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,
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 photocyte 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'-CAtTAyGGNaaYcChGTbCAyCA-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

Semi-quantitative 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 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 samples were treated with RNase-inhibitor (Promega). 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’-TAGTCCATAGGTAGGTTG-3’ and reverse primer 5’-CGAGGGCGGTTGGTGAACA-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’-TCAATCCTGTCTAGGAAAGGAAG-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-[32P]-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 Phospholmager 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'-CGGGGCAGGGGCGACAC-3’ and VvDhn-Reverse: 5'-GCAGAAAAGCTGATGCGAGGCTGC3’; 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.org/protParamTools) 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.oua.gr/PRED-TMR), TMpred (http://www.isrec.isb-sib.ch/software/TMPRED_form.html), NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos_2.0), and ProtParam (http://www.expasy.org/tools/protparam.html) programs. Sequence analysis was performed using DNASTR 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.
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 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>
<thead>
<tr>
<th>aa</th>
<th>Properties</th>
<th>Number</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala (A)</td>
<td>Hydrophobic</td>
<td>6</td>
<td>4.8</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>Strongly basic (+)</td>
<td>4</td>
<td>3.2</td>
</tr>
<tr>
<td>Asn (N)</td>
<td>Polar</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>Strongly acidic (–)</td>
<td>5</td>
<td>4.0</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>Disulfur bonds, polar</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Gln (Q)</td>
<td>Polar</td>
<td>18</td>
<td>14.5</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>Strongly acidic (–)</td>
<td>8</td>
<td>6.5</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>Flexible</td>
<td>23</td>
<td>18.5</td>
</tr>
<tr>
<td>His (H)</td>
<td>Hydrogen bonds</td>
<td>6</td>
<td>4.8</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>Hydrophobic</td>
<td>3</td>
<td>2.4</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>Hydrophobic</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>Strongly basic (+)</td>
<td>12</td>
<td>9.7</td>
</tr>
<tr>
<td>Met (M)</td>
<td>Hydrophobic</td>
<td>6</td>
<td>4.8</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>Cyclic, hydrophobic</td>
<td>7</td>
<td>5.6</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>Polar</td>
<td>7</td>
<td>5.6</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>Polar</td>
<td>10</td>
<td>8.1</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>Polar</td>
<td>3</td>
<td>2.4</td>
</tr>
<tr>
<td>Val (V)</td>
<td>Hydrophobic</td>
<td>1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

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 KGMKEKIKERPG and KGMMEKKEKLPG 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++ but not Ca++, 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 (Blaskovic 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.

<table>
<thead>
<tr>
<th>Site</th>
<th>SUMOylation</th>
<th>N-glycosylation</th>
<th>Casein kinase II phosphorylation</th>
<th>N-myristoylation</th>
<th>Protein kinase C phosphorylation</th>
<th>Amidation</th>
<th>Palmitoylation</th>
<th>MHC binding</th>
<th>S-nitrosylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sites</td>
<td>4</td>
<td>3</td>
<td>9</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
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 (E1f1γ) 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.](image-url)

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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
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.

**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*.

### References


