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Cold-Specific Induction of a Dehydrin Gene Family Member in Barley[1]

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An interval on barley (*Hordeum vulgare* L.) chromosome 7 accounting for significant quantitative trait locus effects for winter hardiness were detected in a winter (Dicktoo) × spring (Morex) barley population (P.M. Hayes, T. Blake, T.H.H. Chen, S. Tragoonrung, F. Chen, A. Pan, and B. Liu [1993] Genome 36: 66–71). Two members of the barley dehydrin gene family, Dhn1 and Dhn2, were located within the region defining the winter hardiness quantitative trait locus effect (A. Pan, P.M. Hayes, F. Chen, T. Blake, T.H.H. Chen, T.T.S. Wright, I. Karsai, Z. Bedö [1994] Theor Appl Genet 89: 900–910). To investigate the possible role of Dhn1 and Dhn2 in winter hardiness, we examined the expression pattern of six barley dehydrin gene family members in shoot tissue in response to cold temperature. Incubation of 3-week-old barley plants at 2°C resulted in a rapid induction of a single 86-kD polypeptide that was recognized by an antiserum against a peptide conserved in the dehydrin gene family. Northern blot analysis confirmed the induction of an mRNA corresponding to Dhn5. The expression patterns of cold-induced dehydrins in shoot tissue for Dicktoo and Morex were identical under the conditions studied, in spite of the known phenotypic differences in their winter hardiness. These results, together with the allelic structure of selected high- and low-survival lines, suggest that the Dicktoo alleles at the Dhn1 and Dhn2 may not be the primary determinants of winter hardiness in barley.

Despite recent advances in identifying genes induced during cold acclimation (see review by Thomashow, 1993), little is understood about the molecular basis of cold hardiness. With the long-term goal of elucidating the genetic control of winter hardiness, we mapped QTL associated with winter hardiness in a population of DH lines derived from the cross of a winter (Dicktoo) and spring (Morex) barley (*Hordeum vulgare* L.) (Hayes et al., 1993). We identified a QTL on the long arm of barley chromosome 7, controlling winter hardiness traits. This region was found to be responsible for 79% of the phenotypic variation in field survival in Bozeman, MT; 39% of the variance for survival at Corvallis, OR; 32% of the variance for LT_{50}, and 22% of the variance for crown fructan content (Pan et al., 1994). This was the only major interval in this population in which QTL effects controlling winter hardiness were located (Hayes et al., 1993; Pan et al., 1994).

Two members of the barley Dhn gene family, Dhn1 and Dhn2, mapped within the confidence interval of the winter hardiness QTL in the Dicktoo × Morex cross. Numerous Dhns have been identified in monocots and dicots in response to ABA or in response to various types of environmental stress or developmental changes involving dehydration (Close et al., 1989, 1993a, 1993b; Dure, 1993). The Dhn family of proteins is characterized by the consensus 15-amino acid sequence EKKGIMDKIKEKLPG found near the carboxy terminus, as well as repeated within the protein, and corresponds to the D-11 family of the late embryogenesis abundant proteins (Dure, 1993).

It is widely accepted that both drought and freezing stress result in desiccation of the protoplasm of plant cells (Steponkus, 1980). During each of these stresses, a lower water potential is established outside the cell, driving the net movement of liquid water out of the cell across the plasma membrane. Levitt (1958) proposed that plants may use a general mechanism of resistance to desiccation, regardless of whether desiccation results from drought or freezing stress. This proposal has been substantiated by numerous physiological studies. For example, gradual dehydration of winter rye seedlings results in the acquisition of both dessication and freezing tolerance (Siminovitch and Cloutier, 1983). Physiological and genetic studies have also shown an association of Dhns with cold acclimation. Dhn-like proteins accumulate during cold acclimation in Arabidopsis (Lin et al., 1990), wheat (Houde et al., 1992a; Guo et al., 1992), spinach (Neven et al., 1993), alfalfa (Wolfraim et al., 1993), potato (Karlsson et al., 1993a), *Brassica rapa* (Teutonico et al., 1993), peach (Arora et al., 1993), and blueberry (Muthalif and Rowland, 1994). Bromegrass and rye plants also accumulate Dhn transcripts during acclimation in the field as well as during acclimation under controlled environment conditions (Robertson et al., 1994). To our knowledge, however, a biochemical function to date has not been assigned to any of the Dhns. Moreover, it is not clear how

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Abbreviations: cM, centiMorgan; DH, doubled-haploid; Dhn, dehydrin; LOD, $\log_{10}$ of ratio between maximal likelihood estimates; LT_{50}, temperature required to kill 50% of the plants; mR, morphological marker of rough awn; QTL, quantitative trait locus; RFLP, restriction fragment length polymorphism.

Dhn gene expression is controlled. ABA is likely to play a role in the expression of at least some Dhn genes in dehydrating seedlings, but there is no clear evidence that ABA uniformly regulates Dhn gene expression in developing seeds (Chandler et al., 1993).

To further our objective of identifying genes on the long arm of barley chromosome 7 controlling components of winter hardiness and to determine whether Dhn1 and Dhn2 play a role in mediating winter hardiness, we analyzed the expression pattern of Dhns in response to cold in Dicktoo and Morex barley. We report here that Dhn5, located on chromosome 6, is induced in 3-week-old barley plants within 24 h of exposure to cold temperatures under controlled environment conditions. Together with data concerning the allelic structure of selected DH lines at the Dhn1 and Dhn2 loci, these data suggest that Dhn1/Dhn2 are probably not the primary determinants of low-temperature stress resistance in Dicktoo barley, as measured under controlled environment conditions.

MATERIALS AND METHODS

Dicktoo is a six-row winter feed barley of unknown ancestry and mixed description released by the Nebraska Agricultural Experimental Station in 1952. Morex is a six-row spring malting barley released in 1978 by the Minnesota Agricultural Experimental Station. DH lines from a Dicktoo × Morex cross were developed as described by Chen and Hayes (1989).

Mapping

RFLPs detected by Himalaya barley Dhn1, Dhn2, Dhn3, Dhn4, and Dhn6 (cDNA clones) and Dhn5 (a genomic clone) (Chandler et al., 1993) were used, together with an additional 70 markers, to construct the linkage map shown in Figure 1. Marker analysis procedures, map construction, and marker nomenclature were described by Pan et al. (1994). The linkage map was constructed using Mapmaker/EXP 3.0 (Lander et al., 1987; Lincoln et al., 1992a). Seventy-four markers provide relatively uniform coverage over the seven barley chromosomes. Assignments of linkage groups to the seven chromosomes of barley were made based on previously mapped markers. QTL analyses were conducted using the interval mapping procedures of Mapmaker/QTL 1.1 (Lincoln et al., 1992b). The minimum LOD threshold was specified at 3.0. QTL effects for low-temperature survival and winter growth habit traits were previously discussed by Pan et al. (1994).

Treatments

Barley seeds were surfaced sterilized, soaked for 2 d in the dark in Petri dishes on a layer of moist filter paper, and then transferred to a 23°C incubator with a 16-h light/8-h dark cycle for 3 to 7 d as described by Close et al. (1989). Alternatively, seeds were planted in a soil-less mixture (1:1:1 peat:vermiculite:perlite) in pots and grown in a 23°C greenhouse for 3 weeks prior to cold treatments. For cold-temperature treatments, 3-week-old plants in pots, or seedlings in Petri dishes, were transferred to growth chambers maintained at 23 or 2°C with a 16-h light/8-h dark cycle for 1 to 8 d. After the cold treatments, plant material was harvested, frozen in liquid nitrogen, and stored at −80°C.

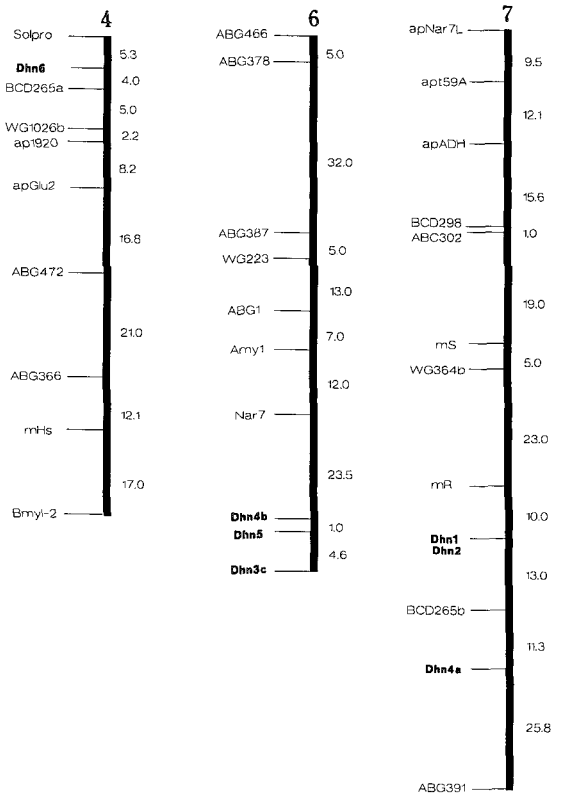

Figure 1. Dhn1 and Dhn2 map to chromosome 7. Barley Dhn1 and Dhn2 were mapped previously to the long arm of chromosome 7 in the interval flanked by markers mR and BCD256b. This interval has been identified by QTL analysis to contain genes controlling winter hardiness. The location of Dhn genes Dhn3 through Dhn6 are also indicated. The linkage map was based on 100 DH lines derived from the cross of Dicktoo × Morex (units are cM). Chromosomes 1, 2, 3, and 5 do not contain known Dhn genes and therefore are not shown in this diagram.

Immunoblots

Crude protein extracts were prepared from shoot tissue as described by Close et al. (1989) except that the extraction buffer contained 30 mM Tris, pH 7.5, 200 mM NaCl, 1 mM PMSF. Protein concentration was determined by the bicinchoninic acid method (Brown et al., 1989) using BSA as a standard. Proteins were separated in 12.5% polyacrylamide in a Mini-Protein II cell (Bio-Rad) and transferred to nitrocellulose. Nitrocellulose filters were blocked with 5% nonfat dry milk powder in TBST and incubated with rabbit anti-peptide antibodies, followed by anti-rabbit IgG peroxidase conjugate. An enhanced chemiluminescence detection system (Amersham) was used to develop the immunoblots. Expression and purification of DHN5 protein in *Escherichia coli* will be described elsewhere.

Northern Analysis

RNA was extracted from samples of shoot tissue using a hot phenol buffer (phenol-0.1 M LiCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS [1:1]). RNA (5 μg) was electrophoresed in a 1.2% agarose-formaldehyde gel (Sambrook et al., 1989) and transferred to a ZetaProbe membrane (Bio-Rad) according to the manufacturer's instructions. Hybridization probes were labeled with radioactive nucleotide using purified cDNA fragments and a random-primed labeling kit (Ambion, Austin, TX). cDNAs for Himalaya barley Dhn genes Dhn1 to Dhn4 have been described (Close et al., 1989). Dhn5 and Dhn6 are genomic clones (Close et al., 1995; T. Close, unpublished data, respectively). Blots were hybridized for 16 h at 65°C in 250 mM $NaHPO_4$, 7% SDS, followed by washing at low stringency (2× SSC, 0.1% SDS at 65°C for 1 h; 0.1× SSC at 50°C for 20 min) or high stringency (2× SSC, 0.1% SDS at 78°C for 1 h; 0.1× SSC at 60°C for 20 min) (Close et al., 1989).

RESULTS

Linkage Mapping of Dhn1 and Dhn2

The Dhn1 and Dhn2 clones revealed RFLPs that mapped to the minus arm of chromosome 7 (Pan et al., 1994). Alleles at these two RFLP loci showed complete co-segregation in the mapping population of 100 DH lines of a Dicktoo × Morex cross, indicating that the two genes are tightly linked. The Dhn1 and Dhn2 loci are 10.1 cM distal to the mR locus and 13.3 cM proximal to the BCD265b locus (Fig. 1). Before the Dhn loci were added to the linkage map, the QTLs for three measures of low-temperature stress (field survival in Oregon, field survival in Montana, and LT_{50}) mapped between mR and BCD265b (Hayes et al., 1993). When the Dhn1 and Dhn2 data were added to the map, QTL resolution was not increased. The LOD score for the QTL peak between Dhn1/Dhn2 and BCD265b was somewhat higher than the QTL peak between mR and Dhn1/Dhn2 (LOD = 27.1 versus 25.2), but confidence intervals overlapped. Thus, with the current population size and phenotype data sets, further resolution of the QTL position is not possible.

Cold-Induced Expression of Dhn5

Previously, we demonstrated that phenotypic variation for winter hardiness in Dicktoo and Morex observed under natural conditions can also be induced under controlled environment conditions (Hayes et al., 1993). After acclimation at 2°C for 5 weeks under controlled laboratory conditions, the LT_{50} values for Dicktoo and Morex were −7.5 and −3.8°C, respectively (Hayes et al., 1993). We therefore used the same temperature and photoperiod regime in these experiments. Tissue was sampled over a 3-week period. Reasoning that genes controlling winter hardiness may be (a) induced by cold temperatures and (b) expressed in vegetative (crown) tissues, we asked whether Dhn1 and Dhn2 are induced by cold-temperature treatment in Dicktoo and Morex plants. First, we analyzed soluble protein extracts prepared from cold-treated shoot tissue of 3-week-old Dicktoo and Morex plants by immunoblotting. A single Dhn species of approximately 86 kD was induced by cold treatment in both Dicktoo and Morex shoots (Fig. 2A). The Dhn-specific antibody failed to react with extracts prepared from control plants maintained at 25°C. Detectable, albeit very low, levels of the 86-kD polypeptide were observed within 1 d of exposure to low temperature (Fig. 2A, lanes 4 and 5) and increased to easily detectable levels in both Dicktoo and Morex shoot tissue upon longer exposure to low temperature (lanes 6–13). However, within each sampling set, there was no significant difference in the amount of 86-kD protein present in Dicktoo versus Morex shoot tissue (for example, compare lanes 6 and 7 or 8 and 9). Equal protein loading was assessed by Coomassie blue staining of replicate gels (Fig. 2B). Preimmune serum did not react with the 86-kD polypeptide (data not shown).

Nucleic acid hybridization and immunological data suggest that the barley genome encodes approximately 10 Dhns (Close and Chandler, 1990). The nucleotide sequences for Himalaya barley Dhns (Close et al., 1989, 1995; Close and Chandler, 1990; T.J. Close, unpublished data) and the corresponding map locations in the DH lines derived from a Dicktoo × Morex cross (Pan et al., 1994) have been determined for Dhn1 through Dhn6 (refer to Fig. 1). To determine which member of the Dhn gene family was induced by the cold-temperature treatment, we probed blots of Dicktoo and Morex RNA with Himalaya barley Dhn cDNAs and genomic clones. As Close et al. (1989) have demonstrated in Himalaya barley, hybridization

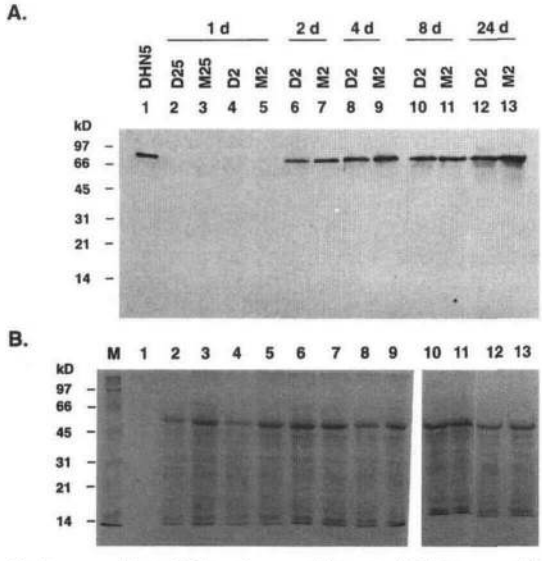

Figure 2. Immunoblot of Dhns from cold-treated Dicktoo and Morex shoots. A, Protein samples were prepared from 3-week-old Dicktoo (D) and Morex (M) shoots that had been incubated at either 25°C (lanes 2 and 3) or 2°C (lanes 4–13) for 1 to 24 d. Total protein (12.5 μg) was loaded per lane on a 12.5% SDS-polyacrylamide gel. Lane 1 contains DHN5 protein purified from *E. coli*. Immunoblot analysis was performed using a crude anti-Dhn immune serum (diluted 1:500), followed by a peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The sizes of Bio-Rad prestained molecular mass standards are indicated. B, Replicate gels were stained with Coomassie blue.

stringency can be manipulated to the point at which all of the Dhn genes are detected by any one Dhn probe or at which only one Dhn gene is detected by each probe. Northern analysis of total RNA prepared from cold-treated 3-week-old Dicktoo and Morex shoots using Dhn1 as probe under low-stringency conditions detected a primary transcript of approximately 2.1 kb (Fig. 3A). In addition, a second, fainter band migrating at a lower molecular mass was apparent in both Dicktoo and Morex cold-treated shoots. Under high-stringency conditions, only the Dhn5 probe hybridized to the 2.1-kb transcript (Fig. 3B). A weak signal was also detected for Dhn4; however, the transcript migrated at the same position as that detected by Dhn5 and may result from cross-hybridization of Dhn4 with Dhn5. In addition to the 2.1-kb transcript, the Dhn5 probe hybridized to several higher molecular mass RNA species. Under the same high-stringency conditions used for Dhn5, Dhn1, Dhn2, Dhn3, and Dhn6 probes did not hybridize detectably to RNA prepared from cold-treated Dicktoo and Morex shoots (Fig. 3B).

The predicted molecular mass of the polypeptide encoded by Dhn5 is 58.5 kD. Whereas northern analysis under high-stringency conditions detected a primary transcript of 2.1 kb corresponding to Dhn5, immunoblot analysis of cold-temperature-induced polypeptides detected a Dhn polypeptide species at 86 kD, considerably larger than predicted for a transcript of 2.1 kb. To resolve the discrepancy between the predicted and apparent molecular mass of Dhn5, we compared the migration of the 86-kD protein to purified Himalaya DHN5 protein produced in *E. coli*. As shown in Figure 2A (lane 1), purified DHN5 migrated slower than the predicted 58.5 kD and co-migrated with the cold-induced 86-kD Dhn polypeptide. Thus, both the northern and western analyses support the cold-temperature induction of Dhn5 in both Dicktoo and Morex barley shoots. Similarly, low-temperature induction of Dhn5 has been observed in Himalaya barley seedlings (M. Robertson, personal communication).

Developmental expression and tissue-specific expression of Dhns have been observed in both pea and cereals (Chandler et al., 1993; Close et al., 1993b). We were interested in determining whether Dhn5 could also be induced by low-temperature treatment in young seedlings as well as mature vegetative tissues. As shown in Figure 4, Dhn5 gene

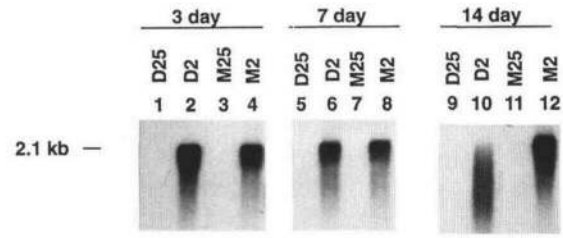

Figure 4. Developmental and environmental regulation of Dhn5. Total RNA (10 μg/lane) prepared from shoot tissue of 3-, 7-, and 14-d-old Dicktoo (D) and Morex (M) seedlings that had been incubated at 25°C (25) or 2°C (2) for 48 h as described in "Materials and Methods" was analyzed by northern blotting using Dhn5 as a probe under high-stringency conditions.

expression was induced by cold treatment in 3-, 7-, and 14-d-old Dicktoo and Morex shoots. Dhn5 protein was also detected in root and crown tissue of both Dicktoo and Morex plants following exposure to low temperature (data not shown). In preliminary experiments, we also observed that Dhn5 was present in an array of Dhn polypeptides induced by water stress in Dicktoo and Morex (K. van Zee, unpublished data).

Map Location of Dhn Genes and Relationship to QTL Effects

The six Himalaya Dhn clones revealed RFLPs in the Dicktoo × Morex population (Fig. 1). As previously described, Dhn1 and Dhn2 map to the minus arm of chromosome 7. Dhn3 revealed a complex banding pattern: with the available markers, only one band (Dhn3c) could be mapped, and it defines the terminal position on the minus arm of chromosome 6. Dhn4 also revealed a multiband polymorphism. One locus (Dhn4b) mapped to the minus arm of chromosome 6 and the other (Dhn4a) mapped to the minus arm of chromosome 7, distal to the Dhn1 and Dhn2 loci. Dhn5 mapped to the minus arm of chromosome 6, between the Dhn4b and Dhn3c loci. Dhn6 mapped to the plus arm of chromosome 4. Pan et al. (1994) mapped QTL for low-temperature tolerance and a range of winter growth habit-related phenotypes in this same population. The only point of coincidence for QTL effects for these

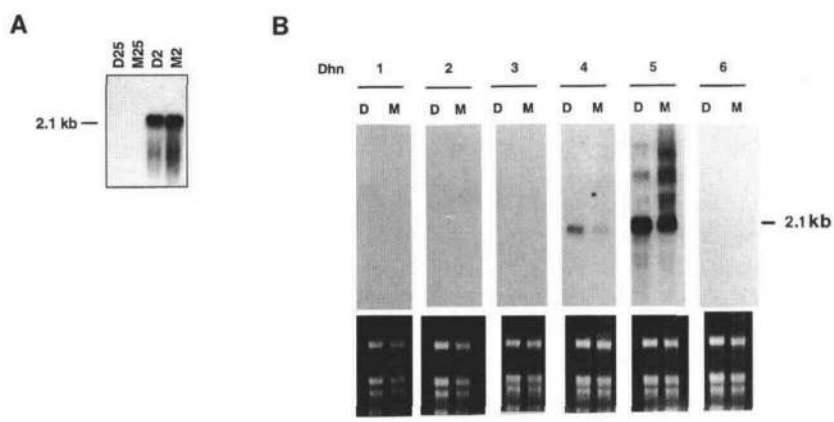

Figure 3. Northern analysis of cold-induced Dhn mRNAs. A, Total RNA prepared from shoot tissue of 3-week-old Dicktoo (D) and Morex (M) plants that had been incubated at 25°C (25) or 2°C (2) for 48 h was separated on a 1.2% gel and transferred to Zeta-Probe. Total RNA (10 μg) was loaded per lane. Blots were cut into strips and probed with Dhn1 under low-stringency conditions. B, Replicate strips containing 10 μg of total RNA prepared from Dicktoo (D) and Morex (M) shoot tissue that had been incubated at 2°C for 48 h were probed with Dhn1 through Dhn6 under high-stringency conditions. Ethidium bromide staining of the RNA samples prior to blotting is shown below each autoradiograph.

traits and the mapped Dhn loci was on chromosome 7, in the vicinity of Dhn1/Dhn2.

Although Dhn1 and Dhn2 polypeptides are not detectably induced by cold temperatures in shoots under the conditions used, the map location of these genes suggests that they may play a role in mediating winter hardiness. To explore the possible direct associations of the Dhn1 and Dhn2 loci with low-temperature tolerance, the most hardy and least hardy 20 DH lines that were in common to the two measures of field survival were identified. Within the 20 most low-temperature-sensitive lines, there were 2 lines that had Dicktoo alleles at the Dhn1 and Dhn2 RFLP loci. The representative data are summarized in Table I. One line (DH-29) had the Morex allele at the distal marker (BCD265b), and the second line (DH65) had Dicktoo alleles at the Dhn1/Dhn2 and BCD265b marker loci. Within the 20 most low-temperature-resistant lines, 1 line (DH-67) had Morex alleles at the Dhn1 and Dhn2 loci and the Dicktoo allele at BCD265b, the distal locus. If Dhn1 and/or Dhn2 were the principal determinants of low-temperature response in this population, complete co-segregation of marker genotype and phenotype would be expected. If one assumes that the gene or genes underlying low-temperature response lie in the interval defined by Dhn1/Dhn2 and BCD265b, then DH-29 and DH-67 may represent the products of single crossovers distal and proximal, respectively, to the gene or genes underlying low-temperature response, and DH-65 may represent a double crossover event. The QTL analysis procedures assume strictly additive gene action. The presence of phenotypic transgressive segregants suggests that more complex interactions, i.e. additive × additive epistasis, may be operative.

DISCUSSION

Dhns are a family of closely related proteins that accumulate in plants in response to a dehydration component and an elevated ABA level. Although the occurrence of Dhn proteins has been correlated with plant acquisition of drought, salt, and cold tolerance, direct evidence that any of the Dhn proteins play a causal role in stress acclimation has not been established. Our interest in the cold-temperature regulation of Dhn gene expression lies in the finding that two Dhn genes, Dhn1 and Dhn2, map to a region on barley chromosome 7, where QTL effects for low-temperature resistance were previously detected (Pan et al., 1994).

In this report, we have demonstrated that exposure of barley plants grown in a controlled environment to cold temperatures results in the strong induction of one of the six Dhn genes tested in shoot tissues. Cold-temperature treatment of seedlings and mature plants resulted in the induction of a Dhn-specific polypeptide with an apparent molecular mass of 86 kD (Fig. 2A). Northern analysis under high-stringency conditions detected one primary cold-induced transcript, which hybridized to the Dhn5 probe (Fig. 3B). Although the predicted molecular mass of the polypeptide encoded by Himalaya barley Dhn5 is 58.5 kD, DHN5 purified from *E. coli* migrates at an apparent molecular mass of 86 kD and co-migrates with the 86-kD cold-induced Dhn polypeptide (Fig. 2A). Taken together, the northern and western analyses identify this cold-induced Dhn polypeptide as Dhn5. An additional transcript migrating with a lower molecular mass than the 2.1-kb transcript discussed above was detected in cold-treated Dicktoo and Morex shoot tissue using Dhn1 as a probe under low-stringency conditions (Fig. 3A). This lower molecular mass transcript was also visible as a faint band when Dhn5 was used as a probe under high-stringency conditions; however, neither Dhn1 nor Dhn2 probe bound detectably to this transcript under the high-stringency conditions used (Fig. 3B). Additional higher molecular mass RNA species were sometimes observed in samples prepared from both Dicktoo and Morex shoot tissue when Dhn5 was used as a probe under high-stringency conditions (Fig. 3B). None of the other Dhn probes we tested bound to these RNA species. Although we cannot rule out that the lower or higher molecular mass transcripts correspond to another Dhn gene family member, we have no direct evidence to suggest that other Dhn polypeptides accumulate in cold-treated shoot tissues of Dicktoo and Morex. Using an antibody prepared against the wheat protein WCS 120, Houde et al. (1992b) also reported the cold-temperature induction of a

Table I. *Marker genotypes for loci on the minus arm of chromosome 7 of representative DH lines from the Dicktoo × Morex population and field survival in Oregon and Montana*

D, Dicktoo allele; M, Morex allele; mS, morphological marker of smooth awn.

Genotype	mS	WG364b	mR	Dhn1/Dhn2	BCD265b	Field Survival	
						Oregon	Montana
						%	%
Dicktoo	D	D	D	D	D	89	42
Morex	M	M	M	M	M	18	0
DH-26	M	M	M	M	M	29	0
DH-29	D	D	D	D	M	25	0
DH-45	M	M	M	M	D	20	0
DH-65	M	M	M	D	D	33	3
DH-68	M	M	M	M	M	10	0
DH-85	D	D	M	M	D	21	0
DH-67	M	M	M	M	D	100	73
DH-69	D	D	D	D	D	99	57
DH-72	D	—	D	D	D	100	38

protein of approximately 85 kD in barley. Dhn5 has homology to WCS 120. In wheat, members of the protein family recognized by an antibody to WCS 120 accumulate to higher levels in freezing-tolerant genotypes than in less-tolerant ones (Houde et al., 1992b). However, in Dicktoo and Morex we have not observed significant differences in either the kinetics of Dhn5 protein accumulation or in the relative amounts of Dhn5 protein between these two freezing-tolerant and freezing-sensitive genotypes.

Although there were no significant differences in the cold induction of Dhn5 in Dicktoo and Morex, there were significant differences in the pattern of lower molecular mass Dhns induced by dehydration stress in Dicktoo and Morex (data not shown). Estimates based on genomic cloning indicate that there are approximately 10 Dhn genes in Himalaya barley (Close et al., 1993b), and to date every barley cultivar examined contains a unique pattern of Dhn polypeptides on both SDS-PAGE and immunoblots (Close et al., 1993a). The fact that one primary Dhn RNA transcript and one Dhn polypeptide were detected in northern and western analyses suggests that other Dhn genes in barley are not induced to detectable levels by cold temperature in shoot or root tissues of seedlings and 3-week-old plants under the conditions used in these experiments.

The regulation of Dhn expression appears to be complex, in that previous studies have demonstrated that Dhn genes are under both developmental and environmental control (Chandler et al., 1993; Close et al., 1993a). For example, investigations of the Dhn protein profile at different stages of cereal development suggested that ABA may regulate Dhn gene expression in dehydrating seedlings but not in developing seeds or mature plant tissues (Chandler et al., 1993). However, when examining the expression of the pLE4 gene of tomato, Bray et al. (1993) established a role for ABA in response to drought. Analysis of Dhn expression in the ABA-deficient *viviparous* mutants of maize also indicated that Dhn expression in vegetative tissues may be under different control than during embryogenesis (Pla et al., 1989). In Arabidopsis, cold-regulated (*cor*, also referred to in the literature as *lti* and *kin*) genes are responsive not only to cold temperature but also to drought and ABA (Hajela et al., 1990; Nordin et al., 1991; Thomashow, 1993). Although the relationship between cold-, drought, and ABA-regulated gene expression is not understood, accumulating evidence suggests that independent signal-transduction pathways may operate in response to different environmental signals (Nordin et al., 1991, 1993; Yamaguchi-Shinozaki and Shinozaki, 1994). For example, the cold-regulated Arabidopsis gene *kin*1 is responsive to ABA but not drought (Kurkela and Borg-Franck, 1992). Whereas the expression of Lti 78 is mainly responsive to low temperature, the expression of Lti 65 is responsive mainly to drought and ABA (Nordin et al., 1993).

The multiband polymorphism observed with Dhn3 and Dhn4, the co-segregation of Dhn1 and Dhn2, and the clustering of Dhn loci on chromosomes 6 and 7 suggest a degree of conservation among the Dhn loci. Of the Dhn loci, we found that under our experimental conditions only Dhn5 was specifically induced by low temperature, and yet no QTL effects for low-temperature tolerance or other winter growth habit-related traits were detected in the vicinity of this locus. Dhn1/Dhn2 were not detectably induced by low temperature under our conditions, and yet QTL effects for low-temperature tolerance were detected in the vicinity of these loci. Furthermore, there was not a complete association of Dhn1/Dhn2 allele type and field survival phenotype. Thus, these loci do not appear to be the primary determinants for the low-temperature tolerance QTL observed on chromosome 7. Alternatively, the coincident genome location of these loci with QTL effects may be evidence for a multilocus cluster of genes controlling a common phenotype, in this case winter hardiness, as postulated by Allard (1988). The availability of this immortal reference population of DH lines will allow for the repeated phenotyping and genotyping that will be required for genetic dissection of winter hardiness. Experiments designed to characterize Dicktoo and Morex alleles at the Dhn loci and examine Dhn gene expression in field-acclimated conditions are underway and should contribute to our understanding of this complex phenotype.

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