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

Yazhou Yang1,2,3, Mingyang He1,2,3, Ziguo Zhu1,2,3, Shuxiu Li1,2,3, Yan Xu1,2,3, Chaohong Zhang1,2,3, Stacy D Singer4 and Yuejin Wang1,2,3*

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

* Correspondence: wangyj@nwsuaf.edu.cn

1 College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, China
2 Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northwest Region), Ministry of Agriculture, Northwest A&F University, Yangling, Shaanxi 712100, China
3 Full list of author information is available at the end of the article

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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 DHNs 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 YnSKn, YnKn, SKn, Kn and KnS [12]. All DHNs possess at least one K-segment (EKKGDMDKKEKLPG), 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 YSKn-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, LT130 [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 LT129 (SKn) and LT130 (Kn) were up-regulated in Arabidopsis under low temperature conditions, only LT130 was up-regulated following salt treatments [1]. Similarly, while ten barley DHNs 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 DHNs increased in response to various stressors (including dehydration, high salinity, and wounding), 16 kDa and 18 kDa DHNs 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 Y2SK2-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_003631828], 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:F896520, 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 VyDHN1 and VyDHN2 were located on chromosomes 4 and 18, respectively, VyDHN3 and VyDHN4 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 Y2SK2- (DHN1), SK2- (DHN2), SK3- (DHN3), or Y2SK3- 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 YnSKn-type DHNs, possessed a higher pl than the SKn-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 YnSKn-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 YnSKn-, SKn-, Kn-, and KS-type proteins (Figure 3), where the Arabidopsis YK-type DHN (At4g39130) was included within the YnSKn 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 YnSKn-type DHNs of Arabidopsis and barley, while the grapevine DHN2 and DHN3 proteins were grouped with the SKn-type DHNs of Arabidopsis and barley. Interestingly, both grapevine species lacked KS- and Kn-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.
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,
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

![Figure 2](image_url)

**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.

| Name      | Type        | No. of Residues | MW (kDa) | pl  | GRAVY | PKC No | CK2 No | SnRK2 No | Expression              | Expression          |
|-----------|-------------|-----------------|----------|-----|-------|--------|--------|----------|-------------------------|---------------------|
| VyDHN1    | Y2SK2       | 130             | 13.9     | 9.36| −1.425| 2      | 1      | 1        | Stress + Seed            | Stress + Seed       |
| VvDHN1    | Y2SK2       | 130             | 13.9     | 9.27| −1.459| 2      | 1      | 1        | Stress + Seed            | Stress + Seed       |
| VyDHN2    | SK2         | 205             | 23.4     | 5.21| −1.527| 1      | 6      | 1        | Constitutive + Stress + Seed | Constitutive + Stress + Seed |
| VvDHN2    | SK2         | 206             | 23.5     | 5.20| −1.514| 1      | 6      | 1        | Constitutive + Stress + Seed | Constitutive + Stress + Seed |
| VyDHN3    | SK3         | 166             | 18.8     | 5.81| −1.736| 3      | 6      | 0        | Seed (weak)              | Seed (weak)         |
| VvDHN3    | SK3         | 166             | 18.9     | 5.92| −1.739| 3      | 6      | 0        | Seed (weak)              | Seed (weak)         |
| VyDHN4    | Y3SK2       | 191             | 20.1     | 6.35| −0.959| 10     | 1      | 0        | Seed                    | Seed                |
| VvDHN4    | Y3SK2       | 191             | 20.1     | 6.26| −0.930| 10     | 1      | 0        | Seed                    | Seed                |

MW (molecular weight), pl (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.
In 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
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
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

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**Figure 5** Expression of DHNs 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.
**Figure 6** (See legend on next page.)
several involved in stress-response, seed development, and hormone signaling (Table 2). In the case of the *VvDHN2* 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 *VvDHN3*, *VvDHN3*, *VvDHN4* 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 *VvDHN4* 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 YeSKn-type DHNs (*DHN1* and *DHN4*) and two SKn-type DHNs (*DHN2* and *DHN3*). Neither Kn- 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/At1g676180, 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 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 (C, D), 40 °C (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.

**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 |
|-------------------|----------------|-------------------------|-----------------------------------|------------|
| 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               | AGAAAAATTCG    | 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   | ATTTCTTCA      | 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       | CCACTTCTTTT    | 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 YnSKn-type DHNs (DHN1 and DHN4) possessed a higher pI than the SKn-type DHNs (DHN2 and DHN3). Since positively charged DHN proteins bind negatively charged membranes with a high affinity [13], it follows that the YnSKn-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 YnSKn-type DHNs are mainly phosphorylated by PKC, while SKn-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 YnSKn-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 SKn- and KnS-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 (YnSKn-type), as well as DHN2 and DHN3 (SKn-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 DHNs in plants is believed to have been associated with the acquisition of desiccation
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.

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.
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 DHNs 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 25 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 VyDHNI coding sequence was amplified from drought-treated leaves of V. yeshanensis cv. 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 T11VA (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 T11VA and S476 (CCAAAGCTGACC), followed by separation on a 6% polyacrylamide gel. Differential fragments were recovered, amplified, by a second round of PCR using the same primers as the first, and cloned into the pGEM-T easy vector (Promega). The 5' end of the VyDHNI 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 VyDHNI sequence was then deduced through alignment of the original DDRT-PCR fragment and 5’ RACE sequence.

The encoded protein sequence of the VyDHNI 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 scanning 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 (Promega) 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 ΔΔCT 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 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), pl (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; MeA: Methyl jasmonate; MeA-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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Author details
1 College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, China. 2 Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northwest Region), Ministry of Agriculture, Northwest A&F University, Yangling, Shaanxi 712100, China. 3 State Key Laboratory of Crop Stress Biology in Arid Areas, Northwest A&F University, Yangling, Shaanxi 712100, China. 4 Department of Agricultural, Food and Nutritional Science, 4–10 Agriculture/Forestry Centre, University of Alberta, Edmonton, Alberta T6G 2P9, Canada.

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