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Classification and expression diversification of wheat dehydrin genes

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ABSTRACT

Dehydrins (DHNs) are late embryonic abundant proteins characterized by the dehydrin domains that are involved in plant abiotic stress tolerance. In this study, fifty-four wheat DHN unigenes were identified in the expressed sequence tags database. These genes encode seven types of dehydrins (KS, SK_3, YSK_2, Y_2SK_2, K_n, Y_2SK_3, and YSK_3) and separate in 32 homologous clusters. The gene amplification differed among the dehydrin types, and members of the YSK_2- and K_n-type DHNs are more numerous in wheat than in other cereals. The relative expression of all of these DHN clusters was analyzed using an *in silico* method in seven tissue types (*i.e.* normal growing shoots, roots, and reproductive tissues; developing and germinating seeds; drought- and cold-stressed shoots) as well as semi-quantitative reverse transcription polymerase chain reaction in seedling leaves and roots treated by dehydration, cold, and salt, respectively. The role of the ABA pathway in wheat DHN expression regulation was analyzed. Transcripts of certain types of DHNs accumulated specifically according to tissue type and treatment, which suggests their differentiated roles in wheat abiotic stress tolerance.

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1. Introduction

Dehydrins, also called group 2 late embryonic abundant (LEA) proteins, are among the most studied dehydration-induced water-soluble proteins. The dehydrin family is characterized by a highly conserved Lys-rich motif that consists of 15 amino acid residues (consensus EKKGIMDKIKEKLPG), referred to as the K-segment [1–3]. K-segments are predicted to form amphipathic α-helices [2,4] and are usually found in more than one copy and in combination with domains rich in Gly and a Ser stretch, the S-segment. The N terminal region of many dehydrins contains another conserved sequence (V/T) DEYGNP, the Y-segment, which shares significant homology with the nucleotide-binding site of plants and bacterial chaperones [5]. K-segments are essential for enzyme protection by their supposed function of preventing abiotic stress-imposed protein aggregation [6]. Dehydrins are subdivided into several classes according to a combination of these conserved segments [2].

Conserved expression of plant *DHNs* was revealed in certain abiotic stress response. The expression of YSK_2-type *DHNs* in barley was revealed to be up-regulated by drought stress but not cold stress, while the expressions of SK_3-, K_n-, and KS-type *DHNs* were induced by both low-temperature stress and drought stress [7,8]. Similar results were revealed in rice and wheat [9–16], Experiment GSE6901, Rice Genome Annotation Project, http://rice.plantbiology.msu.edu/cgi-bin/generate_experiment_page.pl?experiment=GSE6901]. It was noted that *DHN* expression levels were higher in the more tolerant cultivar than that in the less tolerant cultivar [17]. During long-term cold acclimation, the accumulation of dehydrin is significantly affected by Vrn1/Fr1 locus and the expression of the major vernalization gene *VRN1*, respectively [18]. The transcript abundance of wheat *DHNs* was correlated with tissue water content and acquired frost tolerance [12,18]. The role of dehydrins in plant abiotic stresses has been verified by transgenic experiments. Overexpression of wheat YSK_2-type *DHNs* in rice and *Arabidopsis* enhanced their tolerance to salt and osmotic stresses [19,20]. The overexpression of cold-induced *DHNs WCOR410* (SK_3-type) and *CuCOR19* (K_3S-type) in strawberry and tobacco plants, respectively, resulted in improved cold tolerance [21,22].

Gramineous food crops including wheat, rice, maize, sorghum, and barley provide >90% of the human food supply throughout the world. Unlike summer crops such as rice, maize, and sorghum, wheat experiences a low temperature phase in the winter during its seedling stage and usually experiences hot and dry weather in its late growth stages. As such, wheat has broader climate adaptability, especially strong resistance to low temperatures during the

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seedling stage. However, knowledge about the molecular basis for wheat abiotic stress tolerance is limited [23]. Plants usually possess a dehydrin family. For example, Wang et al. [10] identified eight *DHNs* in the rice genome by scanning pseudomolecules of the japonica genome (release 4) from TIGR (http://www.tigr.org). Thirteen *DHNs* have been identified in barley [7,8,17], while ten *DHNs* have been identified in *Arabidopsis* [24]. Although nearly ten *DHNs* have been identified in wheat [11–13,15,25,26], the composition of the gene family in this crop remains unclear. The objective of this study was to explore the composition and diversification of wheat *DHNs* and analyze their expression diversification under abiotic stress conditions. The composition of *DHNs* among the major cereals was also compared.

2. Materials and methods

2.1. Plant materials, growth conditions, and treatments

Spring wheat cv. Kehan 15, a drought tolerance variety bred in the Wheat Research Institute of Heilongjiang Academy of Agricultural Sciences, China, was used for the gene abiotic stress responsive analysis in this study. This cultivar was selected for its higher tolerance to dehydration at the seedling stage as demonstrated in our pre-experimental analysis.

Kehan 15 seedlings were grown in petri dishes at 25/18 °C (day/night) with a 15-h photoperiod for the tissue harvest. For the dehydration treatment, Kehan 15 seedlings grown under hydroponic condition for 10 days were placed on dry filter paper under 70% humidity and dim light; for the cold treatment, 10-day seedlings were transferred to 4 °C with a water supply; and for the salt treatment, 10-day seedlings were transferred to petri dishes with 250 mM NaCl. In each treatment, the leaves and roots were harvested at 24 and 48 h, respectively, after the treatment. In ABA treatment, 10-day seedlings of Chinese Spring grown in petri dishes were transferred to petri dishes with 0.5 mM ABA. Leaf and root tissues were harvested 24 h after the transfer. For all of the experiments, the corresponding tissues were collected from untreated plants as controls.

2.2. RNA extraction, first-strand cDNA synthesis, and PCR

RNA was extracted with the Trizol kit (GIBCO BRL, the United States of America/USA). First-strand cDNA was synthesized using 2 μg total RNA and M-MLV RT reverse transcriptase (Promega, USA) according to the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) for the expression analyses was conducted in 25-μL reactions containing 5 ng of the template, 5 pmol of each primer, 5 nmol of each dNTP, 37.3 nmol of $MgCl_2$, 0.5 U rTaq DNA polymerase (Takara, Japan) and 1× PCR buffer (supplied with the enzyme). The thermal cycle was 94 °C 3 min, then cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 50 s, followed by 72 °C for a 5-min extension. PCR products were resolved in 1.5% gels and visualized with ethidium bromide staining. RT-PCR amplification of the *β-tubulin* gene was used to indicate the amount of cDNA employed in the PCR reactions. The RT-PCR reactions were repeated independently at least three times to ensure reproducibility. The contig (Unigene) cluster-specific primers (Table 1) were designed with Macvector 9.0 (Accelrys, Oxford, USA) using the default parameters. Since *DHN* clusters can share high homology at the nucleotide level, some primers may be shared by different clusters, but at least one primer was cluster-specific within each primer pair to ensure cluster-specific amplification.

2.3. Expressed sequence tags database (dbEST) mining and in silico expression analysis

To collect EST coding information for the wheat dehydrins, the 50 Group 2 LEA proteins (dehydrins) [27] were used to query against the GenBank wheat dbEST with the cutoff parameter of >30% identity in a >20-amino acid overlap. After removing vector sequence contamination and adaptors of the hits, contigs were assembled using the parameters of >100-bp overlap and >97% identity. Contigs derived from cross hits in different queries were removed. In the *in silico* analysis, wheat *DHN* contigs were queried against the wheat dbEST using the criteria of >97% identity and >100-bp overlap. All hits were checked to avoid multiple counting of the same clones caused by double sequencing or repetitive submission. EST hits were then classified according to tissue type. Those from SSH and normalized libraries were excluded. The hierarchical clustering method [28] was employed to compare the EST tissue distribution profiles among the contig clusters. The EST tissue distribution profile is displayed based on the frequency of EST members within a contig cluster.

2.4. Open reading frames (ORFs) and dehydrin domain prediction

The ORF of each contig was predicted with Macvector 9.0 (Accelrys) using the default parameters. The dehydrin domain within each predicted protein was detected using the PFAM domain prediction method (http://smart.embl-heidelberg.de/). Those predicted proteins that lacked a dehydrin domain were removed.

2.5. Multiple sequence alignment and phylogenetic tree construction

Peptide sequences were aligned with Macvector 9.0 (Accelrys) using the Clustal W method [29]. Phylogenetic trees were constructed using the neighbor-joining method, and pictures of them were drawn using the MEGA4 program (http://www.megasoftware.net/mega.html) [30].

3. Results

3.1. Identification of wheat DHNs

We identified the wheat *DHNs* by querying the GenBank wheat dbEST using the 50 different *DHN* sequences representing all known *DHN* types [27] as the query sequences. The unique hits were assembled into 54 dehydrin contigs (Supplemental Table S1), 51 of which had full ORFs of 279–1410 bp. The predicted proteins were sorted into seven types (KS, SK_3, YSK_2, Y_2SK_2, K_n [n = 1, 2, 3, 4, 6 and 7], Y_2SK_3, and YSK_3 types) according to the combination of the conserved segments (Table 2). These dehydrins were grouped into 32 homologous clusters, among which one had five contigs, seven had three contigs, four had two contigs, and the rest had only one member each. We designated these contig clusters as *TaDHNx.y*, where *x* represents the cluster accession and *y* represents the contig accession in each cluster. Among the members in each cluster, the coding regions have >89% identity and the coded peptide sequences have >90% homology. Sequence alignment revealed that the contigs/unigenes included the 14 wheat *DHN*s registered in the NCBI database (Table 2).

3.2. Comparing the DHN family between wheat and other major cereals

As shown in Table 3, the KS, SK_3, and Y_2SK_2 types are common in the five cereals, but the K_n- and Y_2SK_3-type dehydrins exist in wheat and barley only, and the K_n-type *DHNs* exist in higher

Table 1
The wheat *DHN* primers used in the expression analysis.

Contig cluster	Forward primer (5′–3′)	Backward primer (5′–3′)
TaDHN1	CCATTAGCATCGCCATTTTCC	ACCACACGCTCCAAACCCTG
TaDHN2	GTGCAGACCCACCATGACAC	CCTCCCACCTTGACACCAAC
TaDHN3	CGGAAGGAGGAAGAAGGGAATC	CAAACAACACGCAGACACGGTC
TaDHN4	CGGAAGGAGGAAGAAGGGAATC	GCAAGTCACACCACACAACAAAAG
TaDHN5	CGGAAGGAGGAAGAAGGGAATC	ACGGGGGTTGAATGACCAGG
TaDHN6	GCCACAAGGACAACCAGCAG	GTAACAGACACAACGCACGCTG
TaDHN7	GAGGAAGAAGAAGGGAATGAAG	CTGACACGGATGCGATAAGA
TaDHN8	CAGGAGGAAGAAGGGGATGAAG	GACGGAACATAGAGAAGACACGC
TaDHN9	CAGGAGGAAGAAGGGGATGAAG	ACACGGGGTCCACACATACG
TaDHN10	CCAGTTCGTCTGAGGATGATG	CTCACTCTATTTCGGGAAGTTCA
TaDHN11	CCAGTTCGTCTGAGGATGATG	CTGTCGGTGCCAGCCATT
TaDHN12	CCAGTTCGTCTGAGGATGATG	CACACATCGAAGTACAGAGGAGG
TaDHN13	CCAGTTCGTCTGAGGATGATG	TCAAGTAGCTGCCGTGGGA
TaDHN14	CCAGTTCGTCTGAGGATGATG	ACCGGCACCTCAAACTTCG
TaDHN15	CCAGTTCGTCTGAGGATGATG	CATACTAGGAGCCCACATTCAAC
TaDHN16	GAGAAAGAAGGGCATCAAAGAG	GACGATGCTGTAGAAATAGACCC
TaDHN17	GCAGCACGGCACTGACAC	CTTACCTGTACGCGAAGCATAC
TaDHN18	GATTTCCCGAGTGACAGGTTGAG	AGCAAAGATACATTCGCTCCTCC
TaDHN19	CAGCAGAACCAGTGTCAGATTTCC	TCCCGACTTCCCGTAGTTGC
TaDHN20	CAACACTGAGAAAAAGGGCGTC	GTATGTGGCTGTGGTGCTATGC
TaDHN21	TGACCGATAAGGAAACACAGGG	CTTCTCCAACGACCAAGTGAGC
TaDHN22	TGACCGATAAGGAAACACAGGG	CAAGATACATTCGCTTCTCTGAACC
TaDHN23	AACGACTGGCACTGGGACACAC	ATACATTCGCTCCTCCAACGGG
TaDHN24	AACGACTGGCACTGGGACACAC	TACTCCATCCTCTCCTCCAACGAG
TaDHN25	GCACCCACGAGAAGAAAGGC	CGATTCGCATCAGAGAAGTAGTTG
TaDHN26	CGAGAAGAAAGGTATTATGGGCAAG	CTACTCCAACGCTACACACAAACG
TaDHN27	GCGGAGAACATCAATGACAATC	CTTAGGCACCATCAGTGGCA
TaDHN28	AGCAGGGACACACCACTACG	GTGTCCGTGCTGCCTGAA
TaDHN29	TGGGAACCACCAGCAGACAG	GCACAGTGGTGTTGCCAGTG
TaDHN30	CGTGAAAACAGAGGGACAACCG	GGGGGGGGTAAATACAACACAC
TaDHN31	GCGGTTGTGTTTATTCCAGATG	CCTTCTTCTTGTGCTCCTCGTG
TaDHN32	GCACGCATCAGCCAGAAAAAG	TGACACCCTTCGTCTCGTCG

quantities in wheat than in barley. The YSK_3-type dehydrin was detected in neither rice nor maize. Although YSK_2-type dehydrins are common among the cereals, more members were detected in wheat and barley than in the other cereals. Unexpectedly, there were greater quantities of the YSK_2-type dehydrins in rice than in maize or sorghum.

3.3. Phylogenesis of wheat dehydrins

The peptides of deduced dehydrins from each *DHN* cluster were aligned with the dehydrins from barley and rice. For each wheat *DHN* cluster, only one deduced dehydrin sequence was used in the sequence alignment. In the phylogenetic tree constructed with the multiple alignment data, the dehydrins form three distinctive groups, *i.e.* A, B, and C (Fig. 1). Group A and Group B contained dehydrins from all three species, but members in Group C were all derived from the two *Triticeae* crops. Group A is the largest of the three groups and includes all YSK_2- and Y_2SK_2-type dehydrins of the three species as well as the Y_2SK_3-type dehydrins of wheat and barley. This group contains five and three times more members of wheat YSK_2-type dehydrins (Table 2) than those of rice and barley, respectively. Group B contained SK_3- and KS-type dehydrins from the three plants as well as two wheat K_n-type dehydrins. The SK_3- and KS-type dehydrins were further separated into two subgroups. Group C contained only one barley K_n-type *DHN* but 18 wheat K_n-type *DHNs* (Table 2). Two YSK_3-type *DHNs* of wheat and barley were also included in this group. Supplementary_Fig. S1 shows the sequence alignment of the 18 wheat K_n-type dehydrins.

3.4. Expression diversification of wheat DHN clusters

3.4.1. In silico expression analysis

Since the transcripts of wheat *DHNs* within a same cluster usually share similar tissue distributions, we used each *DHN* cluster as a unit in the *in silico* expression analysis. The EST frequency derived from each of the seven tissue types was scored for each of the 32 *DHN* clusters and produced a two-way expression profile, *i.e.*, gene cluster *vs.* tissue type. In those 224 gene cluster-tissue type combinations, gene expression was detected in 83 plots (37.1%) (Suppl._Table S3). *TaDHN28*, *TaDHN29*, and *TaDHN31* had no ESTs in the detected tissues and were excluded from the subsequent study. Based on the EST frequency constituent matrix, hierarchical clustering was performed according to the method of Eisen et al. [28]. A virtual display of the expression profile is shown in Fig. 2. In addition to gene clusters expressed in a tissue-specific manner, most genes expressed at low levels in normal growth root and shoot tissues and several genes had no transcripts within the detected libraries. The transcripts of wheat *DHNs* were mainly distributed in the following three groups: the abiotic stress responsive group, reproductive tissue preferentially expressed group, and seed preferentially expressed group. The abiotic stress responsive group was further separated into four subgroups according to the correlation distance (Fig. 2). Subgroup a, the largest subgroup, contained 12 *DHN* clusters and showed preferential expression in drought-stressed shoot tissues. *DHNs* in this subgroup all belong to the YSK_2-type except *TaDHN22*, *TaDHN27*, and *TaDHN30*, which are K_n-type *DHNs*. Subgroup b was preferentially expressed in cold-stressed shoots, and all members but *TaDHN20* were also expressed in drought-stressed shoots. All members but *TaDHN1* (KS-type) in this subgroup are K_n-type *DHNs*. Subgroup c contained two K_n-type, one YSK_2-type, and one SK_3-type *DHN* cluster and its members were preferentially expressed in drought- and cold-stressed shoots. Subgroup d contained only one Y_2SK_3-type cluster with preferential expression in the cold-stressed shoots as well as in developing and germinating seeds. Subgroup f contained one YSK_3-type cluster and two YSK_2-type clusters and was preferentially expressed in developing seeds, while subgroup g contained only one Y_2SK_2-type cluster that was preferentially expressed in germinating seeds.

Table 2
Summary of the wheat *DHN* members.

Contig cluster	Contig[a]	Gene accession	Dehydrin type	Protein length (aa)	Molecular weight (kD)	Isoelectric point
TaDHN1	*TaDHN1.1*		KS	106	11.92	6.49
	TaDHN1.2		KS	102	11.49	6.96
TaDHN2	*TaDHN2.1*	U73211	SK_3	259	27.94	5.03
	TaDHN2.2	U73210	SK_3	268	28.82	5.09
	TaDHN2.3	L29152	SK_3	262	28.15	5.03
TaDHN3	*TaDHN3.1*		YSK_2	160	16.17	9.04
TaDHN4	*TaDHN4.1*		YSK_2	152	15.4	8.73
TaDHN5	*TaDHN5.1*		YSK_2	149	14.83	6.7
TaDHN6	*TaDHN6.1*	AM180930	YSK_2	150	15.22	9.22
	TaDHN6.2		YSK_2	152	15.34	9.38
	TaDHN6.3	AM180929	YSK_2	150	15.18	9.38
	TaDHN6.4		YSK_2	149	15.22	9.19
	TaDHN6.5		YSK_2	150	15.29	9.4
TaDHN7	*TaDHN7.1*		$Y2SK_2$	215	22.24	6.7
	TaDHN7.2		$Y2SK_2$	217	22.3	7
	TaDHN7.3	EU584500	Y_2SK_2	213	21.83	6.27
TaDHN8	*TaDHN8.1*		YSK_2	138	14.22	8.24
	TaDHN8.2		YSK_2	133	13.93	8.71
TaDHN9	*TaDHN9.1*		YSK_2	143	14.51	7.44
	TaDHN9.2		YSK_2	143	14.43	8.24
	TaDHN9.3		YSK_2	143	14.57	8.24
TaDHN10	*TaDHN10.1*		YSK_2	144	14.51	9.06
TaDHN11	*TaDHN11.1*	X78429	YSK_2	158	15.84	9.01
TaDHN12	*TaDHN12.1*	EU395844	YSK_2	152	15.63	8.27
TaDHN13	*TaDHN13.1*		YSK_2	162	16.2	7.5
	TaDHN13.2		YSK_2	162	16.1	9.06
	TaDHN13.3		YSK_2	162	16.2	8.79
TaDHN14	*TaDHN14.1*		YSK_2	167	16.71	7.5
TaDHN15	*TaDHN15.1*	AF453444	YSK_2	166	16.7	8.27
TaDHN16	*TaDHN16.1*		YSK_2	160	16.25	8.29
TaDHN17	*TaDHN17.1*	AK331525	YSK_2	231	23.23	9.15
	TaDHN17.2		YSK_2	231	23.02	8.88
	TaDHN17.3	AY619566	YSK_2	221	22.05	8.99
TaDHN18	*TaDHN18.1*	AM180932	K_2	93	9.66	6.92
	TaDHN18.2		K_2	93	9.66	6.92
	TaDHN18.3		K_2	93	9.61	7.5
TaDHN19	*TaDHN19.1*		K_3	124	12.78	6.65
	TaDHN19.2	AM180933	K_3	124	12.83	7.52
	TaDHN19.3	AB272228	K_3	112	11.53	6.32
TaDHN20	*TaDHN20.1*[b]		K_4	–	–	–
TaDHN21	*TaDHN21.1*		K_3	147	15.39	6.32
TaDHN22	*TaDHN22.1*		K_4	250	26.16	5.79
TaDHN23	*TaDHN23.1*		K_6	391	38.82	6.98
	TaDHN23.2	P46525	K_6	391	38.90	7.09
TaDHN24	*TaDHN24.1*	P46526	K_7	470	46.87	6.85
TaDHN25	*TaDHN25.1*		Y_2SK_3	383	37.23	8.95
	TaDHN25.2[b]		Y_2SK_3	–	–	–
TaDHN26	*TaDHN26.1*[b]		YSK_3	–	–	–
TaDHN27	*TaDHN27.1*		K_3	221	21.93	5.9
TaDHN28	*TaDHN28.1*		K_2	153	15.55	6.83
TaDHN29	*TaDHN29.1*		K	172	17.06	6.38
TaDHN30	*TaDHN30.1*		K_2	228	24.62	9.84
TaDHN31	*TaDHN31.1*		K	111	13.17	4.72
TaDHN32	*TaDHN32.1*		K_2	138	14.65	5.81

[a] Sequence and NCBI accession numbers of the supporting ESTs see Suppl_Table S1.
[b] Those contigs did not reach the full open reading frames of the corresponding *DHNs*, and their dehydrin types were speculated by corresponding homologs.

3.4.2. Semi-quantitative RT-PCR analysis

To verify and complement the *in silico* expression data, we conducted a semi-quantitative RT-PCR expression analysis of all *DHN* clusters within the Kehan 15 seedling leaves and roots using dehydration, cold, and salt treatments. As shown in Fig. 3, the expression of all *DHNs* was low in normal leaves and roots, a finding that was in accordance with the *in silico* expression analysis results. With the exception of *TaDHN21* and *TaDHN30*, all *DHN* clusters expressed in the drought-stressed shoots of the *in silico* data showed induced expression in Kehan 15 in the dehydrated seedling leaves; furthermore, the expressions of *TaDHN3*, *TaDHN7*, *TaDHN20*, *TaDHN25*, and *TaDHN26* were also induced in Kehan 15 dehydrated seedling leaves. Among the dehydration-induced genes, *TaDHN2*, *TaDHN6*, *TaDHN7*, *TaDHN11*, *TaDHN17*, *TaDHN18*, *TaDHN22*, and *TaDHN23* showed relatively higher transcript levels. All of the dehydration-induced *DHNs* except for *TaDHN1*, *TaDHN3*, and *TaDHN19* showed their highest expression levels 48 h after the dehydration. The three exceptions showed their highest transcript levels in 24-h dehydrated seedling leaves. In contrast to most *DHNs* with induced expression within the dehydrated seedling leaves, only *TaDHN1* and *TaDHN2* showed obviously induced expression, whereas all other *DHNs* showed null or weak expression within the dehydrated seedling roots.

The *DHNs* induced by low temperature showed similar expressions between the leaves and roots of the chilled seedlings. With the exception of *TaDHN1* and *TaDHN2*, the *DHNs* induced in the chilled seedlings were all K_n-type. It was noted that the cold-induced expressions of *TaDHN28* and *TaDHN29* were not detected in the *in silico* analysis. However, all of the other cold-induced *DHNs* were detected in both experiments. Compared with the drought or

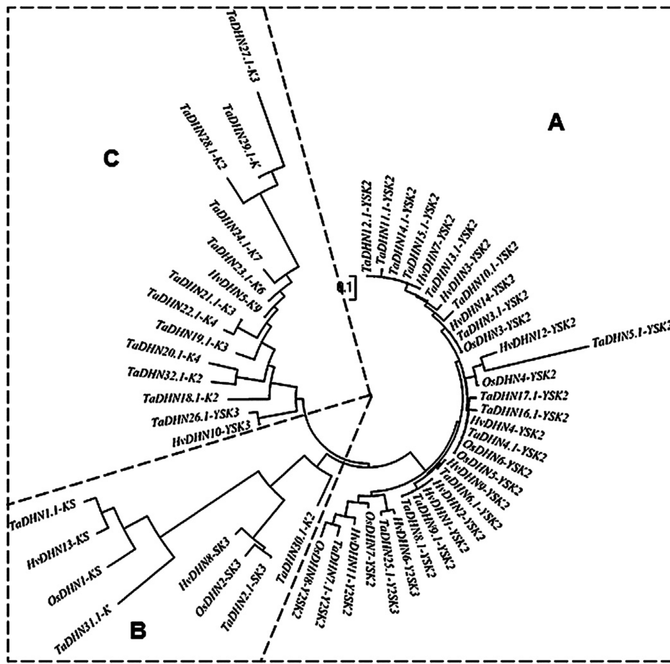

Fig. 1. Phylogenetic tree of dehydrins from wheat, barley, and rice. The barley and rice dehydrin sequences were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/) with the Seq IDs of P12951 (HvDHN1), AF181452 (HvDHN2), AAD02255 (HvDHN3), AAF01692 (HvDHN4), AAQ55320 (HvDHN5), AAD02257 (HvDHN6), AAD02258 (HvDHN7), AAD02259 (HvDHN8), AAD02260 (HvDHN9), AAD02261 (HvDHN10), AAD02252 (HvDHN11), AAD38400 (HvDHN12), AAT81473 (HvDHN13); Os03g0655400 (OsDHN1), Os02g0669100 (OsDHN2), Os11g0454300 (OsDHN3), Os11g0453900 (OsDHN4), Os11g0454000 (OsDHN5), Os11g0454200 (OsDHN6), Os11g0451700 (OsDHN7), and Os01g0702500 (OsDHN8). HvDHN14 here was the C-terminal 79-amino-acid peptide deduced from a contig comprising the expressed sequence tags (ESTs) BY838371 and BY860262 obtained through EST data mining using the method described for wheat gene mining in Section 2.

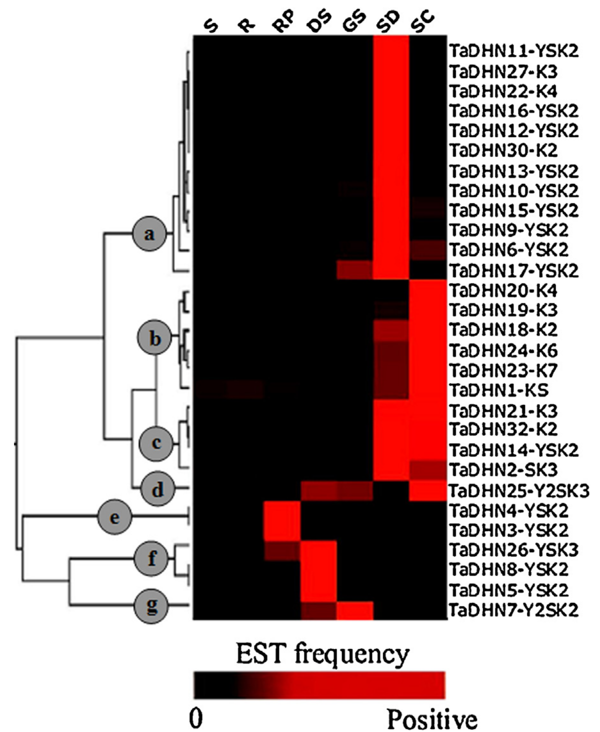

Fig. 2. Clustered correlation display of expressed sequence tag frequency of wheat *DHN* clusters. At the top of the panel, the seven types of tissues from which the cDNA libraries were constructed included the following: S, shoot tissues, including the shoot, leaf, and crown; R, roots; RP, reproductive tissues including the anther, pistil, ovary, spikelet, and whole spike before and during flowering; DS, developing seeds; GS, germinating seeds; SD, shoot desiccated for >24 h or down to <80% leaf relative water content; SC, shoot at <5 °C for more than 24 h. The expression profile of each gene cluster is represented by a single row of colored boxes, while that of each tissue type is represented by a single column. The brackets on the left show the clusters that formed based on similar expression patterns. Letters a–g indicate gene clusters showing similar expression patterns.

cold treatment, salt stress mainly induced *DHN* within the seedling roots. *TaDHN2*, *TaDHN7*, *TaDHN9*, *TaDHN13*, *TaDHN14*, *TaDHN17*, and *TaDHN20* were the main *DHN*s induced by salt stress. The expression of *TaDHN2* was also induced by salt stress in the seedling leaves, but other *DHN*s showed null or weak expression in this tissue type.

Since the wheat *DHNs* showed similar type-specific expression patterns in the 24- and 48-h stress-response analysis, we selected one *DHN* from each of the seven wheat *DHN* types and detected their expressions in 24-h ABA-treated seedling leaves and roots. As shown in Fig. 4, all selected *DHNs* but *TaDHN1* (KS-type) and *TaDHN23* (K_n-type) showed induced expression in both the leaves and the roots of the ABA-treated seedlings. *TaDHN23* also showed induced expression in ABA-treated seedling roots. Differences in the ABA-induced expression were observed among the different *DHN* types. Compared with the weakly induced expression of *TaDHN2* (SK_3-type), *TaDHN17* (YSK_2-type) expression was strongly induced in the both tissue types in the ABA-treated seedlings.

Table 3
Numbers of wheat *DHN* unigene clusters (unigenes) classified by dehydrin types and corresponding homolog numbers in barley, sorghum, maize and rice.

Dehydrin type	Species				
	Wheat[a]	Barley[b]	Sorghum[a]	Maize[a]	Rice[b]
K_n	8 (18)	1			
KS	1 (2)	1	1	1	1
SK_3	1 (3)	1	1	1	1
Y_2SK_2	1 (3)	1	1	1	1
YSK_3	1 (1)	1	1		
Y_2SK_3	1 (3)	1			
YSK_2	12 (25)	8	1	1	5

Barley DHNs were obtained using the method described for wheat gene mining in Section 2. DHNs of sorghum, maize and rice were from their genome annotation database, which should cover all the present dehyrins in the corresponding species. The supporting gene sequences/accessions and the database hyperlink see Suppl_Table S1(a) and Fig. 1(b).

4. Discussion

4.1. Wheat DHN family

Wheat *DHNs* were identified through exhaustive EST data mining in this study. A similar method was used in wheat fasciclin-like arabinogalactan protein gene identification and homoeologous gene discrimination [31–33]. Our results here indicate that wheat has at least 54 putative *DHNs*. Twenty-two *DHN* clusters were derived based on the sequence identity of the produced peptides, which is more than that in either rice or other cereals. The YSK_2- and K_n-type *DHNs* comprise the major *DHNs* in wheat. It is obvious that these two *DHN* types experienced more duplications than the other *DHN* types (Fig. 1), which led to more *DHNs* in wheat than in the other cereals. Gene duplications, internal deletions, and recombinations were suggested in the evolution of Group 1 LEA genes [34]. The sequence alignment results (as shown in Suppl. Fig. S2) indicated that similar mechanisms could be taken in the evolution of wheat *DHNs*. The greater numbers of duplication of the YSK_2- and K_n-type *DHNs* suggests their important roles in wheat environment adaptation. SK_n-type *DHNs* are common among the major cereals

Fig. 3. Expression profile of wheat *DHNs* in the leaves and roots of Kehan 15 seedlings over a 48-h course of dehydration, 4 °C chilling, and 250 mM NaCl treatment. *β-tubulin*, 22 cycles; *DHN*, 25 cycles. TT, treatment time (h).

and exist in gymnosperms [35], a finding that suggests that they could be fundamental to the biological processes in these species and that they could be the ancestors of other *DHN* types.

Wheat is an allohexaploid species with three homoeologous subgenomes (A, B, and D), each of which has seven chromosomes. Our previous research revealed that highly homoeologous contigs clustered together could be homologs from the three homoeologous subgenomes [33]. With the exception of the seven clusters with three homologous contigs each, all of the dehydrin clusters had more or fewer than three contigs (Table 2). We speculate that high conservation among the paralogs could lead to more (>3) contigs in a cluster. Otherwise, high rate differentiation among the homologs could lead to fewer (<3) contigs in a cluster. Moreover, gene loss could also lead to fewer (<3) contigs in a cluster.

Fig. 4. Expression of wheat *DHNs* in the leaves and roots of Chinese Spring seedlings in a 24-h course of 0.5 mM ABA treatment. *β-tubulin*, 22 cycles; *DHN*, 27 cycles. TT, treatment time (h).

4.2. Expression diversification of wheat dehydrins

The expression diversification of the wheat *DHNs* suggests their differentiated roles in wheat life. Previous studies suggest that the synthesis of Y_nSK_m dehydrins is induced by drought but not low temperatures, whereas the dehydrins of the K_nS, SK_n, and Y_2K_n types are preferentially accumulated in plants in response to low temperatures [5]. The *in silico* and RT-PCR analysis results revealed that the expression of wheat *DHNs* usually differentiated with abiotic stresses and/or tissue types (Figs. 2 and 3). The SK_3-type *TaDHN2* showed similar induced expression in leaves and roots of dehydration-, cold-, and salt-stressed seedlings. The SK_3-type *DHN* in rice was induced by cold as well as drought [9]. Overexpression of this type of *DHN* improves freezing tolerance in transgenic strawberry plants [21]. The wheat KS-type *DHN* is constitutively expressed in seedling tissues, reproductive tissues, and seeds, and it showed considerably increased transcripts when the plants were subjected to chilling and dehydration, which is supported by the results of its ortholog in barley [7]. It was reported that the presence of a KS-type dehydrin can reduce the formation of reactive oxygen species from Cu and inhibit the generation of hydrogen peroxide and hydroxyl radicals in the Cu-ascorbate system [36].

YSK_2-type *DHNs* are mostly induced in dehydrated seedling leaves (Fig. 3), which is supported by the results of their orthologs in rice (Fig. 1 and Table 3, Experiment GSE6901, Rice Genome Annotation Project, http://rice.plantbiology.msu.edu/cgi-bin/generate_experiment_page.pl? experiment=GSE6901). Overexpression of the wheat YSK_2-type dehydrin (AY619566) in *Arabidopsis* enhances the osmotic stress tolerance of the transgenic plants [20]. The expression of most K_n-type dehydrins is induced by dehydration and cold stress. The positive genomic amplification of the K3 dehydrin variants selected by their superior tolerance to freezing and the induction of their expression at low temperatures strongly supports a link with cold adaptation in alfalfa (*Medicago sativa spp. sativa* L.) [37]. As the major *DHN* in wheat, the duplication and induced expression of YSK_2- and K_n-type *DHNs* must be very important or essential to the stress tolerance of wheat. The specific expressions of *TaDHN3*, *TaDHN4*, *TaDHN5*, and *TaDHN8* in reproductive tissues or developing seeds suggest that YSK_2-type dehydrins could also protect these tissues under dehydration. Their orthologs in barley showed similar preferential expression in seed embryos [8]. Except for the YSK_2-type *DHNs*, the Y_nSK_m-type *DHNs* (YSK_3, Y_2SK_2, and Y_2SK_3) also induced expression in dehydrated seedling leaves (Fig. 3), which suggests their roles in wheat dehydration tolerance. A set of these types of *DHNs* induced in salt-stressed seedling roots could also play a role in wheat salt stress protection.

4.3. Putative stress signals in wheat DHN expression regulation

The induced expressions of wheat *DHNs* varied with dehydrin types, tissues, and abiotic stresses, which suggest different signals in wheat *DHN* expression regulation. The expression of SK_3-type *DHN* was weakly induced in the leaves and roots of ABA-treated seedlings, which was also revealed in the *in silico* analysis of wheat normal and ABA-treated shoot libraries (data not shown) but was strongly induced in both leaves and roots under all the three abiotic stresses (Figs. 3 and 4). As such, its induced expression by abiotic

stresses could be controlled by ABA and an ABA-independent pathway. The expression of rice SK_3-type *DHN* was revealed under the regulation of both ABA (The Rice Expression Profile Database [RiceXPro], http://ricexpro.dna.affrc.go.jp) and the CBF/DREB stress-signaling pathway [7]. The cis-acting DRE element of *Arabidopsis rd29A* and the transcription factors DREB1 and DREB2 binding to it are responsible for the reactions to drought, low-temperature, and high-salt stresses [38,39]. The wheat KS-type *DHN* seems to have constant expression in ABA-treated seedlings (Fig. 4), which suggests that its induced expression by abiotic stresses could be mainly controlled by the ABA-independent pathway. The expression of its rice ortholog *OsDHN1* showed weakly induced expression in seedling roots after 12-h ABA treatment (RiceXPro).

The selected Y_nSK_m-type (YSK_2, Y_2SK_2, YSK_3, and Y_2SK_3) *DHNs* all showed induced expression in the leaves and roots of ABA-treated seedlings. All of the rice Y_nSK_m-type *DHNs* also showed dramatically induced expression in ABA-treated seedling roots (RiceXPro). So the abiotic stress-induced expression of Y_nSK_m-type *DHNs* could be conducted partially by the ABA signal. However, we cannot rule out the role of the ABA-independent pathway in Y_nSK_m-type *DHN* regulation. The K_n-type *DHNs* showed weak or no induced expression in ABA-treated seedlings; rather, they were strongly induced in chilled and dehydrated seedlings. It can be speculated that the induced expression of K_n-type *DHNs* by abiotic stresses could be mainly conducted through the ABA-independent pathway. The induced expressions of the SK_3, YSK_2, and K_7 *DHNs* in ABA-treated seedlings suggest that the salt stress-responsive *DHNs* could be also regulated by the ABA pathway.

It was noted that the expression pattern of some *DHNs* in the semi-quantitative RT-PCR analysis seems to be inconsistent with the *in silico* data such as *TaDHN3*, *TaDHN4*, *TaDHN21*, and *TaDHN25* (Figs. 2 and 3). Possible reasons include the limitation of the size of the EST libraries used in the *in silico* analysis, which could lead to failure to detect low-expressed *DHNs*. Meanwhile, alleles of the *DHNs* may also lead to the different expressions detected between the *in silico* and semi-quantitative RT-PCR analysis. Although we tried to avoid designing primers at conserved segments, some primers gave a high background and produced non-specific products. Bands of specific products were selected for these primers according to the predicted size. Our study explored the composition, classification, and different abiotic stress response of wheat dehydrins. The results revealed the correlation between expression and structural diversification of wheat dehydrins. It also suggests a contribution of the *DHN* family to the increased stress adaptability of wheat. Further studies on the precise physiological functions of the different types of dehydrins, especially the YSK_2- and K_n-type dehydrins, would reveal their detailed molecular mechanisms in wheat dehydration and cold tolerance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.10.005.

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