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Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress

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Abstract

Background: Dehydrins (DHNs) protect plant cells from desiccation damage during environmental stress, and also participate in host resistance to various pathogens. In this study, we aimed to identify and characterize the *DHN* gene families from *Vitis vinifera* and wild *V. yeshanensis*, which is tolerant to both drought and cold, and moderately resistant to powdery mildew.

Results: Four *DHN* genes were identified in both *V. vinifera* and *V. yeshanensis*, which shared a high sequence identity between the two species but little homology between the genes themselves. These genes were designated *DHN1*, *DHN2*, *DHN3* and *DHN4*. All four of the DHN proteins were highly hydrophilic and were predicted to be intrinsically disordered, but they differed in their isoelectric points, kinase selectivities and number of functional motifs. Also, the expression profiles of each gene differed appreciably from one another. Grapevine *DHN1* was not expressed in vegetative tissues under normal growth conditions, but was induced by drought, cold, heat, embryogenesis, as well as the application of abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MeJA). It was expressed earlier in *V. yeshanensis* under drought conditions than in *V. vinifera*, and also exhibited a second round of up-regulation in *V. yeshanensis* following inoculation with *Erysiphe necator*, which was not apparent in *V. vinifera*. Like *DHN1*, *DHN2* was induced by cold, heat, embryogenesis and ABA; however, it exhibited no responsiveness to drought, *E. necator* infection, SA or MeJA, and was also expressed constitutively in vegetative tissues under normal growth conditions. Conversely, *DHN3* was only expressed during seed development at extremely low levels, and *DHN4* was expressed specifically during late embryogenesis. Neither *DHN3* nor *DHN4* exhibited responsiveness to any of the treatments carried out in this study. Interestingly, the presence of particular *cis*-elements within the promoter regions of each gene was positively correlated with their expression profiles.

Conclusions: The grapevine *DHN* family comprises four divergent members. While it is likely that their functions overlap to some extent, it seems that *DHN1* provides the main stress-responsive function. In addition, our results suggest a close relationship between expression patterns, physicochemical properties, and *cis*-regulatory elements in the promoter regions of the *DHN* genes.

Keywords: Grapevine, Dehydrin, Stress-induced expression, Powdery mildew, Promoter

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Background

Dehydrins (DHNs) are a class of hydrophilic, thermostable stress proteins with a high number of charged amino acids that belong to the Group II Late Embryogenesis Abundant (LEA) family. Genes that encode these proteins are expressed during late embryogenesis, as well as in vegetative tissues subjected to drought, low temperature and high salt conditions [1-3]. Intriguingly, over-expression of *DHN* genes in transgenic plants has been found to enhance resistance of the transgenic lines to various adverse environments, such as cold, drought, salinity and osmotic stress [4-7], which has raised significant interest in their putative application for crop improvement. Furthermore, it has recently been shown that reduced levels of dehydrins in transgenic Arabidopsis seeds leads to reduced seed longevity [8], emphasizing their importance to seed survival in addition to their influence on vegetative stress tolerance.

While it is generally accepted that DHNs function to protect cells from damage caused by stress-induced dehydration [9], their precise mechanism remains elusive. However, it has been proposed that they may carry out their function through membrane stabilization by acting as chaperones to prevent the aggregation and/or inactivation of proteins under dehydration or high temperature conditions [5,10,11].

Classification of DHNs is based upon structural features of the proteins, such as the presence and copy number of certain conserved motifs, such as the K-, S-, and Y-segments. To date, these proteins have been divided into 5 subclasses, including Y_nSK_n, Y_nK_n, SK_n, K_n and K_nS [12]. All DHNs possess at least one K-segment (EKKGIMDKIKEKLPG), which is generally located at the C-terminal end of the protein and has the ability to form an amphipathic helix-like structure that may play a role in its interaction with membranes and proteins [13,14]. The S-segment consists of a track of serines that can be modified through phosphorylation and may function in the regulation of protein conformation and ion-binding activity [15-17]. The Y-segment (DEYGNP) is located near the N-terminus and shows partial amino acid identity to the nucleotide binding site motif of chaperone proteins from various organisms [12].

Several other conserved regions have also been identified in a subset of DHNs. For example, lysine-rich segments (Lys-segments) contain a cluster of lysines that are generally located between the S- and K-segments [18,19] and have been suggested to participate in the binding of DHNs to DNA or RNA [19]. Nuclear localization signals (NLSs), which bear an RRKK motif, have been found specifically in YSK_n-type DHNs and play a role in their localization to the nucleus [15,16,20]. Furthermore, phosphorylation has been found to be an important factor for substrate binding of DHNs

[16,17,21], and recently a His switch has been found to be involved in the regulation of membrane binding of the *Arabidopsis thaliana* DHN, LTI30 [22].

At the functional level, *DHN* family members often exhibit sub-functionalization, with different genes displaying differential expression profiling throughout development and under stress conditions. For example, while both *LTI29* (SK_2) and *LTI30* (K_6) were up-regulated in Arabidopsis under low temperature conditions, only *LTI30* was up-regulated following salt treatments [1]. Similarly, while ten barley *DHN*s were found to be up-regulated by drought, only three were up-regulated by low temperatures [23], and in *Oleae europaea*, although expression levels of 40 kDa and 42 kDa *DHN*s increased in response to various stressors (including dehydration, high salinity, and wounding), 16 kDa and 18 kDa *DHN*s were mainly induced by salt stress [24]. These differences in expression patterns imply functional diversification within this gene family; however, at present, the relationship between subgroup classification and expression profile is unclear [9].

Grapevine is one of the most important fruit crops in the world and while the majority of grape varieties are directly cultivated from *Vitis vinifera* L., this species is relatively susceptible to powdery mildew (*Erysiphe necator*). Conversely, *V. yeshanensis* is a wild species of grape native to the Yanshan mountain in Hebei province, China, that is highly tolerant to both cold and drought [25,26], and is also resistant to *E. necator* [27]. Previously, two highly similar putative Y_2SK_2-type *DHN* genes (*DHN1a* and *DHN1b*) were identified in *V. vinifera* and their expression was found to be induced by multiple types of stress, such as drought, cold and high salinity [28,29]. In this study, we aimed to identify the members of *DHN* gene family in *V. vinifera*, as well as their homologous equivalents in *V. yeshanensis*. In doing so, we were able to investigate the functional divergence of this gene family in these two species through comparisons of their expression profiles and putative physicochemical characteristics. Furthermore, we also assessed possible relationships between specific *cis*-elements within *DHN* promoter sequences and the regulation of their expression under various conditions.

Results

Identification of *DHN* family members in *V. vinifera* and *V. yeshanensis*

A 280-bp fragment of a *DHN* cDNA was cloned from drought-treated leaves of *V. yeshanensis* acc. Yanshan-1 using differential display reverse transcription-PCR (DDRT-PCR; Additional file 1). Subsequently, the full-length sequence was determined using 5' rapid amplification of cDNA ends (RACE) and was termed *VyDHN1* [GenBank:JF900497]. The putative protein sequence of

this gene was then utilized to detect *DHN* genes from the published *V. vinifera* cv. Pinot Noir clone PN40024 genome sequence [30] via BLAST analysis. Four *DHN* genes were identified, all of which contained a K-segment. These genes were designated *VvDHN1* (corresponding in sequence to the previously identified *V. vinifera DHN1a*) [GenBank:XM_03631828], *VvDHN2* [GenBank:XM_002285883], *VvDHN3* [GenBank:CAN73166], and *VvDHN4* [GenBank:XM_002283569].

The three remaining *DHN* genes were cloned from *V. yeshanensis* acc. Yanshan-1 seed-specific cDNA using primers derived from the *V. vinifera* sequences and were designated *VyDHN2* [GenBank:JQ408442], *VyDHN3* [GenBank:JQ408443], and *VyDHN4* [GenBank:JQ408444]. While only 25 amplification cycles were required to clone both *VyDHN2* and *VyDHN4*, 40 cycles were required in the case of *VyDHN3*. Subsequently, all four *V. yeshanensis* genes were amplified from genomic DNA to identify intronic regions [GenBank:JF896520, JF896556, JF896557, and JF896558, respectively]. In both species, all four *DHN* genes consisted of two exons separated by one intron present within the S-segment.

In terms of nucleotide similarities, virtually no sequence identity was detected between the four *DHN* coding sequences. However, high levels of homology were noted between matching genes belonging to the two different species, with 97% (*DHN4*) and 99% (*DHN1*, *DHN2* and *DHN3*, respectively) identity at the nucleotide level. In terms of chromosomal localization, while *VvDHN1* and *VvDHN2* were located on chromosomes 4 and 18, respectively, *VvDHN3* and *VvDHN4* were mapped to chromosome 3 in opposite orientations (Figure 1).

Characterization and comparison of deduced DHN proteins

Protein sequences were deduced from the corresponding *V. vinifera* and *V. yeshanensis DHN* cDNA sequences, and were composed of 130–206 amino acids exhibiting 97-99% identity at the amino acid level between the two species. Both K- and S-segments were found to be highly conserved between members of the *V. vinifera* and *V. yeshanensis* DHN families, whereas remaining regions displayed relatively low amino acid identity between the four genes. Furthermore, while NLS domains were identified in both DHN1 and DHN4 proteins, a Lys-rich segment was only present in DHN2 (Figure 2). Based on the presence and number of K-, S- and Y-motifs (Figure 2), the four DHNs from each species were classified as either Y_2SK_2- (DHN1), SK_2- (DHN2), SK_3- (DHN3), or Y_3SK_2-type (DHN4) proteins (Figure 2; Table 1).

All members of the DHN family in the two grapevine species analyzed were found to be highly hydrophilic, with GRAVY values ranging from –0.959 to –1.527 and

theoretical pIs from 5.20 to 9.36 (Table 1). DHN1 and DHN4, which were Y_nSK_n-type DHNs, possessed a higher pI than the SK_n-type DHNs (DHN2 and DHN3) in both species. In terms of acidity, our analyses indicated that DHN1 was the sole basic protein, while DHN2 was the most acidic.

Many phosphorylation sites were also predicted within each of the DHN protein sequences analyzed, with DHN1 and DHN4 containing a higher number of putative protein kinase C (PKC) phosphorylation sites than casein kinase 2 (CK2) phosphorylation sites, and DHN2 and DHN3 containing a higher number of CK2 sites than PKC sites (Table 1; Figure 2). In addition, a recently identified conserved motif (LXRXXS) phosphorylated by an Snf1-related kinase (SnRK2-10) [31] was identified in both DHN1 and DHN2 proteins.
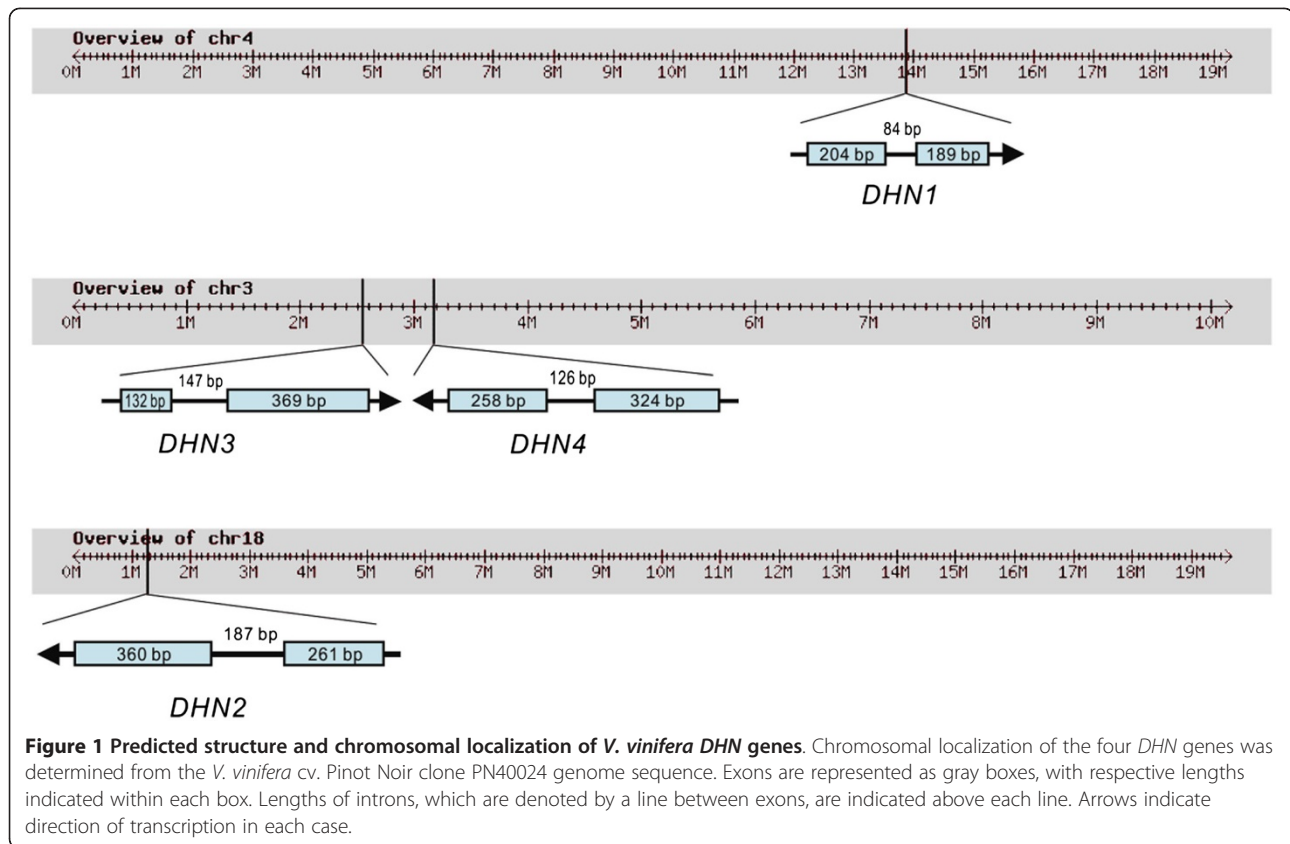
While all four grapevine DHNs were found to be rich in disordered regions and contained relatively few helix or strand motifs (see Additional file 2), the Y_nSK_n-type proteins (DHN1 and DHN4) displayed the highest disorder index and least helix/strand-motifs. Additionally, of the few helix motifs identified, most were located within K-segments, which is consistent with the findings of a previous study of DHN protein structure [14].

Phylogenetic analysis of *V. vinifera* and *V. yeshanensis* DHNs

To date, DHN families from both barley and Arabidopsis have been thoroughly characterized at the genomic level [18,32-35]. Therefore, to provide a further understanding of the relationships between the *V. vinifera* and *V. yeshanensis* DHNs, we conducted a phylogenetic analysis of these genes via comparison with those from barley and Arabidopsis. Overall, we found the number of *DHN* genes in the grapevine species (four) to be smaller than that in either barley (thirteen) or Arabidopsis (ten). Based on our phylogenetic results, the DHNs could be divided into four groups, corresponding to Y_nSK_n-, SK_n-, K_n-, and KS-type proteins (Figure 3), where the Arabidopsis YK-type DHN (At4g39130) was included within the Y_nSK_n group. As expected from our classification of the grapevine DHN sequences based on the presence of various conserved segments, the grapevine DHN1 and DHN4 proteins were grouped together with the Y_nSK_n-type DHNs of Arabidopsis and barley, while the grapevine DHN2 and DHN3 proteins were grouped with the SK_n-type DHNs of Arabidopsis and barley. Interestingly, both grapevine species lacked KS- and K_n-type DHNs; groups which are present in both barley and Arabidopsis.

Expression profiles of *DHN* transcripts in various tissues and developmental stages

To elucidate the physiological functions of different members of the *DHN* family in *V. vinifera* and *V.*

Figure 1 Predicted structure and chromosomal localization of *V. vinifera DHN* genes. Chromosomal localization of the four *DHN* genes was determined from the *V. vinifera* cv. Pinot Noir clone PN40024 genome sequence. Exons are represented as gray boxes, with respective lengths indicated within each box. Lengths of introns, which are denoted by a line between exons, are indicated above each line. Arrows indicate direction of transcription in each case.

yeshanensis, the expression of *DHN* genes was investigated at veraison in roots, stems, leaves, seeds, and fruit peels using semi-quantitative RT-PCR (Figure 4). Both species exhibited highly similar expression profiles for their matching *DHN* genes. Results suggested that under normal growth conditions, *DHN1* was mainly expressed in seeds, with very low levels present in the roots. *DHN2* was constitutively expressed in all tissues; however, weaker levels of expression were noted in leaves and stems than in the other organs tested. *DHN3* was undetectable under the current experimental conditions, suggesting that it was not expressed, or was at levels too low to be detected using this method, in these tissues. The *DHN4* genes, on the other hand, were expressed specifically in seeds. These results indicate that the four *DHN* genes that make up the *V. vinifera* and *V. yeshanensis DHN* gene families, respectively, exhibited very distinct expression patterns in the organs tested.

Intriguingly, reactions in which *DHN1*, *DHN2* and *DHN4* were amplified often exhibited two bands: one that corresponded in size to a spliced transcript, and one that seemed to be closer in size to the band amplified from genomic DNA (ie. containing an unspliced intron). The fact that no amplification products were obtained in negative control reactions lacking reverse transcriptase confirmed that template RNA was free from

contaminating genomic DNA. Therefore, it seems that these larger transcript variants resulted from the presence of unspliced *DHN* transcript variants (either mRNA or pre-mRNA) within the total RNA pool.

To gain a more precise understanding of grapevine *DHN* expression during the process of seed development, inflorescences and berries of *V. vinifera* were harvested from flowering to veraison and used for qRT-PCR expression analyses. *DHN1* and *DHN2* were found to be expressed in floral buds even 6 d before opening of the flowers (Figure 5A and B), after which time their transcripts decreased during the middle stages of embryogenesis, and were strongly up-regulated once again during later stages of embryogenesis. Low levels of *DHN3* were detected in both mid- and late-stages of seed development (Figure 5C) while *DHN4* transcripts were only detectable during late stages of embryogenesis, with peak expression developing just prior to veraison (Figure 5D).

Response of *DHN* gene expression to various abiotic and biotic stresses

In an attempt to determine whether *DHN1*, *DHN2*, *DHN3* and *DHN4* exhibited stress-responsiveness, we analyzed the expression levels of all four genes in the leaves of three *V. vinifera* and *V. yeshanensis* plants,
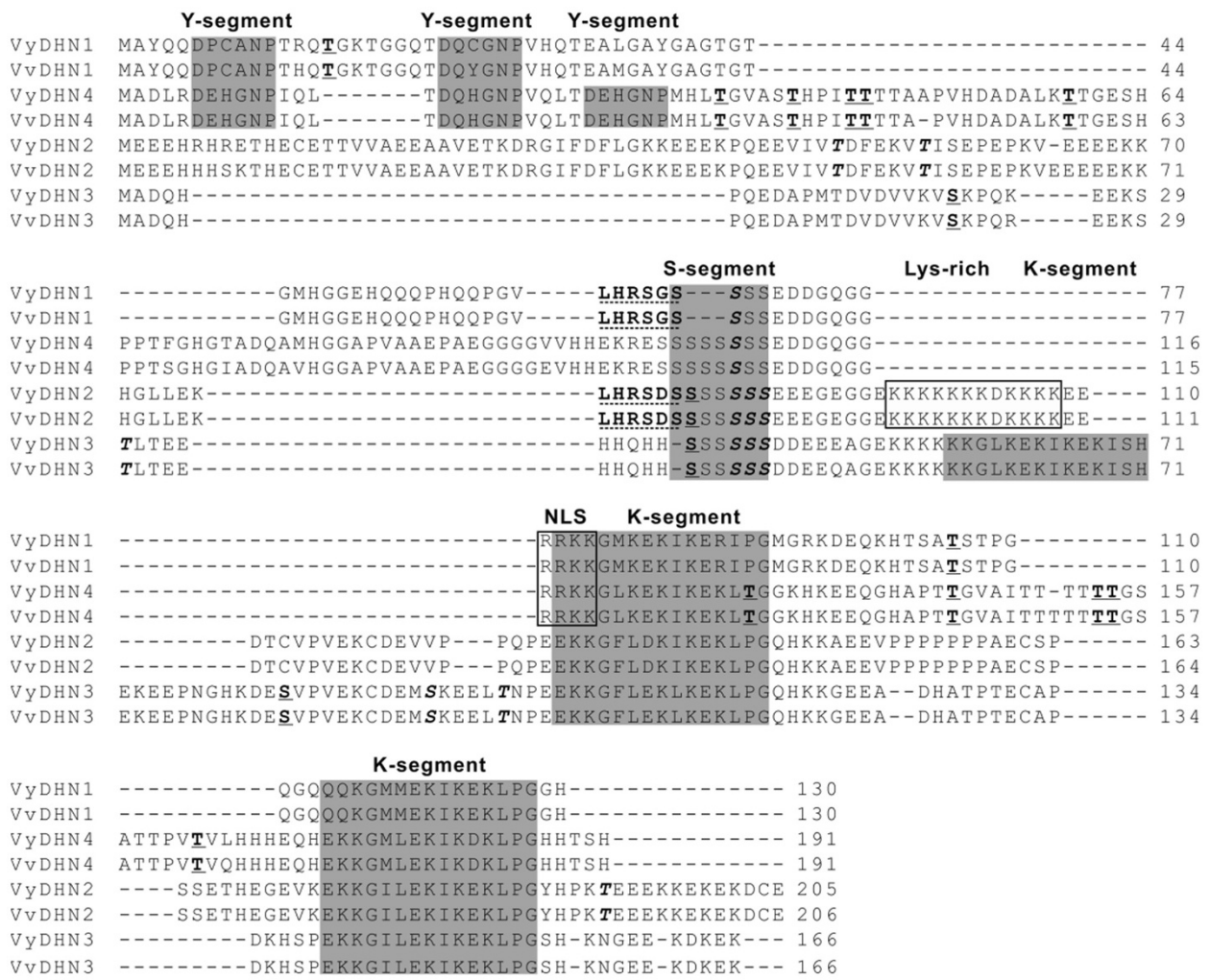
Figure 2 Sequence alignment of DHN proteins from *V. yeshanensis* and *V. vinifera*. Y-segments, S-segments and K-segments are denoted by gray shading. NLS and Lys-rich segments are framed by a black line. Phosphorylation sites are in bold, with PKC sites underlined with a single line, CK2 sites in italics, and SnRK-10 sites underlined with a dotted line.

respectively, that had been subjected to various stress conditions using real-time qRT-PCR. Neither *DHN3* nor *DHN4* from either species exhibited detectable levels of expression under any of the conditions tested here, including drought, cold, heat, or *E. necator* infection; therefore, only *DHN1* and *DHN2* will be discussed further in

Table 1 Characteristics of DHN proteins in *V. yeshanensis* and *V. vinifera*

Name	Type	No. of Residues	MW (kDa)	pI	GRAVY	PKC No	CK2 No	SnRK2 No	Expression
VyDHN1	Y_2SK_2	130	13.9	9.36	−1.425	2	1	1	Stress + Seed
VvDHN1	Y_2SK_2	130	13.9	9.27	−1.459	2	1	1	
VyDHN2	SK_2	205	23.4	5.21	−1.527	1	6	1	Constitutive + Stress + Seed
VvDHN2	SK_2	206	23.5	5.20	−1.514	1	6	1	
VyDHN3	SK_3	166	18.8	5.81	−1.736	3	6	0	Seed (weak)
VvDHN3	SK_3	166	18.9	5.92	−1.739	3	6	0	
VyDHN4	Y_3SK_2	191	20.1	6.35	−0.959	10	1	0	Seed
VvDHN4	Y_3SK_2	191	20.1	6.26	−1.030	10	1	0	

MW (molecular weight), pI (isoelectric point) and GRAVY (grand average of hydropathy) were predicted based on amino acid composition. PKC, CK2 and SnRK2 refer to specific phosphorylation sites. Expression information is based on the RT-PCR experiments described in Figures 4 through 7.
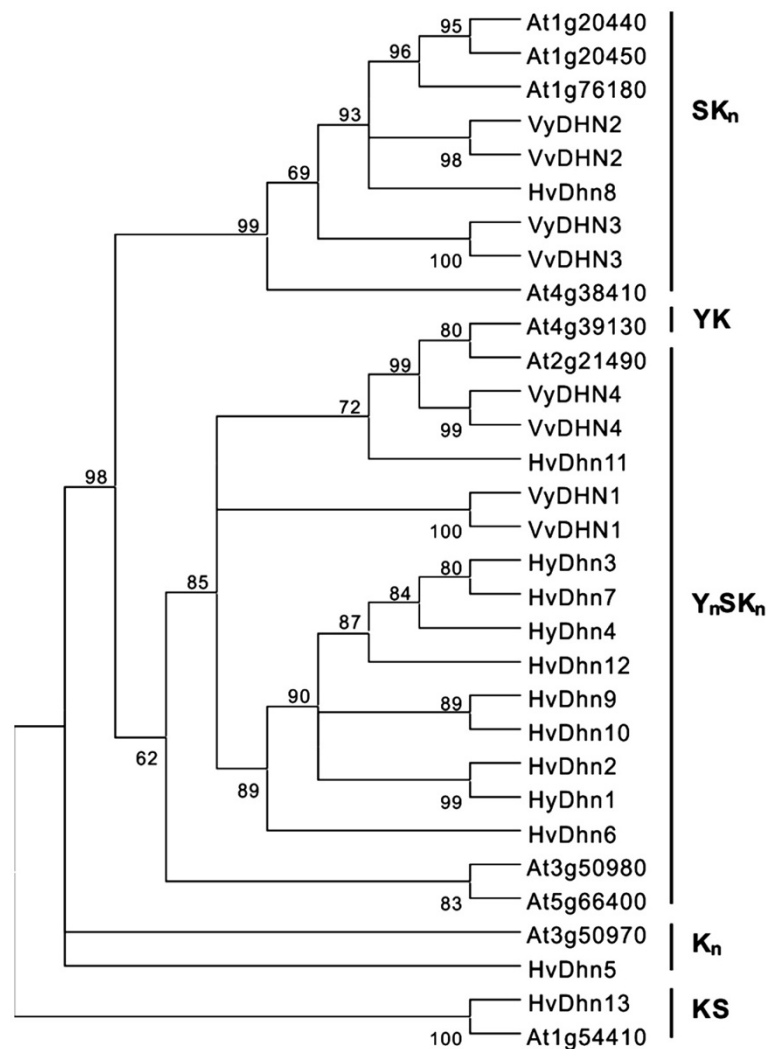
Figure 3 Phylogenetic relationships between grapevine, barley and Arabidopsis DHN proteins. The unrooted dendrogram was constructed with the PhyML tool using the maximum likelihood method based on a complete protein sequence alignment of DHNs from *Arabidopsis thaliana* (At), *Hordeum vulgare* (Hv), *V. vinifera* (Vv), and *V. yeshanensis* (Vy). The bootstrap value is given for each node. GenBank accession numbers are as follows: VvDHN1 [XM_03631828], VvDHN2 [XM_002285883], VvDHN3 [CAN73166], VvDHN4 [XM_002283569], VyDHN1 [JF900497], VyDHN2 [JQ408442], VyDHN3 [JQ408443], VyDHN4 [JQ408444], At1g20440 [AY114699], At1g20450 [AF360351], At1g54410 [NM_104319], At1g76180 [AF339722], At2g21490 [BT000900], At3g50970 [NM_114957], At3g50980 [NM_114958], At4g38410 [NM_120003], At4g39130 [NM_120073], At5g66400 [AY093779], HvDhn1 [AF043087], HvDhn2 [AF181452], HvDhn3 [AF181453], HvDhn4 [AF181454], HvDhn5 [AF181455], HvDhn6 [AF181456], HvDhn7 [AF181457], HvDhn8 [AF181458], HvDhn9 [AF181459], HvDhn10 [AF181460], HvDhn11 [AF043086], HvDhn12 [AF155129], HvDhn13 [AY681974].

this section. In the case of drought conditions, although we found that *DHN1* was induced by this treatment in both species (Figure 6A), we noted slight differences between the two species. While *DHN1* transcripts increased in *V. yeshanensis* between 1–2 d after the drought treatment began and reached a maximum induction of a 237-fold increase compared to baseline expression levels 5 d after treatment, its homologue in *V. vinifera* was delayed in its exhibition of a response (between 2–3 d) and reached a higher maximum induction of 366-fold compared to baseline levels. Conversely, *DHN2* did not appear

to respond to drought treatment in either of the two species tested (Figure 6B). Interestingly, transcripts of both *DHN1* and *DHN2* also exhibited up-regulation in both grapevine species shortly after rehydration (Figure 6 A and B), with a maximum level of expression achieved after approximately 2 h.

Following cold and heat treatment, both *DHN1* and *DHN2* were induced in *V. vinifera* and *V. yeshanensis*. While *DHN2* transcripts increased gradually between 0 h and 48 h following initiation of cold stress in both species, *DHN1* transcripts exhibited a more sudden
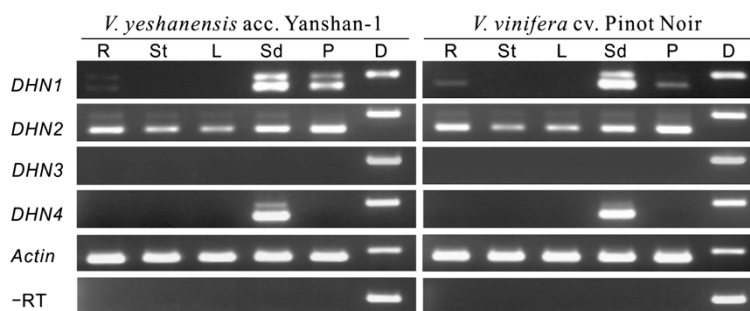
Figure 4 Expression of *DHNs* in various organs of *V. yeshanensis* and *V. vinifera*. Total RNA was isolated from root (R), stem (St), leaf (L), seed (Sd) and fruit peel (P) at veraison, and was quantified using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE, USA). 1 μg DNase-treated total RNA was used as template for first-strand cDNA synthesis in a final volume of 20 μl, and subsequently 1 μl of this reaction was utilized for PCR amplification in a volume of 25 μl. Genomic DNA (D) was utilized as the positive control. RNA without reverse transcriptase was used as the negative control. The grapevine *actin1* fragment was amplified as an internal control. 15 μl of PCR products were separated on a 1.5 % agarose gel containing ethidium bromide in each case.

onset of up-regulation in response to cold beginning between 6–12 h after treatment (Figure 6 C and D). In the case of heat stress, both *DHN1* and *DHN2* transcripts increased to maximum levels 24 hours following the initiation of treatment in both species, and then subsequently decreased (Figure 6 E and F).

To determine whether grapevine *DHNs* were responsive to biotic stress, the levels of *DHN* gene expression were tested in the leaves of *V. vinifera* and *V. yeshanensis* inoculated with *E. necator*, which is the causative agent of grapevine powdery mildew. Results suggested that only *DHN1* was induced by *E. necator*, whereby transcripts increased gradually to a maximum at 3 d post-inoculation (dpi) and then decreased slowly after this point in both species. Interestingly, *V. yeshanensis* exhibited a higher peak level of expression (18-fold increase compared to expression levels immediately prior to treatment) than *V. vinifera* (10-fold increase), as well as an additional sharp increase in *DHN1* expression 6 dpi (Figure 6G). In contrast, *DHN2* did not appear to respond to *E. necator* inoculation in either grapevine species (Figure 6H).

Response of *DHN* gene expression to levels of various signaling molecules
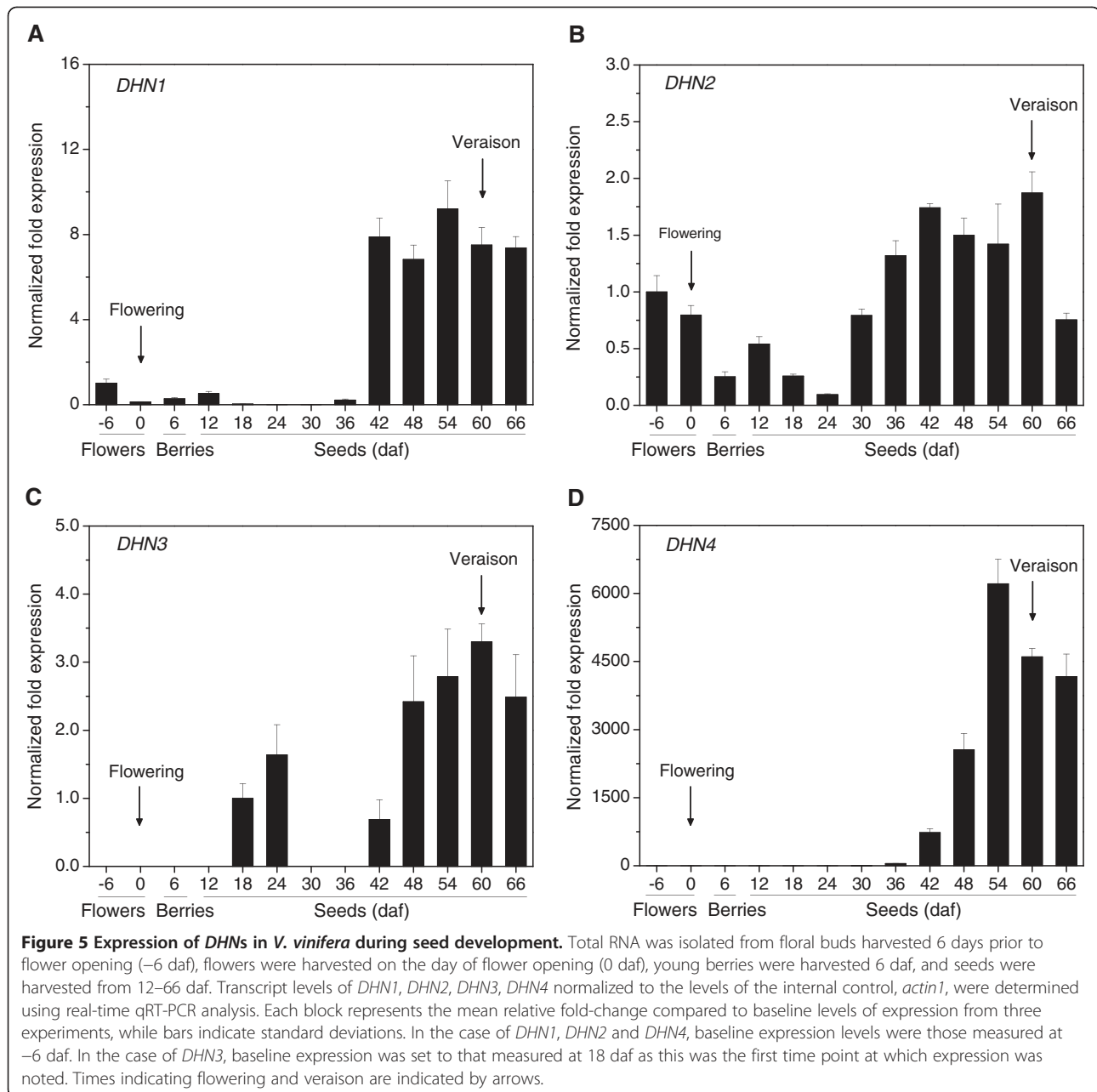
The responses of plants to abiotic and biotic stress are generally mediated by abscisic acid (ABA) and salicylic acid (SA)/jasmonic acid (JA), respectively. To determine whether the induction of grapevine *DHNs* under stress conditions was related to any of these signaling molecules, *DHN* expression was investigated in leaves from three *V. vinifera* plants treated with ABA, SA or MeJA, respectively. As was the case for studies involving abiotic and biotic stress treatment, no detectable levels of *DHN3* or *DHN4* expression could be detected in leaves treated with any of these chemicals. While *DHN1* was induced by ABA, SA and MeJA (Figure 7A), the most

considerable up-regulation (160-fold compared to baseline levels) was noted 8 h following application of ABA. *DHN1* transcripts from leaves treated with MeJA reached a maximum induction of ~20-fold compared to baseline levels approximately 4 h following application, whereas a similar level of induction was reached 8 h following treatment with SA. In the case of *DHN2* transcripts, expression reached a maximum 5-fold induction compared to baseline levels 4 h after application of ABA. Although slight modifications were also noted in *DHN2* expression levels following SA and MeJA applications, respectively, they differed only slightly from changes observed in untreated samples (Figure 7B).

Comparison of *cis*-regulatory elements in the upstream regulatory regions of grapevine *DHN* genes

All four *DHN* promoters (including 1500-bp of sequence upstream of the translational start codon) were cloned from *V. yeshanensis* acc. Yanshan-1 [GenBank: JF899925 for *VyDHN1*, GenBank: JX110839 for *VyDHN2*, GenBank: JX110840 for *VyDHN3*, and GenBank: JX110841 for *VyDHN4*] using primers derived from the corresponding *V. vinifera* sequences. High levels of homology were detected between matching promoters from the two different species, with 94% (*DHN1*), 96% (*DHN2*), and 92% (*DHN4*) identity at the nucleotide level. The promoter of *DHN3*, on the other hand, only exhibited 84% identity between the two species due to a 205-bp deletion in this sequence from *V. yeshanensis*.

To elucidate whether the differential expression patterns of the four grapevine *DHN* genes correlate with transcriptional regulation via their promoters, upstream regions of each gene from *V. yeshanensis* and *V. vinifera* were scanned for putative *cis*-regulatory elements using the PlantCARE database [36]. Nucleotide sequences including 1500-bp upstream of each start codon were obtained from the *V. vinifera* cv. Pinot Noir clone

Figure 5 Expression of *DHN*s in *V. vinifera* during seed development. Total RNA was isolated from floral buds harvested 6 days prior to flower opening (−6 daf), flowers were harvested on the day of flower opening (0 daf), young berries were harvested 6 daf, and seeds were harvested from 12–66 daf. Transcript levels of *DHN1*, *DHN2*, *DHN3*, *DHN4* normalized to the levels of the internal control, *actin1*, were determined using real-time qRT-PCR analysis. Each block represents the mean relative fold-change compared to baseline levels of expression from three experiments, while bars indicate standard deviations. In the case of *DHN1*, *DHN2* and *DHN4*, baseline expression levels were those measured at −6 daf. In the case of *DHN3*, baseline expression was set to that measured at 18 daf as this was the first time point at which expression was noted. Times indicating flowering and veraison are indicated by arrows.

PN40024 genome database [30] , while those from *V. yeshanensis* were obtained directly via cloning, and *cis*-regulatory elements were classified into three groups according to their potential responsive functions: abiotic stress-related elements, biotic stress-related elements, and seed development-related elements. Abiotic stress-related elements comprised ABA-responsive elements (ABRE), dehydration-responsive elements (DRE), heat shock-responsive elements (HSE), and low temperature-responsive elements (LTR). Biotic stress-related elements included MeJA-responsive elements (MeJA-RE), salicylic acid-responsive elements (TCA), as well as stress- and defense-responsive elements (TC-rich repeats). Seed development-related elements comprised only endosperm-specific expression elements (Skn-1 motif).

All *cis*-regulatory elements found in the *V. yeshanensis* and the *V. vinifera DHN* promoters, which exhibited a similar composition and distribution of putative regulatory elements between corresponding promoters, are shown in Table 2. There were obvious differences in the abundance and distribution of *cis*-regulatory elements in the four *DHN* promoters analyzed (Figure 8). The *VyDHN1* and *VvDHN1* promoters had the most diverse collection of putative *cis*-regulatory elements, including
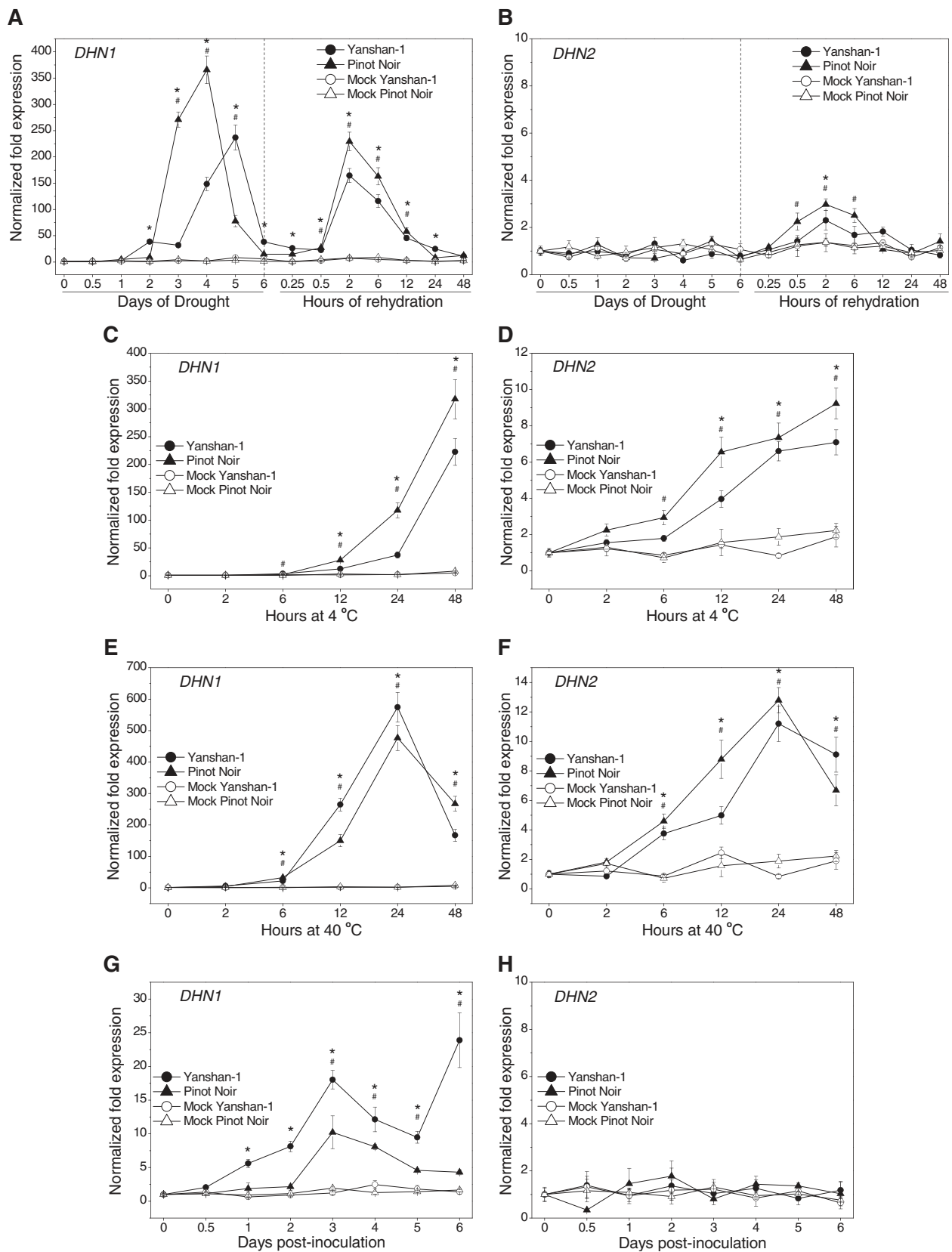
Figure 6 (See legend on next page.)

(See figure on previous page.)
Figure 6 Expression profiles of grapevine *DHN1* and *DHN2* under abiotic and biotic stress. Total RNA was extracted from the leaves of *V. yeshanensis* and *V. vinifera* treated with drought-rehydration (**A**, **B**), 4 ℃ (**C**, **D**), 40 ℃ (**E**, **F**), and inoculation with *E. necator* (**G**, **H**). Samples were taken at the indicated times, with time zero samples harvested immediately prior to treatment. Normalized transcript levels of *DHN1* and *DHN2* were determined by real-time qRT-PCR analysis, with the *actin1* gene serving as an internal control. Each point represents the mean relative fold-change compared to baseline levels of expression from three experiments, while bars indicate standard deviations. Baseline expression levels were those measured just prior to treatment at time = 0. Asterisks (*) and number signs (#) indicate significant increases ($p < 0.05$) in expression levels of target transcripts from *V. yeshanensis* and *V. vinifera*, respectively, compared to the mock-treated controls.

several involved in stress-response, seed development, and hormone signaling (Table 2). In the case of the *VyDHN2* and *VvDHN2* promoters, HSE, LTR and TCA elements were lacking when compared to *VyDHN1* and *VvDHN1*. Only a small number of potential *cis*-elements were identified in the *VyDHN3*, *VvDHN3*, *VyDHN4* and *VvDHN4* promoters. In the *VvDHN3* promoter, an ABRE, Skn-1 and two TC-rich elements were scattered evenly throughout the promoter, while the ABRE element was absent in that of *VyDHN3*. In *VvDHN4*, two ABREs, a Skn-1 and a MeJA-RE were concentrated in the 3' region of the promoter, while a TCA element was present in the 5' region. A similar distribution was noted in the *VyDHN4* promoter, with an additional Skn-1 motif present 765-bp upstream of the translational start codon.

Discussion

Dehydrins are believed to play a fundamental role in the response of plants to various abiotic and biotic stresses. They make up a multigene family with 10 members in Arabidopsis [18,35], 8 members in rice [45], 13 members in barley [23], and 11 members in poplar [46]. However, only 4 *DHN* genes were identified in the published *V. vinifera* genome sequence [30,47], including two Y_nSK_n-type DHNs (*DHN1* and *DHN4*) and two SK_n-type DHNs (*DHN2* and *DHN3*). Neither K_n- nor KS-type DHNs were found in this species, which differs from the *DHN* gene families from other plant species characterized to date and suggests that these types of genes may have been lost in grape species (Figure 2).

Expansion of the *DHN* family has generally occurred through tandem duplication events and whole-genome duplications. For example, At1g20440/At1g20450, At3g50970/At3g50980, and At4g38140/at4g39130 arose from tandem duplications, while At1g20450/At1g76180, At2g21490/At4g39130, and At3g50970/At5g66400 arose from a whole-genome duplication event in Arabidopsis [18], which together resulted in an increase of 6 *DHN* genes. Similarly, whole-genome and tandem duplication events were responsible for an increase of 3 and 2 *DHN* genes, respectively, in poplar [46]. At least 3 *DHN* genes arose from tandem duplication events in rice [45], and it is possible that the two clusters of *DHN* genes on chromosomes 5 H and 6 H in *H. vulgare*, respectively [32], which show a high level of sequence identity within each cluster, may have resulted from tandem duplication events.

While the genomes of poplar, rice and Arabidopsis have undergone at least one recent whole-genome duplication event, the grapevine genome has not [30]. Instead, the four grapevine *DHNs* have likely arisen from
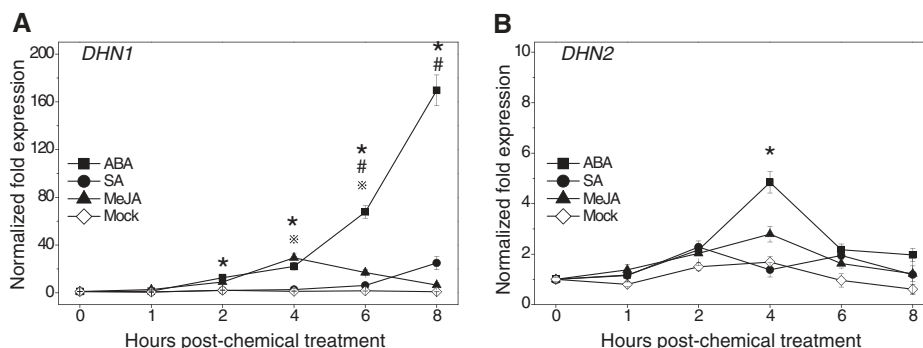
Figure 7 Expression profiles of grapevine *DHN1* and *DHN2* following treatment with various hormones. Total RNA was extracted from the leaves of *V. vinifera* sprayed with 50 μM MeJA, 100 μM SA and 100 μM ABA (**A**, **B**). Samples were taken at the indicated times, with time zero samples harvested immediately prior to spraying. Normalized transcript levels of *DHN1* and *DHN2* were determined by real-time qRT-PCR analysis, with the *actin1* gene serving as an internal control. Each point represents the mean relative fold-change compared to baseline levels of expression from three experiments, while bars indicate standard deviations. Baseline expression levels were those measured just prior to treatment at time = 0. Asterisks (*), number signs (#) and reference marks (※) indicate significant differences ($p < 0.05$) in expression levels of target transcripts from *V. vinifera* treated with ABA, SA and MeJA, respectively, compared to the mock-treated controls.

Table 2 Regulatory elements involved in stress-, pathogen- and embryogenesis-responsiveness in grapevine *DHN* promoter regions

Cis-element	Sequence	Number of *cis*-elements[*]				Function	References
		DHN1	*DHN2*	*DHN3*	*DHN4*		
ABRE	CACGTG	4/4	6/6	0/1	2/2	Abscisic acid responsiveness	[37]
DRE	ACCGAC	1/1	1/1	0/0	0/0	Drought and cold responsiveness	[38]
HSE	AGAAAATTCG	1/2	0/0	0/0	0/0	Heat stress responsiveness	[39]
LTR	CCGAAA	1/1	0/0	0/0	0/0	Low-temperature responsiveness	[40]
TC-rich repeats	ATTTTCTTCA	0/0	0/0	2/2	0/0	Stress and defense responsiveness	[41]
MeJA-RE	CGTCA	1/1	2/3	0/0	1/1	MeJA-responsiveness	[42]
TCA element	CCATCTTTTT	1/1	1/0	0/0	1/1	Salicylic acid responsiveness	[43]
Skn-1_motif	GTCAT	2/2	1/1	1/1	2/1	Endosperm expression	[44]

[*] The first number indicates the number of *cis*-regulatory elements within the *V. yeshanensis DHN* promoter while the second number indicates the number of *cis*-regulatory elements within the *V. vinifera DHN* promoter.

an ancestral genome [30], which is consistent with the low level of sequence similarity between the four sequences. However, *DHN3* and *DHN4* lay in close proximity on chromosome 3 in *V. vinifera*, which implies that one of them may have arisen through a tandem duplication event despite their low level of identity. Therefore, it seems that the relatively low number of *DHN* genes in grapevine may simply be due to a lack of duplication events in this genus. Indeed, it has been suggested that gene family expansion in grapevine has been selective, occurring mainly in those genes involved in aromatic features [30].
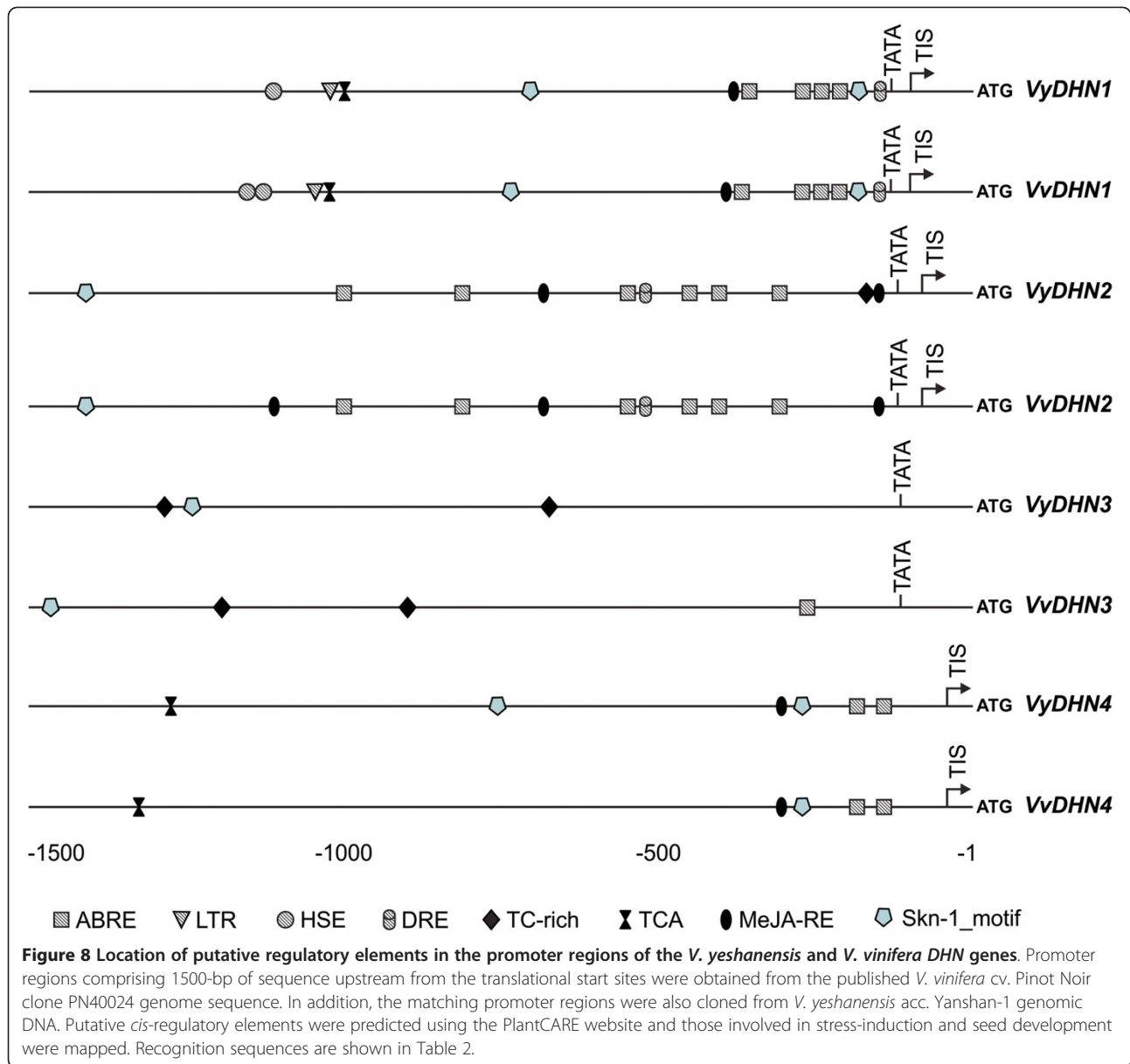
In silico characterization of *V. vinifera* and *V. yeshanensis* DHN protein sequences suggested they were all highly hydrophilic and disordered, but with distinct differences in pI, kinase specificity and content of functional motifs. The two Y_nSK_n-type DHNs (DHN1 and DHN4) possessed a higher pI than the SK_n-type DHNs (DHN2 and DHN3). Since positively charged DHN proteins bind negatively charged membranes with a high affinity [13], it follows that the Y_nSK_n-type DHNs, and especially DHN1, could very well bind with the cell membrane in grapevine. It has been suggested that the binding of DHNs to membranes may be modulated by phosphorylation through an alteration of net charge [22]. The DHN1 and DHN4 proteins from grapevine were found to contain a higher number of putative PKC sites than CK2 sites, whereas DHN2 and DHN3 bore a higher number of putative CK2 sites than PKC sites (Table 1; Figure 2). This finding is in agreement with a previous suggestion that Y_nSK_n-type DHNs are mainly phosphorylated by PKC, while SK_n-type DHNs are mainly phosphorylated by CK2 [22].

DHN proteins with similar physicochemical properties often also exhibit similar expression patterns. For example, while genes encoding alkaline Y_nSK_n-type DHNs, such as *At5g66400*, *HvDhn1*, *HvDhn2*, *HvDhn3*, *HvDhn4*, *HvDhn7*, *HvDhn9* and *HvDhn10*, are generally induced by both embryogenesis and various types of stress [18,23], those encoding acidic SK_n- and K_nS-type DHNs, such as *At1g20440*, *At1g20450*, *At1g76180*, *At1g5410*, *HvDhn8* and *HvDhn13*, are expressed constitutively in vegetative tissues and are also up-regulated by various types of stress [18,23,35]. The expression patterns of grapevine *DHN1* and *DHN2* agree with those predicted by their classification, which suggests that this holds true in the species analyzed here.

Even though the grapevine DHN1 and DHN4 (Y_nSK_n-type), as well as DHN2 and DHN3 (SK_n-type), proteins are grouped into only two classes, all four members of the grapevine *DHN* family exhibited very distinct patterns of expression (Figure 4, Figure 5, Figure 6, and Figure 7). We found grapevine *DHN1* to be induced by drought, cold, heat, *E. necator*, and to be expressed during late stages of embryogenesis, which corresponds well with previous reports [28,29]. Conversely, *DHN2* was found to be constitutively expressed in vegetative tissues and was up-regulated under cold and heat conditions, as well as during late embryogenesis (Figure 4, Figure 5, Figure 6, and Figure 7). In contrast, very low levels of *DHN3* expression were only detected during seed development with no induction observed in vegetative tissues following any of the stress or signaling molecule treatments studied here. Correspondingly, although no *DHN3* transcripts could be identified in GenBank's EST database, a large number (tens to hundreds) of the remaining grapevine *DHN* genes were (data not shown), which suggests that *DHN3* is expressed at undetectable levels in most tissue types. Likewise, *DHN4* was also specifically expressed during late embryogenesis, but at far higher levels than *DHN3* (Figure 4 and Figure 5). These results suggest that the function of the grapevine *DHN* genes is likely divergent, but may also exhibit some level of overlap.

The accumulation of *DHN*s in plants is believed to have been associated with the acquisition of desiccation

Figure 8 Location of putative regulatory elements in the promoter regions of the *V. yeshanensis* and *V. vinifera* DHN genes. Promoter regions comprising 1500-bp of sequence upstream from the translational start sites were obtained from the published *V. vinifera* cv. Pinot Noir clone PN40024 genome sequence. In addition, the matching promoter regions were also cloned from *V. yeshanensis* acc. Yanshan-1 genomic DNA. Putative *cis*-regulatory elements were predicted using the PlantCARE website and those involved in stress-induction and seed development were mapped. Recognition sequences are shown in Table 2.

tolerance in these organisms [12] and expression levels of these genes in vegetative tissues has generally been found to be higher in drought-tolerant cultivars than in their susceptible counterparts [48-51]. However, this is not always the case, as differences in expression levels between tolerant and sensitive genotypes are often dependent on the type of *DHN*, as well as the duration of the stress. While both *V. yeshanensis* and *V. vinifera* have been found to exhibit some tolerance to drought, the former exhibits a higher tolerance than the latter and also displays resistance to cold [25,26]. In the case of induction via temperature stress, both *DHN1* and *DHN2* exhibited cold and heat responsiveness; however, *DHN1* appeared to be far more responsive than *DHN2* (Figure 6 C-F). Interestingly, induction tended to be

higher in *V. vinifera* than *V. yeshanensis*, which is contrary to the levels of temperature sensitivity in these two species.

Conversely, among the four grapevine *DHN* genes tested, only *DHN1* was induced by drought stress in vegetative tissues. This gene was up-regulated between 1–2 d after the initiation of drought conditions in *V. yeshanensis*, while its expression level at this time remained unchanged in *V. vinifera*, suggesting that the expression of *DHN1* was quicker to respond to drought in the tolerant genotype. However, *V. yeshanensis* did not show a higher level of *DHN1* expression than *V. vinifera* at 3 and 4 d following treatment (Figure 6 A and B). A similar situation has been observed in barley, where the *HvDhn6* gene was expressed earlier in tolerant cultivars

than susceptible cultivars under drought conditions, but at lower levels than the susceptible cultivars at time points that were further from the commencement of drought conditions [49,50].

Generally, *DHN* genes are up-regulated under drought stress and down-regulated following rehydration [52-55]. However, in this study, the grapevine *DHN1* and *DHN2* genes also displayed induction 2 h post-rehydration (Figure 6 A and B). In line with this, it has been found previously that leaf ABA content increases during early phases following re-hydration [56]. Therefore, the up-regulation of grapevine *DHNs* after rehydration may correspond to a change in leaf ABA content, since both genes were found to be responsive to this plant hormone (Figure 7 A and B).

Recent studies have indicated that *DHN*s are also responsive to pathogen infection. For example, a *DHN* gene can be utilized to predict blast resistance in rice [57], and the Arabidopsis *LTI30* and *RAB18* genes have been found to be up-regulated by inoculation with powdery mildew [18]. This pathogen-induced expression of *DHNs* may provide another important function for this type of gene in disease resistance. In the current study, only *DHN1* was found to be up-regulated in *V. yeshanensis* and *V. vinifera* following inoculation with *E. necator*, which is the causative agent of grapevine powdery mildew (Figure 6G). Intriguingly, the expression level of *DHN1* was higher in the resistant *V. yeshanensis* than in the susceptible *V. vinifera*, and a second induction event was also apparent in *V. yeshanensis* that was lacking in *V. vinifera*. These results suggest that *DHN1* may participate in powdery mildew resistance in *V. yeshanensis*.

DHN1 from *V. vinifera* was also induced by the signaling molecules SA and MeJA, which are known to be involved in defense response, providing further evidence that it could play a role in systemic acquired resistance [58]. It has been demonstrated previously that a number of pathogen-responsive genes were up-regulated in transgenic Arabidopsis plants overexpressing *DHN-5*, which implies that DHNs might act as stress signaling molecules that regulate defense genes [11]. This may also be the case for the *DHN1* genes from grapevine (Figure 7A), although further experiments will be necessary to show this definitively.

The expression of stress-responsive genes depends upon the presence of *cis*-regulatory elements in their promoter regions [59], as has been shown to be the case for barley *DHN* genes [32]. The four grapevine *DHN* promoters exhibited distinct differences in the composition and distribution of putative regulatory elements held within them. ABREs, which are one of the most common *cis*-elements in the *DHN* promoters, likely played a role in the induction of *DHN1* by ABA, mediating its expression under drought conditions. Indeed,

when taken together, all of the putative regulatory elements identified within both the *DHN1* and *DHN2* promoters could account for their up-regulation by a variety of stresses and their corresponding signal molecules (Figure 8). In contrast, relatively few regulatory elements were found in the *DHN3* and *DHN4* promoters, which corresponds with the fact that neither of these genes were found to be induced by any of the stresses or signaling molecules analyzed.

The quantity and location of regulatory elements could also have a profound effect on grapevine *DHN* expression. It has been found previously that a single copy of an ABRE is not sufficient for ABA-responsive induction of transcription [59]. In this study, a higher number of ABRE elements were located in *DHN1* and *DHN2* promoters than in *DHN3* and *DHN4* promoters (Figure 8); correspondingly, the two former genes were responsive to induction by ABA, whereas *DHN3* and *DHN4* were not. Furthermore, the Skn-1 motif, which has been shown previously to confer a promoter with endosperm-specific expression [60], was found in all four grapevine *DHN* promoters. However, these motifs were located much nearer to the translational start codon in *DHN1* and *DHN4* promoters than in *DHN2* and *DHN3* promoters, which may provide an explanation for increased up-regulation of *DHN1* and *DHN4* during late embryogenesis (Figure 4 and Figure 5).

Conclusions

The *DHN* gene family was identified in a genome-wide search of the published genome sequence of *V. vinifera*, and the corresponding homologues were isolated from *V. yeshanensis*. A large expansion of the *DHN* family apparently did not take place in grapevine, although it has been a common occurrence in other plants. The four grapevine *DHN* genes shared a low sequence identity, and exhibited clear differences in physicochemical properties and expression profiles, which indicates functional diversification within the grapevine *DHN* family. *DHN1* appeared to be the principal stress-responsive gene in grapevine species, and was induced not only by various abiotic stresses but also by *E. necator*. The small size and distinct expression profiles of the grapevine *DHN* gene family makes it an excellent model to elucidate functional differentiation within this gene family, which should contribute to the further understanding of these genes in plants.

Methods
Plant materials
V. yeshanensis acc. Yanshan-1 and *V. vinifera* cv. Pinot Noir were obtained from the Grapevine Repository of Northwest A&F University, Yangling, Shaanxi, China. One-year old rooted seedlings of

Yanshan-1 and Pinot Noir were maintained in a greenhouse, and were utilized for stress experiments. For expression analysis in different plant tissues, root, stem, leaf, seed and fruit peel samples were harvested from three representative veraison-stage *V. vinifera* and *V. yeshanensis* plants, respectively, that had been grown in the field. For expression analysis during seed development, flower buds were harvested from three Pinot Noir plants grown in the field 6 days before flower opening (−6 daf). Flowers were collected at flower opening (0 daf) and young berries were harvested at 6 daf. In addition, seeds were collected from 12–66 daf. Samples were frozen in liquid nitrogen and stored at −80°C.

RNA extraction and first-strand cDNA synthesis
Total RNA was isolated from plant tissues using the method described by Reid et al. [61]. Subsequently, RNA was treated with 10 units RQ1 RNase-free DNase (Promega, Shanghai, China) in the presence of 100 units RNase inhibitor at 37°C for 30 min, followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). RNA was then precipitated with ethanol and dissolved in RNase-free water. First-strand cDNA synthesis was carried out using 1 μg total RNA and the PrimeScript™ RT reagent Kit (TaKaRa Biotechnology, Dalian, China) with 2.5 μM oligo dT primer and 2.5 μM random 6mer primer. The reaction mixture was incubated at 37°C for 40 min and the reverse transcriptase was then inactivated at 85°C for 5 s.

Cloning of DHN genes from V. yeshanensis and V. vinifera
A fragment of the *VyDHN1* coding sequence was amplified from drought-treated leaves of *V. yeshanensis* acc. Yanshan-1 using differential display reverse-transcription PCR (DDRT-PCR) as described by Lin et al. [62]. Briefly, first-strand cDNA was synthesized from 1 μg total RNA isolated from leaves subjected to drought at 0, 2, 3, 4, and 5 d following onset of treatment using primer $T_{11}VA$ (V = A, C, G) at 37°C for 1 h with 200 units of M-MLV (Promega) according to the manufacturer's instructions. This was followed by PCR amplification with primers $T_{11}VA$ and S476 (CCAAGCTGCC), followed by separation on a 6% polyacrylamide gel. Differential fragments were recovered, amplified by a second round of PCR using the same parameters as the first, and cloned into the pGEM-T easy vector (Promega). The 5' end of the *VyDHN1* cDNA was obtained by 5' rapid amplification of cDNA ends (5' RACE) using the BD SMART RACE cDNA Amplification Kit (Clontech, CA, USA) with primer VD1-GSP1 (see Additional file 3 for primer sequence) according to the manufacturer's recommendations. The full-length *VyDHN1* sequence was then deduced through alignment of the original DDRT-PCR fragment and 5' RACE sequence.

The encoded protein sequence of the *VyDHN1* gene was used to identify four *V. vinifera DHN* genes containing putative K-segments via BLAST analyses. BLAST analyses were also performed against the predicted grapevine *DHN* genes using ten previously identified Arabidopsis DHN proteins as query sequences [18]. These results were further validated by searching for *Vitis* DHN sequences in the Pfam database [63].

For the remaining *DHN* genes, seeds were harvested from *V. yeshanensis* at veraison. The *DHN* genes were amplified from cDNA using PrimeSTAR® HS DNA polymerase (TaKaRa) with primers that were designed based upon the *DHN* genes of *V. vinifera* cv. Pinot Noir (see Additional file 3). Cycling parameters for amplification of *VyDHN2* and *VyDHN4* were as follows: 94°C for 3 min, 25 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by a final elongation at 72°C for 5 min. The same parameters were utilized in the case of *VyDHN3* except that 40 cycles were necessary for its amplification. Subsequently, all four *V. yeshanensis DHN* genes were also amplified from genomic DNA to identify intronic regions using the same primers.

V. yeshanensis DHN promoters, including 1500-bp of upstream sequence in each case, were amplified from genomic DNA using PrimeSTAR® HS DNA polymerase (TaKaRa) with primers that were designed based upon the *DHN* genes of *V. vinifera* cv. Pinot Noir (see Additional file 4). Cycling parameters were as follows: 94°C for 3 min, 30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 2-3 min, followed by a final elongation at 72°C for 5 min. PCR products were cloned into pGEM-T easy (Promoga) and three clones were sequenced per *DHN* gene.

Treatment of plants with various hormones, as well as abiotic and biotic stress
Drought experiments were conducted by withholding water from *V. yeshanensis* and *V. vinifera* seedlings. Leaves were harvested 0, 0.5, 1, 2, 3, 4, 5, and 6 d following onset of treatment. Subsequently, the stressed plants were watered to soil saturation and leaves were collected 0.25, 0.5, 2, 6, 12, 24, and 48 h after watering. Plants grown under a normal watering regime were used as a control. For cold- and heat-stress induction, *V. yeshanensis* and *V. vinifera* seedlings were maintained in a growth chamber at either 4°C or 40°C and leaves were harvested 0, 2, 6, 12, 24 and 48 h after treatment. Mock-treated control plants were kept in a growth chamber at 22°C. Pathogen treatment was carried out by inoculating the leaves of *V. yeshanensis* and *V. vinifera* with *E. necator* as previously described [27]. Prior to inoculation, leaves were sprayed with sterile water, and leaves

were harvested 0, 0.5, 1, 2, 3, 4, 5, and 6 d after inoculation. Control plants simply underwent the sterile water spray and were not inoculated.

For signaling molecule treatment, 100 μM ABA [29], 100 μM SA [64], and 50 μM MeJA [65] were sprayed on the leaves of *V. vinifera* and leaf samples were harvested 0, 1, 2, 4, 6, 8 h post-treatment. Leaves sprayed with 0.05% Tween 20 solution were used as a negative control. All stress-induction experiments were performed on three independent plants for each treatment.

Semi-quantitative and real-time RT-PCR analysis

Semi-quantitative RT-PCR was performed using Premix Ex Taq® Version2.0 (TaKaRa) and *DHN*-specific primers that were designed to anneal to either side of an intron in both grapevine species (see Additional file 5 for primer sequences). Genomic DNA was utilized as a size control for unspliced transcripts. Reactions lacking reverse transcriptase were utilized as a negative control to exclude DNA contamination. Each experimental reaction (25 μl final volume) contained 1 μl of template cDNA along with 400 nM of each primer. Cycling parameters were as follows: 94°C for 3 min, 25 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by a final elongation at 72°C for 5 min. The *actin1* transcript [GenBank:AF369524] was utilized as an internal control using primers designed to anneal to both *V. vinifera* and *V. yeshanensis* sequences and the same general parameters as the *DHN* transcripts, except 20 cycles were utilized for amplification. PCR products were separated on a 1.5% agarose gel containing ethidium bromide, and photographed using a Bio Imaging System (Syngene, Cambridge, UK). At least two technical replications were conducted in each case.

Real-time quantitative RT-PCR was conducted via the $\Delta\Delta C_T$ method using the SYBR® Premix *Ex Taq* II Kit (TaKaRa) with primer pairs designed to anneal within the second exon of each *DHN* gene in both grapevine species (see Additional file 6 for primer sequences). The reactions were carried out in triplicate using 1 μl template cDNA in a final volume of 25 μl in an iQ5 Real Time PCR System (Bio-Rad, CA, USA) with the following thermal parameters: 95°C for 10 s, followed by 40 cycles of 94°C for 5 s and 60°C for 30 s, with a final melting gradient from 60°C to 95°C at a rate of 1°C per min. The grapevine *actin1* gene was utilized as an internal control. Relative expression levels of each *DHN* gene from both species were analyzed using the IQ5 software and were denoted as the fold-difference from expression present at baseline levels. Paired t-tests were conducted using Origin Pro 8.0 software (OriginLab Corp., Northampton, MA, USA) to assess the significance of expression level differences between treated samples and the mock controls. Differences were deemed significant at $p < 0.05$. Baseline expression signifies that which was present prior to treatment (ie. the first time point where relative expression is set to 1), except in the case of *DHN3*, where no expression was noted until 18 daf and therefore this time point was utilized as the baseline.

In silico analysis of *DHN* genes and their encoded proteins

Chromosomal locations of *VvDHN* genes were predicted using the BLAT server through the Genoscope Genome Browser (http://www.genoscope.cns.fr/blat-server/cgi-bin/vitis/webBlat). To identify putative *cis*-acting elements within the *V. vinifera DHN* promoters, contigs containing the respective genes were obtained using BLAST. Intergenic regions between the *DHN* genes and their upstream genes were determined according to annotations provided in GenBank. In the case of *V. yeshanensis DHN* promoters, sequences were obtained directly by cloning. The presence of regulatory elements in 1500-bp of sequence upstream of each translational start codon was determined using the PlantCARE database [36].

Protein MW (molecular weight), pI (isoelectric point) and GRAVY (grand average of hydropathy) were predicted using the ProtParam program (Expasy tools) based on their amino acid compositions. Predictions of intrinsic disorder within each *DHN* gene from both species were conducted using the PONDR-FIT tool [66]. Protein secondary structures were predicted using the PSIPRED v3.0 program [67]. The sequence algorithm NetPhosK (Expasy), with its probability limit set to 60%, was utilized to predict phosphorylation sites in VvDHN and VyDHN proteins.

Phylogenetic analysis was carried out by performing multiple alignments of full-length DHN protein sequences from *V. vinifera*, *V. yeshanensis*, *H. vulgare* and Arabidopsis using MEGA5 [68]. DHN sequences from Arabidopsis and barley were obtained from previous reports [18,32-34]. An unrooted dendrogram was constructed based on the alignment with PhyML using the maximum likelihood method [69].

Additional files

Additional file 1: Cloning of the *DHN1* coding sequence from drought-treated leaves of *V. yeshanensis*

Additional file 2: Structure prediction of DHN proteins from *V. yeshanensis* and *V. vinifera*

Additional file 3: Sequence of primers used for cloning *DHN* genes in grapevine

Additional file 4: Sequence of primers used for cloning *DHN* promoters from *V. yeshanensis*

Additional file 5: Sequence of primers used for semi-quantitative RT-PCR in grapevine

Additional file 6: Sequence of primers used for real-time qRT-PCR in grapevine

Abbreviations

ABA: Abscisic acid; ABRE: ABA-responsive element; CK2: Casein kinase 2; Daf: Days after flowering; DDRT-PCR: Differential display reverse-transcription PCR; DHN: Dehydrin; Dpi: Days post inoculation; DRE: Dehydration-responsive element; HSE: Heat shock-responsive element; LTR: Low temperature-responsive element; MeJA: Methyl jasmonate; MeJA-RE: Methyl jasmonate-responsive element; NLS: Nuclear localization signal; PKC: Protein kinase C; qRT-PCR: Quantitative reverse-transcription PCR; SA: Salicylic acid; SnRK: Snf1-related kinase.

Authors' contributions

YY contributed to the design of the study, conducted the majority of experiments and drafted the manuscript; MH contributed to the powdery mildew treatment experiment; ZZ contributed to the signaling molecule treatment experiment; SL contributed to the seed development experiment; YX and CZ were involved in the design of the study and preparation of the manuscript; SDS participated in analysis of results and preparation of the manuscript; YW conceived, designed and directed the study and contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

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