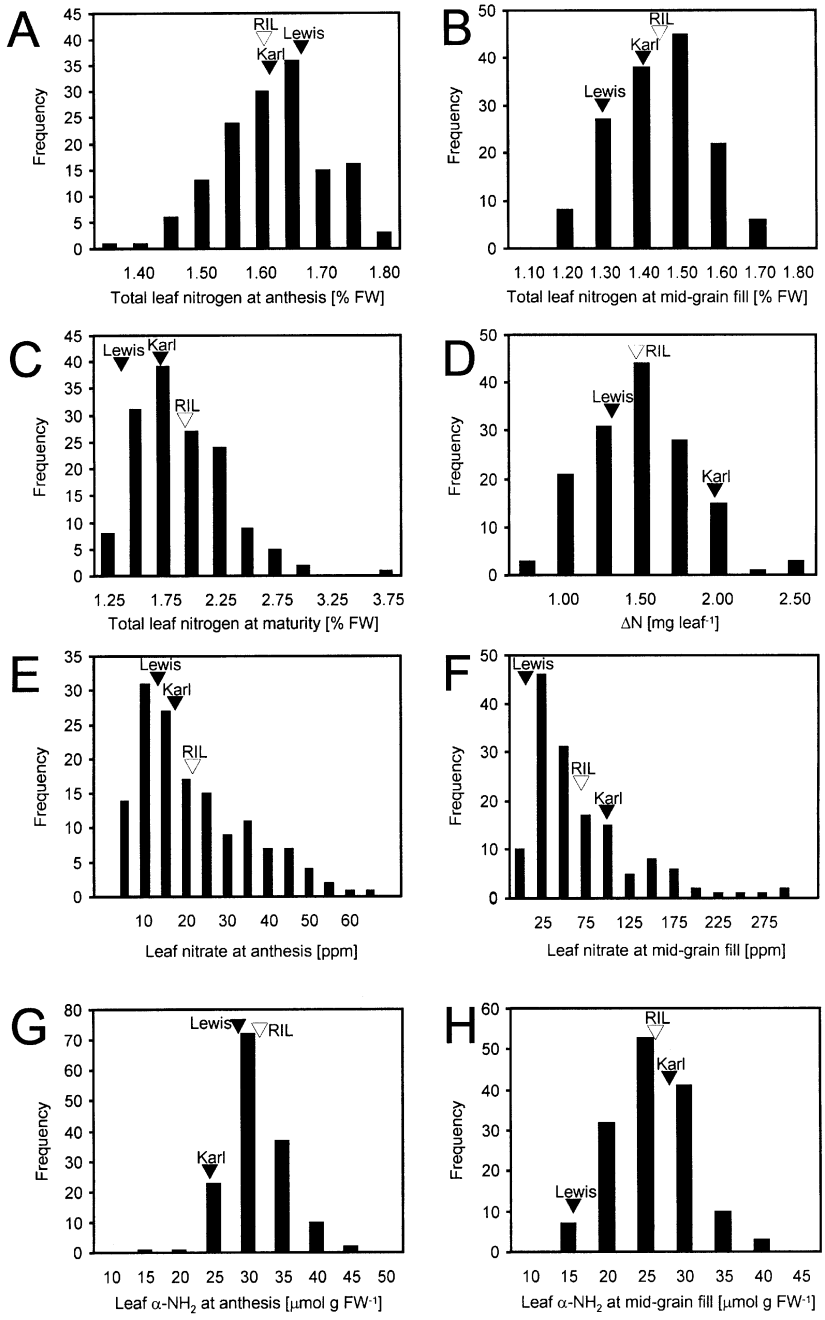

Fig. 1. Frequency distribution of leaf nitrogen parameters in the 146 recombinant inbred barley lines used for this study. Mean values for the recombinant inbred lines (RILs, open triangle) as well as the parental lines, 'Lewis' and 'Karl' (closed triangles) are shown.

(mg leaf^{-1}), it was found to be 49%, for nitrate (ppm) at anthesis 19%, but only 13% at mid-grain fill. The value for soluble α-NH_2 nitrogen was 38% at anthesis and 34% at mid-grain fill. Narrow-sense heritability was 65% for leaf weight.

An analysis of variance was performed on all leaf nitrogen parameters. This analysis indicated that sufficient variation exists between RILs to detect genetic mechanisms controlling the traits (data not shown). Only leaf nitrate at mid-grain fill did not exhibit significant (at P <0.05) genotypic variation, a result of a larger range of values in 2000 relative to 2001. Significant (P <0.01) genotype by environment interactions were detected for nitrogen at maturity, difference in leaf nitrogen concentration between anthesis and maturity, leaf nitrate at mid-grain fill and leaf weight. Phenotypic and rank correlations between years were highly significant and positive for the traits with genotype by environment

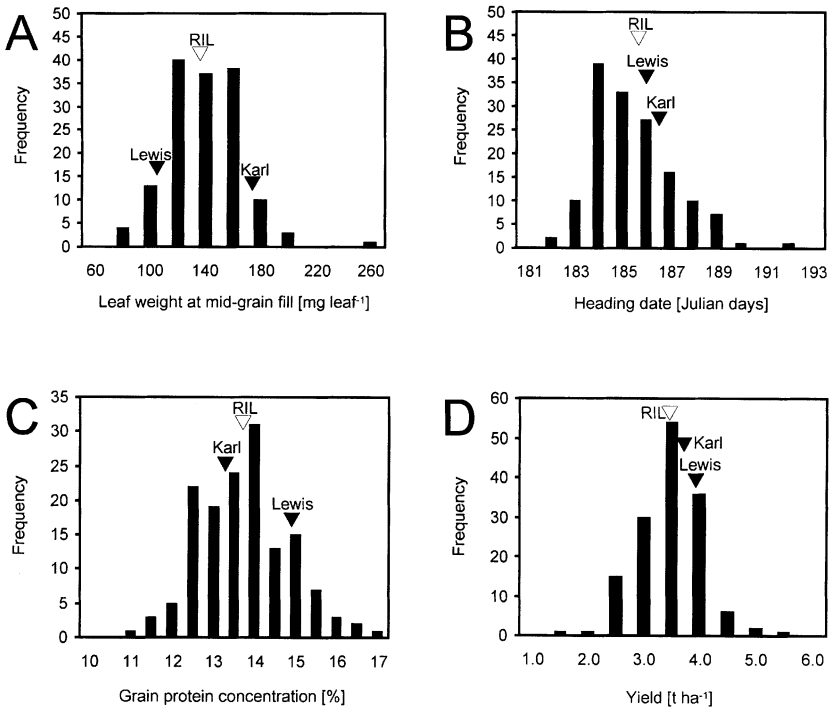

Fig. 2. Frequency distribution of developmental parameters and grain protein concentration in the 146 recombinant inbred barley lines used for this study. Mean values for the recombinant inbred lines (RILs, open triangle) as well as the parental lines, 'Lewis' and 'Karl' (closed triangles) are shown.

interactions. No crossover interactions were observed and interactions were mainly associated with differences in magnitude of effects between years. Genotypic variance exceeded genotype by environmental variance for all traits with the exception of leaf nitrate at mid-grain fill. As the G×E interactions detected were predominantly caused by differences in magnitude between years, data were combined across environments for further analysis.

Correlation analysis

The focus of this work was the evaluation of the genetic control of nitrogen storage and remobilization in barley leaves, as related to grain protein concentration and protein yield. To achieve this goal, simple statistical correlations (Table 1) were determined in addition to genetic analysis in the population of 146 RILs.

Interestingly, nitrogen remobilization efficiency from flag leaves (measured both by residual N in leaves at plant maturity and by the difference in leaf nitrogen content between anthesis and maturity, ΔN) was not correlated with grain protein concentration (Table 1). On the other hand, nitrogen remobilization efficiency was positively correlated with total yield and protein yield (calculated from protein concentration and yield) suggesting that remobilized nitrogen positively influences yield, but does not control grain protein concentration. Poor flag leaf nitrogen remobilization became apparent at a relatively early time point, since total leaf nitrogen as well as soluble inorganic (nitrate) and organic nitrogen pools were higher at mid-grain filling in those lines retaining high nitrogen concentrations in leaves at maturity. Total leaf nitrogen at anthesis was positively correlated with soluble α-NH_2 nitrogen at the same time point and total leaf nitrogen at maturity, but negatively correlated with nitrate at mid-grain fill.

As noticed by other researchers (Forster *et al*., 2000; See *et al*., 2002), developmental parameters have a strong influence on physiological traits. Heading date was negatively correlated with nitrogen remobilization from leaves and yield, but positively correlated with grain protein concentration. Nitrogen was more efficiently retranslocated in barley lines with large flag leaves; accordingly, leaf weight was also positively correlated with yield.

Overall, the picture emerging from simple correlative analysis of the 146 RILs used in this study indicates that, while nitrogen remobilization from leaves is important for total yield and protein yield, grain protein concentration is not controlled by this parameter. The correlations found between heading date and nitrogen pool sizes in leaves indicate that nitrogen remobilization is more complete in those lines with a larger time span between flowering and plant maturity.

Table 1. *Trait correlations*

Mean values of two years were used to correlate leaf nitrogen (% of FW) at anthesis, mid-grain fill and maturity, difference in leaf nitrogen content (ΔN, mg leaf^{-1}) between anthesis and maturity, leaf nitrate (ppm) at anthesis and mid-grain fill, soluble α-NH_2 nitrogen (μmol g^{-1} FW) in leaves at anthesis and mid-grain fill, grain protein concentration (%), yield (t ha^{-1}), heading date (Julian days), leaf weight (mg leaf^{-1}) and protein yield (kg ha^{-1}).

Trait	Nitrogen at anthesis	Nitrogen at mid-grain fill	Nitrogen at maturity	ΔN	NO_3^- at anthesis	NO_3^- at mid-grain fill	α-NH_2 at anthesis	α-NH_2 at mid-grain fill	Leaf weight	Heading date	Grain protein	Yield	Protein yield
Nitrogen at anthesis													
Nitrogen at mid-grain fill	0.148												
Nitrogen at maturity	0.220**	0.609**											
ΔN	–0.036	–0.454**	–0.640**										
NO_3^- at anthesis	0.042	–0.196*	0.039	0.234**									
NO_3^- at mid-grain fill	–0.213**	0.385**	0.422**	–0.275**	0.390**								
α-NH_2 at anthesis	0.373**	0.148	0.072	–0.045	0.035	0.014							
α-NH_2 at mid-grain fill	0.135	0.690**	0.429**	–0.311**	–0.047	0.397**	0.139						
Leaf weight	–0.382**	–0.332**	–0.480**	0.650**	0.110	–0.115	–0.106	–0.295**					
Heading date	–0.145	0.457**	0.291**	–0.287**	–0.365**	0.221**	0.129	0.324**	–0.069				
Grain protein	–0.039	–0.018	–0.035	–0.070	–0.185*	–0.061	0.069	–0.057	–0.002	0.465**			
Yield	0.160	–0.419**	–0.393**	0.231**	0.054	–0.344**	–0.110	–0.387**	0.170*	–0.484**	–0.279**		
Protein yield	0.154	–0.443**	–0.417**	0.212*	–0.022	–0.377**	–0.086	–0.433**	0.036	–0.293**	0.181*	0.890**	

*, ** Significant at $P < 0.05$ and $P < 0.01$, respectively.

QTL analysis

Physiological and morphological traits may be positively or negatively correlated for several reasons. Species like barley that are predominantly self-pollinated contain varieties that differ for a plethora of traits, and mere comparison of inbred lines fails to uncover which of these correlations are the result of pleiotropy or linkage, and which are the result of divergent gene fixation in different lineages. Distinguishing between these two alternative explanations of trait correlation is important because pleiotropy and tight linkage make selection for correlation-breaking individuals difficult. If an allele resulting in low foliar nitrate content pleiotropically results in low grain or forage yield, then that allele will be eliminated in crop improvement programmes. If, however, one genotype contains alleles at two unlinked genes that independently contribute to poor forage yield and low foliar nitrate content, then selection of desirable high-yielding, low foliar nitrate recombinants becomes straightforward.

The purpose of this study was the analysis of relationships between genes controlling variation in leaf nitrogen metabolism and grain protein accumulation, using a combination of quantitative genetic and biochemical tools. Since data from the two years of experimentation were pooled, QTL × environment interactions were analysed. Such interactions were detected for nitrogen at mid-grain fill, nitrogen at maturity, and leaf nitrate at mid-grain fill. In all cases, the interaction was associated with decreased effects of the QTL in 2001 attributed to decreased variance for the traits in 2001 relative to 2000.

Based on total flag leaf nitrogen levels at plant maturity and/or differences in total leaf nitrogen between anthesis and maturity (ΔN), several statistically significant QTL for nitrogen remobilization were detected on the seven barley chromosomes (Table 2; Fig. 3), with chromosomes 3, 6 and 7 each contributing at least two loci. The regions around markers acat466 and acag135 on chromosome 3, acag273 on chromosome 4, TB2122 on chromosome 5, and acgc132 and acgt517 on chromosome 6 appear to be of special interest, since the support intervals of QTL relevant for residual leaf N or nitrogen remobilization overlap with those of other QTL relevant for nitrogen metabolism (Fig. 3), namely with total or partial (nitrate, soluble α-NH_2) leaf nitrogen pools at earlier assay dates. For the loci on chromosomes 3 and 6, alleles associated with low residual N at leaf maturity were also associated with depressed total or soluble organic N levels at earlier harvest dates, while high residual N correlated with higher N levels at earlier dates. This relationship was inverted at acag273 on chromosome 4, where those lines carrying the 'Karl' allele, demonstrating higher residual leaf N at maturity, had depressed soluble α-NH_2 nitrogen levels at mid-grain fill, and at TB2122 on chromosome 5, where lines with lower residual leaf N at maturity had enhanced leaf N at anthesis (Table 2; Fig. 3). In the latter region, an interesting additional observation is that both total leaf

Table 2. *Quantitative trait loci (QTL) for leaf nitrogen parameters*

Total leaf nitrogen (% of FW) at anthesis, mid-grain fill and maturity, difference in leaf nitrogen content (ΔN, mg leaf^{-1}) between anthesis and maturity, nitrate (ppm) at anthesis and mid-grain fill, and α-NH_2 nitrogen (μmol g^{-1} FW) in leaves at anthesis and mid-grain fill are shown. Analyses were based on mean values from two years. Allelic effect shows the effect of carrying the 'Karl' as opposed to the 'Lewis' allele in the respective position, using the units indicated.

Trait	Number of QTLs	Chromosome number	Nearest marker	Support interval (cM)	LoD	Explained variance (%)	Total explained variance (%)	Allelic effect
Nitrogen at anthesis	2	5	actc410	124–184	2.97	8.9		0.046
		6	acgc132	66–90	4.40	13.0	11.9	–0.037
Nitrogen at mid-grain fill	6	3	acat466	182–208	3.44	10.3		0.031
		3	acag135	298–318	6.77	19.2		0.049
		5	acgc424	54–70	3.08	9.3		0.033
		6	actt298	0–12	5.67	16.6		0.040
		6	acgc132	70–102	3.40	10.3		–0.036
		7	actc55	24–28	3.32	10.0	43.3	0.122
Nitrogen at maturity	8	3	acat466	176–208	3.52	10.5		0.113
		3	acag135	260–308	4.99	14.6		0.197
		4	acag273	206–238	2.93	8.9		0.130
		5	TB2122	172–194	4.45	13.1		–0.126
		6	acgg515	54–76	2.83	8.6		–0.120
		6	acgt517	140–152	2.97	8.9		–0.147
		7	acaa389	42–56	3.13	9.5		–0.169
		7	rachi	142–190	2.66	8.1	29.1	0.177
ΔN	3	6	acgc132	68–90	4.70	13.9		0.142
		7	acgc140	32–46	4.26	12.7		0.151
		7	pinb1	52–64	3.59	10.8	20.0	0.148
NO_3^- at anthesis	6	1	actg256	282–306	3.03	9.2		3.321
		3	acaa158	14–59	3.23	9.7		–3.950
		3	acag135	252–322	2.58	7.8		–3.616
		4	HVM40	0–36	3.70	12.1		–3.819
		6	actt298	0–20	2.78	8.5		3.906
		7	acaa270	212–244	3.49	10.6	34.9	4.219
NO_3^- at mid-grain fill	1	6	actt298	0–16	5.51	16.2	16.2	34.549
α-NH_2 at anthesis	1	2	VVLOCI	140–154	4.69	13.8	13.8	–2.761
α-NH_2 at mid-grain fill	8	3	acat466	178–210	2.66	8.1		1.170
		3	actg385	306–332	4.73	13.9		1.740
		3	actc135	348–366	3.29	9.8		1.258
		3	acag175	496–512	3.00	9.1		–1.160
		4	acag273	220–238	4.14	12.3		–1.381
		6	actt298	0–14	3.45	10.4		1.333
		6	acgt517	134–160	3.01	9.1		–1.464
		7	acaa327	240–244	5.53	16.3	37.7	1.627

nitrogen at anthesis and yield are enhanced in presence of 'Karl' alleles. Additional QTL associated with N remobilization from flag leaves were found around markers acaa389 and Rachi on chromosome 7, but these were not associated with other N metabolism-relevant QTL.

It is well-known from the literature that most of the nitrogen found in proteins of mature cereal grains is remobilized and retranslocated from senescing vegetative plant parts (Peoples and Dalling, 1988; Feller and Fischer, 1994). In this study, correlation analysis, while demonstrating a positive correlation between nitrogen remobilization and total yield as well as protein yield, did not link N remobilization (ΔN) from leaves with grain protein concentration (expressed as % of grain weight), suggesting that grain protein concentration is not controlled by the amount of nitrogen remobilized from the leaves. Accordingly, no overlaps of support intervals for these traits were found by QTL analysis. On the other hand, while there was also no overlap of support intervals for ΔN and yield, support intervals of QTL for yield and leaf nitrogen at maturity (in % of FW) overlap on chromosomes 3 (near acag155/acat466) and 5 (actc410/TB2122). On chromosome 3, alleles associated with high leaf N at maturity are associated with low yield, while on chromosome 5, alleles associated with low residual leaf N are also associated with high yield.

Besides QTL associated with leaf nitrogen remobilization, a few additional loci interesting for nitrogen metabolism were identified. Several chromosomal regions involved in nitrate accumulation were found on chromosomes 1 (around marker actg256), 3 (acaa158 and acag135), 4 (HVM40), 6 (actt298) and 7 (acaa270). This information may prove useful for further investigations into nitrogen acquisition, long-distance and cellular transport, and for breeding low-nitrate forage barley varieties. From a basic point of view, the area at actt298 on

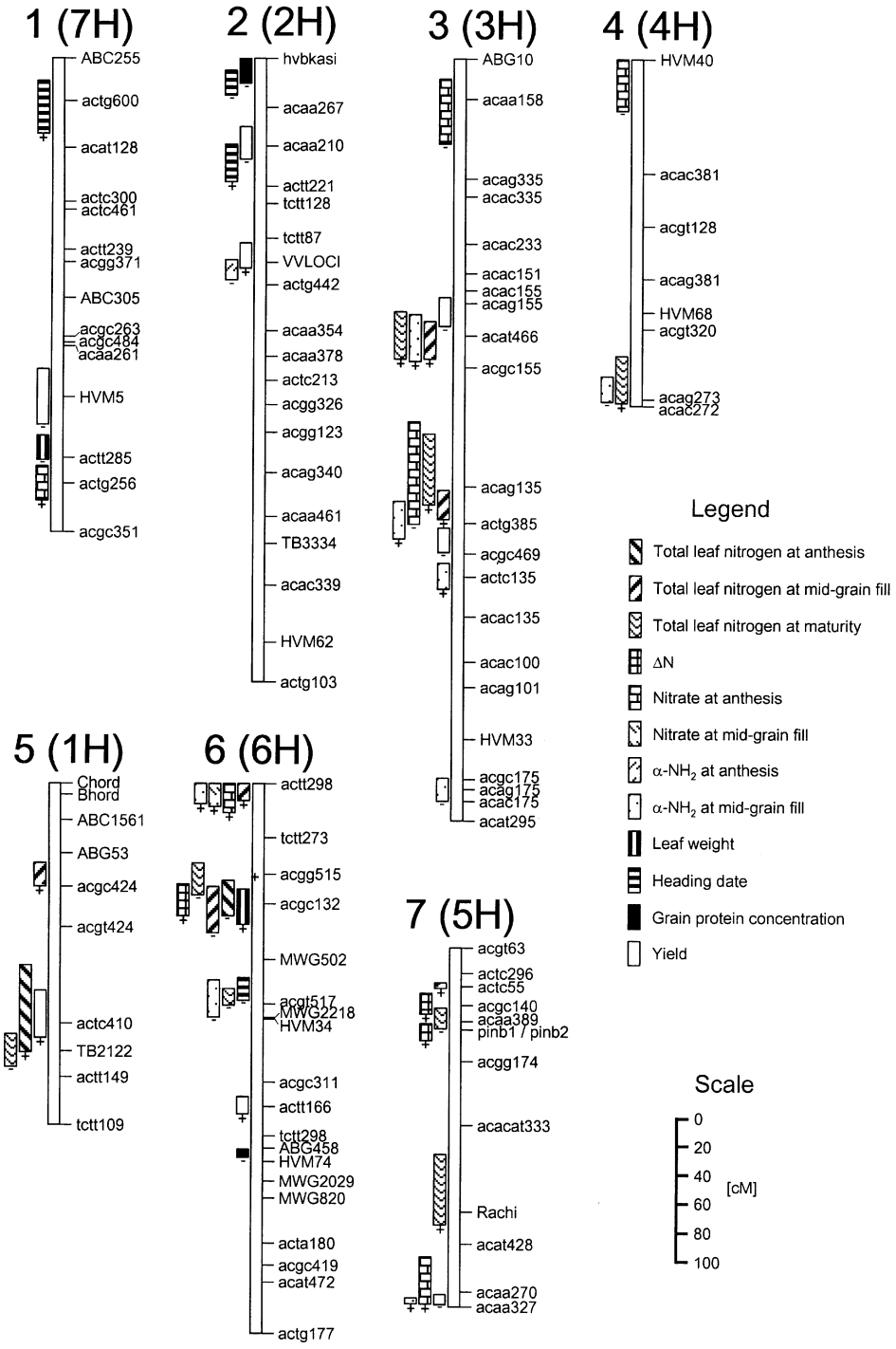

Fig. 3. Linkage map of RILs showing the location of QTL associated with the contents of total leaf nitrogen (% of fresh weight) at anthesis, mid-grain fill and maturity; differences in total leaf nitrogen content (ΔN, mg leaf^{-1}) between anthesis and maturity; leaf nitrate (ppm) at anthesis and mid-grain fill; soluble α-NH_2 nitrogen (μmol g FW^{-1}) at anthesis and mid-grain fill; leaf weight (mg leaf^{-1}) at mid-grain fill; heading date (Julian days); grain protein concentration (%) and yield (t ha^{-1}). Length of bar corresponds to support interval for each QTL, determined as outlined in 'Materials and methods'. The influence of the presence of the 'Karl' allele (+ or –) on the observed trait is indicated below the support interval of each QTL.

chromosome 6 is especially interesting, as several QTL for leaf nitrate at anthesis and mid-grain fill, soluble α-NH_2 and total leaf nitrogen at mid-grain fill overlap, suggesting that this region of chromosome 6 may contain gene(s) involved in leaf nitrogen acquisition and accumulation.

Table 3. *Quantitative trait loci (QTL) for leaf weight (mg leaf^{-1}), heading date (Julian days), grain protein concentration (%) and yield (t ha^{-1})*

Analyses were based on mean values from two years. Allelic effect shows the effect of carrying the 'Karl' as opposed to the 'Lewis' allele in the respective position, using the units indicated.

Trait	Number of QTLs	Chromosome number	Nearest marker	Support interval (cM)	LoD	Explained variance (%)	Total explained variance (%)	Allelic effect
Leaf weight	2	1	actt285	262–278	4.62	13.6		–10.546
		6	acgc132	72–96	4.54	13.4	17.7	8.899
Heading date	4	1	actg600	16–52	3.36	10.1		0.495
		2	acaa267	8–26	7.42	23.0		–1.050
		2	acaa210	60–68	2.57	5.8		0.621
		6	acgt517	132–148	5.75	16.6	35.5	–1.012
Grain protein	2	2	hvbkasi	0–18	8.66	26.3		–0.606
		6	HVM74	250–256	19.50	45.9	50.2	–0.727
Yield	8	1	HVM5	216–254	3.59	10.8		–0.147
		2	acaa210	48–70	4.13	12.3		–0.227
		2	VVLOCI	128–146	3.66	11.0		0.275
		3	acag155	166–186	4.56	13.4		–0.201
		3	acgc469	324–340	7.18	20.3		–0.249
		5	actc410	142–174	4.05	12.0		0.170
		6	actt166	214–226	4.85	14.2		0.176
		7	acaa327	238–244	4.40	13.2	38.1	–0.162

Correlative analysis (Table 1) pointed to a positive correlation between heading date and grain protein concentration, and to negative correlations between heading date and nitrogen remobilization (ΔN) and yield. This is reflected by confidence interval overlaps of QTL for heading date and grain protein on chromosome 2 (at hvbkasi), for heading date and yield also on chromosome 2 (at acaa210), and for heading date and leaf N at maturity near acgt517 on chromosome 6 (Fig. 3). For all these loci, the observed allelic effects (Tables 2, 3) confirm the results of correlative analysis, and demonstrate the influence of plant development on physiological traits.

The region around the major grain protein QTL at HVM74 on chromosome 6 (Table 3; Fig. 3; compare See *et al*., 2002) was analysed in more detail. While no additional QTL above the threshold of 2.5 were detected in this chromosomal region using mean values of two years of experimentation (2000 and 2001), detailed separate analysis of each year (data not shown) allowed some interesting observations. The 2001 field data showed a QTL peak with a LoD of 5.51 and a support interval from 246–258 cM for nitrates at mid-grain fill, and a QTL peak with a LoD of 3.68 and a support interval from 246–254 cM for soluble α-NH_2 nitrogen. No significant peak was found for nitrates at mid-grain fill analysing the 2000 data; while a peak for soluble α-NH_2 nitrogen was found close to HVM74 in this dataset (LoD=3.4, support interval from 266–278 cM), it did not overlap with the support interval of the grain protein QTL. Interestingly, for the 2001 data, 'Karl' alleles associated with low grain protein concentration at maturity were associated with higher nitrate and soluble α-NH_2 nitrogen at mid-grain fill, and RILs carrying the 'Karl' allele in the adjacent QTL for soluble α-NH_2 nitrogen in 2000 also showed depressed values. While these QTL peaks are not stable under changing environmental conditions, it appears intriguing that, for 2001, they co-localize with the grain protein concentration peak explaining 40% of the variation in this trait, and their interpretation may prove helpful in understanding the regulation of cereal grain protein concentration.

Discussion

The focus of this paper was to map QTL important for nitrogen storage and recycling from senescing leaves to the grains after anthesis. These data, especially if combined with QTL data for grain protein yield and grain protein concentration, can be used for ongoing breeding efforts aimed at influencing grain protein concentration while maintaining maximal yield. In the past, several studies have pointed to a negative correlation between these two parameters in cereals (Beninati and Busch, 1992; and references cited therein). Typically, a low grain protein concentration is advantageous if barley is used for malting, while high grain protein is a trait usually sought if this crop is used for food or animal feed (Weston *et al*., 1993). Additionally, the approach chosen here represents a first step towards the identification of genes important for nitrogen redistribution from senescing vegetative tissues. Gene identification should be facilitated by recently published results from rice genome sequencing and synteny between the different cereal genomes (Smilde *et al*., 2001; Goff *et al*., 2002).

Nitrogen remobilization from barley flag leaves

Several QTL influencing nitrogen remobilization (ΔN) and/or nitrogen concentration in leaves at maturity were identified on chromosomes 3, 4, 5, 6 and 7 (Table 2; Fig. 3). Confidence intervals for some of these QTL overlapped with QTL for other nitrogen metabolism-related parameters and for developmental parameters (heading date, leaf size).

Chromosomes 3 and 6 appear to be of special interest in this context. Both contain regions where support intervals of QTL for total leaf N at maturity overlap with QTL for leaf nitrogen at earlier analysis dates. Interestingly, alleles associated with low N in fully senesced leaves at these loci are also associated with low soluble organic or total leaf N at anthesis or mid-grain fill and vice versa; therefore, genes either directly involved in nitrogen remobilization or genes regulating this process may be present in those chromosomal regions. A draft sequence of the rice genome has recently been published and used to study synteny among the cereal genomes (Goff *et al*., 2002; and supplementary data to this article published online regarding synteny between rice, corn and other cereals including barley). The most interesting finding of these authors for the present study is the fact that a high degree of synteny exists between barley chromosome 3 and rice chromosome 1; this result confirms an earlier publication by Smilde *et al*. (2001). Some aspects of the genetics of leaf nitrogen accumulation and recycling have been studied by other authors in rice (Obara *et al*., 2001; Ishimaru *et al*., 2001; Yamaya *et al*., 2002). QTL determining the content of Rubisco, soluble proteins and NADH-GOGAT as well as a structural gene for NADH-GOGAT have been identified on rice chromosome 1 (Ishimaru *et al*., 2001; Obara *et al*., 2001). Considering the synteny between rice chromosome 1 and barley chromosome 3, it appears possible that some of these rice QTL are due to the same gene(s) or group of genes as the QTL identified in this study. Additional work will be needed to identify the regions of the rice genome syntenous to the parts of barley chromosome 6 discussed here, as the data of Goff *et al*. (2002) indicate that regions of a number of rice chromosomes contain markers derived from barley chromosome 6. So far, a combination of biochemical and molecular approaches has led to the identification of a large number of genes involved in nitrogen recycling (Buchanan-Wollaston, 1997; Quirino *et al*., 2000), but for many of them, their exact cellular function remains elusive. It appears realistic that a combination of biochemical and genetic approaches, as used here, will contribute to the improvement of this situation.

In addition to the information gained from N metabolism-related QTL overlaps, the influence of developmental parameters (heading date, leaf size) on nitrogen recycling appears intriguing. Correlative analysis indicates a positive correlation between heading date and leaf N at maturity (i.e. late-flowering lines are less efficient at N remobilization), and a negative correlation between leaf N concentration at maturity and leaf size (i.e. lines with large leaves are more efficient at N remobilization). Regions with support interval overlaps for these traits were found on chromosomes 6 (acgc132, acgt517) and 7 (acaa389). Pleiotropic effects of developmental genes on physiological parameters have been described in the literature (Forster *et al*., 2000), and See *et al*. (2002; compare Fig. 3) observed the influence of heading date on grain protein concentration. These observations could be explained if it is assumed that the velocity of nitrogen export from vegetative barley tissue does not segregate in the barley population used, therefore making the time available for nitrogen recycling more important. In this context, it is interesting that Dreccer *et al*. (1997) found that they were unable to affect the rate of N concentration decline in wheat stems and leaves using a variety of different treatments; based on these results, they discussed the possibility of an 'intrinsic' rate of nitrogen export.

Leaf nitrogen metabolism and yield parameters

The influence of nitrogen remobilization from senescing vegetative plant parts on grain protein concentration and yield has been investigated by different research groups (Van Sanford and MacKown, 1987; Papakosta and Gagianas, 1991; Beninati and Busch, 1992; Youngquist and Maranville, 1992; Barneix and Guitman, 1993; Oscarson *et al*., 1995; Lohaus *et al*., 1998; and references cited therein). While the specific results depend on the species/cultivar and the experimental system (field versus controlled conditions) chosen, it appears reasonable to conclude that leaf nitrogen metabolism influences grain protein concentration and/or grain protein yield.

In this study, no correlation between nitrogen remobilization efficiency and grain protein concentration was detected (Table 1). On the other hand, a strong positive correlation was found between N remobilization and total yield as well as protein yield. As 50–90% of the nitrogen found in cereal grains at harvest are derived from nitrogen remobilized from vegetative plant parts (Van Sanford and MacKown, 1987; Peoples and Dalling, 1988; Papakosta and Gagianas, 1991) and photosynthetic proteins such as Rubisco are usually degraded early during leaf senescence (Feller and Fischer, 1994), leading to a decrease in photosynthetic rates, this result may appear contradictory. However, there is good experimental evidence that leaf lipids, especially from plastidial membranes, are remobilized as well during leaf senescence (Gut and Matile, 1988; McLaughlin and Smith, 1995); therefore, the results obtained here may be explained if the lines more efficient in N remobilization are efficient at lipid degradation and C retranslocation as well. Considerable variation in the

contribution of pre-anthesis assimilates to grain yield has been found among different wheat varieties (Papakosta and Gagianas, 1991), and Gallagher *et al*. (1975, 1976) concluded that pre-anthesis storage of carbohydrates was important for grain yields in barley and wheat.

As discussed for nitrogen remobilization, both grain protein concentration and yield are influenced by heading date. This is reflected by a negative correlation between heading date and yield, a positive correlation between heading date and grain protein concentration (Fig. 3) and by the co-localization of QTL for these traits. One of the major QTL for grain protein concentration overlaps with a QTL influencing heading date (on chromosome 2), and the support interval for a heading date QTL overlaps with a yield QTL near marker acaa210, also on chromosome 2. Therefore, it appears that in late-flowering lines, there is a more severe reduction in carbohydrate (starch) than in protein yield, leading to fewer or smaller grains, but with a higher protein concentration.

The single most important QTL for grain protein concentration on chromosome 6 (at marker HVM74) does not co-localize with any other significant QTL, as determined from two years of experimentation. However, the fact that significant QTL peaks, overlapping with the support interval of this QTL for grain protein concentration, were found for both leaf nitrates and soluble organic nitrogen at mid-grain fill in the 2001 dataset appears intriguing. For both nitrates and soluble α-NH_2, leaf levels at mid-grain fill were higher in those lines carrying the 'Karl' allele, leading to lower grain protein concentration at maturity (data not shown). It is tempting to speculate that developing grains of lines carrying the 'Lewis' allele might be stronger N sinks; however, as narrow-sense heritability of soluble leaf nitrogen pools (especially nitrate) at mid-grain fill is lower than some of the other measured parameters, this effect may be difficult to observe. Since the recently characterized grain protein QTL from *Triticum turgidum* (Joppa *et al*., 1997; Chee *et al*., 2001) represents a potential homologue (See *et al*., 2002), further genetic analysis of this chromosomal region appears important, both from a basic (mechanistic understanding of grain protein accumulation) and applied (importance of grain protein concentration as a quality factor) point of view.

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