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The barley (*Hordeum vulgare* L.) dehydrin multigene family: sequences, allele types, chromosome assignments, and expression characteristics of 11 *Dhn* genes of cv Dicktoo

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Abstract Dehydrins (LEA D11 proteins) have been identified in both higher and lower plants, and are associated with tolerance to, or response to the onset of, low temperature or dehydration. Several studies have suggested that specific alleles of *Dhn* genes may contribute to a number of phenotypic traits, including the emergence of seedlings in cool or saline soils and the frost tolerance of more-mature plants. However, an incomplete collection of the *Dhn* multigene family in any system and nucleic acid cross-hybridization between *Dhn* gene-family members have limited the precision of these studies. We attempted to overcome these impediments by determining the nucleotide sequences of the entire *Dhn* multigene family in barley and by developing gene-specific probes. We identified 11 unique Dicktoo *Dhn* genes. Seven appear to be alleles of *Dhn* genes identified previously in other barley cultivars. Another, *Dhn*9, appears to be orthologous to a *Triticum durum Dhn* gene. A statistical analysis of the total collection of genomic clones brings the estimated size of the barley *Dhn* gene family to 13. Allelic differences in the protein-coding regions appear to result principally from duplications of entire Φ-segments or single amino-acid substitutions, suggesting that polypeptide structural constraints have been a strong force in the evolution of *Dhn* alleles. Chromosome mapping by PCR with wheat-barley addition lines established the presence of *Dhn* genes in four barley chromosomes (3H, 4H, 5H, 6H). RT-PCR demonstrated that the *Dhn* genes are differentially regulated under dehydration, low temperature and ABA treatment, consistent with putative regulatory elements located upstream of the respective *Dhn* coding regions. This whole-genome, gene-specific study unifies what previously seemed to be disparate-mapping, expression, and genetic-variation data for *Dhn* genes in the Triticeae and other plant systems.

Key words Dehydrin · Multigene family · LEA · COR · RAB · Barley · Triticeae

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Introduction

In order to cope with environmental fluctuations, plants have developed strategies to adapt to stress by a number of physiological and biochemical modifications, reflected at the level of gene expression. Drought and low temperature are among the most severe environmental stresses limiting the growth and yield of plants, and many genes have been demonstrated to respond to these stresses (Hughes and Dunn 1996; Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 1996). Induced proteins include several which are thought to function in physically protecting cells from water deficit or temperature change, or in the regulation of gene expression (Dure 1993; Shinozaki and Yamaguchi-Shinozaki 1997). The dehydrins (LEA D11 proteins), some of which have been referred to as RAB or COR proteins, are among the most frequently observed (Close 1997).

Dehydrins comprise an immunologically distinct protein family, and typically accumulate during the maturation-drying phase of seed development, or in seedlings or more mature plants, in response to low temperature, drought, salinity or ABA application. Dehydrins contain abundant charged and polar amino-acids, are usually Gly-rich and free of Cys and Trp, and contain consensus amino-acid sequence domains. Dehydrins appear unstructured in aqueous

solutions (Lisse et al. 1996). The most highly conserved domain is the Lys-rich 15 amino-acid consensus EKKGIMDKIKEKLPG (K-segment), which may form an amphipathic α-helix reminiscent of the lipid-binding class-A amphipathic α-helices of apolipoproteins (Segrest et al. 1990). The K-segment occurs in all dehydrins but varies from 1 to 11 copies within a single polypeptide. Between and preceding the K-segments there are less conserved domains (Φ-segments), which are generally rich in polar amino-acids with either Gly or a combination of Ala and Pro. The other domains are a phosphorylatable tract of Ser residues (S-segment) and a Y-segment. The Y-segment (T/VDEYGNP), when present, occurs in 1–3 tandem copies near the N-terminus. Several immunolocalization and subcellular-fractionation studies have established that dehydrins can be present in the nucleus or the cytoplasm (reviewed in Close 1997). Two recent studies have provided evidence that dehydrins are peripheral membrane proteins, including the major embryo dehydrin of maize which is associated with a cytoplasmic endomembrane (Egerton-Warburton et al. 1997) and an acidic dehydrin in the vicinity of the plasma membrane (Danyluk et al. 1998). It was suggested by these authors, and others, that dehydrins act as stabilizers of membranes or proteins under water-stress conditions (reviewed in Close 1997).

Most of the *Dhn* genes that are up-regulated by dehydration, salinity, or low temperature are also induced by application of exogenous ABA, which increases in concentration in plants under various stress conditions and acts as a mobile stress signal (Merlot and Giraudat 1997). However, there are examples of *Dhn* genes that are induced by dehydration or low temperature, but are not responsive to ABA (Houde et al. 1992; Kiyosue et al. 1993; Whitsitt et al. 1997). ABA-dependent and ABA-independent signal-transduction pathways account for these variations (reviewed in Shinozaki and Yamaguchi-Shinozaki 1997). Several *cis*-acting elements are involved in the ABA-response (ABRE) or the dehydration response (DRE; also known as C-repeats). These *cis*-acting elements are present in one to multiple copies in the promoters of many drought- and cold- induced plant genes (Shinozaki and Yamaguchi-Shinozaki 1997). From *Arabidopsis*, Stockinger et al. (1997) isolated a DRE-binding protein, CBF1. Over-expression of CBF1 causes the synthesis of a suite of COR proteins, including an *Arabidopsis* SK_3 dehydrin, COR47 (Jaglo-Ottosen et al. 1998). Similarly, Abe et al. (1997) reported that MYC- and MYB- related proteins, rd22BP1 and ATMYB2, function as transcriptional activators in the dehydration and ABA-responsive expression of the *Arabidopsis* drought- and cold-inducible gene, *Rd22*. Recent studies have also suggested that expression of the cold-induced wheat *Dhn* gene *Wcs120* may involve a negative regulator(s) modulated by phosphorylation (Sarhan et al. 1997). Indeed, there are numerous permutations of expression patterns for *Dhn* genes, rather than one constant pattern. This may reflect the presence of different combinations of regulatory elements in the promoter regions of various *Dhn* genes and their alleles.

A question that has often arisen during the half century since dehydrins and other dehydration- and cold-induced proteins were first discovered (Siminovitch and Briggs 1953) has been, "what is the fundamental biochemical role of these proteins"? Given that dehydrins are encoded by a multigene family, and that there are several other distinct families of dehydration-related proteins (Hughes and Galau 1989), reductionist approaches to this question have been problematic. Transgenic studies have been hampered by a general uncertainty over where to begin, in regard to both which gene to manipulate and what trait to measure. Even in cases where overexpression of a single transgene correlates with a change in the stress tolerance of a transgenic plant (Xu et al. 1996; Thomashow et al. 1997), or with the induction of a suite of genes together with altered stress tolerance (Jaglo-Ottosen et al. 1998), the experimental variables and phenotypic pleiotropy have as yet been insufficiently explored to warrant a sound conclusion that there is any relationship in nature between the overexpressed gene and genetic variance in stress tolerance.

An alternative is to explore natural variation. Our working hypothesis is that if dehydrins do indeed play a major role in naturally occurring variation in environmental-stress tolerance, then there must exist natural allelic variation in *Dhn* genes, dehydrin proteins, or the regulation of *Dhn* gene expression, which underlies some of the phenotypic traits associated with environmental-stress tolerance. Several examples appear to be consistent with this hypothesis (reviewed in Campbell and Close 1997), the first of which was the mapping of the major winter-hardiness traits of barley (including *Sh2*) to the same region of the genome as a 24-cM cluster of *Dhn* genes in chromosome 5H (Pan et al. 1994). Analysis of a number of doubled-haploids, representing recombination in this interval, indicated that the *Dhn1* and *Dhn2* genes can be ruled out as major determinants of the major QTL effect in this mapping population (van Zee et al. 1995). A similar conclusion can be drawn from a study of chromosome-5A recombinants in wheat (Galiba et al. 1995), where the *Aba2* [*Dhn1* (Georgie 139)] marker was found to be separate from the *Vrn1* and *Fr1* phenotypic determinants. However, the association of every *Dhn* gene in this region has not been tested because a complete collection of 5H *Dhn* genes has not been available. We have attempted to overcome this limitation, and similar limitations related to the apparent co-segregation of *Dhn* genes with other phenotypic determinants (reviewed in Campbell and Close 1997), by assembling a complete collection of barley *Dhn* genes from the entire barley genome.

Materials and methods

Plant materials

Winter barley (*Hordeum vulgare* L. cv Dicktoo) seeds were obtained from Dr. Patrick Hayes (Oregon State University, Corvallis, Oregon) and were increased at the University of California, Riverside. Seeds of Chinese Spring wheat (*Triticum aestivum* cv Chinese Spring), Betzes barley (*Hordeum vulgare* L. cv Betzes) and the six wheat-barley addition lines from these two parents (Islam et al. 1981), were provided by Dr. Adam Lukaszewski (University of California, Riverside, Calif.)

Seeds were surface-sterilized in 70% ethanol for 10 min and 1% sodium hypochlorite and 0.01% Tween 20 for 30 min, then rinsed with sterile water. Sterilized seeds were germinated on moist filter paper in sterile Petri dishes at room temperature in the dark. For cold acclimation, barley seedlings with 3-cm-long coleoptiles were transferred to 5°C for 48 h. For ABA treatment, the roots of intact barley seedlings were immersed in 20 μM of ABA (Sigma Chemical Co., St Louis, Mo.) solution for 24 h. For drought stress, barley seedlings were placed in the gas space of an airtight glass desiccator containing a saturated $MgSO_4$ solution (approximately 90% relative humidity) and incubated for 24 h at room temperature. After each treatment, shoots were cut off, rapidly frozen in liquid nitrogen, and stored at −80°C. Wheat-barley addition lines were grown in a growth chamber.

DNA isolation

Genomic DNA was prepared using a modified method of Martienssen et al. (1989). Two grams of etiolated seedling tissue were ground to a fine powder in liquid nitrogen and incubated at room temperature in 5 ml of extraction buffer (100 mM Tris HCl pH 8.5, 100 mM EDTA, 250 mM NaCl, 2% SDS, and 100 μg/ml of Proteinase K) for 90 min. The lysate was extracted with 10 ml of phenol : chloroform (1 : 1) twice and precipitated with ethanol. DNA was re-dissolved in 1 ml of TE buffer containing 75 μg/ml of DNase-free RNase. DNA was extracted with phenol : chloroform, and chloroform, adjusted to a concentration of 0.3 M sodium acetate, and ethanol-precipitated. The purified DNA was dissolved in sterile water and stored at 4°C.

Genomic library construction and screening

Genomic DNA from etiolated Dicktoo barley seedling tissue was partially digested with the restriction endonuclease *Mbo*I and size-fractionated on 10–40% glycerol gradients to isolate molecules in the range of 13–23 kb. The DNA was then de-phosphorylated, ligated to *Bam*HI/*Eco*RI double-digested EMBL3*cos* (Whittaker et al. 1988), and packaged in vitro using restriction endonuclease-free packaging extracts (Gigapack II Gold, Stratagene, La Jolla, Calif.). The library was titered on *Escherichia coli* strain PMC128 [*e14-(mcrA)* *Δ*(mcrBC-hsdRMS-mrr)102 recB21 recC22 sbcB15 sbcC201]. EMBL3*cos* was obtained from Dr. Noreen Murray (Edinburgh, UK) and the vector DNA was produced according to standard procedures (Sambrook et al. 1989). *E. coli* strain PMC128 was purchased from the American Type Culture Collection. The library was screened with a mixture of ^{32}P-labelled *Dhn* (*Dhn1*, *Dhn2* and *Dhn4*) cDNA- and *Dhn* (*Dhn5* and *Dhn6*) genomic- DNA probes from Himalaya barley (Close et al. 1989, 1995).

Restriction-enzyme mapping

Restriction-enzyme maps of each λ clone were constructed using partial restriction endonuclease digestions and the 12-base (5′-AGGTCGCCGCCC-3′) oligonucleotide complementary to *cosL* (Whittaker et al. 1988). Lambda DNA was isolated from plate lysates using Qiagen (Hilden, Germany) columns, and partially digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII or *Sal*I. The *cosL* oligonucleotide was labeled with γ-^{32}P-ATP by T4 polynucleotide kinase, and annealed to partially digested DNA in 10 μl of 0.1 M NaCl at 45°C for 30 min. DNA fragments were separated in an 0.7% agarose gel in Tris-Phosphate buffer (36 mM Tris-HCl pH 7.7, 30 mM NaH_2PO_4 and 1 mM EDTA) at 1.5 V/cm for 20 h. DNA fragments were transferred to a nylon membrane and exposed to X-ray film.

DNA sequencing and analysis

DNA segments that hybridized with Himalaya dehydrin probes were subcloned into pTZ18R and sequenced on both strands, in their entirety, using the dideoxy chain-termination method. The nucleotide sequence and deduced amino-acid sequences were analyzed with the DNASIS and PROSIS programs (Hitachi Software Engineering Ltd, San Bruno, Calif.) and compared with sequences in GenBank and EMBL databases using the BLAST server. Amino-acid sequence alignments of the deduced dehydrin polypeptides were performed using the CLUSTAL V program. Introns were recognized by comparing known sequences of eight *Dhn* cDNAs with the genomic sequences. Where this was not possible (*Dhn5*, *Dhn 10*, *Dhn11*), introns were assumed to be bounded by GT and AG and located between the SSS and $S_{(1\ to\ 3)}$ED amino-acids, which was the case for all other dehydrins. The accession numbers are given in Table 1.

Primer synthesis

Oligonucleotide primers were designed from DNA sequences using the program PRIMER-MASTER. The 5′-end and 3′-end primers were designed from different exons to produce different-size PCR products from DNA and RNA templates. All Dicktoo *Dhn* genes include an intron, except for *Dhn5*. Oligonucleotide primers were synthesized at Heligen Laboratory (Huntington Beach, Calif.). The nucleotide sequences of the gene-specific primers are listed in Table 1.

Genomic DNA amplification

Genomic DNA amplifications were performed in a 100-μl reaction containing 2.5 units of Ampli*Taq* DNA polymerase (Perkin Elmer, Foster City, Calif.), 1 × PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl_2, 1.5 mM $MgCl_2$), 200 μM of each dNTP, 0.3 μM of primer, and 30 ng of genomic DNA from the wheat-barley addition lines. PCR reactions were initiated at 95°C for 5 min, followed by 32 cycles at 95°C for 1 min, 64°C for 30 s, 72°C for 30 s, and terminated at 72°C for 10 min using a GeneAmp PCR System 9600 (Perkin Elmer, Foster City, Calif.).

RT-PCR

Total RNA was prepared from a mixture of 4–5 seedlings using either a RNeasy Total RNA Plant Kit (Qiagen, Hilden, Germany) or the small-scale procedure described by Verwoerd et al. (1989). First-strand cDNA was made from 1 μg of total RNA in a 20 μl reaction containing 2.5 unit/μl of MuLV reverse transcriptase (Perkin Elmer, Foster City, Calif.) in a buffer of 10 mM HCl pH 8.3, 50 mm KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 1 unit/μl of RNase inhibitor and 0.2 μM of gene-specific 3′-end primer. Reactions were performed at

Table 1 Gene-specific primers for Dicktoo barley *Dhn* genes

Dhn genes	Accession number	5′-primer	3′-primer
Dhn1	AF043087	GACGAGGGATGGCCACAAGACTGA	AGTAACGCATGGCTGCGGATGCTA
Dhn2	AF043088	CCAGCCGACCAGGGACGACCACAA	TTTCGAGCCATCGTACGCAAAGGATG
Dhn3	AF043089	AGGCAACCAAGATCAACACCACCTG	GCGGAAGTTTTACTGCATCTCCATC
Dhn4	AF043090	CGGCAGCGCAAGATGGAGTACCAG	CCCCTCCAACAGCCAAGTGAGCTA
Dhn5	AF043096	AAATGACTGGCATGGGGAGGCATA	CTCCACCAACGAAAGTGAGCTAGG
Dhn6	AF043091	TGACGTCGTGGCACACACCCTC	ACCAGGCCATGTCACAGTACTGC
Dhn7	AF043092	GTCATTTCCAGCCGACGAGGAAGG	CGGGTCCATACAAGAAGCCATATT
Dhn8	AF043093	TCATGGAGGATGAGAGGAGCACCCA	GGCTCTGAGTAGTGGCCTGGAGGTA
Dhn9	AF043094	ATGGAGTTCCAAGGGCAGCACGAC	AGGCTTCGACGCGTAGCTATGCAA
Dhn10	AF043095	GCCAAGAGGCAGCAAGATGGAATACC	TCGGCTTATTGCTCCACCTCCGCTCA
Dhn11	AF043086	AAGAGTTGAAGCACCGTCGAGGG	CGTACATGGTCAAAGAACCGTGT

42°C for 15 min and then heated at 99°C for 5 min. The resulting single-strand cDNAs were amplified using Ampli*Taq* DNA Polymerase (Perkin Elmer, Foster City, Calif.) or *Taq* DNA Polymerase (Qiagen, Hilden, Germany) in a GeneAmp PCR System 9600 (Perkin Elmer, Foster City, Calif.) using gene-specific 5′-end and 3′-end primers. PCR reactions were performed for 35 cycles, each consisting of 95°C for 30 s, 64°C for 30 s, 72°C for 15 s, and terminated at 72°C for 10 min. PCR products were separated on 1.2% agarose gels and stained with ethidium bromide for photography. All gene-specific amplifications were performed using the same seedling RNA samples.

Results

Isolation of the *Dhn* genes from Dicktoo barley

Dicktoo and Morex were the two cultivars of barley used as the parents of the doubled-haploid (DH) mapping population in which the major control of winter-hardiness mapped to chromosome 5H (Pan et al. 1994). A Dicktoo barley genomic library was produced and screened (see Materials and methods) with Himalaya barley *Dhn* probes, which initially identified 26 positive genomic clones. When DNAs from these clones were probed with a mixture of Himalaya barley *Dhn1*, *Dhn2*, *Dhn3*, *Dhn4*, *Dhn5* and *Dhn6*, all Dicktoo clones hybridized. However, only one clone (Dc13-1) hybridized strongly with the known chromosome-5H *Dhn* probes, *Dhn1* and *Dhn2*. The Dicktoo genomic library was screened again using a mixture of probes including Himalaya *Dhn1* and *Dhn2*, plus insert DNA of clone Dc13-1, yielding another 20 hybridizing clones. Further analysis revealed that 10 of these 20 clones hybridized to the Himalaya *Dhn* probes, but the other ten did not. These latter clones presumably were detected by DNA adjacent to the *Dhn* gene on clone Dc13-1 and were not further utilized. In total, 36 *Dhn* clones were identified from the Dicktoo library.

Restriction endonuclease cleavage-site maps of the 36 *Dhn* clones were constructed (see Materials and methods). On the basis of these maps and nucleic acid-hybridization data, the 36 λ clones were sorted into ten contiguous maps (contigs) (Fig. 1). Two *Dhn* genes (*Dhn3* and *Dhn4*) are closely linked, separated by only about 8 kb, and are in the same transcriptional orientation (see sequence analysis for further details on gene identities). The other nine contigs each contain only one *Dhn* gene. All DNA fragments that hybridized with *Dhn* probes were subcloned and sequenced in both strands over the entire *Dhn* gene and variable lengths of the 5′ and 3′ flanking DNA. All of the barley *Dhn* genes contain a single intron adjacent to an exon that encodes the S-segment, except for *Dhn5* which contains no intron. The *Dhn3* and *Dhn7* genes, which encode polypeptides closely related to each other, contain an intron with 88% nucleotide-sequence homology. The intron sequences of the remainder of the *Dhn* genes are not closely related to each other.

Estimation of the *Dhn* gene copy number in barley

To estimate the number of unique *Dhn* genes in the barley genomic libraries that we screened in this and other work, each of the 36 genomic clones from Dicktoo barley (this study), 20 additional *Dhn* genomic clones from Morex barley (D.-W. Choi, unpublished) and three Himalaya barley genomic clones, *Dhn5* (Close et al. 1995), *Dhn6* (T.J. Close, unpublished) and *Dhn1-2* (Lang et al. 1998), were assigned a number between 1 and 59. Of these 59, seven clones contained two *Dhn* genes (*Dhn3* and *Dhn4* in every case). The clones were randomly sampled 50 times to derive a relationship between the sample size and the number of unique types of *Dhn* genes. The asymptotic limit of the number of unique *Dhn* genes, Y, was calculated by the equation:

$$Y = x/(\alpha + \beta x),$$

where x is the number of genomic clones, and the constants α and β are computed by fitting the non-linear function using the SAS package (SAS Institute 1988; Dr. Shizhong Xu, Riverside, Calif.). As the number of samples increases toward infinity, Y approaches $1/\beta$. This analysis provides an estimate that the barley

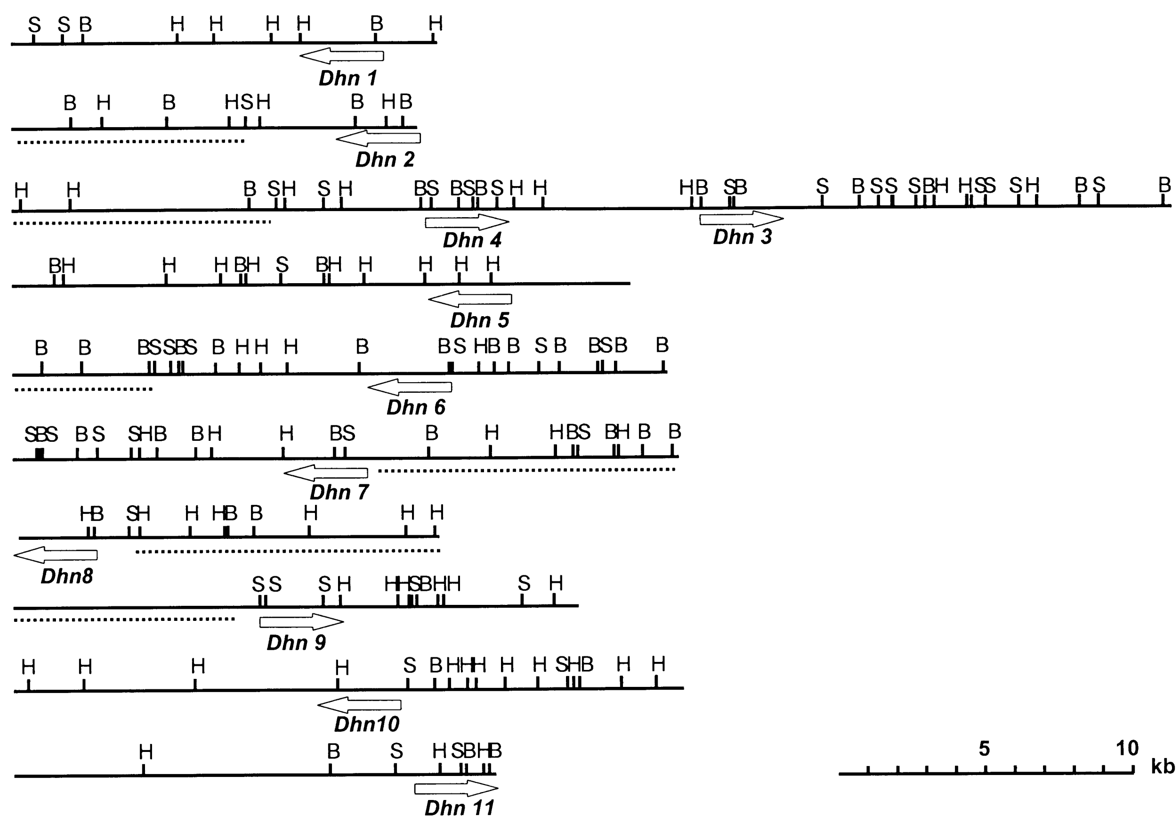

Fig. 1 Restriction maps of the Dicktoo *Dhn* contigs. *Arrows* indicate the position and orientation of each *Dhn* gene. A barley retroelement (BARE) sequence was discovered in the neighboring sequences of one *Dhn* gene (see Discussion). A fragment containing BARE sequences was then used as a hybridization probe to identify the approximate location of additional BARE sequences. The *dashed lines* indicate the region of hybridization with the BARE probe. Restriction enzyme sites are shown as *B* (*Bam*HI), H (*Hin*dIII) and S (*Sal*I)

genomic libraries constructed by the methods that we employed contain 13 *Dhn* genes that can be identified by the screening methods that we used. Therefore, we are confident that our collection of 11 Dicktoo *Dhn* genes represents a nearly complete set. Genes that cannot be propagated in the λ vector, if any, are not included in this consideration.

Sequence analysis of Dicktoo barley dehydrins

Database searches using deduced amino-acid sequences of Dicktoo dehydrins indicated in every case the highest homology to DHN/COR/RAB and other LEA D11 proteins. The typical features of dehydrins can be described by the "YSK" shorthand, as described in the Introduction (Close 1997). The deduced amino-acid sequences from Dicktoo *Dhn* genes, and amino-acid sequence alignment with previously identified dehydrins, are shown in Fig. 2. At this point we introduce a gene and allele nomenclature that we have found to be most convenient. Each gene is referred to by the three-letter mnemonic *Dhn* and each distinct *Dhn* gene is given a unique number. Sequential numbering of *Dhn* genes, beginning with *Dhn1*, was suggested several years ago by Dr. Penny Von-Wettstein-Knowles (Copenhagen, Denmark) and has its precedent in the naming of barley *Dhn1* through *Dhn6*, the names of which we maintain in this work. We indicate the allele of each *Dhn* gene parenthetically by listing the cultivar name followed by the number of amino-acids in the encoded DHN polypeptide. For example Himalaya *Dhn4* becomes *Dhn4* (Himalaya 225). The polypeptide names follow the same convention, except that the protein names are not italicized and the letters are capitalized.

Dicktoo clones Dk14 and Dc13-1 encode YSK_2-type dehydrins identical to DHN1 (Himalaya 139) (NCBI ID #118483) and DHN2 (Himalaya 143) (NCBI ID #118486), and are designated DHN1 (Dicktoo 139) (14.2 kDa, pI 9.64) and DHN2 (Dicktoo 143) (14.4 kDa, pI 9.03), respectively (Fig. 2 A and B). The DHN1 and DHN2 polypeptides appear to be quite closely related to each other, with similarities in their Φ-segment anchor points, as indicated in Fig. 2. It has been shown by other investigators (Lång et al. 1998) that the Himalaya barley cultivar carries two segregating alleles of *Dhn1* (NCBI ID #118483 and NCBI ID #421876), which have been referred to as *Dhn1-1* and *Dhn1-2* (previously called *Dhn1* and *Dhn7*), respectively (Robertson et al. 1995). We refer here to the latter

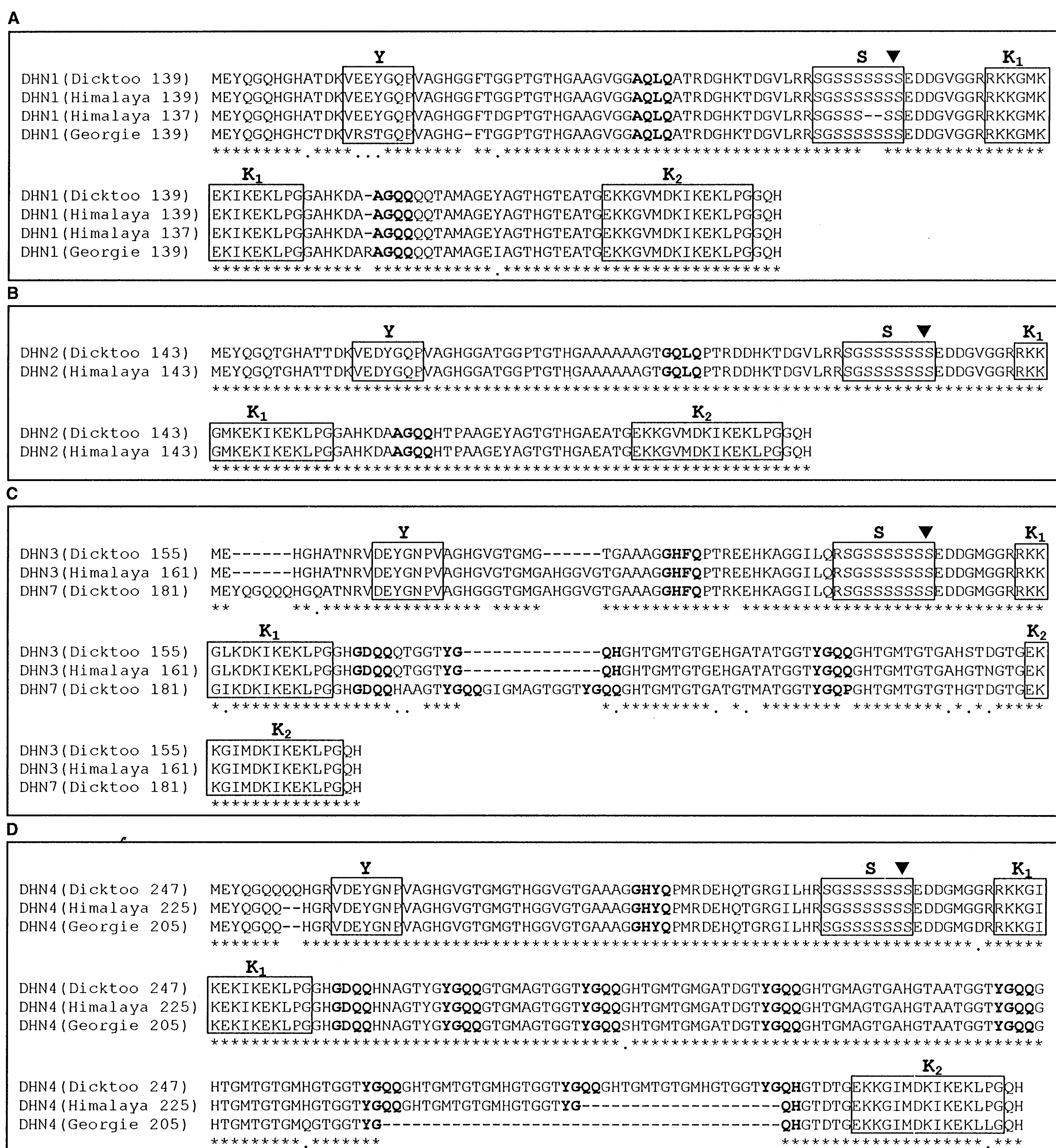

Fig. 2 Comparison of the amino-acid sequences of Dicktoo and previously known dehydrin alleles. Amino-acid residues are designated in *single-letter code*. Asterisks (*) and periods (.) indicate identical and non-identical amino acids, respectively. *Dashes* indicate where a sequence has been expanded to allow optimal sequence alignment. The *arrows* indicate the intron position. The Y-, S- and K-segments are boxed and the Φ-segment landmarks are in *boldface*. Alignments of the deduced amino-acid sequences were performed using the CLUSTAL V program

(*Dhn1-2*) as *Dhn1* (Himalaya 137). Amino-acid sequence alignments show that, relative to DHN1 (Himalaya 139) and DHN1 (Dicktoo 139), two S residues are absent in the S-segment of DHN1 (Himalaya 137) and one amino-acid residue is substituted (G replaced by E). Another allele of the *Dhn1* gene, which has been referred to as *Aba2* in Georgie barley (NCBI ID #1771015), has some extraordinary distinctions from other deduced DHN1 polypeptides, including the presence of a C, the lack of a good Y-segment consensus,

E

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                          K1                                                           K2
DHN5(Dicktoo 575)   MEHQAHVAGEKKGIMEKIKEKLPGGHGDHKQAADTHGQQGHAGTGTHGAPATGGAYEQQGRTGVTGTGLNGADAGEKKGLMEK
DHN5(Himalaya 575)  MEHQAHVAGEKKGIMEKIKEKLPGGHGDHKQAADTHGQQGHAGTGTHGAPATGGAYEQQGRTGVTGTGLNGADAGEKKGLMEK
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                     K2                                                                       K3
DHN5(Dicktoo 575)   IKDKLPGGHGDQQTTAGTYGQQGHTGTAAHGTPAGGGTYDQEGHTGMSGMGAHDTHTTGGAYGQHAHTGGTGTGTHGTGEKKG
DHN5(Himalaya 575)  IKDKLPGGHGDQQTTAGTYGQQGHTGTAAHGTPAGGGTYDQEGHTGMSGMGAHDTHATGGAYGQHAHTGGTGTGTHGTGEKKG
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                        K3                                                     K4
DHN5(Dicktoo 575)   VMENIKDKLPGGHGDHQKTSDTYGHQEDTEMTGMGRHSTPATGGSYGQHVHTGQTDMGTHGTGEKKGVMENIKDKLPGGHDDH
DHN5(Himalaya 575)  VMENIKDKLPGGHGDHQKTSDTYGHQEDTEMTGMGRHSTPATGGSYGQHVHTGQTDMGTHGTGEKKGVMENIKDKLPGGHDDH
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                                                                  K5
DHN5(Dicktoo 575)   QKTAGTYGQQGHVGTGTHGTPATGGAYGQHEHTGATGTGTYGTGEKKGIMENIKEKLPGGHGGHQKTGDTYGHQEDTKMTGMG
DHN5(Himalaya 575)  QKTAGTYGQQGHVGTGTHGTPATGGAYGQHEHTGATGTGTYGTGEKKGIMENIKEKLPGGHGDHHKTGDTYGHQEDTKMTGMG
                    **************************************************************.*.******************

                                                 K6
DHN5(Dicktoo 575)   RHSAPATGGSYGQHAHTGETGTGTHGTGEKKGVMENIKEKLPGGHDDHQKTAGTYGQQGHVGTGTHGTPATGGAYGQHEHTGA
DHN5(Himalaya 575)  RHSAPATGGSYGQHAHTGETGTGTHGTGEKKGVMENIKEKLSGGHDDHQKTAGTYGQQGHVGTGTHGTPATGGAYGQHERTGA
                    *****************************************.*************************************.***

                              K7                                                       K8
DHN5(Dicktoo 575)   TGTETYSTGEKKGVMENIKEKLPGGHGDHQQTGGIYGQQAHVGTGTHDTPATGGTYGQHGHTGVTGTGTHGTGEKKGVMENIK
DHN5(Himalaya 575)  TGTETYSTGEKKGVMENIKEKLPGGHGDHQQTGGIYGQQAHVGTGTHDTPATGGTYGQHGHTGVTGTGTHGTGEKKGVMENIK
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                     K8                                                          K9
DHN5(Dicktoo 575)   EKLPGGHSDHQHTTETYGQHRHTGVAGTETHGTTATGGTYGQQAQTGTTGAGTHGTDGTGEKKSLMDKIKDKLPGQH
DHN5(Himalaya 575)  EKLPGGHSDHQHTTETYGQHRHTGVAGTETHGTTATGGTYGQQAQTGTTGAGTHGTDGTGEKKSLMDKIKDKLPGQH
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                                   Y1                                         Y2
DHN6(Dicktoo 502)   MAHFQGQQHGHPATRVDEYGNPVTAGGHGGGVTGTDGLGHFQGQQHGRTTTRLDEYGNPVTAGHGVGAGSTGTEVHGPGHAGY
DHN6(Himalaya)      -----------------------------------------------------------------------------------

DHN6(Dicktoo 502)   GSTGTNDTGGHGRQVGYGATGTGTHDAGGYGGSGIAPRHGGAGTGVHDAGGLGHTTGHGATSTHGTGHTAGYGSTGTGMTGTH
DHN6(Himalaya)      ------------------------------------------------------------------------------MTGTH
                                                                                              *****

DHN6(Dicktoo 502)   GTGHTAGYDATGTGITGTHGTGHTTGYGGTGT-----HGTGHTAGYDATGTGIAGTHGTGRTAGYGGTGTGITGTHGTGHTTG
DHN6(Himalaya)      GTGHTAGYDATGTGITGTHGTGHTTGYGGTGTGITGTHGTGHTAGYDATGTGIAGTHGTGRTAGYGGTGTGITGTHGTGHTTG
                    ********************************     **********************************************

DHN6(Dicktoo 502)   YGGGATGTGITGTHGTGHTAGLGGTGTGITGTHGTGHTTGYGGGVTGTGITGTHGTGHTTGLGGTGTGITGTHGTGHTAGLGG
DHN6(Himalaya)      YGGGATGTGITGTHGTGHTAGLGGTGTGITGTHGTGHTTGYGGGVTGTGITGTHGTGHTTGLGGTGTGITGTHGTGHTAGLGG
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                                                      S ▼                  K1
DHN6(Dicktoo 502)   TGTGITGTHDVVGTHPHGGLGEHKTGGILHRSGSSSSSSSSSEDDGMGGRRKKGMKQKIKEKLPGGNKEQTTATGGYGPGYTG
DHN6(Himalaya)      TGTGITGTHDVVGTHPHGGLGEHKTGGILHRSGSSSSSSSSSEDDGMGGRRKKGMKQKIKEKLPGGNKEQTTATGGYGPGYTG
                    ***********************************************************************************

                                  K2                                                       K3
DHN6(Dicktoo 502)   TTGTGGAHGATEGTHEKKGVMEKIKEKLPGGHKDNPPHTTATTGGYGAGTTGTGGYGAGTTGTGGTYGATEGTHEKKGMMEKI
DHN6(Himalaya)      TTGTGGAHGATEGTHEKKGVMEKIKEKLPGGHKDNPPHTTATTGGYGAGTTGTTGYGAGTGGTAGTYGATEGTHEKKGMMEKI
                    *****************************************************.******.**.*******************

                      K3
DHN6(Dicktoo 502)   KEKLPGGHH
DHN6(Himalaya)      KEKLPGGHH
                    *********
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Fig. 2 Continued

and both the insertion and deletion of single amino-acid residues. We refer to the corresponding polypeptide as DHN1 (Georgie 139).

Dicktoo clone Dc15-130 encodes a 155 amino-acid YSK_2 polypeptide (15.7 kDa, pI 9.07). This polypeptide is very similar to DHN3 (Himalaya 161) (NCBI ID #118487) (Fig. 2 C). A six amino-acid sequence (AHGGVG), that is not present in the Dicktoo polypeptide, and two amino-acid substitutions near the C-terminal K-segment entirely account for the differences. We designate the protein encoded by Dc15-130 as DHN3 (Dicktoo 155) on the premise that it represents the product of the Dicktoo allele of the *Dhn3* gene.

The polypeptide sequence deduced from clone Dk8 shows considerable similarity to Dicktoo barley DHN3 (Fig. 2 C), but cannot represent the product of a *Dhn3*

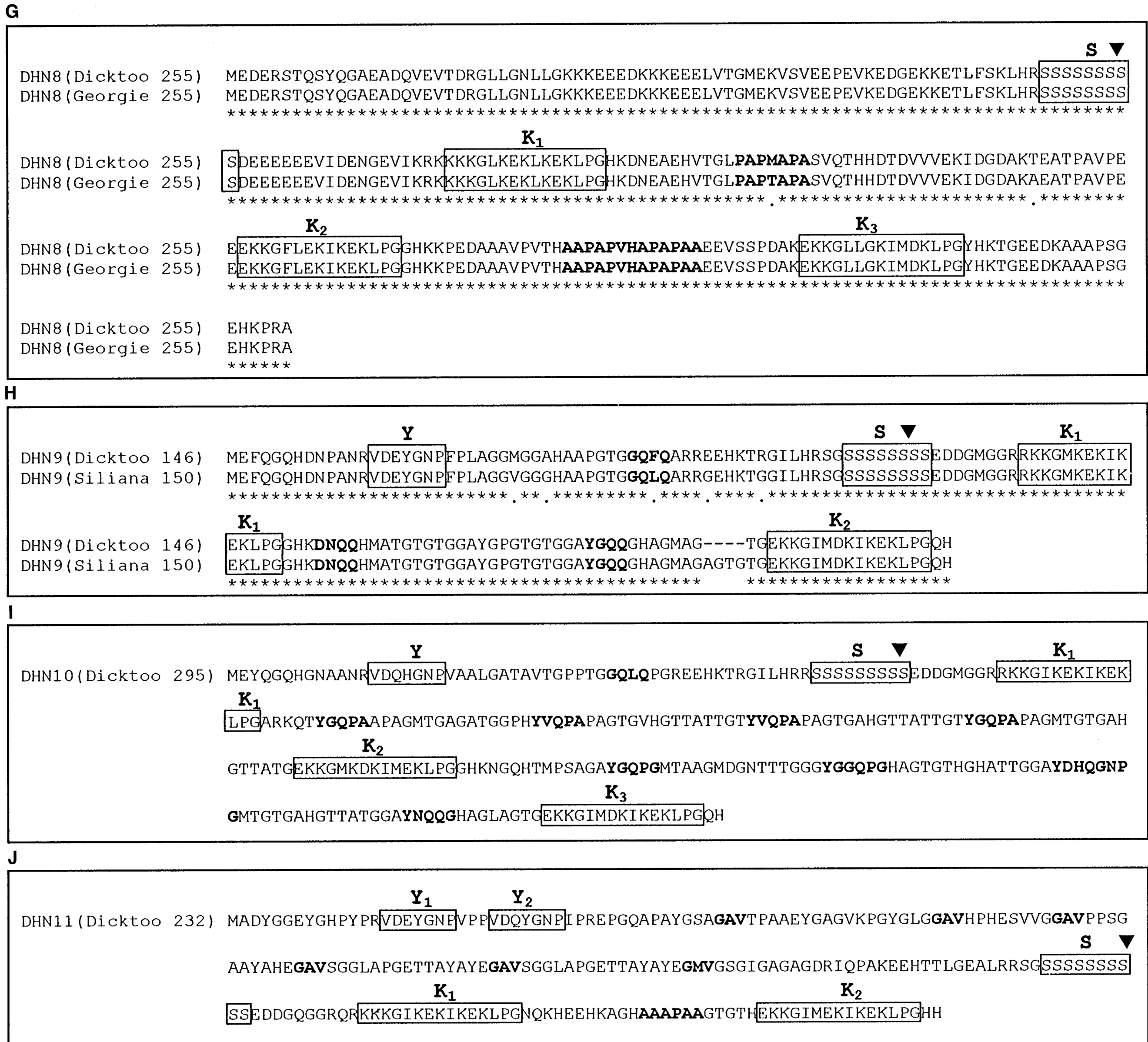


Fig. 2 Continued

allele because the Dicktoo seed that we used for DNA isolation has a long history of single-seed propagation (unlike Himalaya barley which is maintained as an inbred line with known variation between individuals in the line). We refer to this polypeptide as DHN7 (Dicktoo 181), which is a YSK_2 polypeptide of 181 amino acids (18.1 kDa, pI 10.13). There are four Φ-segments between the two K-segments of DHN7 (Dicktoo 181), whereas DHN3 (Himalaya 161) and DHN3 (Dicktoo 155) each have only three Φ-segments. The DHN7 polypeptide can be distinguished from DHN3 polypeptides by the presence of an extra amino-acid sequence (YQGQQQ) near their N-terminus (also present in DHN4 polypeptides). Also, there are 15 amino-acid substitutions in Dicktoo DHN7 relative to the Dicktoo DHN3 polypeptide.

Clone Dc15-108 encodes a YSK_2 polypeptide of 247 amino-acids (24.7 kDa, pI 9.02) with great similarity to both DHN4 (Himalaya 225) (NCBI ID #118489) and a polypeptide encoded by a cDNA called ABA3 from Georgie barley (Gulli et al. 1995) (NCBI ID #1006670), which could also be called DHN4 (Georgie 205) (Fig. 2 D). We refer to the Dicktoo polypeptide as DHN4 (Dicktoo 247). This polypeptide differs from DHN4 (Himalaya 225) and DHN4 (Georgie 205) mainly in the number of duplicate copies of the last Φ-segment (QQGHTGMTGTGMHGTGGTYG). This Φ-segment is present as only one copy in DHN4 (Georgie 205), two copies in DHN4 (Himalaya 225), and three copies in DHN4 (Dicktoo 247). Other small distinctions between these three apparent alleles are also shown in Fig. 2 D. Two extra Q residues are present in

the N-terminal region of DHN4 (Dicktoo 247) and amino-acid substitutions exist at four positions in DHN4 (Georgie 205).

Clone Dk4, which we refer to as DHN5 (Dicktoo 575), encodes a K_9 dehydrin (58.5 kDa, pI 6.68) very similar to the K_9 dehydrin DHN5 (Himalaya 575) (NCBI ID #282985) (Fig. 2 E). The number of K-segments has been found to be variable in previously described K_n dehydrins of the Triticeae (Close 1997). The *Dhn5* gene is the only known *Dhn* gene in barley that lacks an S-segment.

Clone Dc15-2 encodes a 502 amino-acid Y_2SK_3 polypeptide (47.6 kDa, pI 8.04). We refer to this protein as DHN6 (Dicktoo 502) since it is very similar to the available partial sequence of DHN6 determined previously from Himalaya barley (T. J. Close, unpublished data) (Fig. 2 F). The DHN6 protein is distinct from all other DHN proteins in that there is an extensive region to the N-terminal side of the S-segment containing a series of 16 Gly- and Thr-rich Φ-segments punctuated by the consensus sequence (A/T/G)G(Y/L)(G/D).

Clone Dc18-4 encodes an SK_3 dehydrin distinct from DHN6, and which we refer to as DHN8 (Dicktoo 255). This protein is a 255 amino-acid polypeptide (27.7 kDa, pI 5.1). DHN8 (Dicktoo 255) is very similar to the protein encoded by a cDNA from Georgie barley called PAF93 (Grossi et al. 1995) (NCBI ID #642246), which could also be called DHN8 (Georgie 255). The Dicktoo and Georgie DHN8 proteins differ only by amino-acid substitutions at two positions (Fig. 2 G). One characteristic of these proteins is the presence of a unique K-rich sequence (KKKEEEDKKKEEE) distinct from the conserved K-segment, positioned N-proximal to the S-segment. Another distinguishing feature is that the Φ-segments between the K-segments are rich in A and P rather than G, particularly between the last two K-segments.

Clone Dc15-4 encodes a YSK_2 dehydrin of 146 amino-acid residues (15.1 kDa, pI 10.34). We refer to this protein as DHN9 (Dicktoo 146). The most closely related dehydrin in the database was a durum wheat protein, which has been named TdDhn15 (Fig. 2 H) (Labhilili et al. 1995; NCBI ID #1181296). The only differences between the two are that four amino acids (AGTG) in the second Φ-segment of DHN9 (Siliana 150) are absent from DHN9 (Dicktoo 146), and four other amino-acid residues are substituted. The bread wheat RAB15 clone (NCBI ID #231983) seems also to represent a *Dhn9* ortholog based on DNA sequence-relatedness, even though this relationship is not so readily evident at the deduced polypeptide sequence level due to an apparent abnormality in the reading frame of the RAB15 protein between the Y-segment and the S-segment.

Clone Dc10-2 encodes a 295 amino-acid YSK_3 dehydrin (29.2 kDa, pI 10.51) (see Fig. 3 I), which we refer to as DHN10 (Dicktoo 295). Clone Dc25-1 encodes a 232 amino-acid Y_2SK_2 dehydrin (23.5 kDa, pI 6.75) (Fig. 2 J), which we refer to as DHN11 (Dicktoo 232). Neither of these polypeptides is sufficiently similar to any previously identified dehydrin to infer allelic relationships. Two unique characteristics of the DHN10 protein are that it is the only dehydrin that contains a Y-segment and more than two K-segments, and that it also contains Φ-segments rich in all three amino acids G, A and P. Two unique characteristics of the DHN11 polypeptide are a longer-than-usual span containing six Φ-segments between the Y-segment and the S-segment, and a very short span between two K-segments (Fig. 2 J).

Chromosome location of Dicktoo *Dhn* genes

We unambiguously determined the chromosome location of each of the 11 Dicktoo *Dhn* genes described in this report by PCR-amplification of genomic DNA from wheat-barley addition lines with gene-specific primers

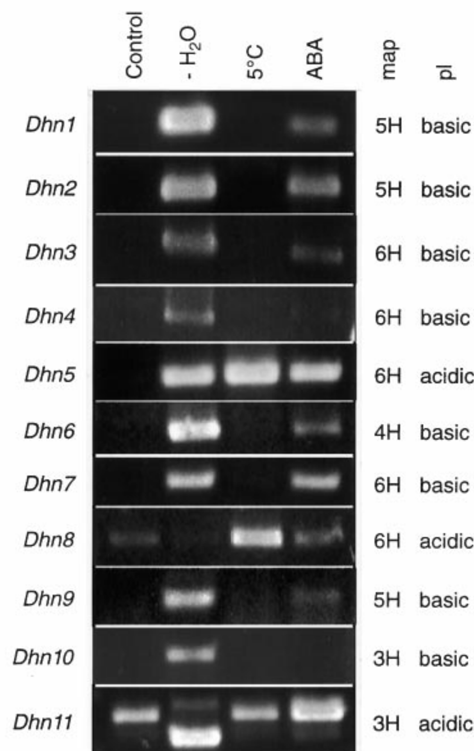


Fig. 3 Expression of each *Dhn* gene under several conditions. Five-day old barley seedlings grown at room temperature (*control*) were either dehydrated at 90% relative humidity for 1 day ($-H_2O$), cold acclimated at 5°C for 2 days (5°C), or treated with 20 μM of ABA for 2 days (*ABA*). Total RNA was isolated and reverse transcribed with a 3′-primer, and then amplified with gene-specific primer sets. The gene-specific primer sets are listed at Table 1. Annealing temperatures were adjusted for some primer sets in order to optimize the PCR reactions. RT-PCR products were electrophoresed in a 1.2% agarose gel. The prominent band present in three of the four *Dhn11* samples is not present in samples treated with DNase prior to RT-PCR reactions (data not shown), so it is presumed not to be derived from mRNA

(Table 1). Figure 3 summarizes the results. Chromosomes 3H, 4H, 5H and 6H carry these *Dhn* genes.

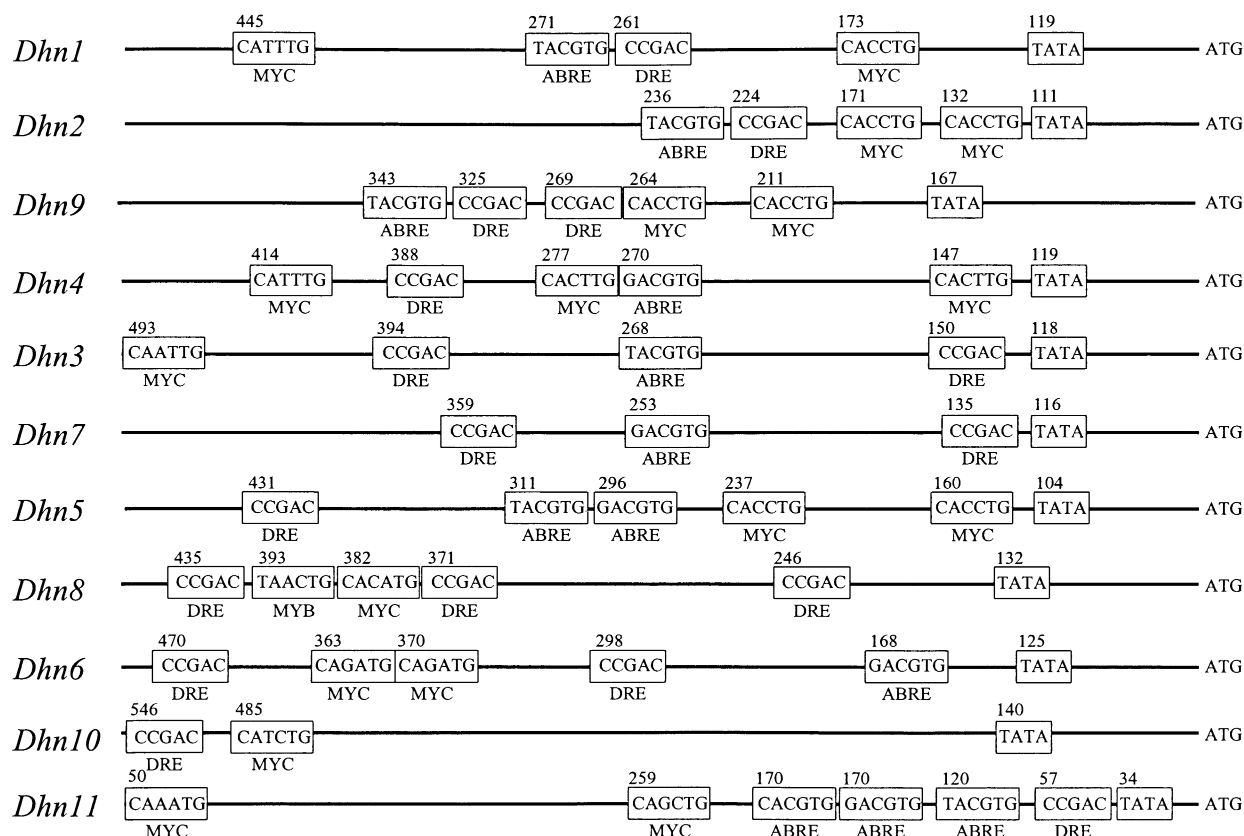
Expression of Dicktoo *Dhn* genes

To determine the expression pattern of each barley *Dhn* gene, we amplified *Dhn* mRNA using dehydrin gene-specific primers and RT-PCR (see Materials and methods). Total RNA was isolated from barley seedling shoots following several treatments, including control, dehydration, cool temperature, and ABA treatment (see Materials and methods). We utilized RT-PCR because Northern-blot hybridization is fraught with ambiguities associated with hybridization of the various *Dhn* genes to each other, as discussed previously with regard to a study of the Himalaya barley *Dhn1-1* and *Dhn1-2* alleles (Lång et al. 1998). Having the complete nucleotide sequences of all 11 *Dhn* genes, and in most cases more than one allele, enabled us to choose primers that would avoid the cross-hybridization problem without imposing allele-specificity. As with the wheat-barley addition-line mapping, RT-PCR required considerable trial and error with primers and reaction conditions.

The results show that expression of the *Dhn* genes is differentially regulated under several treatment conditions. In most cases, expression of the *Dhn* genes was not observed under normal growth conditions, but one exception was *Dhn8*. All 11 *Dhn* genes except *Dhn8* are up-regulated (but to different extents) by dehydration, and most of the *Dhn* genes are also up-regulated by ABA treatment, but there is not a strict correlation between the magnitude of up-regulation by dehydration and ABA. The latter generally evokes a lower level of expression. Some notable constants are that: (1) all of the *Dhn* genes that encode YSK_2 dehydrins (*Dhn1*, *Dhn2*, *Dhn3*, *Dhn4*, *Dhn6*, *Dhn7* and *Dhn9*) are up-regulated by both dehydration and ABA treatment, but not by a 5°C treatment, and all have a high predicted pI ranging from 9.02 to 10.58, and (2) the *Dhn* genes up-regulated by a 5°C treatment (*Dhn5* and *Dhn8*) encode acidic dehydrins with predicted pIs of 6.68 and 5.10, respectively.

Despite these underlying consistencies in expression of the "basic" versus "cold-regulated" dehydrins, there are differences within each group. For example, *Dhn5* is up-regulated by cold treatment, dehydration and ABA treatment, whereas *Dhn8* is expressed at a detectable basal level under normal conditions, up-regulated by

Fig. 4 Putative cis-acting elements in the 5′-flanking region of 11 Dicktoo *Dhn* genes. About 500-bp of the 5′-flanking region of each Dicktoo *Dhn* gene were analyzed for putative ABRE, DRE, MYC and MYB regulatory elements. The consensus sequences are (Py)ACGTGGC for ABRE, CCGAC for DRE, CANNTG for the MYC binding site, and (Py)AAC(Py)(Pu) for the MYB recognition site. *Numbers* on the boxes indicate nucleotides relative to the site of the start codon (ATG). The putative TATA boxes (TATAAA) were identified from 34 to 167 bases upstream of the initiation codon



cold and ABA, and down-regulated by dehydration (under the conditions that we examined). Grossi et al. (1995) reported that *Paf93* [*Dhn8* (Georgie 255)] responded rapidly to dehydration, prior to an increase in ABA levels, and then that the expression of this gene was reduced after 24 h of dehydration. Another outlier to the general patterns of expression noted above is *Dhn10*, which is induced by dehydration, but not by cold, and very weakly if at all by ABA.

We note the presence of typical regulatory element sequences related to ABA-, drought-, and cold-response in the 5′ flanking regions of each of the 11 *Dhn* genes (Fig. 4). In some examples there appears to be a strong correlation between the putative regulatory elements and the observed expression pattern. For example, we found one or more putative ABRE and DRE elements in the promoter region of *Dhn5*, *Dhn6*, *Dhn11*, and all of the *Dhn* genes that encode YSK_2 dehydrins (*Dhn1*, *Dhn2*, *Dhn3*, *Dhn4*, *Dhn7* and *Dhn9*); all of which are up-regulated by both dehydration and ABA. In addition, all of these *Dhn* genes, except *Dhn7*, also contain MYC-related elements. MYC- and MYB-related elements are involved in dehydration- and ABA-responsive expression of the *Arabidopsis* drought- and cold-inducible gene *Rd22* (Abe et al. 1997). Another good correlation is provided by *Dhn8*. The *Dhn8* gene [*Paf93*, Grossi et al. (1995)], which is up-regulated only transiently by dehydration, weakly by ABA, and mainly by cold treatment, has three DRE-, one MYC-, one MYB- and no ABRE-related elements. Similarly, the *Dhn10* gene has no ABRE-, one DRE- and one MYC-related element (both located more than 400 bases upstream of the presumed initiation site) and is principally dehydration- and weakly ABA-induced. In summary, there are some strong positive correlations between the presence of typical regulatory elements and observed expression patterns, but the locations and number of putative regulatory elements in the 5′-upstream regions are highly variable from one *Dhn* gene to another. A mechanistic study of these elements is outside the scope of this report, as is any description of the differences that have been found (D.-W. Choi, unpublished) in the 5′ regulatory regions of various alleles of a given *Dhn* gene.

Discussion

We identified 11 Dicktoo *Dhn* genes within ten contigs derived from a total of 36 genomic clones identified from 2×10^6 λ recombinants. Given the size of the haploid barley genome (approximately 5×10^6 kb) and an insert size of 18 kb, it is theoretically necessary to screen only about 1.3×10^6 λ recombinants in order to reach a probability of 0.99 for finding any given gene. Close and Chandler (1990) suggested that the barley genome encoded approximately ten *Dhn* genes from nucleic acid-hybridization and immunological data. A statistical analysis of our current data refines this estimate to a minimum of 11 (cloned) and possibly 13 barley *Dhn* genes. Since hexaploid wheat contains three homoeologous Triticeae genomes, this also means that hexaploid wheat contains 33–39 *Dhn* genes.

The Dicktoo barley *Dhn* gene collection may represent nearly all of the Triticeae *Dhn* genes present in all Triticeae genomes. The 11 barley *Dhn* genes that we have described account for all but one of the known Triticeae *Dhn* genes. The lone exception is the gene that encodes the *Triticum durum* TdDHN9.6 (Labhilili et al. 1995; NCBI ID #1181294), which is a 93 amino-acid K_2 polypeptide most closely related to a segment of DHN5. Alternatively, there may exist additional dehydrin genes with such a low nucleotide sequence similarity to the 11 identified barley *Dhn* genes that they would not have been detected in our screening procedure, or else could not be propagated in the λ cloning vector. Among the 11 barley *Dhn* genes that were identified, seven appear to be Dicktoo alleles of *Dhn* genes previously reported in other cultivars of barley, while another four (*Dhn7*, *Dhn9 Dhn10*, *Dhn11*) are new to barley. The Dicktoo barley *Dhn9* gene encodes a polypeptide most similar to a polypeptide encoded by a cDNA identified in durum wheat (Fig. 2; Labhilili et al. 1995). The relationship seems close enough to conclude that they represent orthologues. There are additional examples that can be considered to be cases of orthologous Triticeae *Dhn* genes. For example, genes encoding the major cold-induced WCS120 proteins of wheat, which map to 6AL, 6BL and 6DL, could be considered orthologues to the major cold-induced *Dhn5* gene of barley, which maps to 6HL (Houde et al. 1992; Limin et al. 1997). Also, four very similar, acidic SK_3 Triticeae dehydrins have been identified previously, and may represent orthologues of barley *Dhn8*. These are the Esi35 cDNA of *Lophopyrum elongatum* (NCBI ID #308896) and the COR410 proteins of *Triticum aestivum* (NCBI ID #1169018, 1657842, 1657844), the latter of which appear to be peripheral membrane proteins associated with the plasma membrane (Danyluk et al. 1998). In addition, in durum wheat (Labhilili et al. 1995), another deduced polypeptide (NCBI ID #1181291) is closely related to barley DHN7, DHN3 or DHN4, the allelic forms of which seem to represent nearly a continuation of related variation. There are at this time no known counterparts of the barley *Dhn10* and *Dhn11* genes.

The distinguishing features between dehydrin alleles at the protein level are principally deletion or duplication of Φ-segments and single amino-acid substitutions (Fig. 2). In comparison with the frequency of these two types of allelic variation, single amino-acid insertions or deletions are very rare. This suggests that the various domains (Y-, S-, K- and Φ-segments) represent structures with considerable constraint in the length of the polypeptide chain. Therefore, the vision of unordered

polypeptides in a native state (Lisse et al. 1996), which was based on measured properties of purified proteins in an aqueous environment, is probably incorrect. The native state might actually be quite structured, but involve binding to lipids or other ligands, similar to apolipoproteins (reviewed in Close 1997).

The *Dhn3* and *Dhn4* genes are more closely linked than are other *Dhn* genes, being separated by only about 8 kb. Tandemly repeated *Dhn* genes have been described previously in *Arabidopsis* and rice (Nordin et al. 1993; Welin et al. 1994), which have much smaller genomes than barley. In rice, four *Rab16* (responsive to abscisic acid) genes are tandemly arrayed in a segment some 30 kb in length (Yamaguchi-Shinozaki et al. 1989). Though the *Dhn3* and *Dhn7* genes must have a greater physical separation than do *Dhn3* and *Dhn4*, the *Dhn3* and *Dhn7* genes appear to be more closely related to each other evolutionarily, since they contain very similar introns. Possibly the *Dhn3* and *Dhn7* genes participate in unequal recombination events, leading to some homogenization of their allelic forms.

In the course of determining the Dicktoo *Dhn* gene sequences, a number of neighboring sequences were also identified, and among these sequences a barley retroelement (BARE) was discovered (Mamminen and Schulman 1993). Following the identification of one BARE, DNA hybridization of all ten contigs was conducted to identify the locations of additional BAREs. Several BAREs were found, and some are located near *Dhn* genes (Fig. 1). This result suggests that the evolution of the barley *Dhn* gene family has involved transpositions, duplications (White et al. 1994) and other transposon-related rearrangements, and further suggests that the insertion of BAREs may have had consequences for *Dhn* gene expression. Duplications and transpositions have also been suggested to play a role in the evolution of the LEA B19 (Group 1) gene family proposed by Stacy et al. (1995).

We developed gene-specific primers to determine the chromosome location and gene expression pattern of each *Dhn* gene. PCR results demonstrated that the 11 *Dhn* genes that we found in Dicktoo are located in four chromosomes (3H, 4H, 5H, 6H) in barley. These results confirm previous results of *Dhn* location by DNA-blot hybridization using wheat-barley addition lines or mapping populations (reviewed in Close 1996). It was previously reported that *Dhn1* and *Dhn2* map to barley chromosome 5H, *Dhn3* and *Dhn5* map to 6H, *Dhn6* maps to 4H, and a *Dhn4* probe detects loci on both and 5H and 6H (reviewed in Campbell and Close 1997). One recent study has also detected a locus on 7H using a *Dhn2* probe (Teulat et al. 1998). Additional PCR results in this report showed that the new barley dehydrin genes, *Dhn10* and *Dhn11*, are located in chromosome 3H, *Dhn9* is in chromosome 5H, and *Dhn7* and *Dhn8* are in chromosome 6H. The presence of *Dhn* genes in chromosome 3H was previously unknown, although Werner-Fraczek and Close (1998) found preliminary evidence of a *Dhn* locus on Thatcher wheat chromosome 3D. Previous studies suggested that 5H and 6H *Dhn* genes exist as clusters in chromosomes 5H and 6H (Pan et al. 1994; Campbell and Close 1997). Pan et al. (1994) reported that two *Dhn* genes, *Dhn1* and *Dhn2*, in chromosome 5H are located some 24 cM from another 5H *Dhn* locus detected by the *Dhn4* probe, and that three *Dhn* loci detected by *Dhn3*, *Dhn4* and *Dhn5* probes in chromosome 6H seemed to be separable from each other by recombination. Our results demonstrate that the *Dhn3* and *Dhn4* genes are very closely linked, since they are separated by only about 8 kb. The presence of five cross-hybridizing *Dhn* genes on 6H may have led to some inaccuracies in the mapping of this region. The availability of gene-specific primers for each of these genes, together with an expanded collection of 145 new Dicktoo × Morex DH lines (Patrick Hayes, Corvallis, Oregon, personal communication) and other DH mapping populations, can now be used to resolve lingering questions regarding recombinational distances within this cluster of *Dhn* genes.

As noted above, we are particularly interested in the *Dhn* cluster on chromosome 5H. Further efforts are underway to determine if any 5H *Dhn* gene is a candidate gene for the winter-hardiness QTL in 5H. Since we have not joined the three contigs that carry the three known 5H *Dhn* genes, it may be necessary to utilize a recently developed 7X Morex barley BAC library (A. Kleinhofs, personal communication) to be certain that we have identified all of the *Dhn* genes in this region. Fluorescence in situ hybridization might also help determine the physical distances between 5H *Dhn* genes. We are currently re-examining the relative map positions of the barley 5H *Dhn* genes (*Dhn1*, *Dhn2* and *Dhn9*) using gene-specific PCR primers with Dicktoo × Morex (Pan et al. 1994) and other doubled-haploid mapping populations.

RT-PCR results show that expression of the *Dhn* gene transcripts is differentially regulated under conditions such as dehydration, cold- and ABA-treatment (Fig. 3), as noted in the Results section. Another consideration is that some *Dhn* and other cold-inducible genes may be regulated by post-transcriptional control mechanisms (Hajela et al. 1990; Phillips et al. 1997). For example, the protein encoded by the potato *ci7* gene encoding an SK_3 dehydrin does not accumulate at low temperature despite the presence of *ci7* transcripts (Kirch et al. 1997). Also, under low temperature, GUS activity was not detected in transgenic tomato plants carrying a chimeric gene consisting of the *ci7* promoter fused to the GUS reporter gene, despite the presence of GUS transcripts. Kirch et al. (1997) suggested that the stability of *ci7* transcripts may increase at low temperature, and that the translation efficiency of *ci7* transcripts and the turnover of CI7 protein may also be controlled in a temperature-dependent manner. In order to avoid the cellular damage that dehydrins

might normally help minimize, post-transcriptional control may be an important means of rapidly producing these proteins when plants are exposed to stress conditions.

The results presented in this manuscript provide a foundation which can facilitate studies to address new and lingering questions related to the *Dhn* genes, including mechanisms of gene expression, the nature and evolution of allelic variation, and cause-and-effect relationships between specific alleles of *Dhn* genes and stress tolerance traits.

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