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A newly identified barley gene, *Dhn12*, encoding a YSK_2 DHN, is located on chromosome 6H and has embryo-specific expression

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Abstract Dehydrins are water-soluble lipid-associating proteins that accumulate during low-temperature or water-deficit conditions, and are thought to play a role in freezing- and drought-tolerance in plants. *Dhn* genes exist as multi-gene families in plants. Previously, we screened lambda genomic libraries of two barley cultivars in an effort to isolate all of the barley *Dhn* genes. We identified 11 unique *Dhn* genes and estimated a total of 13 *Dhn* genes in the barley genome. To extend the collection, we used an alternative source of clones, a 1.5×Morex barley BAC library. In this library, we found nine *Dhn* genes that we described previously and one new *Dhn* gene, *Dhn12*. The *Dhn12* gene encodes an acidic YSK_2 dehydrin. The *Dhn12* gene is located on chromosome 6H, and shows a different expression pattern from all other *Dhn* genes identified previously. RT-PCR results show that *Dhn12* expression is embryo-specific. *Dhn12* is not expressed in seedling shoots under any of the conditions tested, including non-stressed as well as dehydrated, or cold-, ABA- or NaCl-treated seedlings.

Key words Bacterial artificial chromosome (BAC) library · Dehydrin · DHN · Embryo-specific expression · *Hordeum vulgare*

Introduction

Dehydrins (DHNs) are an immunologically distinct protein family (a.k.a. LEA D-11) and typically accumulate during the maturation phase of seed development or in response to low temperature, drought, salinity or ABA application (Close 1996, 1997; Campbell and Close 1997). Dehydrins typically have an abundance of charged and polar amino-acids, are usually Gly-rich and free of Cys and Trp, and contain consensus amino acid sequence domains termed Y-, S- and K-segments. The K-segment is the most-highly conserved with a consensus EKKGIMDKIKEKLPG. The S-segment is a phosphorylatable tract of Ser residues. The Y-segment (T/VDEYGNP), when present, occurs near the N-terminus. Numerous permutations of these domains are reflected in a range of DHNs (e.g., Y_nSK_n, SK_n, K_n, Y_nK_n and K_nS), with lengths ranging from 82 to 575 amino acids (reviewed in Campbell and Close 1997). There is DNA sequence and immunological evidence of dehydrins in a wide range of photosynthetic organisms including higher and lower plants (reviewed in Close 1997).

Immunolocalization studies have shown that dehydrins can be present in the nucleus or cytoplasm (reviewed in Close 1997). A recent study of wheat acidic SK_3-type dehydrins (WCOR410) showed that these DHNs are located in the vicinity of the plasma membrane (Danyluk et al. 1998). It has been suggested by several investigators that DHNs act as stabilizers of membranes or proteins under freezing or water-stress conditions. Genetic studies have also been consistent with *Dhn* genes being associated with environmental stress tolerance (reviewed in Campbell and Close 1997). For example, Pan et al (1994) established that the barley *Dhn1* and *Dhn2* loci on chromosome 5H are within a major QTL interval (including *Sgh2*) controlling freezing tolerance in the Dicktoo and Morex barley double-haploid mapping population.

We have attempted to find all of the *Dhn* genes in the barley genome (Choi et al. 1999). Our previous studies identified 11 *Dhn* genes and provided an estimate of 13 in the genome. In our efforts to complete the collection of barley *Dhn* genes, a 1.5× BAC library from Morex barley was used as an alternative source of cloned genes. In this report, we present the discovery of another barley *Dhn* gene, *Dhn12*, in this library. *Dhn12* encodes an atypical YSK_2 DHN and shows only seed-specific expression.

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Materials and methods

Plant materials

Morex barley (*Hordeum vulgare* L. cv Morex) seeds were obtained from Dr. Patrick Hayes (Oregon State University, Corvallis, Ore.). Seeds were surface-sterilized and germinated on moist filter paper in sterile Petri dishes as described (Choi et al. 1999). Cold, dehydration and ABA treatment were performed as described (Choi et al. 1999). For NaCl treatment, the roots of intact barley seedlings were immersed in 0.5 M NaCl solution for 1 day. Immature embryos were harvested from maturing Morex barley seeds 40 days post-anthesis. Seeds of Chinese Spring wheat (*Triticum aestivum* cv Chinese Spring), Betzes barley (*H. 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.). Wheat and barley plants were grown in a greenhouse. Leaves and shoots were cut off, frozen in liquid nitrogen, and stored at –80°C.

BAC library screening

A 1.5× Morex barley BAC library filter set was obtained from Dr. Andris Kleinhofs (Washington State University, Pullman, Wash.). The filters were rinsed twice with 2× SSC and pre-hybridized in hybridization buffer containing 1% BSA-fraction V, 1 mM EDTA (pH 8.0), 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS, 10 µg/ml of sheared and denatured salmon sperm DNA at 65°C for 4 h (Woo et al. 1994). The solution was exchanged with fresh hybridization buffer and pre-hybridization was carried out for an additional 8 h. DNA fragments from ten different *Dhn* genes (omitting *Dhn3*) of the 11 known *Dhn* genes of Morex barley (Choi and Close, unpublished data) were mixed and used as a probe. This mixture was ^{32}P-labelled. Hybridization was carried out at 65°C for 32 h. After hybridization the filters were washed with 0.5× SSC, 0.1% SDS at 65°C for 40 min. The filters were autoradiographed using Fuji film with a single intensifying screen at –20°C for 2 days.

BAC DNA isolation and analysis

DNAs from positive BAC clones were isolated by an alkaline-lysis procedure (Sambrook et al. 1989) from 5-ml overnight cultures containing 12.5 µmg/ml of chloramphenicol. BAC DNAs were digested with *Bam*HI and *Sal*I, and the fragments separated in a 0.7% agarose gel. The total size of each BAC clone was calculated as the sum of the size of all DNA fragments produced by *Bam*HI digestion. Enzyme-digested DNAs were blotted onto a nylon membrane and probed with ^{32}P-labelled *Dhn1*-, *Dhn4*- and *Dhn9*-DNA probes according to standard procedures (Sambrook et al. 1989). The identity of the *Dhn* gene carried on each positive BAC DNA was verified by PCR using the 11 *Dhn* gene-specific primer sets described previously (Choi et al. 1999).

DNA sequencing and analysis

BAC-clone DNA fragments that hybridized with *Dhn* 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 programs (Hitachi Software Engineering Ltd, San Bruno, Calif.) and compared with sequences in DNA databases using the BLAST server. Amino-acid sequence alignments of deduced dehydrin polypeptides were performed using the CLUSTAL V program. From the *Dhn12* gene sequence, we designed a *Dhn12* gene-specific oligonucleotide primer set composed of 5′-GATGATCC AGCAGCAAACTCA-3′ (5′-primer) and 5′-TCAGCTCGAGCTT-GACGACT-3′ (3′-primer). Oligonucleotides were synthesized at Genosis (The Woodlands, Tex.). These primers were used for both genomic DNA amplification and RT-PCR.

Genomic DNA amplification

Genomic DNAs from Chinese Spring wheat, Betzes barley, and the six wheat-barley addition lines from these two parents were purified using DNAzol according to instructions provided by the manufacturer (Life Technologies, Gaithersburg, Md.). Genomic DNA amplifications were performed using a GeneAmp PCR System 9600 (Perkin Elmer, Foster City, Calif.) in a 100-µl reaction containing 2.5 units of *Taq* DNA polymerase (Qiagen, Hilden, Germany), 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl_2, 1.5 mM $MgCl_2$), 1× Q-solution, 200 µM of each dNTP, 0.3 µM of primer, and 100 ng of genomic DNA. PCR reactions were initiated at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, and terminated at 72°C for 10 min. Amplified DNAs were electrophoresed in a 1.2% agarose gel.

RT-PCR

Total RNA was prepared from seedlings grown at room temperature and several stress conditions (see Plant materials) using the small-scale hot-phenol procedure described by Verwoerd et al (1989). RNA was treated with RNase-free DNase I (Life Technologies, Gaithersburg, Md.) at room temperature for 15 min in buffer containing 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$ and 50 mM KCl. DNase I was inactivated by adding EDTA (final concentration 2.5 mM) and heating to 65°C for 10 min. To synthesize the first strand cDNA, 0.25 µg of total RNA was used in a 20-µl reaction containing 50 units/µl of MuLV reverse transcriptase (Life Technologies, Gaithersburg, Md.) in a buffer of 10 mM HCl pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM each dNTP, 1 unit/µl of RNase inhibitor and 0.75 µM of *Dhn12*-specific 3′-end primer. Reactions were performed at 42°C for 30 min and then heated at 99 C for 5 min. The resulting first-strand cDNAs were amplified using *Taq* DNA Polymerase (Qiagen, Hilden, Germany) with the *Dhn12*-specific primer set employing the same conditions as for genomic DNA amplification. RT-PCR products were electrophoresed in a 1.2% agarose gel and stained with ethidium bromide.

Results

Screening of a barley BAC library

A total of 31 BAC clones putatively hybridizing with the probe were further examined. Their DNAs were digested with *Bam*HI and *Sal*I and analyzed by DNA hybridization using *Dhn1*, *Dhn4* and *Dhn9* gene probes. The DNA hybridization results showed that 15 of these BAC clones contained *Dhn* DNA (Table 1). Further analysis showed that all 15 BAC clones hybridized to the single probe, *Dhn1*, at low stringency. Presumably this cross hybridization is caused by all of the *Dhn* genes having highly conserved K-segment domains. DNA fragments of the remaining 16 did not hybridize with *Dhn1* (data not shown).

To determine which BAC clone contained which *Dhn* gene, each of the 15 positive BAC clones was examined by PCR using *Dhn* gene-specific oligonucleotides (Choi et al. 1999). Restriction-digestion patterns and PCR-amplification results showed that the 15 BAC clones represent eight groups of contiguous overlapping clones (contigs) encoding ten *Dhn* genes. The results are summarized in Table 1. Three BAC clones, 33I7, 34D13 and 112L24, overlap each other and seemed to carry a previously unidentified *Dhn* gene. We chose BAC clone 34D13 for

Table 1 Summary of BAC clones carrying the *Dhn* gene[a]

Contig	BAC clone address	*Dhn* gene	Insert DNA size (kb)
1	46-D-19	*Dhn1*	50
	17-K-24		34
2	113-M-7	*Dhn3*, *Dhn4*, *Dhn7*	117
	113-O-9		
3	52-O-2	*Dhn5*	65
4	152-O-10	*Dhn6*	110
	162-M-6		107
5	86-H-8	*Dhn9*	142
	161-L-21		86
6	22-M-19	*Dhn10*	52
	115-I-12		101
7	73-O-22	*Dhn11*	119
8	33-I-7	*Dhn12*	142
	34-D-13		87
	112-L-24		137

[a] No BAC clones carrying *Dhn2* or *Dhn8* were identified in the 1.5× library

further analysis because this clone contained the smallest insert DNA fragment (approximately 87 kb).

DNA sequence analysis

From BAC clone 34D13 we subcloned a 7-kb *Sal*I fragment and a 5-kb *Sph*I fragment, each of which hybridized to a *Dhn* probe. Figure 1 A shows the restriction map. A 2.9-kb *Xba*I fragment of the 5-kb *Sph*I fragment was subcloned and sequenced. Comparison of the DNA sequences with other genomic sequences of *Dhn* genes identified a single intron, from +184 to +274 (numbering from ATG), defined by the boundary consensus sequences (GT/AG) that flank the introns of plant genes. This fragment encodes a polypeptide consisting of 141 amino acids (14.2 kDa, pI, 6.72). We refer to this polypeptide as DHN12 (Morex 141). DHN12 contains a high percentage of Gly (23.4%), and Ala (11.6%). The deduced amino-acid sequence of DHN12 shows the highest similarity (79.7%) to barley DHN9, which is a YSK_2-type dehydrin (Fig. 1B) located on chromosome 5H. Inspection of the *Dhn12* nucleotide sequences upstream of the open reading frame revealed two ACGT core sequences of G-box elements recognized by a basic leucine-zipper (bZIP) transcription factor involved in seed specific-expression of storage proteins (Schmidt et al. 1992). These two positions are at –217 and –238 (upstream of ATG). No abscisic acid response element (ABRE) or drought response element (DRE), both which are present in the 5′ -flanking region of most of the *Dhn* genes, was detected. These results suggested that expression of the *Dhn12* gene may be different from the *Dhn* genes identified previously.

We designed a *Dhn12* gene-specific primer set from the DNA sequence, and used these primers to determine the chromosome location of *Dhn12* by PCR-amplification. The PCR results indicate that *Dhn12* is on chromosome 6H (Fig. 2).

RT-PCR

To determine the expression pattern of the *Dhn12* gene, we amplified *Dhn12* mRNA with *Dhn12* gene-specific primers by RT-PCR. Total RNA was isolated from barley seedling shoots following several treatments, including room temperature, dehydration, cool temperature, ABA and salt treatment, from 40 DPA embryos. For comparison, *Dhn3* mRNAs from the same RNA samples were amplified with *Dhn3* gene-specific primers. The results

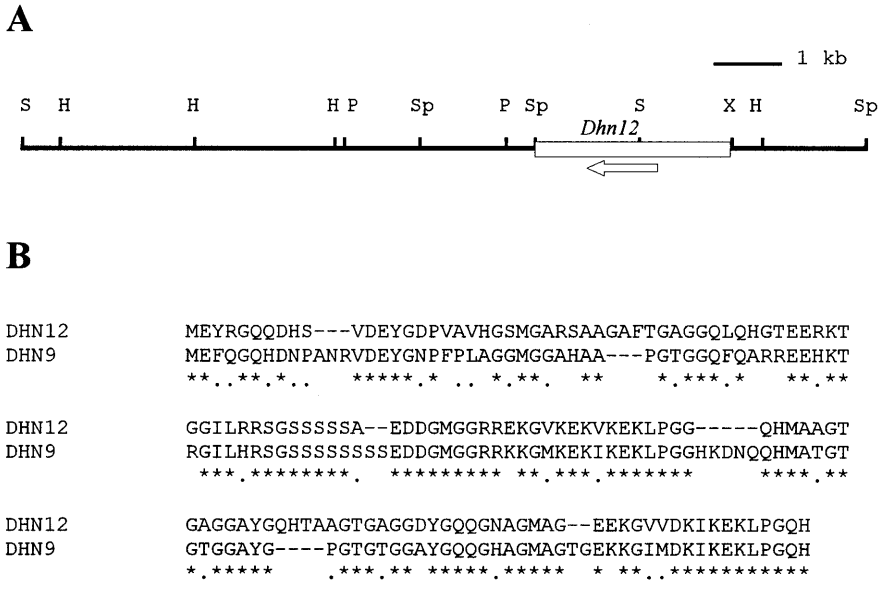

Fig. 1 **A** Restriction map of a fragment of BAC clone 34D13 carrying *Dhn12*. The *open box* indicates a 2.9 kb *Sph*I/*Xba*I fragment which was subcloned and sequenced. The *arrow* shows the orientation of *Dhn12*. Restriction enzyme sites are shown as *H* (*Hin*dIII), *P* (*Pst*I), *S* (*Sal*I), *Sp* (*Sph*I) and *X* (*Xba*I). **B** Comparison of the DHN12 amino-acid sequence with barley DHN9. Amino-acid residues are designated in single letter code. *Asterisk* (*) and *period* (.) indicate identical and non-identical amino acids, respectively. Dashes indicate where a sequence has been expanded to allow optimal sequence alignment. Alignments of the deduced amino-acid sequences were performed using the CLUSTAL V program

Fig. 2 Chromosome assignment of the *Dhn12* gene. PCR reactions were performed on genomic DNA from Chinese Spring wheat (*CS*), Betzes barley (*BB*) and six wheat-barley chromosome addition lines (*2H, 3H, 4H, 5H, 6H* and *7H*) using *Dhn12* gene-specific primers. Amplified DNAs were electrophoresed in a 1.2% agarose gel

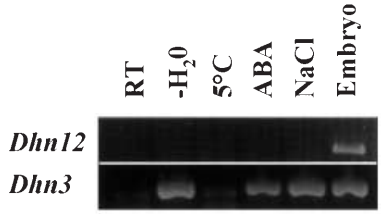

Fig. 3 Five-day old barley seedlings grown at room temperature (RT) were either dehydrated at 90% relative humidity for 1 day ($-H_2O$), cold acclimated at 5°C for 2 days (*5°C*), or treated with 20 µmM ABA for 2 days (*ABA*) or 0.5 M NaCl for 1 day (*NaCl*). Embryos were collected from developing seeds 40 days post-anthesis (*Embryo*). Total RNA was treated with RNase-free DNase I, reverse transcribed with the *Dhn12* gene-specific 3′-primer, and then amplified with the *Dhn12* gene-specific 5′-primer. RT-PCR products were electrophoresed in a 1.2% agarose gel

as expected show that the *Dhn3* gene was induced by drought stress, ABA, and salt treatment, as well as in developing seeds, but that transcripts of the *Dhn12* gene were detected only in developing seeds (Fig. 3). These results demonstrate that expression of the *Dhn12* gene is embryo-specific and is not induced in seedlings by dehydration or cold-, salt- and ABA-treatment.

Discussion

DHN12 is an atypical YSK_2 dehydrin, composed of 141 amino-acid residues with a slightly acidic isoelectric point (pI, 6.72). DHN12 shows the highest amino-acid sequence similarity to DHN9, a YSK_2-type dehydrin located on chromosome 5H (Choi et al. 1999). *Dhn12* differs from all other *Dhn* genes in gene-expression pattern. *Dhn12* is induced in developing seeds but not in seedlings by dehydration, salt-, cold- or ABA-treatment. Previous studies show that all other barley *Dhn* genes that encode YSK_2 dehydrins are up-regulated by both dehydration and ABA treatment as well as during the late stage of embryo development, but not by cold treatment, and all have a basic isoelectric point (Choi et al. 1999). In contrast, *Dhn5* and *Dhn8*, which are up-regulated by cold treatment, encode acidic DHNs with predicted pIs of 6.68 and 5.10, respectively, and are not expressed in developing seeds (Choi and Close, unpublished data). Analysis of the 5′ flanking region of each *Dhn* gene shows that there are positive correlations between the presence of putative *cis*-acting regulatory elements, such as ABRE and DRE, and observed expression patterns under both stress and ABA treatment (Choi et al. 1999). The *Dhn12* promoter does not containing ABRE or DRE elements. The absence of these *cis*-acting regulatory elements is consistent with *Dhn12* not being expressed under dehydration, cold, salt- and ABA-treatment conditions. Further inspection of the *Dhn12* promoter identified ACGT core sequences of G-box elements involved in the seed-specific expression of storage proteins (Schmidt et al. 1992, Williams et al. 1992). One well-studied regulatory protein which binds to the ACGT core sequence and induces seed-specific expression is *Opaque-2*, which regulates endosperm-specific expression of the zein storage protein gene in maize (Schmidt et al. 1992). The ACGT core sequences are also found in other *Lea* genes expressed in seeds, as well as in barley *Dhn* genes identified previously (Choi et al. 1999, Prieto-Dapena et al. 1999). The *Dhn* genes are expressed preferentially in the embryo, and not in the endosperm during seed development (Asghar et al. 1994). It has not been tested whether ACGT core sequences are related to embryo-specific expression. Other *cis*-acting elements for seed-specific expression, such as the RY repeat (CATGCATG) in a legumin gene (Baumlein et al. 1992), are not present in the 5′-flanking region (within 1 kb) of *Dhn12*. The detailed regulatory mechanism of the embryo-specific expression of the *Dhn12* gene and the specific function of the encoded protein remain to be determined.

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