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ORIGINAL PAPER

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Barley *Dhn13* encodes a KS-type dehydrin with constitutive and stress responsive expression

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Abstract Dehydrins (DHNs) compose a family of intrinsically unstructured proteins that have high water solubility and accumulate during late seed development, low temperature or water deficit conditions, and are thought to play a protective role in freezing and drought tolerance in plants. Twelve *Dhn* genes were previously described in the barley genome. Here, we report an additional member of this multigene family, *Dhn13*. The *Dhn13* gene is located in chromosome 4 near marker MWG634 and encodes a 107-amino acid KS-type DHN. Semi-quantitative reverse transcriptase PCR data indicated that *Dhn13* is constitutively expressed in seedling tissues and embryos of developing seeds. Microarray data were consistent with these results and showed a considerable increase of *Dhn13* transcripts when plants were subjected to chilling and freezing temperatures. The highest transcript levels where observed in anthers. The presence of ABRE, MYC, DRE, and POLLEN1LELAT52 regulatory elements in the putative *Dhn13* promoter region is in agreement with expression data.

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Introduction

Crop production usually is far below genetic potential. Unfavorable environmental conditions typically depress yields more than 70% (Boyer 1982) below the maximum. To increase yields under sub-optimal conditions, an understanding of basic biological mechanisms underlying plant responses to cold, drought, and salinity stresses may be helpful. Cellular dehydration is a commonality among these stresses, and plants exhibit common molecular responses to them. Accumulation of a family of proteins known as dehydrins (DHNs) is one of the prominent components of this adaptive process (Close 1997). DHNs typically accumulate in different tissues in response to low temperature, drought, salinity, or abscisic acid (ABA) application and during the late phase of embryogenesis (Campbell and Close 1997).

DHNs are part of a larger group of proteins known as late embryogenesis abundant, all of which are characterized by high glycine content, high hydrophilicity, and low secondary structure in purified form (Garay-Arroyo et al. 2000). The distinctive feature of DHNs is a conserved, lysine-rich 15-amino acid domain named the "K segment" that can take on an amphipathic α-helix structure (Close 1997). Other DHN domains are the S and Y segments and less-conserved domains rich in polar amino acids (Φ-segments). The S segment is a tract of serine residues. The Y segment, when present, occurs near the N terminus. Permutations in the arrangement of these domains define five sub-classes of DHNs: Y_nSK_n, SK_n, K_n, Y_nK_n, and K_nS (reviewed in Campbell and Close 1997; Svensson et al. 2002).

A positive correlation between DHNs and adaptation to cellular dehydration has been extensively documented in the literature. The specific functions of DHNs remain somewhat unclear, but several hypotheses link the characteristic hydrophilic amino acid composition and secondary structure to molecular functions. DHNs may act as water attractants in cells with low water potential, having a role in osmotic potential regulation (Nylander

et al. 2001). Another proposed function is to stabilize protein and membrane structures by surfactant or chaperone-like activities (Close 1996). A protective interaction with membranes may occur by means of the K segment that, though intrinsically unstructured, takes on an amphipathic helical structure when bound to membranes (Koag et al. 2003). *Arabidopsis* ERD14, an SK_2 DHN, and celery VCaB45, a vacuolar DHN, bind calcium in a phosphorylation dependent manner (Heyen et al. 2002; Alsheikh et al. 2003). In addition, a KS DHN has been shown to transport iron in the phloem of castor bean (Kruger et al. 2002). A question arising from these findings is whether each DHN structural type may have a specific function.

We have sought to find all of the *Dhn* genes in the barley genome (Choi et al. 1999, 2000; Choi and Close 2000). Our previous studies identified 12 barley *Dhn* genes. In ongoing efforts to complete the collection of barley *Dhn* genes, here we describe another, *Dhn13*.

Materials and methods

Plant material

Morex barley (*Hordeum vulgare* L. cv. Morex) seeds were obtained from Dr. Patrick Hayes (Oregon State University, Corvallis, Ore., USA), and then increased in a greenhouse and the field at the University of California (Riverside, Calif., USA). For expression studies, plants were raised in a growth chamber (Model GC-15, EGC Chegrin Falls, Ohio, USA), at 23°C (day) and 20°C (night) temperature, 12-h photoperiod with 148 μmol $m^{-2}s^{-1}$ average photosynthetically active radiation and 70% relative humidity. Eight-day-old seedlings were used in each treatment. For drought stress, water was withheld and samples taken at specific values of soil water content. For low-temperature studies, chilling stress first was imposed and cold acclimation stimulated by dropping the chamber temperature to 4°C for 5 days, followed by a freeze/thaw treatment by cycling the temperature between −10°C (night) and 2°C (day) for 8 days. For ABA treatments, ABA was sprayed onto leaves as a 100-μM solution with 0.05% (v/v) Tween 20. Immature embryos were harvested from developing seeds 20 days after pollination. Germinating seed samples were obtained from seeds imbibed for 24 h on moist paper in Petri dishes. The Oregon Wolfe Barley mapping population that contains 94 F_1-derived doubled-haploid lines (http://barleyworld.org/oregonwolfebarleys/concept.php; Costa et al. 2001) was obtained from Dr. Patrick Hayes (Oregon State University) and propagated at the University of California. Seeds of wheat (*Triticum aestivum*) cv. Chinese Spring, barley cv. Betzes, and six wheat–barley addition lines from these two parents (Islam et al. 1981) were provided by Dr. Adam Lukaszewski (University of California). Plants were grown in a greenhouse, and leaf tissues were cut off, rapidly frozen in liquid nitrogen, and stored at −80°C until use.

Bacterial artificial chromosome library screening

A bacterial artificial chromosome (BAC) library covering 6.3 times the Morex barley genome (Yu et al. 2000) was screened with mixed probes composed of gene fragments of ten known barley *Dhn* genes, following a hybridization procedure previously described (Choi and Close 2000). Forward 5′-CATGGCCGGCATCGTCCACAAGATC-3′ and reverse 5′-GATCTCAGTCGCTGTCGCTGCTG-3′ PCR primers were used to identify BAC clones containing *Dhn13*.

DNA sequencing and analysis

BAC plasmid DNA of clone 258E14 was isolated using a QIAGEN large-construction DNA purification kit (QIAGEN, Valencia, Calif., USA) and digested with *Bam*HI. Digestion fragments were separated in a 0.7% agarose gel, and the 8-kb fragment containing the DHN gene was purified using QIAquick gel extraction kit (QIAGEN) and cloned into pTZ19R, following standard procedures (Sambrook and Russell 2001). The cloned 8-kb fragment was partially sequenced, starting with primers designed from a fragment amplified with maize PCR primers, and then walking out of known sequence in both directions, using additional primers. Sequencing was done at the University of California, Genomics Core Facility, using the dideoxy chain-termination method and an ABI3100 sequencer (Applied Biosystems, Foster City, Calif., USA). Chromatogram trace analysis and sequence assembly was done using the PHRED–PHRAP–CONSED suite (Gordon et al. 1998). GenBank accession AY681974 contains the sequence. Analysis of promoter sequences was performed on 1,000 bp upstream from the translation start codon, using the software Signal Scan (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) and a plant *cis*-acting regulatory DNA elements database (Higo et al. 1999).

Chromosome localization and genetic linkage mapping

To determine which chromosome carries the *Dhn13* gene, DNA from Chinese Spring wheat, Betzes barley, and the six wheat–barley addition lines from these two parents was PCR-amplified using the *Dhn13*-specific primers 5′-CATCATCCACAAGATCGAGGAG-3′ and 5′-CCGTCCTTCTTGTCCTTCTTCT-3′. Reaction conditions were 95°C for 15 min, followed by 30 amplification cycles, with a denaturation step at 95°C for 30 s and annealing first for 30 s at 65°C for 5 cycles, then at 60°C for 5 cycles, then at 55°C for the remaining 20 cycles. In each cycle, the extension step was at 72°C for 30 s. The final step was 72°C for 10 min. Amplified DNA was electrophoresed in a 1.2% agarose gel. Forward 5′-CGAGCAAGTGGGTGAAGAG-3′ and reverse 5′-GGCAGCGATTATTGGGGG-3′ primers

were used to amplify a 5′UTR polymorphic fragment in the genomic DNA of the Oregon Wolfe Barley mapping population. PCR conditions were 40 cycles with an annealing temperature of 55°C for 30 s, extension at 72°C for 60 s, and denaturation at 95°C for 15 s. The genotype of each individual was recorded and used in Map Manager QTX software (Manly et al. 2001) to obtain gene recombination frequencies and the *Dhn13* map location. The Kosambi (1944) mapping function was used. The reference data set was available at http://barleyworld.org/oregonwolfebarleys/maps.php.

Expression analysis by semi-quantitative reverse transcriptase-PCR

Total RNA was isolated from frozen samples (see ''Plant materials'' in the ''Materials and methods'' section), using TRIzol Reagent (Chomczynski and Sacchi 1987). Further purification was done using an RNeasy spin column (QIAGEN, Chatsworth, Calif., USA) with on-column DNase treatment, following the manufacturer's instructions. The first strand of cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems) and random hexamer primers. The resulting cDNA was used for PCR amplification, using 5′-CGGAGAGAAGTAGGGACAG-3′ as forward primer and 5′-GAGGAAGGAGAAGGGGAAG-3′ as reverse primer. PCR conditions were similar to PCR reactions used for mapping (above), except that the annealing temperature was 60°C.

Expression analysis by microarray

Expression data collected using the Affymetrix Barley1 GeneChip from cold, drought, and control experiments were analyzed using GeneSpring (Silicon Genetics, Redwood City, Calif., USA). For each chip, all data were normalized to the 50th percentile, and values less than 0.34 were set to 0.34. Each gene value was normalized using the median of its measurements in all samples. A cross-gene error model was used to estimate sample-to-sample variation. *Dhn13* expression levels were studied using the probe set ''contig4809_at.'' Data came from triplicated experiments.

Results

A BAC clone contig contains a gene coding for a small KS-type DHN

The Morex barley BAC library was screened using *Dhn* probes (see ''Materials and methods''), resulting in 107 *Dhn*-positive BAC clones. Seventy-five yielded PCR amplification products, using primers from previously described *Dhn* genes. To identify clones containing novel *Dhn* genes, DNA from the remaining positive BAC clones was used as template in PCR reactions with primer pairs designed from *Dhn*-like sequences not previously described in barley. Primers designed to amplify a maize KS DHN (Campbell 2000) resulted in amplification of a barley fragment with similar size to the maize sequence from BAC clones 258E14, 268O8, and 651P1. Restriction digestion and Southern blotting, as well as PCR amplification from subclones, showed that a 8-kb *Bam*H1 fragment of clone 258E14 contained a novel DHN gene. This fragment was further subcloned and partially sequenced using primers targeted to the coding region as starting points. The sequenced DNA segment is 2,174 bp and contains an intronless open reading frame (ORF) encoding a deduced polypeptide of 107 amino acids (12,050 Da). This

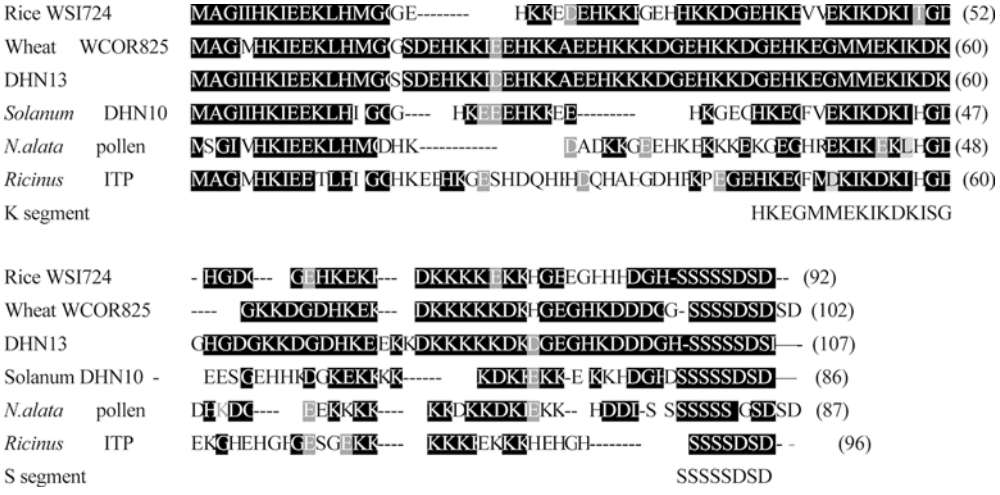

Fig. 1 Comparison of the deduced amino acid sequence of barley DHN13 to similar sequences found in an NCBI blastp search: rice WSI724, a protein induced by water stress; wheat cold-acclimation protein WCOR825 (full sequence from HarvEST:Wheat http://harvest.ucr.edu); *Solanum* DHN10; pollen protein from *Nicotiana alata*; and *Ricinus communis* iron transport protein (*ITP*) 2. Amino acid residues identical to DHN13 are shown in *black boxes*, similar ones in *gray boxes*. The DHN motif K and S segments are shown in the *bottom line*

deduced protein is named DHN13 (Fig. 1). The DHN13 protein is composed of only 11 amino acids, three of which (K, D, and E) account for 57% of the total, while G, H, and M account for 27%. Due to this bias, the overall composition is very hydrophilic. DHN13 contains one K segment (HKEGMMEKIKDKISG) at position 46–61 and one S segment at the carboxy terminus (SSSSSDSD). A blastp search of the GenBank nr database, using the DHN13 deduced amino acid sequence, revealed similarity of DHN13 to several other small proteins containing the KS domain arrangement. DHN13 has highest similarity to the wheat cold-induced protein WCOR825 (T06808) and rice water-stress-induced protein WSI724 (BAA05539). Other KS-type DHNs have been identified as *Solanum* DHN10 (AF542504), *Nicotiana alata* pollen protein (AAN78183), and iron transport protein 2 from *Ricinus communis* (CAC84735).

The *Dhn13* gene is located on chromosome 4H

Dhn13-specific primers were used to determine the chromosome location by PCR amplification of genomic DNA from wheat–barley addition lines (see "Materials and methods"). The results indicated that chromosome 4H carries the *Dhn13* gene (Fig. 2a). To map the *Dhn13* gene, three primer pairs were designed to amplify fragments covering the 3′UTR, ORF, and 5′UTR regions and to search for size polymorphisms among the parental lines of the Morex × Dicktoo, Morex × Steptoe, and Oregon Wolfe Barley mapping populations. A size polymorphism was detected in the 5′UTR between the parents of the Oregon Wolfe Barley population and used to map the *Dhn13* gene. Figure 3 shows the location of the *Dhn13* gene on the short arm of chromosome 4H. It falls 6.6 cM from MWG644 and 19.23 cM from E36M62-78. *Dhn6* is also in this chromosome at another location (Fig. 2b).

Dhn13 is expressed constitutively in several tissues but has higher expression in cold-acclimated plants and anthers

To initially gauge *Dhn13* expression, reverse transcriptase (RT)-PCR analyses were performed on RNA samples isolated from seedling crown tissue after different stress treatments and under normal conditions. Treatments included ABA application, drought, chilling, and freeze/thaw cycles. Other tissues included seedling root and shoot, germinating seeds and 20 days-after-planting embryos from control plants. *Dhn4* cDNA was amplified from the same mRNA samples for comparison (Fig. 3). *Dhn13* transcripts were detected in all samples, while *Dhn4* transcripts were present only in seeds and in ABA- or drought-treated plants, as previously observed. This result indicates that *Dhn13* has constitutive expression in different barley tissues.

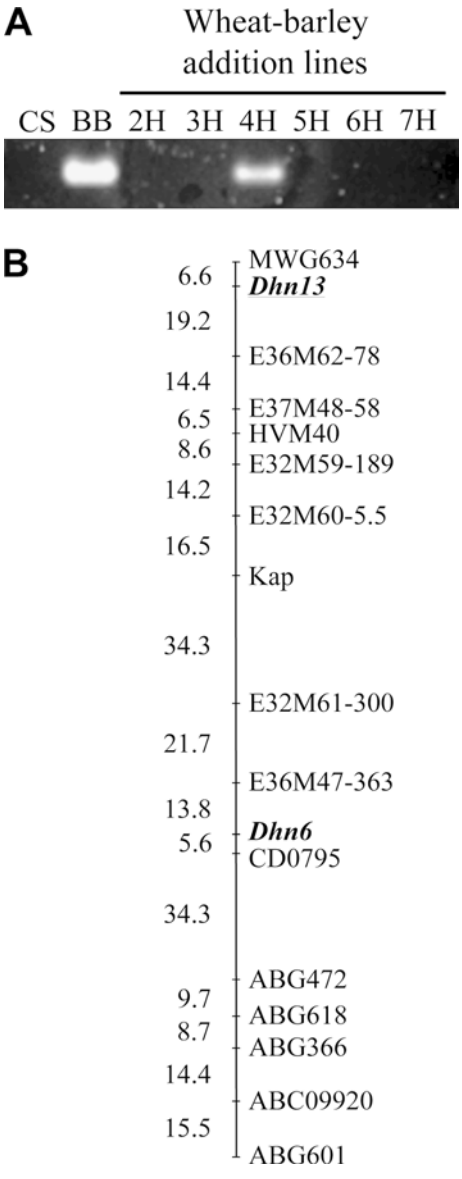

Fig. 2 Location of the *Dhn13* gene. **a** Chromosome assignment. PCR reaction products from genomic DNA of Chinese Spring wheat (*CS*), Betzes barley (*BB*) and six wheat–barley chromosome addition lines (*2H*, *3H*, *4H*, *5H*, *6H*, and *7H*), using *Dhn13* gene-specific primers. **b** Genetic mapping. Linkage map of chromosome 4H with the short arm at the top from the Oregon Wolfe Barley mapping population, based on Costa et al. (2001). Map location of *Dhn13* is shown. *Numbers on the left of the chromosome* indicate the distance in centiMorgans; *symbols on the right* represent genetic markers

To know if there are quantitative differences in *Dhn13* expression, we took advantage of data gathered using the Affymetrix "Barley1" GeneChip (Close et al. 2004) from various stress experiments (E. Rodríguez and J. Svensson, unpublished) and from studies of normally growing plants (A. Druka, unpublished). The probe sets named "contig4809_at" and "contig4808_s_at" on the Barley1 chip represent the *Dhn13* gene, but only the signal from contig4809_at is discussed here (Fig. 5),

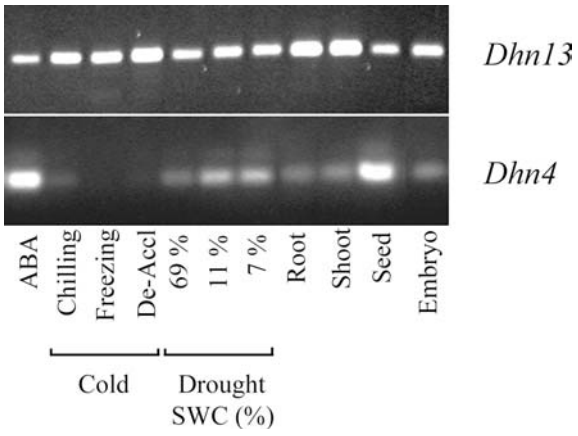

Fig. 3 Reverse transcriptase-PCR amplification of *Dhn13* product. Amplification products from abscisic acid-treated seedlings (*ABA*), chilling treated (*cold-acclimated*), chilling treated and exposed to sub-zero cycles (*freeze/thaw*), de-acclimated at 20°C, or drought-stressed to the indicated soil water content [*SWC/%)*]. Root and shoot samples were from seedlings grown in normal conditions; the seed sample was from seeds imbibed for 24 h; the embryo sample was excised from developing seeds 20 days after planting. A *Dhn4* fragment was amplified from the same RNA samples for comparison

since contig4809_at is a more-specific probe set than is contig4808_s_at (see Harvest:Barley, http://harvest.ucr.edu). Data analysis produced a "present" call for contig4809_at in every hybridization, consistent with *Dhn13* constitutive expression found by RT-PCR. In addition, microarray data showed a strong transcript increase in several samples; for example, chilling-stress samples had a 2.8-fold average *Dhn13* transcript increase over control samples, and plants subjected to sub-zero temperatures had an 8.5-fold increase. There was also somewhat elevated transcript accumulation in plants under drought stress (Fig. 4a). Comparison among tissues showed the highest differences; for example, *Dhn13* transcripts were 9.7 times higher in anthers and five times higher in germinating embryos than in leaf (Fig. 4b).

Dhn13 promoter

Promoter analysis of the 5′-upstream region of *Dhn13* (Fig. 5) identified two putative abscisic acid responsive elements ABRE (Seki et al. 2002) at −836 and −871 bp from the start codon; three putative MYC elements (Abe et al. 2003) at −300, −809, and −873; three putative MYB elements at −284, −400, and −477; three putative core drought responsive elements (DRE) (Xue 2002) at −354, −521, and −851; and three putative POLLEN1LELAT52 elements (Bate and Twell 1998) at positions −155, −452, and −747. Moreover, there are apparent coupling elements (i.e., CE1) that, with ABREs, form an ABRC response complex (Shen and Ho 1995). The presence of these *cis* elements is consistent with the expression data.

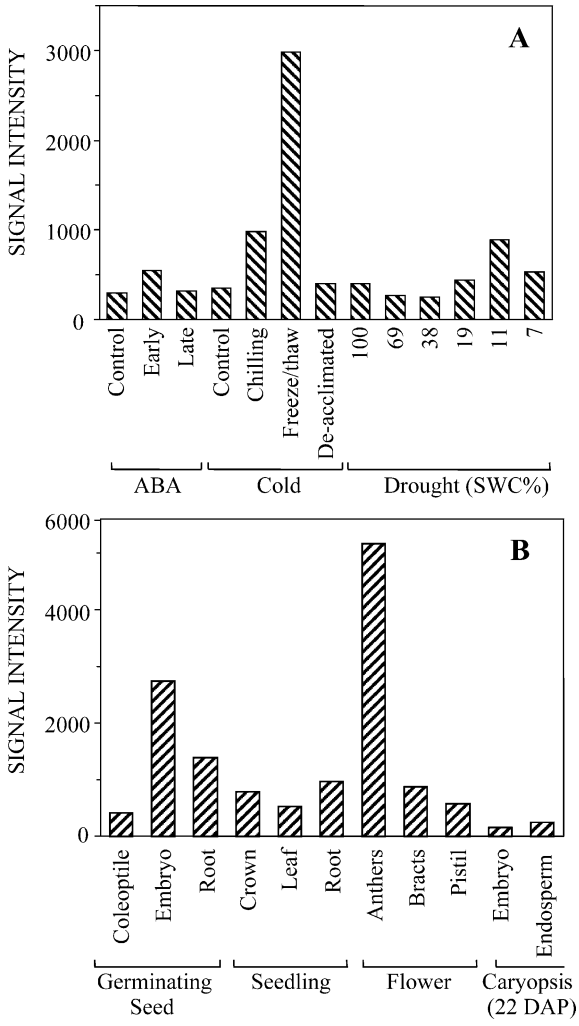

Fig. 4 *Dhn13* transcript detection, using a microarray. Microarray data from stress experiments (**a**) and different tissues and development stages (A. Druka, unpublished, **b**). Data were analyzed using GeneSpring, normalized to the every-chip median value. Signal intensity is the normalized probe-set fluorescence in arbitrary units. *Dhn13* values are shown, as stated in "Materials and methods"

Discussion

The barley *Dhn13* gene encodes a small DHN composed of 107 amino acids with one K segment, a C-terminal S segment, and no Y segment. The S segment in the DHN13 protein is unique among barley DHNs, both because of its C-terminal location and the lack of the consensus sequence $LHRSGS_{4\text{-}10}(E/D)_3$. DHN13 does not have much similarity to other barley DHNs outside of the K segment, but it has the same features of the KS-type subgroup found in other plants, most of which are water deficit or cold stress responsive (Rorat et al. 2004). The gene for wheat cold-induced WCOR825 protein and the rice gene coding the WSI724 protein seem to be the orthologues of barley *Dhn13*.

The only specific physiological role of the KS-type DHNs identified so far is the iron-binding activity of the

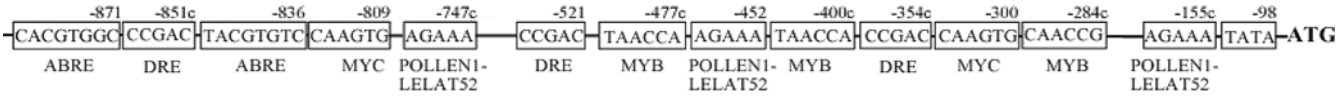

Fig. 5 Putative *cis*-elements in the *Dhn13* promoter region. One thousand base pairs of upstream sequence were analyzed for putative regulatory elements as described in "Materials and methods." Consensus sequences are PyACGTGT/GC for ABRE, CCGAC for DRE, CANNTG for MYC, PyAACNPu for MYB, AGAAA for Pollen1-lelat52, and TATAAA for TATA box. *Numbers on the boxes* indicate nucleotides relative to the site of the start codon

KS-type DHN of castor bean (Kruger et al. 2002). Ferric ions moving in the sieve tubes of castor bean hypocotyls are complexed to a KS-type DHN that has highly specific Fe(III) binding activity. Various other DHNs also bind metals, inferred by the fact that they can be purified using immobilized metal ion affinity chromatography (Svensson et al. 2000). In addition, *Arabidopsis* ERD14, an SK_2 DHN (Alsheikh et al. 2003), and celery VcaB45 (Heyen et al. 2002) have calcium-binding capacity. In contrast with the castor bean DHN, ERD14 has higher affinity for divalent than trivalent cations. Since ERD14 is an SK_2 DHN, this raises the possibility that cation-binding specificity may be related to the specific DHN type.

The iron-binding capacity of KS-type DHNs may be directly related to a protective role, since free iron increases oxidative damage by means of a Fenton reaction, and oxidative stress is a common characteristic of water deficit or cold stress. Possibly, iron binding by constitutively expressed KS DHNs contributes to phloem iron transport under normal growth conditions, but is used as an antioxidative iron-sequestering agent under dehydration or cold conditions, when harmful peroxides are likely to be produced near susceptible membranes. Interestingly, transformed tobacco plants over-expressing a KS-type DHN from *Citrus unshiu* (CuCOR19) had less lipid peroxidation when exposed to chilling, and purified CuCOR19 had an antioxidant effect in soybean liposomes (Hara et al. 2003).

Dhn13 has constitutive expression, but its transcripts accumulate to elevated levels during cold acclimation and after exposure to freezing temperatures, and the highest transcript level is in anthers. This expression pattern seems to be characteristic of KS-type DHNs in different species. A KS DHN has been described recently in potato plants (DHN10). As with barley *Dhn13* transcripts, potato DHN10 protein is present in several tissues but at higher levels in flowers and in response to cold stress (Rorat et al. 2004). The high level of *Dhn13* transcripts in barley anthers also has a relation to pollen expression of KS-type *Dhn* genes from other species. A pollen-expressed protein of *Nicotiana alata* has the KS-type motif (Takebayashi et al. 2003). In addition, publicly available microarray data for the *Arabidopsis* transcriptome in the Nottingham Arabidopsis Stock Centre (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) includes an analysis of microgametogenesis, where the transcript of *At1g53310*, encoding a KS-type DHN, shows high expression in tricellular and mature pollen. Also, a search of the NCBI dbEST database to obtain EST sequences similar to *Dhn13* yielded several ESTs from anther or pollen cDNA libraries of various plants (data not shown).

Promoter analysis of *Dhn13* is consistent with the expression data. Drought, cold, and pollen-related promoter elements parallel the observed expression. As with other barley DHNs, the *Dhn13* upstream sequence contains DRE, ABRC, MYC, and MYB elements that may be related to stress responsiveness. The *Dhn13* promoter region also has three POLLEN1LELAT5 elements, described as necessary for pollen-specific expression (Bate and Twell 1998).

The location of *Dhn13* in barley chromosome 4, near MWG644, does not coincide with any barley trait that has an obvious relationship to *Dhn13* expression data. However, there are interesting mapping correlations near the *Dhn13* rice orthologue, 8360.t04028 (TIGR_TU ID). This rice gene is in chromosome 3 in a region syntenous with barley chromosome 4 (Chen et al. 2003); it encodes the WSI724 protein and is located in BAC clone AC138004. This clone maps close to the positions of *hst7*, *v1*, and *v7* mutants (http://www.gramene.org). The *hst* mutant is related to rice hybrid sterility, and both *virescents* mutants (*v1*, *v7*) show a phenotype of almost-white seedlings. Aberrant DHN13 expression during anther development could possibly compromise pollen viability, explaining the *hst7* phenotype. A chlorotic phenotype is often related to iron deficiency, so it also seems plausible that a DHN13 defect might result in the *v1* or *v7* phenotypes. However, these speculations remain to be tested.

In conclusion, the barley *Dhn13* gene encodes a KS-type DHN with a considerable basal level of expression under normal conditions and elevated expression in anthers and cold-stressed green tissue. DHN13 merits further attention to define its role in cold acclimation, pollen physiology, and iron binding.

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