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Identification and characterization of a seed-specific grapevine dehydrin involved in abiotic stress response within tolerant varieties

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Abstract: To identify and isolate genes related to abiotic stress (salinity and drought) tolerance in grapevine, a candidate gene approach led to the isolation from Cabernet Sauvignon cultivar of a full-length cDNA of dehydrin gene. The latter, named *VvDhn*, which is highly and mainly induced in late embryogenesis in seeds, encodes for a protein of 124 amino acids with a predicted molecular mass of 13.3 kDa. Details of the physicochemical parameters and structural properties (molecular mass, secondary structure, conserved domains and motives, and putative posttranslational modification sites) of the encoded protein have also been elucidated. The expression study of *VvDhn* was carried out within plant organs and tissues as well as under drought and salt stresses. *VvDhn* was not detected in vegetative tissue, whereas it was only expressed during seed development (during late embryogenesis) at extremely high levels and was induced by salt and drought stresses as well as ABA application. Moreover, salt stress induced *VvDhn* expression in the tolerant variety (Razegui) but not the sensitive variety (Syrah), which did not display expression variation during stress; *VvDhn* expression level and salt-stress response depend on regulatory mechanisms that are efficient only in the tolerant variety. On the other hand, under drought stress *VvDhn* was induced in both tolerant and sensitive varieties, with higher levels in the tolerant variety. In addition, stress signal molecules such as ABA (applied alone or in combination with saccharose) induced *VvDhn* expression, even at low levels. Minimal knowledge about the role and functionality of this gene is necessary and constitutes a prerequisite for including *VvDhn* in grapevine abiotic stress tolerance improvement programs.

Key words: Dehydrin, salinity, drought, expression study, ABA, activity regulation, bioinformatic

1. Introduction

Grapevine is one of the most important cultivated plants in the world with nearly 7.5 million hectares of viticultural land under cultivation and 69.1 million tons of grape fruit produced in 2012 (OIV, 2013). Approximately one-third of the world's irrigated soils and a large proportion of soils in dryland agricultural regions are saline (Deinlein et al., 2014; Gupta and Huang, 2014), and since grapevine production and planting area are greatly affected by soil salinity and drought (Hamrouni et al., 2011; Cramer et al., 2013), it is of agricultural importance to analyze and improve the salt and drought tolerance of grapevine (Cramer, 2010; Cramer et al., 2013). Research for grape productivity improvement has mainly focused on the processes of berry development and ripening (Gapper et al., 2014; Seymour and Granel, 2014) and pathogens and disease resistance (Australian Wine Research Institute, www.awri.com.au/); little has been accomplished regarding the improvement of the tolerance of grapevine to abiotic stress, especially drought. In fact, drought tolerance is a complex trait, and the long list of drought-stress–responsive genes seems to support this statement (Cramer et al., 2013). Although many genes are induced by drought, only a few of them seem efficient for stress tolerance (Hanana et al., 2008; Cramer, 2010). Dehydrins figure among several ubiquitous dehydration-stress–responsive protein types in plants and are induced by stimuli that have a dehydrative component such as drought, low temperature, salinity, and ABA (Close, 1997; Hanin et al., 2011; Wang et al., 2014); they belong to the LEA D11 family, which accumulates late in embryogenesis and is distributed in a wide range of organisms including higher plants, algae, yeast, and cyanobacteria (Rorat,

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2006). All dehydrins contain the highly conserved lysine-rich domain (K-segment) characterized by the consensus EKKGIMDKIKEKLPG and other domains such as a track of serine residues (S-segment) and the consensus motif (T/VDE/QYGNP), termed Y-segment, located near the N-terminus (Close, 1997; Vaseva et al., 2014a). The number and order of Y-, S-, and K-segments define 5 different dehydrin subclasses: YnSKn, YnKn, SKn, Kn, and KnS (Close, 1997; Vaseva et al., 2014b). YnSKn-type dehydrins are induced by ABA or by drought but not by low temperature. The acidic or neutral YnKn-, SKn-, and Kn-type dehydrins preferentially accumulate in response to low temperature (Vaseva et al., 2014b). SKn-type dehydrins were shown to bind metals, for example calcium, depending on the phosphorylation of the proteins. KnS-type dehydrins respond to drought and low temperature, bind metals, and participate in iron transport for a long-distance transport of micronutrients (Kruger et al., 2002). They are thought to protect plant proteins and membranes from the loss of water during drought and cold temperatures (Sadder and Al-Doss, 2014). Rorat (2006) presumed that metal binding by dehydrins constitutes a protective mechanism against oxidative damage. Among strategies leading to the genetic improvement and adaptation of plants to these stresses is the identification and the transfer of genes involved in drought and salinity tolerance. In this respect, our study aims to identify and characterize a dehydrin gene (YSK_2 type) isolated from a grapeberry cDNA library and analyze its spatiotemporal expression under different environmental conditions and elicitor treatments. Furthermore, we assessed its expression under salt and drought stresses in contrasting grapevine varieties in order to underline, for the first time in grapes, its implication in abiotic stress adaptation and response.

2. Materials and methods

2.1. Plant material and growth conditions

2.1.1. Grapevine field and hard cuttings

In order to carry out *VvDhn* expression study using RT-PCR, berries from grape (*Vitis vinifera* 'Cabernet Sauvignon') plants were harvested from a vineyard in Domaine du Grand Parc of Bordeaux (France) and organized in samples corresponding to 5 phenological stages of berry development. The stages 1, 2, 3, 4, and 5 represent, respectively, 3, 6, 8 10, and 12 weeks after blooming. These berry development stages were chosen according to criteria including size, titers of soluble sugars, softening, and color of the berries (Downey et al., 2003). After cutting the pedicel, berries were immediately frozen in liquid nitrogen and then stored at –80 °C until use. When harvesting, skin and seeds were separated from the flesh and frozen in the same manner as whole berries (Deluc et al., 2006). Six-month-old rooted plants obtained from grape cuttings (*V. vinifera* L. 'Cabernet Sauvignon') were cultivated in sandy soil in a growth room programmed for 25/20 °C under a 16/8 h light/dark photocycle at 75% relative humidity (Ollat et al., 1998). Young (2–3-cm wide) and old (9–10-cm wide) leaves were also collected to study *VvDhn* expression in these organs (Deluc et al., 2006). The differential expression of *VvDhn* under both salt and drought stresses was assessed using northern blot analysis on 2 pairs of contrasting behavior varieties: Razegui, salt tolerant; Syrah, salt sensitive; Kahli, drought tolerant; and Guelb Sardouk, drought sensitive. Hard cuttings from these varieties harboring at least 2 dormant buds were excised in winter and then soaked at their basal segment with exuberone (indole-3-butyric acid analogue) in order to induce rooting. They were cultivated in crates filled with sandy soil under controlled chamber conditions (temperature, 25 °C; relative humidity, 70%; photoperiod, 16 h; light intensity, 30 µmol photons m^{-2} s^{-1}) and irrigated with Long Ashton solution (Hewitt, 1966). After 2 weeks, rooted plants were transferred to individual pots. Following 2 additional weeks of adaptation, plants were submitted to salt stress (100 mM NaCl), and salt was gradually added to the nutritive solution in a step-wise increase (25 mM every 15 days). Samples of old leaves were harvested after 6 and 24 h of 100 mM NaCl stress exposure (Hamrouni, 2010). Then, salt-stressed plants were transferred back to control conditions and harvested after 72 h, following an additional recovery period of 48 h. For the drought stress experiment, Kahli and Guelb Serdouk varieties were cultivated in well-watered pots for 3 months, and drought stress was applied by stopping irrigation 3 weeks before harvest. Samples of old leaves were frozen in liquid nitrogen and stored at –80 °C until use.

2.1.2. Cell suspension culture

Since the plant hormone abscisic acid (ABA) is produced under water-deficit conditions and is instrumental in the development of tolerance against drought, we studied the expression of *VvDhn* in berry cell suspensions under both ABA and saccharose treatments. The grapeberry cell suspension derived from Cabernet Sauvignon berries was maintained at 25 °C in an orbital shaker (100 rpm) by weekly subculture, according to Decendit et al. (1996). At the exponential growth phase, ABA (10 µM) alone or with saccharose (58 mM) was supplemented to the medium.

2.2. Identification and cloning of *VvDhn* gene

To identify a dehydrin gene from grapevine, we applied a candidate gene approach. The first step was to design degenerate primers (forward: 5'-CArTAyGGnAAyCChGTbCAyCA-3' and reverse: 5'-TChGAhGAhGAhGanCChGA-3') from conserved regions of different plant dehydrin sequences. Then, in

order to amplify the gene, PCR reactions were performed using Taq polymerase (Gotaq, Promega) on a berry cDNA library (véraison stage, corresponding to 8 weeks after flowering, 'B3') constructed using the SMART cDNA library construction kit (CLONTECH). The conditions for amplification were 94 °C for 3 min followed by 30 cycles at 94 °C for 1 min, 54 °C for 30 s, 72 °C for 2 min, and 72 °C for 10 min. The amplified fragments were purified from agarose gels and ligated into a pGEMT-Easy vector (Promega, Madison, WI, USA) and sequenced. On the basis of the sequences of these fragments and in order to obtain both ends of the cDNA, universal SP6 and T7 primers located in the pTriplEX vector (CLONTECH) were used for asymmetric PCR. The assembly of contiguous sequences provided us with a full-length cDNA (876b), *VvDhn*, with an open reading frame (ORF) of 375 nucleotides (GenBank accession no.: AY634281).

2.3. Spatio-temporal expression study of *VvDhn* gene

Semiquantitative RT-PCR was used to assess the spatio-temporal expression of *VvDhn* gene and its expression under ABA and saccharose treatments.

2.3.1. RNA extractions

For RNA extraction from berries harvested during the ripening stages, the method of Asif et al. (2000) was used (seeds and skins). RNA from roots and leaves was extracted using a protocol with CTAB and lithium chloride precipitation, as described by Chang et al. (1993). RNA from cell suspensions was extracted using RNeasy plant kit (Qiagen), according to the manufacturer's instructions. All RNA samples were treated with RNase-free DNase I (Promega) followed by phenol-chloroform extraction, ethanol precipitation, and, finally, suspension in DEPC-treated sterile water (Deluc et al., 2006). No DNA contamination was detected based on PCR amplification. All RNA analyses were performed at least 3 times with 3 independent samples.

2.3.2. RT-PCR reactions

For first-strand cDNA synthesis, RT was run with reverse transcriptase (100 units of MMLV RNase H, Promega) in 20 µL of reaction on 2 µg of total RNA (denatured at 75 °C for 10 min) for 60 min at 42 °C in the presence of 1.2 µM of oligo(dT), RT 5X buffer, 4 mM DTT, 800 µM of dNTPs, and 20 units of RNAsin (ribonuclease inhibitor). After completion of first-strand cDNA synthesis, 5-µL aliquots were taken for PCR. The detection of transcripts was done by using the forward primer 5'-TAGGTCCATAGGTCATAGGTG-3' and reverse primer 5'-CAGATTGGGGGTGGTAACA-3' chosen specifically and preferentially in the 3'-UTR region of the dehydrin mRNA. The expected length of the amplified fragment was 240 bp. PCR, performed in 50 µL reaction with GoTaq (Promega), comprised 24 cycles of 94 °C for 1 min, 54 °C for 30 s, and 72 °C for 1 min. Cycling was preceded by an initial denaturation step (94 °C for 2 min) and followed by a final extension step (72 °C for 10 min). A grapevine elongation factor 1 (EF1γ, accession no.: AF176496) gene, amplified under the same conditions with primers 5'-TCAATCTGTCTAGGAAAGGAAG-3' and 5'-GCGGGCAAGAGATACCTCAA-3', was used as a control (200 bp). The amplification products were then analyzed by agarose gel electrophoresis (1%).

2.3.3. *VvDhn* expression under salt and drought stresses study

RNA was extracted as previously described from leaves of control and treated plants at 2 treatment points (6 and 24 h) and the recovery point (72 h). Total RNAs (10 µg per sample) from control and NaCl-treated leaves were analyzed by blotting on nylon membranes (Hybond N, GE Healthcare Europe GmbH, Munich, Germany). Hybridization was performed at 65 °C in a phosphate-SDS-EDTA buffer system (Church and Gilbert, 1984) using α-[^{32}P]-dCTP-labeled (3000 Ci/mmol) cDNA fragment encoding part of *VvDhn*. This hybridization probe was labeled with the HexaLabel DNA labeling kit (Fermentas, St Leon-Rot, Germany), according to the manufacturer's instructions. After hybridization, membranes were washed with decreasing stringency up to 0.2 × SSC and 1% (w/v) sodium dodecylsulfate at 65 °C and then exposed to the phospho imager screen. Signal intensities were analyzed by a Bio-Rad Molecular PhosphoImager using the Bio-Rad Quantity One software (version 4.6.3) in order to assess the gene expression differences (Daldoul et al., 2009). The *VvDhn* probe was amplified by PCR using the following primers: *VvDhn*-Forward: 5'-CGGGGCAGGGGCAGCAAC -3' and *VvDhn*-Reverse: 5'- GCAGAAAGCTGATGCGAGGCTGC -3', chosen specifically and preferentially in the 3'-UTR region of the dehydrin mRNA, with an amplicon size of 227 bp.

2.4. Computational analysis

Sequence alignment and phylogenetic relationships were analyzed by the CLUSTAL X multiple sequence alignment algorithm (Thompson et al., 1997). The phylogenetic tree was drawn with MEGA software (MEGA 5.2 version) for evolutionary analysis of 115 protein sequences (Tamura et al., 2011). Phylogenetic analysis of dehydrin proteins was performed among prokaryotic and eukaryotic (mainly plant species) organisms whose full-length sequences were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) using 'dehydrin' query search (April 2014). The multiple Em for motif elicitation (MEME) software allowed us to discover, within these previous protein sequences, motifs along with their frequency and position (Bailey and Elkan, 1994). The following molecular and structural studies were completed:

posttranslational modifications analysis were performed using tools and software available on ExPASy (http://www.Expasy.Protparameters.Tools) and Pôle BioInformatique Lyonnais (http://pbil.univ-lyon1.fr/) servers. Topological predictions were made by TopPred0.01 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), DAS (http://www.sbc.su.se/~miklos/DAS/), PRED-TMR (http://o2.biol.uoa.gr/PRED-TMR), TMpred (http://www.isrec.isb-sib.ch/software/TMPRED_form.html), NetPhos 2.0 (www.cbs.dtu.dk/services/NetPhos 2.0), and ProtParam (http://www.expasy.org/tools/protparam.html) programs. Sequence analysis was performed using DNASTAR and Winpep (version 3) (Hennig, 1999), and BLAST search was performed on the NCBI platform (Altschul et al., 1990). Helicoidal representations were drawn by HeliQuest software (version 2) (Gautier et al., 2008). The 5-adjacent genomic sequence to *VvDhn* gene and *VvDhn* chromosomal location were identified by performing a BLAST search of *VvDhn* sequence on the grapevine genome, *V. vinifera* 'Pinot Noir' clone PN40024 genome sequence (Jaillon et al., 2007). The promoter motives and cis-regulatory elements of this putative promoter sequence were analyzed with the programs PLACE Signal Scan (http://www.dna.affrc.go.jp/htdocs/PLACE/signalup.html) and MatInspector (http://www.genomatix.de/matinspector/).

3. Results and discussion

3.1. Molecular characterization and genomic organization of VvDhn

The cloned cDNA of *VvDhn* is 846 nucleotides long (30 polyA nucleotides removed) with a 5' untranslated region of 84 nucleotides, a predicted ORF of 375 nucleotides, and a 3' untranslated region of 387 nucleotides (Figure 1). The predicted ORF encodes a protein of 124 amino acids that shares similarities with other dehydrins, and the phylogenetic analysis we conducted on 115 peptidic sequences showed that dehydrins are organized according to their structure and family (Figure 2). The genomic organization of *VvDhn* is made of a single intron (99 nucleotides) and 2 exons on the fourth chromosome. BLAST analysis led to the identification of a regulatory region located at the upstream part of the transcription start site (TSS) of *VvDhn* that contained putative regulatory elements (Figure 1). This last genomic region (adjacent

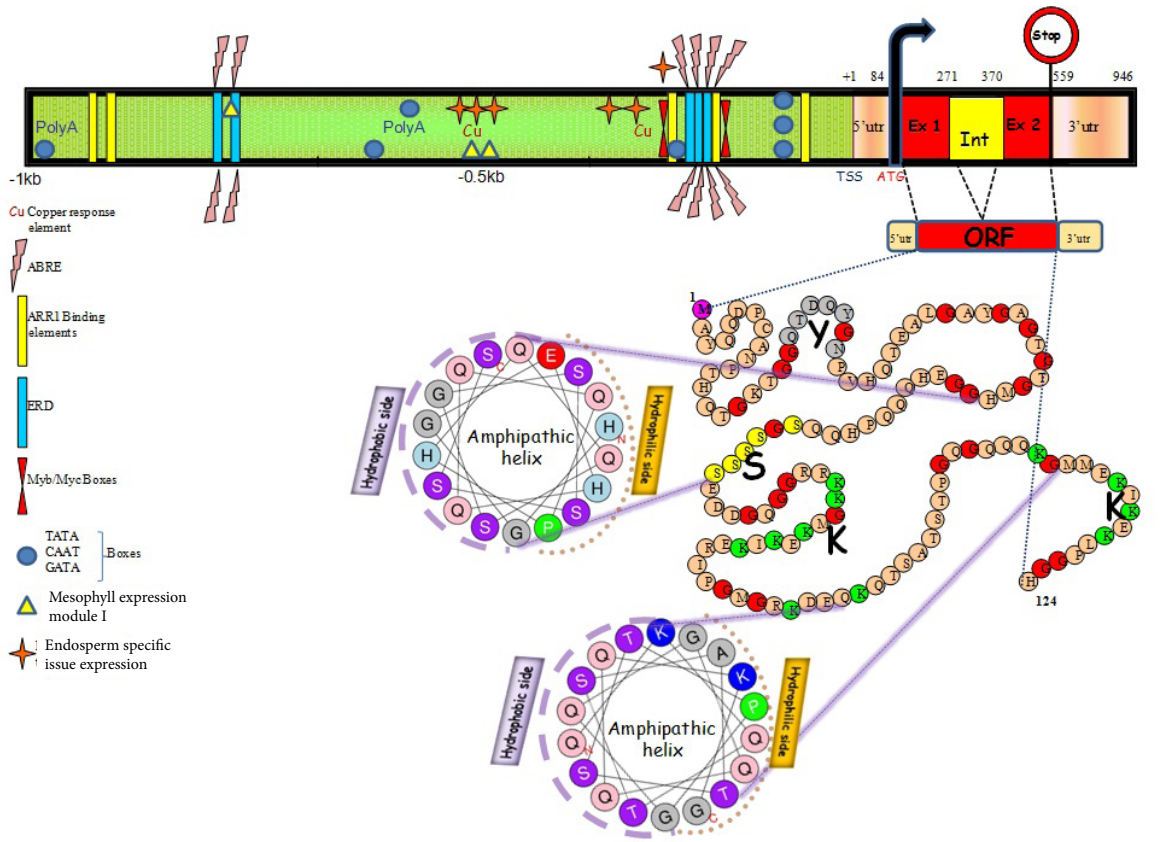

Figure 1. Genomic organization of *VvDhn* and translational process showing structural details of the protein. Gray, yellow, and green boxes of the peptidic chain represent respectively the Y-, S-, and K-segments. High frequency of glycine (G) amino acids shown in red.

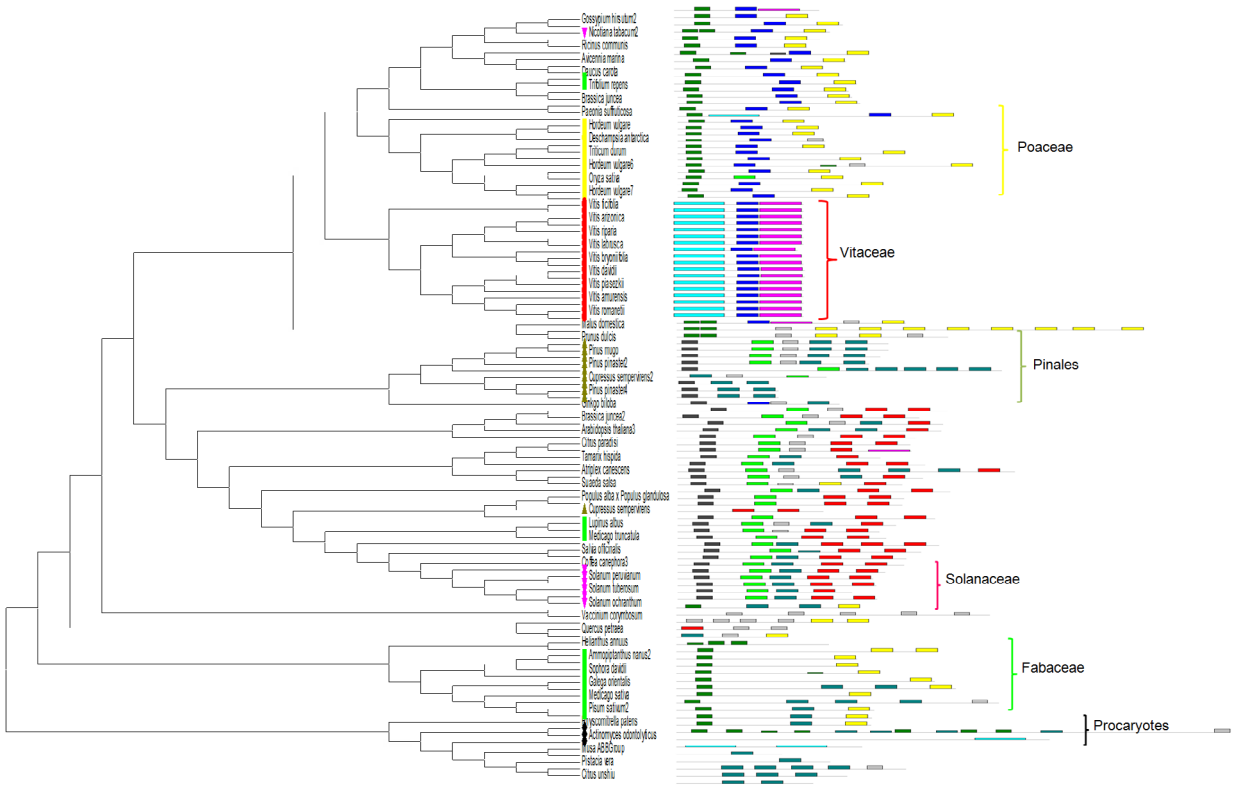

Figure 2. Phylogenetic relationship between 115 dehydrin proteins (phylogenetic tree was drawn using the neighbor-joining method). MEME results show motif categories, positions, and frequencies in each sequence.

and located within *VvDhn*, ~1-kb long) was analyzed in order to find putative regulatory elements within the 2 strands. Thus, several regulatory elements were identified upstream from the *VvDhn* TSS (Figure 1). The first TATA, GATA, and CAAT boxes were located around 200 nucleotides upstream from the transcription initiation site. Almost 45 ABA and ERD (early response to dehydration) responsive genes were recorded in both strands, mainly concentrated around 200-bp upstream of the TSS. A cluster of responsive elements for dehydration is present immediately upstream from the transcription start site; MYB, MYC, and ABRE responsive elements. Moreover, putative cis-elements for plant hormone regulation, i.e. cytokinin-responsive ARR1 binding elements, were also identified in this region. The existence of organ- and tissue-specific (seed, endosperm, and mesophyll) motives in the promoter sequence of *VvDhn* implies a very tight regulation. Among the numerous potential cis-sequences found in the *VvDhn* promoter, the copper-responsive elements seem particularly relevant for further analysis. Indeed, despite these hypothetical copper-responsive motives that appear to be strongly related to copper transport, findings remain speculative as these data have not been experimentally confirmed. The presence of these regulatory and cis-acting elements in this region suggests a role for dehydration tolerance with tissue-specific expression for the *VvDhn* gene. This bioinformatic analysis can be considered a first approach for identifying a range of transcriptional regulatory elements in a promoter; however, the functionality and activity of these grounds must be confirmed experimentally.

3.2. Structural analysis of VvDhn

3.2.1. Primary structure

VvDhn encodes a protein of 124 amino acids with a calculated molecular mass of 13.3 kDa and an isoelectric point of 9.0. Amino acid sequence analysis indicated that VvDhn protein contains the Y-segment close to the N-terminus, a serine cluster (S-segment) in the central part, and 2 repeats of lysine-rich consensus motifs (K-segment) at the carboxy-terminal region, which represents a typical YSK_2 structure of dehydrins (Figure 1). The amino-acidic composition of VvDhn (Table 1) shows high levels of frequency for Gly, which allows flexibility to the protein (18.5%), Gln (14.5%), and Lys (9.7%). Moreover, 33% of the amino-acidic composition is polar; polar amino acids are those with side-chains that prefer to reside in an aqueous (i.e. water) environment, making proteins hydrophilic (Close, 1996). For this reason, one generally

Table 1. Amino-acidic composition of *VvDhn* (http://www.Expasy.Protparameters.Tools.).

aa	Properties	Number	Frequency (%)
Ala (A)	Hydrophobic	6	4.8
Arg (R)	Strongly basic (+)	4	3.2
Asn (N)	Polar	2	1.6
Asp (D)	Strongly acidic (–)	5	4.0
Cys (C)	Disulfur bonds, polar	1	0.8
Gln (Q)	Polar	18	14.5
Glu (E)	Strongly acidic (–)	8	6.5
Gly (G)	Flexible	23	18.5
His (H)	Hydrogen bonds	6	4.8
Ile (I)	Hydrophobic	3	2.4
Leu (L)	Hydrophobic	2	1.6
Lys (K)	Strongly basic (+)	12	9.7
Met (M)	Hydrophobic	6	4.8
Pro (P)	Cyclic, hydrophobic	7	5.6
Ser (S)	Polar	7	5.6
Thr (T)	Polar	10	8.1
Tyr (Y)	Polar	3	2.4
Val (V)	Hydrophobic	1	0.8

finds these amino acids exposed on the surface of a protein. Methionine and cysteine, amino acids responsible for disulfur bonds, are relatively weak (5.6%). The total number of negatively charged residues is 13; there are 16 positively charged residues. Indeed, charge distribution in a protein is crucial within its cytosolic activity. A putative nuclear localization signal consisting of arginine and lysine residues (RRKK) was also found just upstream from the first K-segment. Nuclear localization of dehydrins in plant cells is associated with their protective role in stabilization of transcription machinery in unfavorable conditions (Allagulova et al., 2003).

3.2.2. Secondary structure

Secondary structure prediction showed that VvDhn contains 11% α-helix, 4% extended strand, and 85% random coil and loop. The unique cysteine of the protein is localized at the 30th amino acid from the N-terminal. Repetitive structures KGMKEKIKERIPG and KGMMEKIKEKLPG are respectively found at position [97–109] and [132–144]. Although no addressing signal peptide has been identified within the VvDhn protein, hydrophobicity profile analysis revealed a putative transmembrane region [34–42] that could interact with endomembranes in order to stabilize them. It was demonstrated by Koag et al. (2003) that the maize YSK2 DHN1 displayed in vitro binding activity to phospholipid vesicles as they may protect lipid membranes against peroxidation.

3.2.3. Tertiary structure

Helical wheel representation (Figure 1) of the peptidic regions from 47 to 64 and 94 to 111 residues displayed an amphipathic pattern (both hydrophobic and hydrophilic faces), with the potential for protein solubility, water binding, and hydrophobic interaction (Dure, 1993). The amphipathic α-helices can interact with partly dehydrated surfaces of various proteins and the surfaces of biomembranes (Hanin et al., 2011). The binding of dehydrins to the partly dehydrated surface of other proteins enhances formation of amphipathic α-helices in a dehydrin molecule and protects other proteins from further loss of water envelope. It has been suggested that these interactions between partly dehydrated surfaces of dehydrin molecules and other proteins and/or biomembranes present the basis of dehydrin protective functions (Hanin et al., 2011).

3.2.4. Posttranslational modifications

VvDhn contains several sites of activity regulation and posttranslational modifications (Table 2). Three putative N-glycosylation sites were identified in our sequence. N-glycosylation affects physical and chemical properties of glycoproteins, modifying molecular weight, solubility, and electrical charges. N-glycosylation also enhances the establishment of physiological protein conformation, increases thermostability, and protects against proteolysis. This PTM may also have supplemental activities such as the modulation of the protein half-life and recognition mechanisms (protein–protein, protein–cell, or cell–cell targeting) and positive or negative modulation of enzymatic activity (Schwarz and Aebi, 2011). In addition, 9 putative sites of phosphorylation (casein kinase II) were found in VvDhn; 6 of them were of serine type, mostly concentrated between the 61st and 64th positions (in the "S" domain). According to Alsheikh et al. (2003), casein kinase II phosphorylation activates and regulates the calcium binding activity of celery and Arabidopsis dehydrins, respectively. Thus, the presence of many potential casein kinase II phosphorylation sites in the Ser-cluster domain of VvDhn may point to calcium binding activity. Paradoxically, by using immobilized metal ion affinity chromatography, Hara et al. (2005) demonstrates a specific metal-binding property of citrus dehydrin, mainly Cu^{2+} but not Ca^{2+}, within a histidine-residue–rich region that was not found in the VvDhn peptidic sequence. It has also been demonstrated that the S-segment can be phosphorylated; this phosphorylation is related to the binding of nuclear localization signal peptides to nuclear transport (Goday et al., 1994). Phosphorylation has many functions including important regulation activities, activation of molecules, protein activity inhibition, transformation of nonpolar hydrophobic proteins into polar hydrophilic molecules, and binding proteins to activate or inhibit particular cell-signaling systems. Phosphorylation plays a significant role in a wide range of cellular processes. It can activate or deactivate many protein enzymes, resulting in a conformational change in structure in many enzymes (via interaction with other hydrophobic and hydrophilic residues in the protein), and can allow protein-protein interaction via recognition domains (de Lartigue, 2011). Moreover, VvDhn displays 5 putative N-myristoylation sites. These play a vital role in membrane targeting and signal transduction in plant responses to environmental stress (Moriya et al., 2013). Four putative SUMOylation sites were identified within VvDhn. SUMOylation is a posttranslational modification involved in various cellular processes such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle. SUMOylation of target proteins has been shown to cause a number of different outcomes including altered localization and binding partners (Wang and Dasso, 2009). One site of putative palmitoylation has been detected in VvDhn. Palmitoylation enhances the hydrophobicity of proteins and contributes to their membrane association. Palmitoylation also appears to play a significant role in subcellular trafficking of proteins between membrane compartments and modulating protein–protein interactions. Because palmitoylation is a dynamic, posttranslational process, it is thought to be employed by the cell to alter the subcellular localization, protein–protein interactions, or binding capacities of a protein (Blaskovic et al., 2013). Three major histocompatibility complex (MHC) binding sites were also found in VvDhn, although these molecules are the signal beacons of the immune system. If present in plants, they could be involved in the plant self-incompatibility system (Matton et al., 1994). Two amidation sites are present in VvDhn. Amidation neutralizes negative charges on the C-terminus of the polypeptide and is essential to the biological activity of many neuropeptides and hormones; it could increase protein polarity and, consequently, provide better protection against proteolysis (Lanigan and Sheppard, 2013). One putative S-nitrosylation site has been identified. S-nitrosylation reactions signal a broad spectrum of cellular activities including transcriptional and posttranscriptional regulation of protein expression as well as regulation of membrane, cytosolic, mitochondrial, nuclear, and extracellular protein functions (Gould et al., 2013). All these PTMs suggest various cellular functions and processes including cell cycle regulation; DNA repair; chromosomal maintenance; modification of cytoplasmic signal transduction, nuclear import, and subnuclear compartmentalization; DNA repair; transcription regulation; and stress response.

Table 2. Inventory of putative posttranslational modification sites of *VvDhn*.

Site	SUMOylation	N-glycosylation	Casein kinase II phosphorylation	N-myristoylation	Protein kinase C phosphorylation	Amidation	Palmitoylation	MHC binding	S-nitrosylation
Number of sites	4	3	9	5	1	2	1	3	1
Positions	17-74-91-119	10-22-100	15-25-61-62-63-64-98-101-102	36-39-41-45-60	15	70-88	8	32-57-110	8

3.3. Spatio-temporal expression of *VvDhn*

Spatio-temporal expression analysis of *VvDhn* using semiquantitative RT-PCR revealed a specific expression in seeds, especially at the late stages of maturation (Figure 3). Indeed, its expression appeared in an intensive manner at the third and fourth stages exclusively (8 and 10 weeks after flowering). It is interesting that the appearance of the dehydrins in embryonal tissue correlates with endogenous ABA, the level of which is usually increased during seed formation, especially before maturation. This intense and sudden accumulation of dehydrin could be due to the endogenous content of ABA in the embryo (Allagulova et al., 2003), thus representing an important compound for the dehydration phase preparation of the embryo. It is commonly outlined that LEA proteins (and thus dehydrins) are intensively synthesized during seed development as a part of the embryogenesis program. However, it is the maturation drying that induces drought-stress–related reactions such as expression of dehydrins (Kleinwächter et al., 2014). In other grape tissues and organs, no (or very little) transcript could be detected; some studies show that it can be expressed in buds and leaves, mainly distributed in vascular tissues (Rorat, 2006; Yang et al., 2012). The intensive accumulation of dehydrins in seeds at the late stages of their maturation, during dehydration, suggests the involvement of these proteins in protective reactions promoting maintenance of embryo structures under conditions of water deficit.

3.4. Expression study of *VvDhn* under stress conditions and elicitor applications

3.4.1. Salt stress

Our experiments showed that salt treatment (100 mM NaCl) induced early (6 h) and late (24 h) expression of *VvDhn* in leaves of grape plants. The level of transcripts detected in the tolerant cultivar was twice as high as in the sensitive one. Moreover, this tolerant cultivar was better able to recover after 72 h since there was no further detection of *VvDhn* transcripts. The sensitive cultivar was still expressing *VvDhn* even after 72 h of recovery, meaning that it could not adapt well to this salt constraint. It was previously reported that dehydrins are expressed in grape tissues subjected to drought stress (Xiao and Nassuth, 2006; Yang et al., 2012; Choi et al., 2013); our study found that, in addition, *VvDhn* is expressed upon salt stress. Moreover, we originally showed that *VvDhn* is particularly induced in tolerant varieties, which implies its involvement in the mechanism of salt and drought stress adaptation, not only as a molecular response, as this gene was less expressed in the sensitive variety. This differential expression of *VvDhn* under salt treatment and within the contrasting genotypes suggests its involvement in salt-stress response and adaptation (Figure 4). It is well known that dehydrin genes are upregulated under drought stress and downregulated following rehydration (Yang et al., 2012; Vaseva et al., 2014a). In Mediterranean saltbush, the expression level of the dehydrin gene was enhanced by salinity stress in roots but not in shoots (Sadder and Al Doss, 2014). This was confirmed for salt stress for the first time in grapes in our study, where *VvDhn* was differentially expressed in contrasting varieties.

3.4.2. Drought stress

The expression of *VvDhn* was also investigated under drought stress. Clearly, drought treatment induced accumulation of *VvDhn* in both genotypes; nevertheless, the amount of transcripts in the tolerant genotype was about 3-fold higher than in the sensitive one, showing its implication in drought tolerance (Figure 5). When studying grapevine metabolism response to water deficit in 2 contrasting cultivars, Shiraz and Cabernet Sauvignon, which have different hydraulic behaviors, Hochberg et al. (2013) showed a differential molecular response to stress among genotypes and identified biologically relevant metabolites. It is well known that water deficit also induces the synthesis of protective proteins, such as dehydrins and

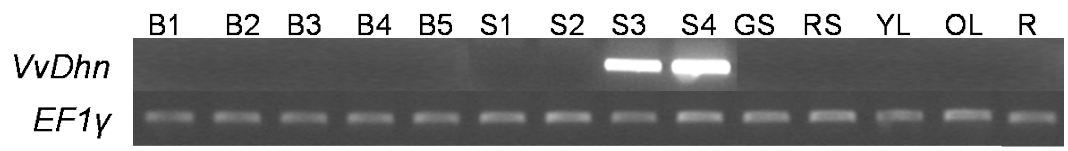

Figure 3. *VvDhn* expression during *Vitis vinifera* berry development. RT–PCR was performed as described in Materials and methods. *Elongation factor-1γ* (*Ef1γ*) was used as an internal control to normalize expression of *VvDhn*. B1 and B2, green (whole) berry (3 and 6 weeks after flowering, respectively); B3, véraison (turning color) stage (8 weeks after flowering); B4 and B5, red (whole) berry (10 and 12 weeks after flowering, respectively); S1, S2, S3, and S4 seeds, (3, 6, 8, and 10 weeks after flowering, respectively); GS, green skin; RS, red skin; YL, young leaves (light green); OL, old leaves (dark green); R, roots.

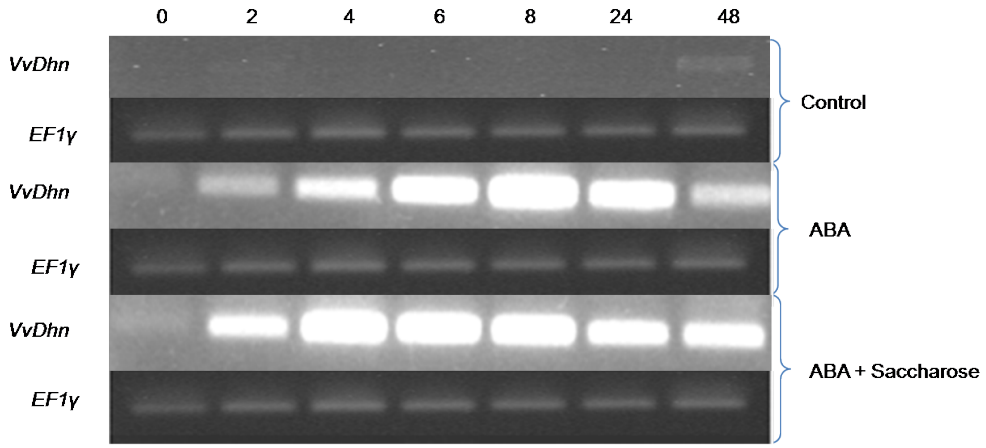

Figure 4. *VvDhn* expression under salt stress (100 mM NaCl) for both tolerant (Razegui) and sensitive (Syrah) varieties. Northern blot was performed as described in Materials and methods. C, control plants; S, stressed plants. Analyses were realized 6 and 24 h after salt treatment and 72 h after recovery.

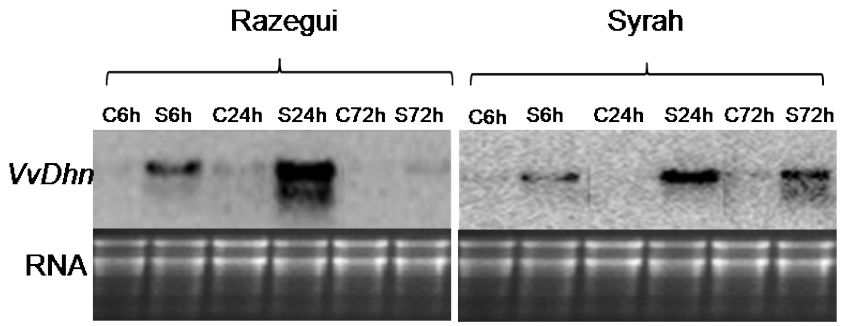

Figure 5. *VvDhn* expression under drought stress for both tolerant (Kahli) and sensitive (Galb Sardouk) varieties. Northern blot was performed as described in Materials and methods. C, control plants; S, stressed plants.

late-embryogenesis–abundant (LEA) proteins (Cramer et al., 2013; Hochberg et al., 2013). Indeed, higher expression levels of dehydrin in tolerant genotypes under drought stress are commonly observed (Hu et al., 2010); nevertheless, this is not always the case, as differences in expression levels between tolerant and sensitive genotypes are often dependent on the type of dehydrin as well as the duration of the stress (Yang et al., 2012). Upon water deprivation, dehydrin transcripts increased in vessel-associated cells of grapevine leaf petioles (Chitarra et al., 2014). In *Trifolium repens*, Vaseva et al. (2014a) suggested that different dehydrin classes have distinct roles in the drought-stress response and vegetative development. The *VvDhn* transcription under drought stress confirms the activity of ERD-responsive elements found in the regulatory region of the gene. Since dehydrin genes are expressed during water-stress responses in plants, it was speculated that by stabilizing membranes they protect plants from damage caused by cell desiccation (Campbell and Close, 1997). Moreover, due to their unfolded state, higher accumulation, and capability to bind water, dehydrin proteins can help maintain the original cell volume, thus preventing cellular collapse (Hanin et al., 2011).

3.4.3. Elicitors

Since the plant hormone ABA is produced under water-deficit conditions and is instrumental in the development of tolerance against drought, we studied the expression of *VvDhn* in berry cell suspensions under both ABA and saccharose treatments. RT-PCR (Figure 6) showed that ABA early and highly induces *VvDhn*. Moreover, combined with saccharose, ABA could enhance the expression of *VvDhn* in kinetics and amount. Saccharose seems to positively interact with ABA to induce the expression of *VvDhn* in grapeberry cells. Our results, indicating that *VvDhn* is inducible by ABA treatment, confirm the

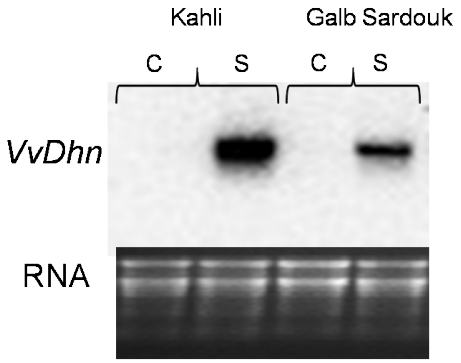

Figure 6. *VvDhn* expression in grapeberry cell suspension under ABA/saccharose treatments during 48 h. RT–PCR was performed as described in Materials and methods. *Elongation factor-1γ* (*Ef1γ*) was used as an internal control to normalize expression of *VvDhn*.

functionality of ABA regulatory elements found in the promoter sequence.

In conclusion, we have characterized a salt- and drought-inducible dehydrin gene from *Vitis vinifera* that was differentially expressed in 2 pairs of varieties that showed contrasting behavior against salt and drought stresses, respectively. The VvDhn protein contains the Y-, S-, and K-conserved domains and belongs to the YSK2 family. *VvDhn* is specifically expressed in seeds at the late stages of maturation and not in vegetative tissues, whereas once a plant is exposed to salt or drought stress dehydrin accumulates in high amounts, particularly in a tolerant genotype. *VvDhn* expression is also regulated by ABA elicitor. Interestingly, the presence of particular cis-elements (mainly, abiotic stress response elements (ABRE)) within the promoter region was positively correlated with *VvDhn* expression profiles, and since *VvDhn* is both induced by drought and salt stresses, particularly among the tolerant varieties, it can be used as a molecular marker and would be an interesting candidate for abiotic-stress–tolerance improvement and trait breeding. Overexpression of this protein through genetic transformation will allow for validation of its function and, consequently, promote improvement in the tolerance of grapevine to drought and salinity. Indeed, genetic transformation of tobacco plants by *VvDhn* under P35S-constitutive promoter greatly improved their tolerance to salt and drought stresses in comparison with wild-type plants.

References

Allagulova ChR, Gilamov FR, Shakirova FM, Vakhitov VA (2003). The plant dehydrins: structure and functions. Biochemistry (Moscow) 68: 945–951.

Alsheikh MK, Heyen BJ, Randall SK (2003). Ion binding properties of the dehydrin ERD14 are dependent upon phosphorylation. J Biol Chem 278: 40882–40889.

Asif MH, Dhawan P, Nath P (2000). A simple procedure for the isolation of high quality RNA from ripening banana fruit. Plant Mol Biol Rep 18: 109–115.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J Mol Biol 215: 403–410.

Bailey TL, Elkan C (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers, Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology, pp. 28–36, AAAI Press, Menlo Park, California, USA.

Blaskovic S, Blanc M, van der Goot FG (2013). What does S-palmitoylation do to the membrane proteins? FEBS J 280: 2766–2774.

Campbell SA, Close TJ (1997). Dehydrins: genes, proteins, and associations with phenotypic traits. New Phytol 137: 61–74.

Chang S, Puryear J, Cairney J (1993). A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11: 113–116.

Chitarra W, Balestrini R, Vitali M, Pagliarani C, Perrone I, Schubert A, Lovisolo C (2014). Gene expression in vessel-associated cells upon xylem embolism repair in *Vitis vinifera* L. petioles. Planta 239: 887–899.

Choi Y-J, Hur YY, Jung S-M, Kim S-H, Noh J-H, Park S-J, Park K-S, Yun H-K (2013). Transcriptional analysis of Dehydrin1 genes responsive to dehydrating stress in grapevines. Hortic Environ Biote 54: 272–279.

Church GM, Gilbert W (1984). Genomic sequencing. P Natl Acad Sci USA 81: 1991–1995.

Close TJ (1996). Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plantarum 97: 795–803.

Close TJ (1997). Dehydrins: a commonality in the response of plants to dehydration and low temperature. Physiol Plantarum 100: 291–296.

Cramer G (2010). Abiotic stress and plant responses from the whole vine to the genes. Aust J Grape Wine R 16: 86–93.

Cramer GR, Van Sluyter SC, Hopper DW, Pascovici D, Keighley T, Haynes PA (2013). Proteomic analysis indicates massive changes in metabolism prior to the inhibition of growth and photosynthesis of grapevine (*Vitis vinifera* L.) in response to water deficit. BMC Plant Biol 13: 49.

Daldoul S, Chenenanoui S, Mliki A, Höfer M (2009). Improvement of an RNA purification method for grapevine (*Vitis vinifera* L.) suitable for cDNA library construction. Acta Physiol Plant 31: 871–875.

de Lartigue J (2011). Phosphorylation: the master switch of the cell. Oncology live. Published Online: Thursday, December 15, 2011.

Decendit A, Ramawat KG, Waffo P, Deffieux G, Badoc A, Mérillon JM (1996). Anthocyanins, catechins, condensed tannins and piceid production in *Vitis vinifera* cell bioreactor cultures. Biotechnol Lett 18: 659–662.

Deinlein U, Stephan AB, Horie T, Luo W, Xu G, Schroeder JI (2014). Plant salt-tolerance mechanisms. Trends Plant Sci 19: 371–379.

Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde JP, Mérillon JM, Hamdi S (2006). Characterization of a grapevine R2R2-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiol 140: 499–511.

Downey M, Harvey J, Robinson S (2003). Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. Aust J Grape Wine R 9: 15–27.

Dure L III (1993). Structural motifs in LEA proteins. In: Close TJ, Bray EA, editors. Plant Responses to Cellular Dehydration During Environmental Stress. Current Topics in Plant Physiology. Vol. 10. Rockville, MD, USA: American Society of Plant Physiologists, pp. 91–103.

Gaper NE, Giovanonni JJ, Whatkins CB (2014). Understanding development and ripening of fruit crops in an 'omics' era. Hort Res 1: 14034. doi:10.1038/hortres.2014.34.

Gautier R, Douguet D, Antonny B, Drin G (2008). HELIQUEST: a web server to screen sequences with specific α-helical properties. Bioinformatics 18: 2101–2102.

Goday A, Jensen AB, Culianez-Macia FA et al. (1994). The maize abscisic acid-responsive protein Rab17 is located in the nucleus and interacts with nuclear localization signals. Plant Cell 6: 351–360.

Gould N, Doulias PT, Tenopoulou M, Raju K, Ischiropoulos H (2013). Regulation of protein function and signaling by reversible cysteine S-nitrosylation. J Biol Chem 288: 26473–26479.

Gupta B, Huang B (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. International Journal of Genomics 214: 1–18.

Hamrouni L (2010). Evaluation de la tolérance au sel chez la vigne en Tunisie. Thèse de Doctorat, Faculté des Sciences de Tunis, pp. 246 (in French).

Hamrouni L, Hanana M, Abdelly C, Ghorbel A (2011). Exclusion du chlorure et inclusion du sodium: deux mécanismes concomittants de tolérance à la salinité chez la vigne sauvage *Vitis vinifera* subsp. *Sylvestris* (var. 'Séjnène'). Biotechnol Agron Soc 15: 387–400 (in French).

Hanana M, Deluc L, Fouquet R, Daldoul S, Léon C, Barrieu F, Ghorbel A, Mliki A, Hamdi S (2008). Identification et caractérisation d'un gène de réponse à la déshydratation rd22 chez la vigne (*Vitis vinifera* L.). CR Biol 331: 569–578 (in French).

Hanin M, Brini F, Ebe C, Toda Y, Takeda S, Masmoudi K (2011). Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms. Plant Signaling and Behavior 6: 1503–1509.

Hara M, Fujinaga M, Kuboi K (2005). Metal binding by citrus dehydrin with histidine-rich Domains. J Exp Bot 56: 2695–2703.

Hennig L (1999). WinGene/WinPep: user-friendly software for the analysis of amino acid sequences. Biotechnology (NY) 26: 1170–1172.

Hewitt EJ (1966). Sand and Water Culture Methods Used in the Study of Plant Nutrition. Technical Communication No. 22. Commonwealth Agricultural Bureaux, Farnham Royal, UK.

Hochberg U, Degu A, Toubiana D, Gendler T, Nikoloski Z, Rachmilevitch S, Fait A (2013). Metabolite profiling and network analysis reveal coordinated changes in grapevine water stress response. BMC Plant Biol 13: 184.

Hu L, Wang Z, Du H, Huang B (2010). Differential accumulation of dehydrins in response to water stress for hybrid and common Bermuda grass genotypes differing in drought tolerance. J Plant Physiol 167: 103–109.

Jaillon O, Aury J-M, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N, Aubourg S, Vitulo N, Jubin C et al. (2007). The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449: 463–467.

Kleinwächter M, Radwan A, Hara M, Selmar D (2014). Dehydrin expression in seeds: an issue of maturation drying. Front Plant Sci 5: 402.

Koag M-C, Fenton RD, Wilken S, Close J (2003). The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity. Plant Physiol 131: 309–316.

Kruger C, Berkowitz O, Stephan UW et al. (2002). A metal binding member of the late embryogenesis abundant protein family transports iron in the phloem of *Ricinus communis* L. J Biol Chem 277: 25062–25069.

Lanigan RM, Sheppard TD (2013). Recent developments in amide synthesis: direct amidation of carboxylic acids and transamination reactions. Eur J Org Chem 2013: 7453–7465.

Matton DP, Nass N, Clarke AE, Newbigin E (1994). Self-incompatibility: how plants avoid illegitimate offspring. P Natl Acad Sci USA 91: 1992.

Moriya K, Nagatoshi K, Noriyasu Y, Okamura T, Takamitsu E, Suzuki T, Utsumi T (2013). Protein N-myristoylation plays a critical role in the endoplasmic reticulum morphological change induced by overexpression of protein lunapark, an integral membrane protein of the endoplasmic reticulum. PLoS ONE 8: e78235.

Ollat N, Geny L, Soyer JP (1998). Les boutures fructifères de Vigne: validation d'un modèle d'étude de la physiologie de la Vigne. J Int Sci Vigne Vin 32: 1–9 (n French).

OIV (2013). International Organization of Vine and Wine: Statistical Report on World Vitiviniculture 2013, pp. 28.

Rorat D (2006). Plant dehydrins—tissue location, structure and function. Cell Mol Biol Lett 11: 536–556.

Sadder MT, Al-Doss AA (2014). Characterization of dehydrin AhDHN from Mediterranean saltbush (*Atriplex halimus*). Turk J Biol 38: 469–477.

Schwarz F, Aebi M (2011). Mechanisms and principles of N-linked protein glycosylation. Curr Opin Struc Biol 21: 576–582.

Seymour GB, Granell A (2014). Fruit development and ripening. J Exp Bot 65: 4489–4490.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar K (2005). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882.

Vaseva II, Anders I, Feller U (2014a). Identification and expression of different dehydrin subclasses involved in the drought response of *Trifolium repens*. J Plant Physiol 171: 213–224.

Vaseva II, Anders I, Yuperlieva-Mateeva B, Nenkova R, Kostadinova A, Feller U (2014b). Dehydrin expression as a potential diagnostic tool for cold stress in white clover. Plant Physiol Bioch 78: 43–48.

Wang Y, Dasso M (2009). SUMOylation and deSUMOylation at a glance. J Cell Sci 122: 4249–4252.

Wang Y, Xu H, Zhu H, Tao Y, Zhang G, Zhang L, Zhang C, Zhang Z, Ma Z (2014). Classification and expression diversification of wheat dehydrin genes. Plant Sci 214: 113–120.

Xiao H, Nassuth A (2006). Stress- and development-induced expression of spliced and unspliced transcripts from two highly similar dehydrin 1 genes in *V. riparia* and *V. vinifera*. Plant Cell Rep 25: 968–977.

Yang Y, He M, Zhu Z, Li S, Xu Y, Zhang C, Singer SD, Wang Y (2012). Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. BMC Plant Biol 12: 140.

Zhang HM, Zhang LS, Liu L, Zhu WN, Yang WB (2013). Changes of dehydrin profiles induced by drought in winter wheat at different developmental stages. Biol Plantarum 57: 797–800.