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Map locations of barley *Dhn* genes determined by gene-specific PCR

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Abstract We previously identified 11 unique barley *Dhn* genes and found, using wheat-barley addition lines, that these genes are dispersed on four chromosomes 3H, 4H, 5H, 6H. In the present work, more precise positions of barley *Dhn* genes were determined using gene-specific PCR and 100 doubled haploid lines developed from a cross of Dicktoo and Morex barley. *Dhn10* is located on 3H between saflp106 and ABG4. *Dhn6* is at the previously determined position on 4H between SOLPRO and BCD265a. *Dhn1* and *Dhn2* are at the previously determined position on 5H between mR and saflp172. The *Dhn* locus previously called *Dhn*4a on barley 5H or *Dhn*2.2 on *T. monococcum* 5A is in fact *Dhn9* and maps to a revised position between BCD265b and saflp218. *Dhn3*, *Dhn4*, *Dhn7* and *Dhn5* each map to the same position on chromosome 6H, suggesting that the previously reported separation of *Dhn3*, *Dhn4* and *Dhn5* may reflect limitations in the accuracy of Southern blot data. In addition to clarifying the map positions of these important stress-related genes, these results illustrate the advantage of gene-specific probes for the mapping of individual genes in a multi-gene family.

Key words Dehydrin · *Dhn* · *Hordeum vulgare* · PCR-based mapping

Introduction

Drought, salinity and low temperature are examples of environmental stresses, that limit the growth and yield of crop plants. Under stress conditions, plants adapt physiologically and biochemically, including the induction of proteins that presumably help minimize damage (Bray 1993, Ingram and Bartel 1996). Dehydrins (DHN, Syn. LEA D-11) are among the most frequently observed proteins induced by dehydration, salinity or low temperature. DHNs also are induced by abscisic acid (ABA) and during late stages of seed development (Close 1996, 1997, Campbell and Close 1997). DHNs contain a highly conserved amino acid sequence domain called the K-segment (Close 1996). Two other conserved domains, the Y- and S-segments are also present in most DHNs. Recent studies suggested that DHNs act as stabilizers of membranes and/or proteins under stress condition (Egerton-Warburton 1997, Danyluk et al. 1998, Ismail et al. 1999). Genetic studies have revealed that some *Dhn* loci are associated with quantitative trait loci (QTLs) for important physiological traits in various species (reviewed in Campbell and Close 1997).

Dhn genes exist as a dispersed multi-gene family in plants. The most complete information on the *Dhn* gene family is in barley (Choi et al. 1999), where it was shown that 11 *Dhn* genes are dispersed on four chromosomes, 3H, 4H, 5H and 6H. Several mapping studies have located the position of *Dhn* genes within barley chromosomes. *Dhn6* is on the short arm of 4H; *Dhn1* and *Dhn2* are on long arm of 5H; *Dhn3*, *Dhn4* and *Dhn5* are on long arm of 6H (for example, Pan et al. 1994). In diploid wheat, *Dhn* genes have been mapped on chromosome 4A, 5A and 6A in *Triticum monococcum* (Dubcovsky et al. 1995) and 5D in *Triticum tauschii* (Gill et al. 1991, Lagudah et al. 1991). Limin et al. (1997) reported that hexaploid wheat (*Triticum aestivum*) *Wcs120* family genes (orthologous to barley *Dhn5)* are located on the long arm of each group 6 chromosome. Werner-Fraczek and Close (1998) assigned DHN proteins to genes on chromosome arms 4DS, 5BL and 6AL in wheat (*Triticum aestivum* L. cv. Chinese Spring) using cytogenetic stocks and a Western blot procedure.

The *Dhn1* and *Dhn2* loci on barley 5H are within a major QTL controlling freezing tolerance (Hayes et al.

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1993, Pan et al. 1994). This QTL is flanked by *mR-bcd265b* on 5H and includes the *Sh2* locus, which is a determinant of vernalization response (Pan et al. 1994, Laurie et al. 1995). The barley *Sh2* gene is orthologus to *Vrn1-A* on 5A of wheat and *Sp1* on 5R of rye (Dubcovsky et al. 1998). Galiba et al. (1995) showed that a wheat *Dhn* gene (detected by barley *Aba2)* is located near *Vrn1-A* and *Fr1* (frost resistance). It appears from genetic linkage data that *Dhn1* and *Dhn2* are not the same as *Sh2*, *Vrn1-A* or *Fr1* (Galiba et al. 1995; van Zee et al. 1995).

Due to cross-hybridization between *Dhn* genes, several bands are typically identified by the Southern blot method, which can be problematic. We hypothesized that there may be some inaccuracies in previously assigned map positions and *Dhn* gene identities. In this report, we present the map position of 9 barley *Dhn* genes using a gene-specific polymerase chain reaction (PCR) and 100 doubled haploid (DH) lines developed from a cross of Dicktoo and Morex barley.

Materials and methods

Plant materials

Winter and spring barley (*Hordeum vulgare* L.) cvs. Dicktoo and Morex, respectively, and 100 F_1-derived DH progeny from a cross between these two parents were used in these studies (http://wheat.pw.usda.gov/ggpages/DxM/). Dicktoo, Morex and DH line seeds were obtained from Dr. Patrick Hayes (Oregon State University, Corvallis, Ore.) and propagated at the University of California, Riverside. Seeds were germinated and grown to young seedlings. Leaf tissues were cut off, rapidly frozen in liquid nitrogen and stored at –80°C until use.

Genomic DNA isolation and amplification

Genomic DNA was prepared from leaves using Plant DNAzol® (BRL, Gaithersburg, Md.) according to instructions from the manufacturer. PCR amplifications were performed using a GeneAmp PCR System 9600 (Perkin Elmer, Foster City, Calif.) in a 50-µl reaction containing *Taq* DNA polymerase (Qiagen, Hilden, Germany), 1 × PCR buffer (10 m*M* TRIS-HCl pH 8.3, 50 m*M* KCl_2, 1.5 m*M* $MgCl_2$), 1 × Q-solution, 200 µ*M* of each dNTP, 0.3 µ*M* primer, and 100–150 ng of genomic DNA. PCR reactions were initiated at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 59–65°C for 30 s, 72°C for 1 min, and terminated at 72°C for 10 min. PCR annealing temperatures were optimized for each *Dhn* gene-specific primer set (Table 1).

Detection of polymorphisms

To develop PCR product polymorphisms as markers of each *Dhn* genotype, we compared 11 *Dhn* gene sequences from Dicktoo (Choi et al. 1999) and Morex (sequences obtained from lambda genomic clones, genomic DNA-derived PCR products and RNA-derived PCR products; Choi and Close, unpublished data) barley. *Dhn* gene-specific oligonucleotides (Table 1) were designed using the program PRIMER-MASTER. In our design, the first priority was to amplify different sized PCR products from the Dicktoo and Morex alleles. Where this was not possible, we utilized restriction endonuclease cleavage sites at positions that differed between the two alleles. To digest amplified DNA, 5 µl of PCR product was digested in a 10-µl reaction volume. Enzyme-digested DNAs were then analyzed by electrophoresis through a 2% agarose or a 7% polyacrylamide gel and ethidium bromide staining. Table 1 shows the *Dhn* gene-specific oligonucleotides and restriction enzymes used to identify each *Dhn* allele.

Linkage analysis

The map location of each *Dhn* gene was determined by aligning our data with existing mapping data for the Dicktoo × Morex barley mapping population available at http://wheat.pw.usda.gov/ggpages/ DxM/dmsor.txt.

Results

Detection of *Dhn* gene polymorphisms

Comparison of deduced amino acid and DNA sequences for each *Dhn* gene from Dicktoo and Morex barley revealed duplications, deletions or single base substitutions. For example, in DHN4 the number of Φ-segments between the K_1- and K_2-segments varies markedly in several cultivars (Choi et al. 1999). There are eight Φ-segments in Dicktoo DHN4, but the 6th and 7th Φ-segment (QQGHTGTGTGMHGTGGTYGQQGHTGMTGTGMHGTGGTYG) are missing in Morex DHN4. Similarly, the 14th Φ-segment (GYGGGVTGTGITGTHGTGHTT) of Dicktoo DHN6 is not present in Morex

Table 1 Primers for *Dhn* gene mapping

Dhn genes	5′-primer	3′-primer	Annealing Temperature (°C)	Restriction enzyme	Polymophism (bp)	
					Dicktoo	Morex
Dhn1	TCACTGTTCGTACTTCGTAGCACC	TCCGCAGTTGCTCCTCCAAT	63	None	1437	1350
Dhn2	CCAGCCGACCAGGGACGACCACAA	TCCGTCGTCCCCTGCATA	59	None	1030	965
Dhn3	AGGCAACCAAGATCAACACCACCTG	GCGGAAGTTTTACTGCATCTCCATC	62	*Nla*III	104	65
Dhn4	CGGCAGCGCAAGATGGAGTACCAG	CCCCTCCAACAGCCAAGTGAGCTA	64	None	998	881
Dhn5	AGCAGCGCTTGTAGATATTCCCGA	CTCCACCAACGAAAGTGAGCTAGG	62	*Sau*3AI	1157	1449
Dhn6	CAATCCGTGAAGCGAAGAGATGG	ACCAGGCCATGTCACAGTACTGC	62	*Kpn*I	976	517
Dhn7	ACCTGTGCAAGATGGAGTACCA	TTTCTGCAGAGGTGGGTACGT	60	None	736	766
Dhn9	CGCCACTAGTAAGCAAGGCATACC	AGGCTTCGACGCGTAGCTATGCAA	65	None	1172	1132
Dhn10	GCCAAGAGGCAGCAAGATGGAAT	GGGACGGTACGTACTGCAGGATA	63	*Ban*I	159	142

DHN6. Also, 10 amino acid residues (EHGGVGTGMG) between the Y- and S-segment of Dicktoo DHN7 are not present in Morex DHN7. Only small differences between alleles exist in the transcribed regions of *Dhn1*, *Dhn2* and *Dhn9*. However, the deletion of some DNA sequences was detected downstream of the 3'-untranslated region of Morex *Dhn1* and *Dhn2*, relative to Dicktoo. For *Dhn9*, 39 bp are absent in the 5'-flanking region of Morex *Dhn9*, relative to Dicktoo *Dhn9*. Based on these flanking sequence differences, we designed *Dhn* gene-specific oligonucleotides to amplify different sizes of DNA fragments from different *Dhn* alleles of *Dhn1*, *Dhn2* and *Dhn9*. For other *Dhn* genes, it was necessary to cleave the PCR product with an allele-specific restriction endonuclease to readily differentiate the two alleles. Table 1 summarizes the *Dhn* gene-specific oligonucleotides and allele-specific restriction endonuclease used for each *Dhn* gene. To facilitate determination of the *Dhn6* genotype, we digested the PCR products with *Kpn*I. Figure 1 A shows the polymorphism for each *Dhn* gene that was mapped. *Dhn8* and *Dhn11* were refractory to these approaches and will require an alternative method to distinguish the alleles.

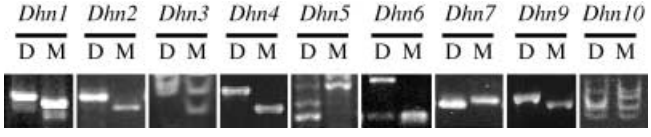

Fig. 1 Genotype of nine *Dhn* genes in the mapping population. The *Dhn* gene polymorphisms are shown, as detected by PCR and, in some cases, also by digestion with endonuclease. *D* Dicktoo; *M* Morex

Dhn gene mapping

To determine the map position of each *Dhn* gene, we aligned these data with existing mapping data for the Dicktoo × Morex barley mapping population available at http://wheat.pw.usda.gov/ggpages/DxM/dmsor.txt. The number of recombinants between the *Dhn* locus and the nearest locus was divided by the number of individuals for which genotype data was available for both markers to give a percentage recombination value. Figure 2 shows the map location determined for each of these 9 *Dhn* genes. The recently identified *Dhn10* gene is located on 3H between saflp106 and ABG4. *Dhn6* mapped to the same position on chromosome 4H as determined previously by Pan et al. (1994). Chromosome 5H contains 3 *Dhn* genes, *Dhn1*, *Dhn2* and *Dhn9*. *Dhn1* and *Dhn2* are completely linked and mapped between marker mR and saflp172, as reported by Pan et al. (1994). *Dhn9* is located between BCD265b and saflp218, which is approximately the same position previously reported for *Dhn*4a (Pan et al. 1994). Since *Dhn*4a was a locus detected by the *Dhn4* probe, possibly there were some inaccuracies in the Southern blot data. Five *Dhn* genes, *Dhn3*, *Dhn4*, *Dhn5*, *Dhn7* and *Dhn8* were mapped previously to chromosome 6H (Choi et al. 1999). The map

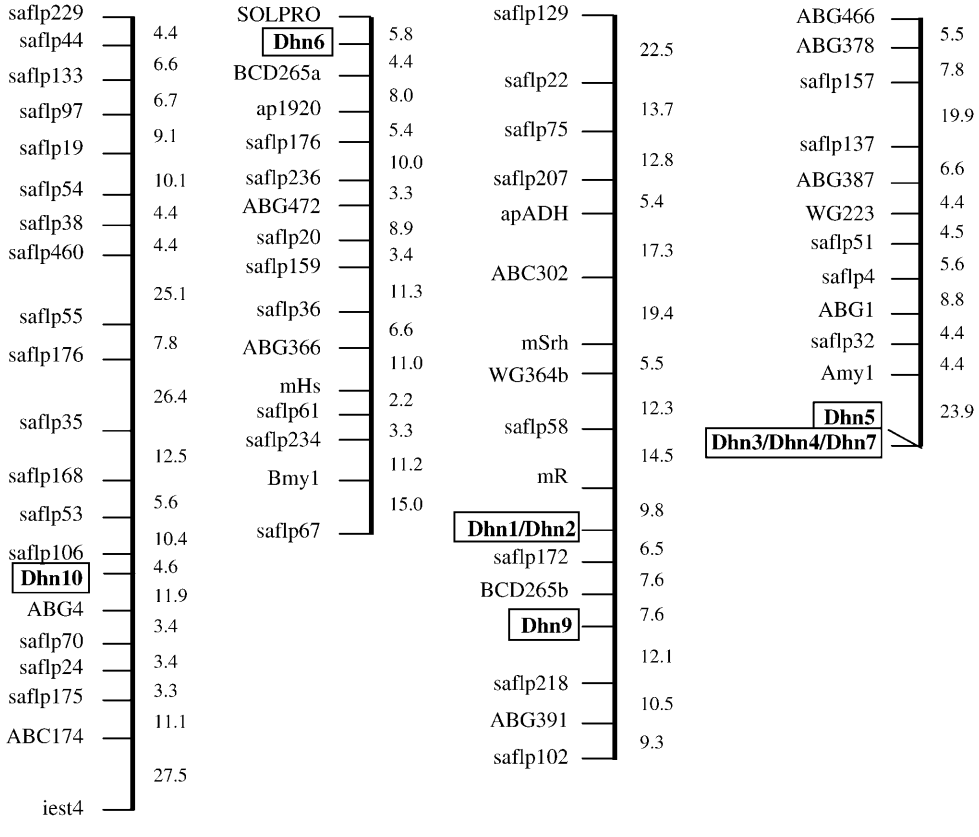

Fig. 2 Map location of the *Dhn* genes. Four barley chromosomes (3H, 4H, 5H and 6H from left to right) carry *Dhn* genes. The map location of each *Dhn* gene is compared with mapping data for the Dicktoo × Morex barley mapping population, which are available at http://wheat.pw.usda.gov/ggpages/DxM/dmsor.txt. The map positions of *Dhn* genes are *boxed* and have been added to the genetic map available at http://wheat.pw.usda.gov/ggpages/DxM/ dmmap896.gif. Units are percentage recombination

positions of *Dhn3*, *Dhn4*, *Dhn7* and *Dhn5* in the current study are identical to each other. This result differs from the data of Pan et al. (1994), where it was reported that three *Dhn* genes, *Dhn3*, *Dhn4* and *Dhn5*, were recombinationally separable in the Dicktoo × Morex mapping population. As with *Dhn9*, we feel that the discrepancy is likely to be due to inaccuracies in Southern blot data.

Discussion

Dispersed multi-gene families pose special problems in regards to genetic mapping and gene expression studies. The *Dhn* multi-gene family is a typical example. Individual probes detect multiple bands on Southern blots, making it difficult to derive accurate mapping data and impossible to ascertain specifically which gene is being mapped. The use of gene-specific probes is essential for resolving the map position of each *Dhn* gene. For example, a *Dhn* locus was detected between BCD265b and saflp218 on chromosome 5H with a *Dhn4* hybridization probe in barley (Pan et al. 1994). At a similar position on chromosome 5A of diploid whaet (*T. monococcum)*, a *Dhn* locus was detected with a *Dhn2* probe (Pan et al. 1994; Dubcovsky et al. 1995). This *Dhn* locus was named *Dhn*4a in barley and *Dhn2.2* in diploid wheat. Our gene-specific data indicates that this locus is *Dhn9* and that the map location of *Dhn9* is nearer to BCD265b than previously reported. Also, the *Dhn*4B, *Dhn*5 and *Dhn*3C loci were mapped on the long arm of chromosome 6H, separated by 1.0 (*Dhn*4B-*Dhn*5) and 4.6 cM (*Dhn*5-*Dhn*3 C), respectively (Pan et al. 1994). Our mapping data from gene-specific PCR demonstrates that the positions of four *Dhn* genes, *Dhn3*, *Dhn4*, *Dhn5* and *Dhn7* in fact cannot be separated in this mapping population.

Physical mapping with genomic clones has confirmed that several *Dhn* genes on chromosome 6H are linked tightly. *Dhn3* and *Dhn4* are separated by only 8 kb in a λ-clone contig containing these *Dhn* genes (Choi et al. 1999). *Dhn7* is also physically linked to *Dhn3* and *Dhn4* in a 110-kb BAC clone isolated from Morex barley (Choi and Close 2000). We have also isolated a BAC clone carrying both *Dhn1* and *Dhn2* in an approximate 90-kb fragment (Choi and Close, unpublished data), confirming that these two loci are tightly linked. There have been previous reports of *Dhn* gene clusters in rice and *Arabidopsis* (Yamaguchi-Shinozaki et al. 1989; Welin et al. 1994; Rouse et al. 1996). For example, four rice *Dhn* genes are linked in a 30-kb DNA fragment and are expressed differentially by osmotic stress (Yamaguchi-Shinozaki et al. 1989). The tandem clustering of *Dhn* genes and duplication of K- or Φ-segments in DHN polypeptides may indicate that these genes have evolved by duplication, as has been suggested for the LEA-1 gene family (Stacy et al. 1995).

The method of gene-specific PCR was suggested previously as a tool for barley genome mapping (Tragoonrung et al. 1992). For 9 of 11 *Dhn* genes, we have succeeded with this approach. We have so far been unable to design allele-specific PCR amplification or restriction endonuclease cleavage strategies to distinguish the genotypes of *Dhn8* and *Dhn11*. To determine the map position of the *Dhn8* and *Dhn11* genes, we require more sequence information from DNA linked to these genes. Alternatively, sequence-specific amplification polymorphism (S-SAP) could be used (Waugh et al. 1997). A complete collection of barley *Dhn* genes and unambiguous determination of the map locations of each *Dhn* gene will help to define the linkage between *Dhn* genes and a number of important agricultural traits in barley and its relatives.

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