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Identification of barley mutants in the cultivar 'Lux' at the *Dhn* loci through TILLING

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Abstract

TILLING is a reverse genetic strategy that allows screening for mutations in genes with known sequences in a plant mutant population. A TILLING population has been developed for the Danish barley variety 'Lux' (*Hordeum vulgare* L.), by using sodium azide to induce mutations. Scoring of four visible phenotypic characters of barley seedling in reference to the parental cultivar 'Lux' in the M_3 plants showed over 3.5% lethality. A series of pool ratios of mixed DNA from mutant lines were tested and 10-fold pools appeared to be the practical mixing ratio for the detection of fragments in the 500–700 bp range. Two of the 13 known dehydrin genes, *Dhn12* and *Dhn13*, respectively, were examined and five independent missense mutations were obtained from a population of 9575 barley mutant plants. This corresponds to a mutation density of approximately one mutation every two and half million base pairs for these two genes. The mutant population of approximately 10 000 lines was screened for mutations in two genes in a short time due to high pooling ratio.

Key words: TILLING — *Hordeum vulgare* — barley — dehydrin — *Dhn12* — *Dhn13*

During the last two decades, the knowledge in the field of molecular biology has increased enormously and important progress in studying gene structures and functions has been achieved. Ample information on the genome sequences is now available in particular from model species such as rice and *Arabidopsis* (Bevan et al. 2001, Goff et al. 2002), but the functions of the predicted genes are largely unknown. Therefore, to fill the gap between the structure and function of genes, many research on reverse genetic techniques have been developed. Induced mutations with molecular marker technology are now playing an important role in this field, leading to a firmed up demand for mutagenized plant material in which certain characters have been changed due to knockout mutations of the responsible genes. Using molecular and genetic tools, a mutated character can then be associated with a DNA sequence of previously unknown function (Henikoff and Comai 2003, Comai and Henikoff 2006).

TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetic strategy developed that allows rapid mutational screening to discover induced lesions in a gene of interest (McCallum et al. 2000). The strategy is based on a PCR screening within known sequences region of interest for variants of heteroduplex analysis or mismatch cleavage to detect mutations. The use of chemical mutagens allows generating an allelic series at the DNA level, resulting in a change of function, reduced activity or specificity or a knockout mutation (Henikoff and Comai 2003).

So far TILLING has been successfully applied to both plants and animals, *Arabidopsis* (Till et al. 2003), *Lotus* (Perry et al. 2003), maize (Till et al. 2004), wheat (Slade et al. 2005), *indica* rice (Wu et al. 2005, Till et al. 2007), barley (Caldwell et al. 2004, Talamè et al. 2008) soybean,(Cooper et al. 2008), *Pisum sativum* (Dalmais et al. 2008), *Drosophila melanogaster* (Bentley et al. 2000), rat (Smits et al. 2004), zebrafish (Wienholds et al. 2003, Draper et al. 2004).

Dehydrins (*Dhn*) are produced in a wide variety of plant species in response to environmental stress caused by a dehydrative component (Campbell and Close 1997). Dehydrin proteins typically accumulate in plants during the late stages of embryogenesis or in response to low temperature, ABA application, or any environmental influence that has a dehydration component, such as drought, salinity or extra-cellular freezing (Campbell and Close 1997). The most complete information on the *Dhn* gene family is available for barley (Choi et al. 1999). In barley, it has been estimated that the total number of dehydrins should be 12–14 genes of which 13 members of the gene family have been cloned so far. (Choi and Close 2000, Choi et al. 2000).

A population of 9575 lines (M_3) of mutant barley has been developed in the Danish variety Lux (Engvild and Rasmussen 2004) and many root hair mutants were isolated in this population.

Here, we demonstrate the utility of the TILLING technique in the detection of induced point mutations in two dehydrin genes, *Dhn12* and *Dhn13* that might be of interest for a research programme on drought effects on plants.

Material and Methods

Development of mutagenized population: The mutagenized population has been developed in the Danish barley variety 'Lux' (Sejet Plant Breeding, Denmark). 'Lux' has high yield potential, very short stiff straw and is moderately resistant to lodging. Lux is resistant to powdery mildew possessing the *Mla12* resistance gene and very resistant to brown rust, but it is moderately susceptible to net blotch. It

is susceptible to *Rhynchosporium*, and it has a late maturing and small grain size.

Approximately 24 000 seed of *Hordeum vulgare* cv. 'Lux' were soaked overnight in demineralised water at room temperature, and then treated with 1.5 mM sodium azide in 0.1 M sodium phosphate buffer pH 3.0, 1 ml/seed for 2.5 h according to the IAEA (International Atomic Energy Agency) manual on mutation breeding (2nd edn). After rinsing in tap water and air drying, these M_1 seeds were sown the same day in the field. Spikes, four to six per M_1 plant, were harvested and propagated to M_2.

Tissue collection, DNA isolation and pooling: The M_3 seeds were planted in the soil to get enough material for DNA isolation, and grown in a greenhouse (24°C 16 h day and 14°C 8 h night) in trays having 5 × 5 cm holes for each plant. One plant each of the 9575 lines in M_3 was scored for four visible phenotypes/characters: abnormal or no growth, chlorophyll defects, dwarfs and necrotic spots on leaves.

After 12–15 days, 0.5 g sample of leave tissue from single plant were collected in liquid nitrogen and stored at −20°C. Freeze-dried tissue was grounded in 2 ml tubes with two 3 mm steel-balls to a fine powder in Retsch Mixer Mill (Mixer Mill MM 200, F. Kurt Retsch, Haan, Germany) for 2 min at a speed of 30 Hz. The extraction of genomic DNA was according to the CTAB procedure (Saghai-Maroof et al. 1984) in deep 96-well plates (2.2 ml capacity per well, Eppendorf). The extracted DNA was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and quantified on 1% agarose gel using λ DNA as a reference.

Following extraction, DNA was normalized to a final concentration of 10 ng/μl. DNA samples were arrayed in a microtitre 96-well format and pools of 2, 4, 8, 10 and 12 DNA samples mixed were tested.

TILLING procedure to detect induced point mutations: Polymerase chain reaction was performed in a 20 μl volume using 1 U of Amplicon *Taq* polymerase in 1X standard buffer (http://www.ampliqon.com), 0.2 mM dNTPs, 0.5 μM primer mix. Unlabelled primers were obtained from MWG Biotech (http://www.mwg-biotech.com), and labelled primers were obtained from Applied Biosystem (Warrington, UK) (Table 1). Primers were mixed in a ratio of 2 : 3 (unlabelled : labelled) to a final primer concentrations of 0.5 μM. Cycling was performed in Applied Biosystem, GeneAmp® PCR System 2700 96-well cyclers. Cycling was followed by CEL I digestion treatment. PCR and CEL I digestion assays were carried out as described by Mejlhede et al. 2006. The DNA was separated by denaturing gel electrophoresis and detected in ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The gel image was analyzed using GenScan 3.1 Software (Applied Biosystems, Foster City, CA, USA).

DNA Sequencing: DNA sequencing of individual samples were performed in 50 μl volumes. The amplified fragments were purified to remove/inactivate the remaining primers and dNTPs by using Sephadex G-50 fine (Amersham Biosciences, AB, Uppsala, Sweden). The eluates were freeze-dried and sent to MWG for sequencing (http://www.mwg-biotech.com). Sequence trace analysis was performed using SEQUENCHER TM 4.1 (Gene Codes, Ann Arbor, MI, USA) software.

A web-based tool for the analysis of polymorphisms in genes 'Project Aligned Related Sequences and Evaluate SNPs programs' (PARSESNP) (Taylor and Greene 2003) was used with sequencing data of *Dhn12* and *Dhn13*. The software determines the translated amino acid sequence from a reference DNA sequence (genomic or cDNA), and the effects of the supplied polymorphisms on the expressed gene product. It provides a Position-Specific Scoring Matrix (PSSM). Difference scores based on alignment analysis were used also for data sequencing analysis.

Results

Phenotypic analysis of mutation population

Previous studies on this population showed that the chlorophyll mutation frequency of the mutagenized population 'Lux' material was 7% of the M_1 progeny and 0.9% of M_2 seedlings.

In the current study, four visible phenotypic characters of seedling (no growth, chlorophyll defects, dwarfs and necrotic spots on leaves) were scored in reference to the parent cultivar 'Lux' in the M_3 generation, showing over 3.5% abnormal plants (Table 2).

DNA preparation and pooling

Screening of pooling ratio experiments were performed using a different positive mixed-fold pooling ratio at the beginning (2, 4, 8, 10, 12), 10-fold pool appeared to be the practical ratio of detection by ABI 377 (Applied Biosystems) for fragments in the 500- to 700-bp range (Fig. 1). Therefore high-throughput TILLING was performed on 10-fold pooled samples for the *Dhn12* and *Dhn13* genes.

High-throughput screening of the mutant population

In this study, 9575 mutant lines were screened and pooled 10-fold in a 96-well format. Primers were designed to yield a 745 bp and a 544 bp product for *Dhn12* and *Dhn13*, respectively. PCR products were loaded on ABI PRISM® 377 DNA Sequencer (Applied Biosystems) and showed three positive pools for *Dhn12* and two positive pools for *Dhn13*, respectively (Fig. 2). These five positive pools were confirmed by screening the 10 individuals after mixing their DNA with Lux wild type DNA and using the CELI method to find the individual mutant in the pool. A single plant was identified from each of

Table 2: Phenotype screening of the M_3 seedlings. Four visible phenotype characters were scored in reference to the parent cultivar (cv. 'Lux') in the M_3 seedling plants

Phenotype	No. of mutants	% Population
No growth	60	0.64
Chlorophyll defects		
White plants	61	0.81
Yellow plants	16	
Dwarfs	153	1.61
Necrotic spots on leaves	49	0.51

Table 1: List of primer sequences and expected DNA fragment length

Primer	Target	Sequence (5′–3′)	Labelled dye	Annealing temperature	Size (bp)
Dhn12 F	Dehydrin12	GTGCAGCGCTACAAACAGAA	5′-Fluorescent label, VIC	68°C	745
Dhn12 R		GATCGCGGGCATCTTATTTA	5′-Fluorescent label, 6-FAM		
Dhn13 F	Dehydrin13	TAAATACCGGCGAAGACGAG	5′-Fluorescent label, VIC	68°C	544
Dhn13 R		CCAACAAGAAGCCAAGAACG	5′-Fluorescent label, 6-FAM		

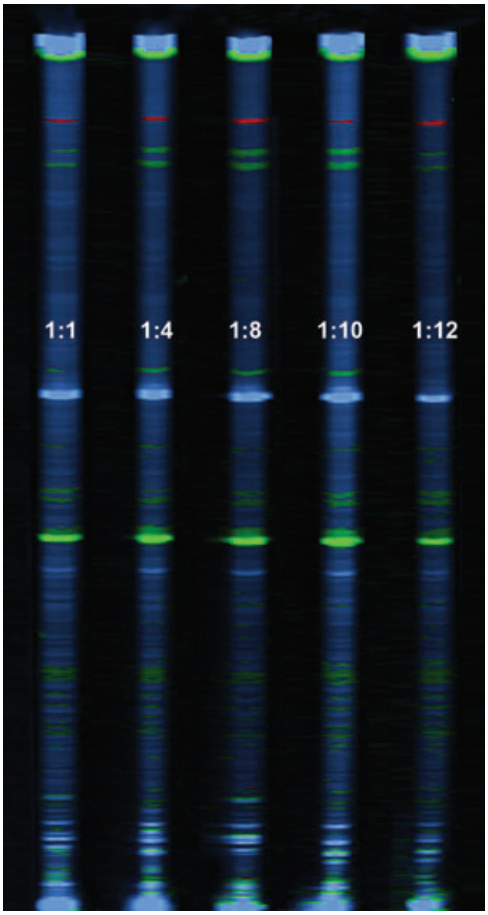


Fig. 1: DNA positive samples were pooled in different ratio (1:1, 1:4, 1:8, 1:10 and 1:12), and the resulting PCR products were visualized using denaturing polyacrylamide gel electrophoresis (ABI PRISMR 377 DNA Sequencer)

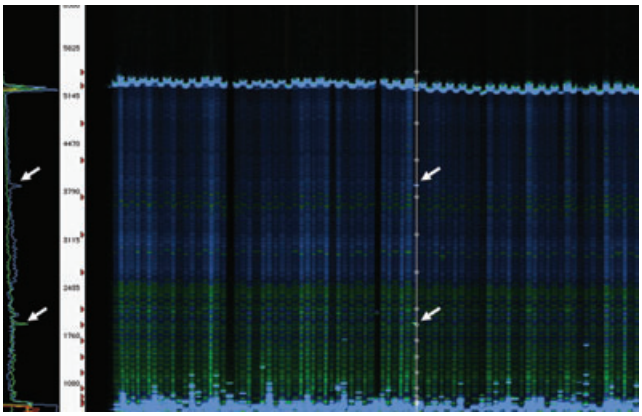


Fig. 2: An example of gel image of mutation detection analyses on an ABI PRISMR 377 DNA Sequencer running the GenScan 3.1 program. Representative example of a gel used to screen barley samples for mutation in the *Dhn12* loci. Each of the 96 lanes of the polyacrylamide gel contains tenfold-pooled CEL I-digested PCR products of 745 bp. Arrowheads indicate an SNP present in the sample. It is labeled at the 5′ terminal with VIC (Green) on one strand and with 6-FAM (Blue) on the other strand

the 10 positive pools, for both genes, that carried a putatively induced mutation. The size of the CELI-cleaved fragment approximated the location of the mutation, which was then determined by single pass sequence from both ends of each fragment.

Sequencing data analysis

The three mutations in *Dhn12* and two in *Dhn13* were allocated to five lines and the PCR products were sequenced for each. Data was submitted to PARSESNP, which automatically analyzes mutations discovered and the graphical and tabular outputs from PARSESNP are shown in Fig. 3. The alignments of sequencing fragments from *Dhn12* and *Dhn13* with wild Lux showed that three of these mutations were in the coding region and all of them were missense resulting in amino acid changes. Two of these missense mutations were G/C-to-A/T mutations, while the third one was C/G-to-A/T. The other two point mutations were located in non-coding region either upstream of the start codon or beyond the stop codon, and both were G/C-to-A/T mutations.

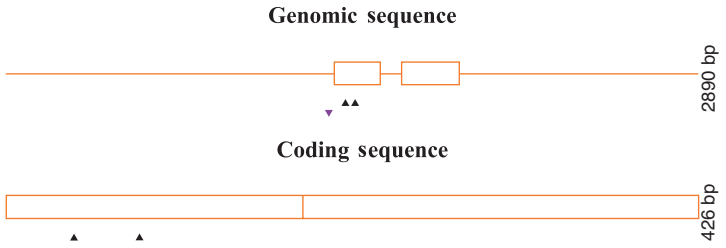
Discussion

TILLING as a reverse genetic strategy is becoming more common for studying gene functions. It has different advantages over other reverse genetic methods as it does not require transformation or RNAi techniques, it could be applied to many crops. The chlorophyll mutation frequency of the mutagenized population material study that done on this population previously was similar to that obtained with other barley cultivars from which low-phytate mutants were isolated (Rasmussen and Hatzack 1998). Another indication of mutation frequency is given by the identification of 58 root hair mutants by screening of approximately 20 000 M_2 seedlings from 2000 M_1 progenies on black filter paper (Engvild and Rasmussen 2004).

Two of the 13 dehydrin genes were screened for within the 'Lux' population in order to investigate the benefits of using TILLING for high throughput mutation discovery. *Dhn* genes were chosen as the full sequences of these genes are available. Another interested purpose for using dehydrin genes is that these genes have been shown to be up-regulated by abiotic stress conditions (drought, cold and salinity) in plants. Creating allelic series of these genes may allow studying their function in the cell and impact on drought tolerance. As they are relevant for drought tolerance, their manipulation may be relevant for breeding drought adapted germplasm.

Pooling ratio of DNA is an effective issue to distinguish the mismatched heteroduplex of the target fragments. It is very important to make a balance between increasing the pooling ratio which reduces the number of assays needed per mutation detection and detection sensitivity. But, as pooling levels increase, the proportion of heteroduplexes decreases, and sensitivity is reduced. Increasing of pooling levels means that the effort in finding the mutant individual within a positive pool exceeds that of screening the pools (Henikoff and Comai 2003). One of our targets in this study was to maximize throughput of the TILLING procedure by increasing pooling but still being able to detect all possible mutations available. We have tested the Lux mutant population with different pooling folds and shown that heteroduplex point mutation detection can be efficiently discovered even in 12-fold pools, while in the most other TILLING organisms' projects four- to eightfold pools have used (Perry et al. 2003, Caldwell et al. 2004, Slade et al. 2005). The increasing detection sensitivity

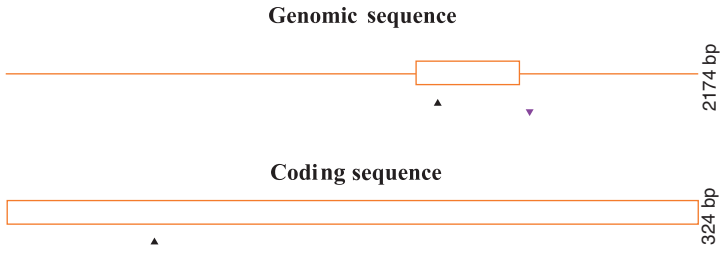
(a)



(b)

#	Nucleotide change	Effect	Restriction enzyme differences from rebase		Description	Zygosity
			Gained in variant	Lost from reference		
1	G125A	Non-coding	MmeI	BbvI, Fnu4HI, NspBII, TseI	E10P10002F	Homo
2	G193A	G15S	TatI	BcefI	E10P6102R	Homo
3	C233A	S28Y		AciI, BsiYI, EciI	A02P4802R	Homo

(c)



(d)

#	Nucleotide Change	Effect	Restriction enzyme differences from rebase		Description	Zygosity
			Gained in variant	Lost from reference		
1	G173A	D24N	MboII	FokI	F11P09801F	Homo
2	G459A	Non-coding		AarI, BsgI, BspMI	A12P04101F	Homo

Fig. 3: PARSESNP output of two *Dhn* genes. Data was provided to PARSESNP. The graphical and tabular outputs from PARSESNP are shown. (a) and (c) is a map showing the positions of three and two independent mutations in the TILLed fragment, based on the gene model (red boxes for exons and lines for introns) of *Dhn12* and *Dhn13* genes, respectively. The table provides information concerning the effect of the mutation and restriction site changes that can be used for genotyping progeny plants. Purple arrowheads that point downwards indicate changes to non-coding regions of DNA. Black arrowheads that point upwards indicate changes that induce missense mutations in the protein product. The effect of each nucleotide change is listed and summarized in tabular form below (b) and (d) for *Dhn12* and *Dhn13*, respectively. Additionally, the restriction sites either gained or lost because of the induced polymorphism are listed in these tables (Taylor and Greene 2003, http://www.proweb.org/parsesnp/)

could be related to the use of labelled primers (6-Fam and Vic) on the ABI 377 Sequencer system (Applied Biosystems). We aim to increase the sensitivity of mutation detection and reach up to 16-fold pools and even more.

Five point mutations were detected yielding a mutation density of approximately one mutation every two and half million base pairs or 2000 mutations per barley genome. All missense mutations resulted in changes of either G/C or C/G to A/T as expected for sodium azide treatment (Taylor and Greene 2003, Talamè et al. 2008). This mutation frequency within those two genes was lower than the one observed in another barley TILLING project (*Hordeum vulgare* L. cv. 'Optic'), where one mutation every one million base pairs was obtained (Caldwell et al. 2004). Besides this, there was one mutation every 374 000 base pairs in Morex TILLING population (Talamè et al. 2008). One reason for this difference with the 'Optic' population might be related to the mutagenesis treatment, because the 'Lux' population was treated with sodium azide while *Hordeum vulgare* L. cv. 'Optic' population was treated with EMS. On the other hand, four thousand DNA samples of this population were screened with two *Dhn* genes (*Dhn*3 and *Dhn*8) showing one mutation per 0.6 Mb and per 1.4 kb, respectively (data not shown). The average point mutation frequency of these four genes is one mutation every one and half million base pairs. This frequency may provide a series of up to 25 alleles within each 1.5 kb fragment of a target gene. This mutation frequency is comparable with other TILLING populations like 1–1.4 mutations per 1 Mb in the 'Optic' barley population and one mutation per Mb in rice IR64 population (Caldwell et al. 2004, Wu et al. 2005), but it is lower than those observed from *Arabidopsis*, *Lotus* and wheat mutant populations (1 per 300 kb, 20 kb and 22 kb, respectively) (Perry et al. 2003, Slade et al. 2005).

These developments in the procedure of the mutation detection technique (such as reaching up to 10-fold pooling and to use the ABI-3100 system; Applied Biosystems) made the work easier and more efficient, as it will increase the

throughput and decrease the cost of rapid mutation discovery in this population when it will be screened with more candidate genes.

We have for the first time demonstrated the successful application of the barley cultivar 'Lux' population to detect mutations in target genes. Construction of TILLING populations in barley and successful application to detect mutations (allelic series of a gene) is not only important for the scientific community but also from a plant breeding perspective, and in particular for breeding for drought tolerance.

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