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ORIGINAL ARTICLE

Genome-wide identification, characterization, and expression analysis of the dehydrin gene family in Asian pear (*Pyrus pyrifolia*)

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Abstract Dehydrins (DHNs) are a complex family of plant proteins that play an important role in protection of higher plant cells from dehydration and desiccation damage during environmental stresses, such as drought, high salinity, and low temperature. However, information on *DHN* genes of Asian pear (*Pyrus pyrifolia*), an economically important fruit crop grown in temperate regions in East Asia, e.g., China and Japan, is limited. To gain insights into this gene family in pear and to elucidate their roles in floral buds under low-temperature conditions, we performed a genome-wide identification, characterization, and expression analysis of *DHN* genes. Seven *PpDHN* genes were identified. Sequence alignment analysis of all putative proteins from these genes showed that all of the proteins contained a typical K-domain. These genes were categorized into SK_n, Y_nSK_n, YK_n, and K_n groups based on gene characterization and phylogenetic relationships. Hierarchical cluster analyses showed that in non-stressed pear, PpDHN genes were expressed in all vegetative tissues except young leaves and shoot tips, in which *PpDHN1*, *PpDHN2*, and *PpDHN4* were not expressed. Transcript levels of four *PpDHN* genes increased significantly in floral buds in response to low-temperature treatment, which indicated that they play important roles during stress adaptation. This study provides evidence that the family of pear *DHN* genes may function in tissue development and stress responses. The data will be valuable for further studies of the functions of *DHN* genes under different stress conditions in pear.

Keywords Asian pear · Dehydrin genes · Phylogenetic tree · Expression · Low-temperature treatment

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Introduction

Dehydrins (DHNs) are a group of hydrophilic, thermostable stress-responsive proteins with a high number of charged amino acids belonging to the Group II late embryogenesis abundant (LEA) family. Genes that encode these proteins are expressed during late embryogenesis as well as in vegetative and reproductive tissues in response to low temperature, drought, and high salinity (Close 1996; Nylander et al. 2001; Allagulova et al. 2003; Kosova et al. 2007; Xu et al. 2008; Kim and Nam 2010). Overexpression of *DHN* genes in transgenic plants has been observed to increase resistance to unfavorable environmental conditions (Puhakainen et al. 2004; Peng et al. 2008; Ochoa-Alfaro et al. 2011; Shekhawat et al. 2011), which has enhanced interest in potential use of *DHN* genes for crop improvement. DHNs are associated with maintenance of protein structure and water binding because they are important components of dehydration tolerance (Farrant et al. 2004; Garnczarska et al. 2008). Generally, it is accepted that the function of *DHNs* is to protect the cell from dehydration and desiccation damage induced by environmental stresses and cellular dehydration (Eriksson and Harryson 2011), but the precise mechanism remains elusive.

Dehydrins are characterized by the presence of highly conserved motifs such as the Y-, S-, and K-segments. The distinctive sequence feature of all DHN proteins is a conserved, Lys-rich 15-residue motif, EKKGIMDKIKEKLPG, named the K-

segment, which is generally located at the C-terminal end of the protein and is able to form an amphipathic helix-like structure that may play a role in its interaction with membranes and proteins (Koag et al. 2009; Hughes and Graether 2011). Optionally, additional motifs in DHNs are the Y-segment ([V/T]D[E/Q]YGNP), which is usually found as one to three tandem copies in the N-terminus, the S-segment containing a track of Ser residues, and the less conserved Φ-segment rich in polar amino acids and interspersed between K-segments (Close 1996; Allagulova et al. 2003). The S-segments can be customized through phosphorylation and may play a role in the regulation of protein conformation and ion-binding activity (Jensen et al. 1998; Riera et al. 2004; Alsheikh et al. 2005). On the basis of the presence and arrangement of the above conserved motifs in a single protein, DHN proteins have been divided into five subclasses, comprising Y_nSK_n, Y_nK_n, SK_n, K_n, and K_nS (Allagulova et al. 2003; Rorat 2006). Several additional conserved regions have been identified in a subset of DHNs. For example, lysine-rich segments (Ly segments) contain a cluster of Lys residues that are generally located between the S- and K-segments (Hundertmark and Hincha 2008; Hara et al. 2009) and have been suggested to participate in the binding of *DHNs* to DNA or RNA (Hara et al. 2009). In addition to these subclasses, some DHNs are assignable to certain transitional forms, such as the SK_3S arrangement in one DHN protein of chickweed (Close 1996). The abovementioned works have explored DHN structure and function in herbaceous model plants such as *Arabidopsis*, maize, and barley, but the genome-wide identification and characterization of DHN subclasses/types have not been extended previously in many woody plants.

Genes that encode *DHNs* are part of a multigene family (Hundertmark and Hincha 2008). At the functional level, DHN family members show various functional activities, as different genes display differential expression profiles during development and under stress conditions. For example, SK_n, YK_n, and K_n-type *DHNs* in *Arabidopsis*, loquat, barley, and apple are upregulated under low-temperature treatment (Choi et al. 1999; Nylander et al. 2001; Liang et al. 2012; Xu et al. 2014). However, the relationship between subgroup classification and expression profile is unclear because of the different expression patterns within this gene family (Eriksson and Harryson 2011).

Pear is an economically important tree crop all over the world. In East Asian, including China, Japan, and Korea, Asian pear (*Pyrus pyrifolia*) is the main pear specie for fruit production. Asian pear plants frequently experience environmental stresses that can affect fruit production. *DHNs* have been reported in woody plant species, including poplar, apple, grape, and loquat (Liang et al. 2012; Liu et al. 2012a, b; Yang et al. 2012; Xu et al. 2014). However, information on the genome-wide identification and characterization of the *DHN* gene family in pear is limited. One *DHN* gene was identified in a transcriptome analysis of pear and its expression pattern was analyzed during dormancy (Liu et al. 2012a, b). In the present study, we identified members of the *DHN* gene family in Asian pear and investigated their putative roles under low-temperature conditions. We in silico identified and characterized the cDNA of seven *DHN* genes from the pear genome sequences (Wu et al. 2013), and further cloned the genes and investigated their spatial and temporal transcriptive patterns, and also the expression pattern in floral bud under low-temperature treatment.

Materials and methods

Plant materials and treatment

Samples of floral buds, leaf buds, young leaves, mature leaves, bark, shoot tips, and roots were collected from pear "Cuiguan" grafted on *Pyrus calleryana* rootstock grown in the orchard of Zhejiang University (Hangzhou, China. E120° 09', N30° 31'). The floral buds, leaf buds, and bark were collected on 15 January 2014; mature leaves and roots were sampled on 15 October 2014; and young leaves and shoot tips were collected on 10 April 2013. Mixed cDNAs from all of the organs and tissues were used for cloning *DHN* genes. Floral buds of *P. pyrifolia* "Cuiguan" were used for expression analysis of cloned *DHN* genes in response to low temperature. One-year-old shoots (about 60-cm-long) were randomly collected from adult trees on 1 November 2012 during leaf fall, before any natural chilling accumulation. These shoots were placed with the base in vials containing water for low-temperature (4 °C) treatment in a growth chamber, with a 12 h light/12 h dark photoperiod under white light (320 μmol m^{-2} s^{-1}) and 75 % relative humidity. Floral buds were collected after 0, 2, 4, 8, 12, 24, and 48 h treatment. Floral bud samples collected at 0 h served as control. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Identification of pear *DHN* gene family members

The profile file (dehydrin.hmm) of the Pfam dehydrin domain (PF00257) was downloaded from the Pfam database (http://pfam.sanger.ac.uk/family?acc=PF00257) and the Hidden Markov models (HMM) search program HMMER were used to search the candidate dehydrion gene in predicted pear protein sequences in the Pear Genome Project database (http://peargenome.njau.edu.cn/default.asp?d=1&m=1). In detail, the hmmsearch (v3.1b1) command was used with an *E* value threshold of 1e-10. To corroborate the identity of the obtained sequences as dehydrin proteins, we manually examined the sequences for the presence of highly conserved Y-, S-, and lysine-rich K-segments. The sequences

that contained these segments were considered as pear dehydrin candidate proteins and served for further analysis.

RNA extraction, cDNA synthesis, and cloning of full-length cDNA

Total RNA was extracted from all organs and tissues using the CTAB method of Zhang et al. (2012) with a slight modification. The quality and quantity of RNA was assessed by electrophoresis in 1 % agarose gel and calculation of the spectrophotometric absorbance ratio A_{260}/A_{280}. Total RNA concentration was measured after DNase-I treatment. First-strand cDNA was synthesized from 4 μg DNA-free RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA), and 2 μl of 10-fold diluted cDNA was used as a template for cloning and quantitative polymerase chain reaction (qPCR) analysis. To clone the full-length cDNA, we mixed the cDNAs from all organs and tissues and used 2 μl of the mixed cDNAs. The PCR reactions were performed with Taq DNA polymerase (Fermentas) in accordance with the manufacturer's recommended protocol. Primers for cloning of seven *DHN* genes were designed based on the genomic sequences (Pear Genome Project database) using Primer 3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are listed in Table 1. The PCR products were gel-purified and cloned into the pMD18T vector (Takara, Japan) and transformed into *Escherichia coli* strain DH5α. After colony PCR, eight clones were sequenced to determine the DHN sequences in *P. pyrifolia* "Cuiguan."

Multiple sequence alignment, phylogenetic, conserved motif, and structural analyses

Multiple sequence alignments of the deduced amino acid sequences were generated using ClustalW and BioEdit software with default parameters. The alignments were refined manually when necessary. Based on alignment of the complete protein sequences of pear, loquat, apple, and grape, an unrooted phylogenetic tree was constructed using MEGA 6.06 software (http://www.megasoftware.net/mega.html) with the neighbor-joining method using p-distances and complete deletion of gap positions (Saitou and Nei 1987). The reliability of the phylogenetic tree was estimated with 1000 bootstrap replicates. To identify the conserved and shared motifs among the DHN protein sequences, we used the online version 4.9.1 of the Multiple Expectation for Motif Elicitation (MEME) tool (http://meme.nbcr.net/meme/intro.html) (Bailey and Elkan 1994; Bailey et al. 2006). The full-length protein sequences were subjected to MEME analysis with the following parameters: the optimum number for each motif was between 2 and 120, occurrences of motif distribution were any number of repetitions, maximum number of motifs was 15, and the optimum widths of motifs were constrained between 8 and 60 residues. The exon and intron structures of *PpDHN* genes were determined from the cDNA sequences and pear genomic sequences alignment using an online software GSDS 2.0 (http://gsds.cbi.pku.edu.cn; Hu et al. 2015).

Real-time quantitative PCR and hierarchical cluster analysis

Real-time quantitative PCR (Q-PCR) analysis was used to study the expression of *DHN* genes in different tissues (floral buds, leaf buds, young leaves, mature leaves, bark, shoot tips, and roots) at different growth stages and in floral buds after low-temperature treatment. The specific forward and reverse primer sequences used for gene expression analysis are shown in Table 1. Primers were designed to amplify an 80–200-bp product. RNA extraction and first-stand cDNA synthesis were performed in accordance with the method described for cloning full-length cDNAs. The total volume for Q-PCR reactions was 15 μl, containing 7.5 μl SYBR Premix Ex Taq™ (Takara, Japan), 3 μl of 1:10 (*v*/*v*) diluted cDNA, 0.5 μl of each primer (10 μM), and 3.5 μl of RNase-free water. Three technical replications for each sample were performed. The Q-PCR reactions were performed on a LightCycler 1.5 (Roche, Germany), starting with a preliminary step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The specificity of the amplifications was verified by obtaining a single melting-curve peak and a single band in agarose gel electrophoresis. *PpyActin* (JN684184) was used as a reference gene. Three biological replicates were used, and three measurements were performed for each replicate. Error bars are standard error of three biological replicates. The relative gene expression of *PpDHNs* was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Hierarchical cluster analysis was used to analyze the transcript abundance levels in different tissues using the TIGR MeV 4.8.1 software package (Saeed et al. 2003). Pearson's correlation analysis and average linkage clustering were employed for data aggregation.

Results

Identification and classification of the dehydrin gene family in Asian pear

Seven candidate Asian pear dehydrin genes (nos. 1–7 in Table 2) were identified using a HMM search of the *Pyrus* x *bretschneiderii* genome using the pfam model "dehydrin." The predicted amino acid sequence contained at least one K-segment, which is an essential motif of dehydrins. The identified genes were designated as *PpDHN1* to *PpDHN7* (*P. pyrifolia DHN*) (Table 2), based on the basic nomenclature of *DHN* genes established by Campbell and Close (1997).

Table 1 Sequence of primers used in this study

Purpose	Genes name	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Gene cloning	*PpDHN1*	ATGGCGAATTATGGTTCAAC	CTACTTCTTGTGGCCGCCCG
	PpDHN2	ATGTCGCATCTGCATAATCA	TTACTTTGTTCTTGTGAGAA
	PpDHN3	ATGGCGGAGGAGTACAACAA	TTAGTAGGAAGGAGTATCCT
	PpDHN4	ATGGCGAATTATGGTTCAAC	CTACTCGGCGTGGCCACCAG
	PpDHN5	ATGGCGCATTTACAAAATCA	TTATATCTTGTGGTGTCCAC
	PpDHN6	ATGGCGCACTATCAGAACCA	TTAGTGGTGTCCACCGGGAA
	PpDHN7	ATGCACAAACACCACATTGA	CTATGATGACTAGGCAACTT
Q-PCR	*PpDHN1*	GGAGAGCCCCAGGAAAAGAA	GGGATGATGATCTACTCGGCG
	PpDHN2	AGGGGATCATGGACAAGATCA	TATTGCGTAGTGTTCGGTGC
	PpDHN3	CCGAGGAAGAACACAAGGACA	AGCCACAAATGACACCACCA
	PpDHN4	GGAGAGCCCCAGGAAAAGAA	AACAGTGCGCAACGATGGAT
	PpDHN5	AGCACCATGAAAAGAAGGGGA	GTAGTGCTCTCGGTGCGTTA
	PpDHN6	CGCACCATGAAAAGAAGGGC	ACGCCAAGCACCGGTTATTA
	PpDHN7	ATTGGAGGGCAAGTCTGGTG	CCATAACCTTGGCGCATGAC
	PyActin	CAGATCATGTTTGAGACCTTCAATGT	CATCACCAGAGTCCAGCACAAT

Furthermore, all seven putative *DHN* genes were confirmed by RT-PCR amplification (Fig. 1), cloning, and sequencing (Table 2). The cloned sequences of *PpDHNs* were blasted against the predicted sequences and showed at least 98 % identity to their corresponding predicted sequences in the pear genome. The coding sequences (CDS) of all *DHN* genes have been submitted to GenBank and were assigned the following accession numbers: *DHN1* (KP260647), *DHN2* (KP260648), *DHN3* (KP260649), *DHN4* (KP260650), *DHN5* (KP260651), *DHN6* (KP260652), and *DHN7* (KP260653). The length of CDS for the *PpDHNs* genes varied from 456 to 1428 bp. The seven deduced PpDHN proteins varied substantially in size and molecular weight, ranging from 135 (PpDHN7) to 286 (PpDHN3) amino acids in length and from 16.78 kDa (PpDHN7) to 33.08 kDa (PpDHN3) in molecular weight. The amino acid sequence alignment showed high identity among each sequence. The theoretical p*I* ranged from 5.04 (PpDHN3) to 10.76 (PpDHN7), suggesting that K_n-type DHNs acquire a higher p*I* than SK_n-, Y_nK_n-, and Y_nSK_n-type DHNs (Table 2).

Multiple sequence alignment, structure, and phylogenetic analysis

The DHN family of seven members is relatively small but similar numbers of *DHNs* are known in loquat (seven), apple (nine), and grape (four). In plants, five different structural types of DHNs are distinguished: Y_nSK_n, SK_n, K_n, Y_nK_n, and K_nS DHNs, where n shows the number of repeats in each domain (Rorat 2006). In the present study, we analyzed the deduced PpDHN sequences for the presence of dehydrin-specific amino acid motifs (Y-, S-, and K-segments). The characteristic segments of the ClustalX alignment of deduced PpDHNs protein sequences is shown in Fig. 1. The K-segment was present as one or more copies in each sequence. The S-segment was present in five deduced PpDHNs (PpDHN1, 2, 3, 4, and 5) but only as one copy in all five DHNs. The Y-segment was present in five PpDHNs (PpDHN1, 2, 4, 5, and 6) with two copies in each PpDHN (Fig. 1). Dehydrin proteins also contain a conserved polypeptide K-motif in which small variations in sequence may be

Table 2 Biochemical properties of all identified pear DHN proteins

Gene	Annotation ID from pear genome data	Genbank accession number	Deduced polypeptide length (aa)	Deduced polypeptide MW (kDa)	Deduced polypeptide (p*I*)	Scaffold	Motif type
PpDHN1	Pbr040481.1	KP260647	242	26.32	8.03	Scaffold 890.0	Y_2SK_3
PpDHN2	Pbr040484.1	KP260648	188	19.87	8.73	Scaffold 890.0	Y_2SK_3
PpDHN3	Pbr005766.1	KP260649	286	33.08	5.07	Scaffold 13.0	SK_3
PpDHN4	Pbr040482.1	KP260650	195	21.16	7.79	Scaffold 890.0	Y_2SK_3
PpDHN5	Pbr040485.1	KP260651	216	22.88	8.04	Scaffold 890.0	Y_2SK_3
PpDHN6	Pbr040480.2	KP260652	201	22.07	7.49	Scaffold 890.0	YK_4
PpDHN7	Pbr015788.1	KP260653	135	16.78	10.76	Scaffold 232.0	K_2

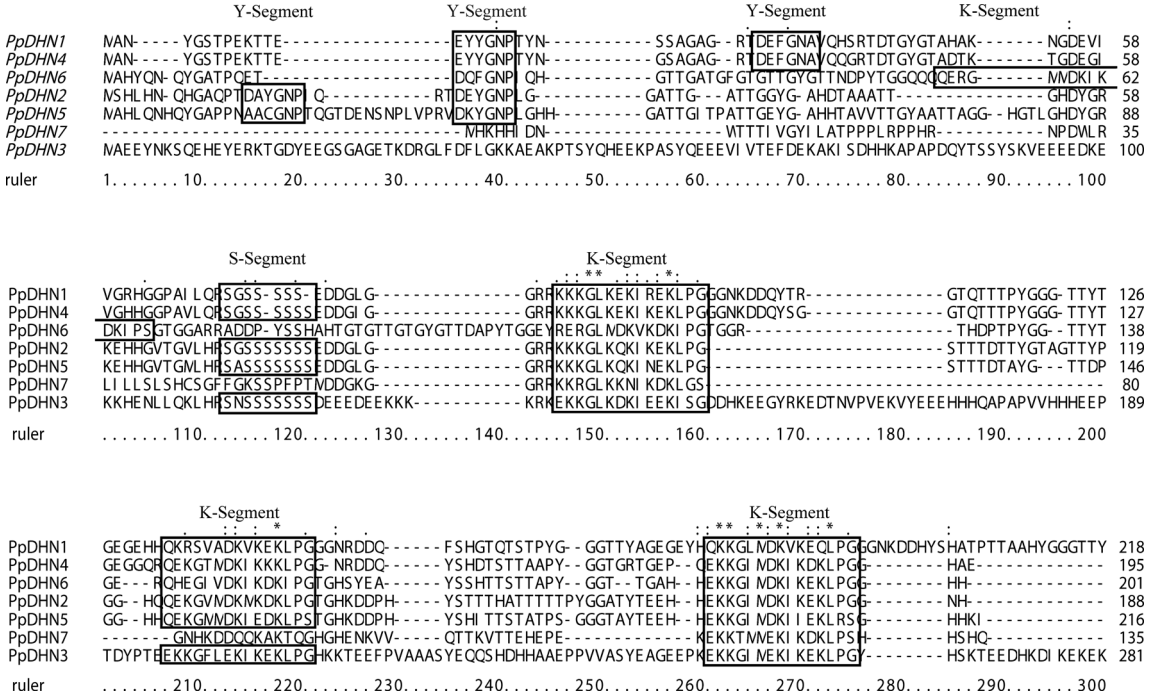

Fig. 1 Amino acid sequence alignment of the seven identified pear DHN proteins. Conserved amino acid sequences are enclosed in *black boxes* for all Y-, K-, and S-segments, which are indicated above the relevant sequence

present in loquat, apple, and grape. We also observed some variation in the amino acid sequence substitutions of the PpDHNs in the conserved K- and Y-motifs (Fig. 1). Four types of DHNs were observed in the present study, comprising the Y_nSK_n type (PpDHN1, 2, 4, and 5; Y_2SK_3), SK_n type (PpDHN3; SK_3), K_n type (PpDHN7; K_2), and Y_nK_n type (PpDHN6; YK_3) (Table 2).

Construction of a neighbor-joining tree resolved the seven PpDHN proteins into four distinct clans including Type I (PpDHN1, 2, 4, and 5), Type II (PpDHN3), Type III (PpDHN6), and Type IV (PpDHN7) (Fig. 2a). Divergence in exon/intron structure often plays a key role in the evolution of gene families. Therefore, the exon/intron structures of the pear DHNs were examined (Fig. 2b) to gain further insights into their possible structural evolution. Intron/exon compositions were determined by aligning the cDNA to genomic sequences of the deduced PpDHN proteins. No intron was present in *PpDHN6*, whereas the other *PpDHN* genes contained one intron and two exons (Fig. 2b).

To examine phylogenetic relationships among the PpDHN proteins and to place them within the established subgroups, we generated an unrooted tree based on sequence alignments of the DHN protein sequences of pear (PpDHN1, PpDHN2, PpDHN3, PpDHN4, PpDHN5, PpDHN6, PpDHN7), loquat

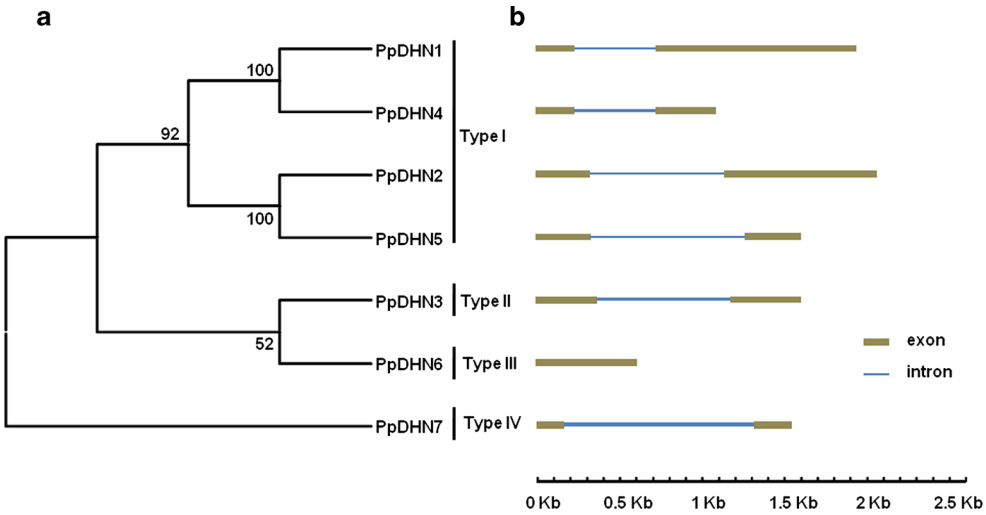

Fig. 2 Phylogenetic relationships and exon–intron structure of pear DHN proteins. **a** Phylogram of the pear DHN proteins constructed with the neighbor-joining method using MEGA 6.0 and derived from an alignment of the complete sequences. **b** The exon–intron structure of pear *DHN* genes. Introns and exons are represented by *light blue lines* and *light yellow boxes*, respectively

(EjDHN1, EjDHN2, EjDHN3, EjDHN4, EjDHN5, EjDHN6, EjDHN7), apple (MdDHN1, MdDHN2, MdDHN3, MdDHN4, MdDHN5, MdDHN6, MdDHN7, MdDHN8, MdDHN9), and grape (VyDHN1, VyDHN2, VyDHN3, VyDHN4). The tree was derived from the deduced protein sequences of seven PpDHNs, seven EJDHNs, nine MdDHNs, and four VyDHNs. The DHNs were divided into four clades designated I, II, III, and IV (Fig. 3). The four clades generally corresponded to the subgroups distinguished by motif analysis. The DHNs placed in the SK_n subgroup (PpDHN3, MdDHN8 and 9, EjDHN2 and 5, and VyDHN2 and 3) were assigned to clade IV, whereas clade III contained one K_n DHN (PpDHN7) and two Y_nSK_n DHNs (VyDHN1 and 4; Fig. 3). Clade I contained four Y_nSK_n DHNs (PpDHN2 and 5, MdDHN1, and EjDHN1), four YK_n DHNs (PpDHN6, MdDHN5 and 6, and EjDHN6), and two K_n DHNs (MdDHN7 and EjDHN4). The four Y_nSK_n DHNs (PpDHN1 and 4, and MdDHN2 and 4), together with two SK_n DHNs (EjDHN3 and 7) and one K_n DHN (MdDHN3) were assigned to clade II (Fig. 3). This phylogenetic analysis

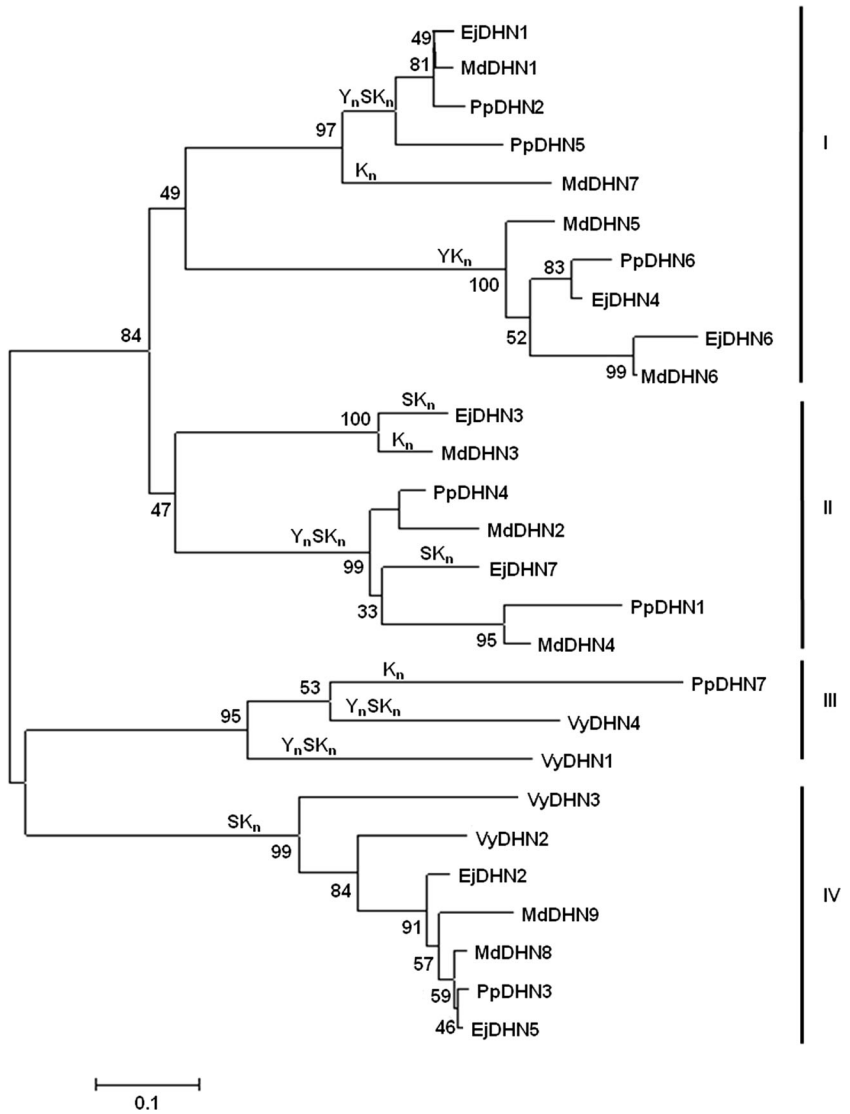

Fig. 3 Phylogenetic relationships among pear (*Pyrus pyrifolia*, *Pp*), loquat (*Eriobotrya japonica*, *Ej*), apple (*Malus domestica*, *Md*), and grape (*Vitus yeshanensis*, *Vy*) DHN proteins. The unrooted phylogram was constructed with the neighbor-joining method using MEGA 6.0 and was derived from an alignment of complete DHN protein sequences for the above species. Proteins were arranged into four subgroups (I, II, III, and IV) of putative orthologs based on their sequence similarities. Bootstrap support values from 1000 replicates are given at each node. GenBank accession numbers of all DHN sequences are as follows: PpDHN1 (KP260647), PpDHN2 (KP260648), PpDHN3 (KP260649), PpDHN4 (KP260650), PpDHN5 (KP260651), PpDHN6 (KP260652), PpDHN7 (KP260653), EjDHN1 (FJ472835), EjDHN2 (FJ472836), EjDHN3 (KF277187), EjDHN4 (KF277188), EjDHN5 (KF277189), EjDHN6 (KF277190), EjDHN7 (KF277191), MdDHN1 (JQ649456), MdDHN2 (JQ649457), MdDHN3 (JQ649458), MdDHN4 (JQ649459), MdDHN5 (JQ649460), MdDHN6 (JQ649461), MdDHN7 (JQ649462), MdDHN8 (JQ649463), MdDHN9 (JQ649464), VyDHN1 (JF900497), VyDHN2 (JQ408442), VyDHN3 (JQ408443), VvDHN4 (JQ408444)

revealed that six of the pears' DHNs were more closely related to apple and loquat, whereas PpDHN7 was closely related to VyDHN1 and 4.

Expression profile of pear *DHN* genes in different tissues

Quantitative RT-PCR was used to estimate the transcript abundance of the seven *PpDHN* genes in seven organs and tissues (floral buds, leaf buds, roots, bark, young leaves, mature leaves, and shoot tips) under normal annual growing conditions. Three genes (*PpDHN3*, *6*, and *7*) were expressed in all tissues (Fig. 4). Expression of three genes (*PpDHN1*, *2*, and *4*) was not detected in young leaves and shoot tips, and expression of *PpDHN5* was not detected in root tissue (Fig. 4). The transcript level of *PpDHN1*, *2, 3*, and *4* was higher in floral buds, roots, leaf buds, and bark than in young and mature leaves and shoot tips. However, pear *DHN* genes of the same type were preferentially expressed in the same tissues under normal growth conditions. For example, *PpDHN1*, *2*, and *4* (all of the Y_nSK_n type) showed similar tissue expression patterns and were preferentially expressed in roots, bark, leaf buds, and floral buds, whereas few transcripts accumulated in mature leaves. The *PpDHN5* transcript level was higher in floral buds, leaf buds, and bark than that in other tissues. The transcript level of *PpDHN6* was high in bark, shoot tips, and young and mature leaves, whereas the transcript level of *PpDHN7* was higher in root, shoot tips, and young and mature leaves than in other tissues (Fig. 4).

Differential expression pattern of pear *DHN* genes in response to low temperature

To determine whether *DHN* genes are responsive to low temperature in pear floral buds, we used Q-PCR to examine the *PpDHN* transcript abundance in response to treatment for 0, 2, 4, 8, 12, 24, or 48 h at 4 °C (Fig. 5). The responses among the *DHN* genes to low-temperature treatment were different. The transcript level of four genes (*PpDHN3*, *4*, *6*, and *7*) was upregulated by low-temperature treatment, whereas the transcript level of three genes (*PpDHN1*, *2*, and *5*) remained constant throughout the low-temperature treatment (Fig. 5). *PpDHN3* transcription was induced by low-temperature treatment for less than 8 h, then increased thereafter and peak at 48 h. Transcription of *PpDHN4*, *6*, and *7* was dramatically induced by low-temperature treatment for 4, 8, and 12 h, respectively, and peaked at 48 h. Abundance of *PpDHN7* transcripts was higher than that of the other six *DHN* genes during low-temperature treatment.

Discussion

DHNs are believed to play an essential role in the response of plants to various stresses, such as cold and drought. *DHN* genes comprise a multigene family with 10 members in *Arabidopsis* (Bies-Etheve et al. 2008; Hundertmark and Hincha 2008), eight members in rice (Wang et al. 2007), 13 members in barley (Tommasini et al. 2008), 11 members in poplar (Liu et al. 2012a, b), nine members in apple (Liang et al. 2012), seven members in loquat (Xu et al. 2014), and

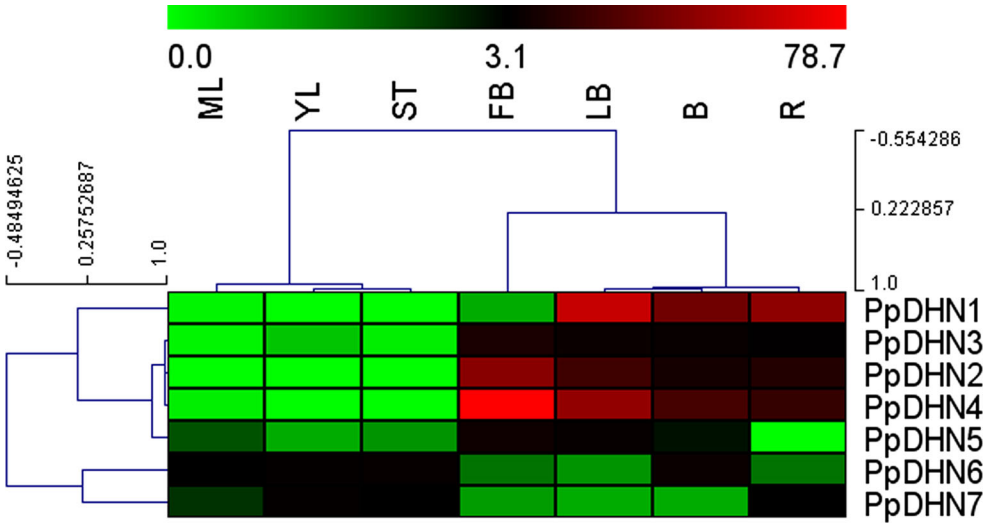

Fig. 4 Hierarchical cluster analysis of the log2 transcript levels of seven *DHN* genes in different tissues of pear. Cluster analysis was performed using Multiple Experiment Viewer (MeV v.4.9.0); Pearson correlation, and average linkage clustering. The *color scale* above each tissue type represents log2 transcript abundance. The brightness is directly proportional to the transcription ratio; *red* represents high levels of transcription and *green* represents low levels of transcription. *FB* floral buds, *R* root, *LB* leaf buds, *B* bark, *ML* mature leaves, *YL* young leaves, *ST* shoot tips

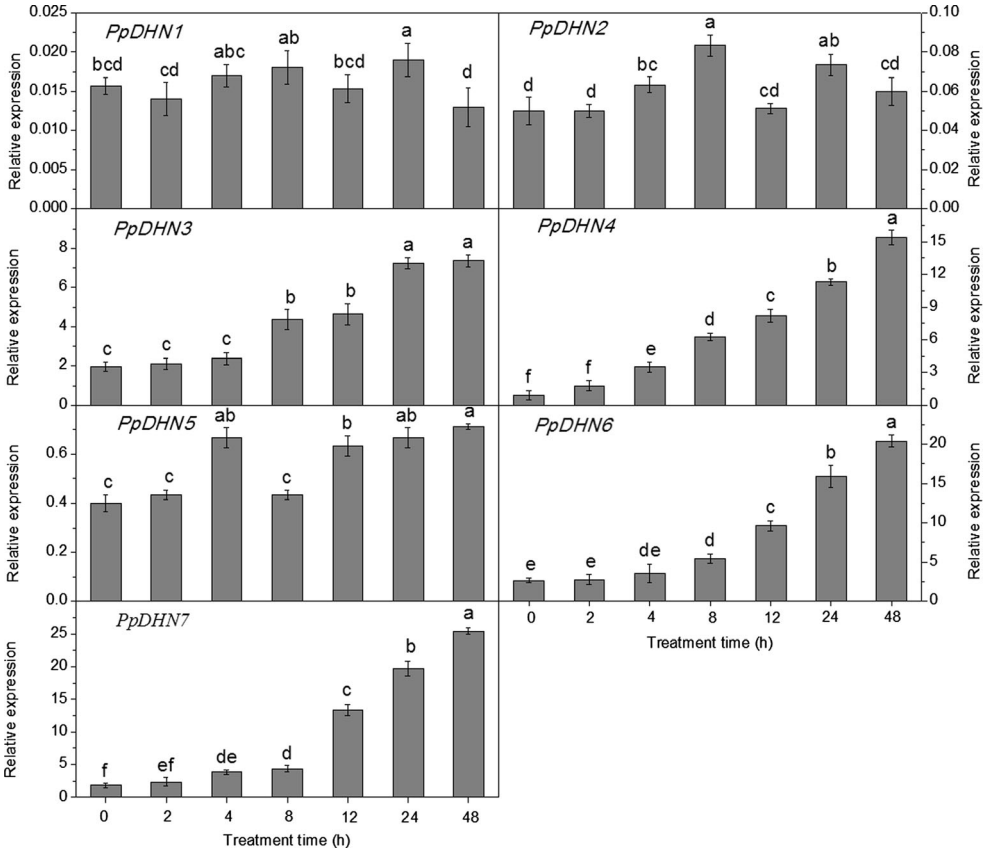

Fig. 5 Relative transcript abundance of pear *DHNs* in floral buds under low temperature (4 °C) treatment. The value for each sample is the mean of three technical replicates, and the *vertical bars* indicate the ±standard error

four members in grape (Yang et al. 2012). In the previous study of *P. pyrifolia* (white pear group) genome sequence, only one *DHN* gene was identified but at that time its conserved segments were unknown (Liu et al. 2012a, b). Based on the presence of a conserved K-segment, we identified seven *PpDHN* genes out of 42,925 genes in the Asian pear genome sequences. With regard to the generally accepted DHN classification (Close 1996; Rorat 2006), the seven pear DHNs were assigned to four of the five subgroups and consisted of four Y_nSK_n-type DHNs (*PpDHN1*, *2*, *4*, and *5*) and one each of SK_n, YK_n, and K_n-type DHNs (*PpDHN3*, *6*, and *7*), respectively (Fig. 1). In pear, the KS-type DHNs were not identified, which differs from the dehydrin gene families from other plant species described to date and suggests that KS-type genes may have been lost in pear. DHN proteins have been classified in five subgroups based on the presence and arrangement of different conserved motifs in a single protein, namely Y_nSK_n, K_n, SK_n, K_nS, and YK_n (Allagulova et al. 2003; Rorat 2006). In addition, some plant species lack the K_n and KS-type DHNs (Liang et al. 2012; Yang et al. 2012; Xu et al. 2014). The number of DHN members in pear is comparable to the seven known in loquat, nine in apple, and four in grape; thus, family sizes are similar among these species. Therefore, through genome-wide identification and characterization, we enhanced and completed the information related to these annotated pear genes for future investigations.

An unrooted tree was generated based on the complete protein sequences of all seven *DHN* genes in pear. The pear *DHN* genes were resolved into four different clans (Fig. 2a). The four different clans including (Type I, Type II, Type III, and Type IV), which reflect evolutionary divergence, generally correspond to subgroups identified by motif analysis. It has been reported previously that *DHNs* can be divided into four classes/types based on their motif analysis (Liang et al. 2012; Liu et al. 2012a, b). To gain additional insights into the structural evolution of the seven pear DHNs, the exon and intron structure was investigated in the present study (Fig. 2b). All Y_nSK_n, SK_n, and K_n types of pear DHNs contained two exons and one intron, whereas the YK_n-type DHN contained only one exon. In a previous study, two exons and one intron were identified in Y_nSK_n and SK_n-type DHNs and one exon was identified in a YK_n-type DHN in apple (Liang et al. 2012).

According to phylogenetic analysis, the PpDHN3 should be placed in group IV, which contains all SK_n-type DHNs, such as MdDHN8 and 9, EjDHN2 and 5, and VyDHN2 and 3 (Fig. 3). The acidic dehydrins from loquat (EjDHN2 and 5), apple (MdDHN8), and grape (VyDHN2 and 3) are actively expressed during low-temperature treatment, whereas the abundance of MdDHN9 transcripts is stable in chilled seedlings of apple (Liang et al. 2012; Yang et al. 2012; Xu et al. 2014). The expression patterns of PpDHN3, 4, 6, and 7 agree with those predicted by their structural classification. DHN

proteins with similar physiochemical properties often also show similar expression patterns. Alkaline and neutral DHNs, such as Y_nSK_n DHNs, are usually responsive to ABA and drought, whereas acidic DHNs, such as K_n, YK_n, and SK_n DHNs, are preferentially provoked by low temperature (Rorat 2006). Genes encoding alkaline and neutral Y_nSK_n-type DHNs, such as EjDHN1; MdDHN1, 2, and 4; and VyDHN1 and 4, are generally induced by both embryogenesis and different types of stresses (Liang et al. 2012; Yang et al. 2012; Xu et al. 2014). The genes encoding proteins with acidic properties, such as Sk_n-, YK_n-, and K_n-type DHNs (EjDHN2, 3, 4, 5, 6, and 7; MdDHN3, 5, 6, 7, 8, and 9; and VyDHN2 and 3) are expressed continuously in vegetative tissues and also upregulated by different stresses (Liang et al. 2012; Yang et al. 2012; Xu et al. 2014).

In plants, accumulation of *DHN* family members is implicated to coincide with tolerance of freezing and low temperature (Dhanaraj et al. 2005; Welling and Palva 2006; Kosova et al. 2007). Characterization and expression pattern analysis of the *PpDHN* genes suggest that they play a positive role in tolerance to environmental stresses. Recently, different DHNs with similar structure have been examined in different field crops, herbaceous and woody plants (Arora and Wisniewski 1994; Wisniewski et al. 1996, 2006; Karlson et al. 2003; Lee et al. 2005; Hinniger et al. 2006; Bies-Etheve et al. 2008; Pulla et al. 2008; Yakovlev et al. 2008; Liang et al. 2012; Perdiguero et al. 2012; Velasco-Conde et al. 2012; Yang et al. 2012; Trygve Devold Kjellsen et al. 2013; Yamasaki et al. 2013; Lim et al. 2014; Falavigna et al. 2015). Pear is among the most economically important fruit crops worldwide, and a variety of environmental stresses severely impact fruit production and quality. Therefore, we performed genome-wide identification of *DHN* genes in pear, and obtained evidence for the gene evolutionary histories and expression patterns under low-temperature treatment. Previous studies have confirmed that *DHN* transcripts accumulate not only during seed desiccation and in response to water deficit induced by drought, low temperature, or salinity, but are also present in almost all vegetative tissues under optimal growth conditions (Rorat 2006; Kosova et al. 2007; Tunnacliffe et al. 2010). We used hierarchical cluster analysis to examine the expression patterns of pear *DHN* genes in different tissues under normal growth conditions. Almost all pear *DHN* genes were expressed in all vegetative tissues except young leaves and shoot tips, in which *PpDHN1*, *2*, and *4* were not expressed (Fig. 4). Similar results have been reported previously (Liang et al. 2012; Liu et al. 2012a, b). These authors observed the expression of poplar and apple *DHN* genes in all tissues analyzed except mature leaves and flowers under normal growing conditions. Furthermore, in the present study, hierarchical cluster analysis indicated that the Y_nSK_n-type pear *DHN* genes showed similar expression patterns (Fig. 4). These results are similar to previous findings by Rorat (2006), Battaglia et al. (2008), and Liu et al. (2012a, b)), who showed that different types of *DHN* proteins can localize to the same tissues during development under normal growth conditions.

It has been proposed that different types of *DHN* proteins are involved in responses to diverse growth conditions. The transcript abundance of all acidic pear *DHN* genes, such as *PpDHN3*, *6*, and *7*, increased dramatically after 4, 8, and 12 h of low-temperature treatment, respectively. The magnitude of the increase during 48 h of treatment was greater for *PpDHN7* than that of the other *PpDHN* genes (Fig. 5). It has been reported previously that K_n-type *DHN* genes are directly involved in cold acclimation (Choi et al. 1999; Dhanaraj et al. 2005). The transcript levels of *PpDHN7* were markedly higher than those of the other *PpDHN* genes, followed by *PpDHN6* (Fig. 5). Therefore, it is suggested that *PpDHN7* and *PpDHN6* may be more important than the other *DHN* genes for resistance to low temperature in pear floral buds. Xu et al. (2014) also observed that a K_n-type *DHN* (*EjDHN4*) is much more responsive to freezing than any other type of *DHN* gene. In the present study, the transcript abundance of all acidic-type *DHN* genes and one basic *DHN* gene (*PpDHN4)* was increased in response to low temperature. Similar results have been reported for acidic-type *DHN* genes in barley, *Arabidopsis*, apple, and loquat that were actively expressed during low-temperature treatment (Gilmour et al. 1992; Koster and Lynch 1992; Choi et al. 1999; Liang et al. 2012; Xu et al. 2014). In apple and loquat, transcripts of basic *DHN* genes (*MdDHN2* and *4*, *EjDHN2* and *3*) were increased under low-temperature stress (Liang et al. 2012; Xu et al. 2014).

In conclusion, extensive research effort has been devoted to characterization of *DHN* genes in herbaceous and woody plants, such as *Arabidopsis*, barley, rice, apple, poplar, loquat, grape, and peach, etc., but such attention has not been extended previously to Asian pear trees. In the present study, we addressed this knowledge gap by undertaking a genome-wide identification, characterization, and expression analysis of *DHN* genes in pear. We identified seven *DHN* genes, all of which contained the conserved K domains in the sequences. The seven pear *DHN* genes were phylogenetically clustered into four groups (SK_n, Y_nSK_n, YK_n, and K_n). Discrimination of four groups was mutually supported on the basis of exon/intron structure. In addition, transcription of the pear *DHN* genes was analyzed in seven vegetative and reproductive tissues. These data provide valuable information for understanding the classification, cloning, and putative functions of the *DHN* gene family. Three of the *DHN* genes were not expressed in young leaves and shoot tips under normal growth conditions, whereas expressions of three DHN genes were not affected by low-temperature treatment. The expression information reported in this study will be useful for further investigations of the functions of the *DHN* genes under different stress conditions.

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Conflict of interest The authors declare that they have no competing interests.

Data archiving statement The pear *DHN* gene family sequences have been submitted to National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) and the accession numbers been shown in Table 2.

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