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The crucial role of Φ- and K-segments in the *in vitro* functionality of *Vitis vinifera* dehydrin DHN1a

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ABSTRACT

Dehydrins (DHNs), group II LEA (Late Embryogenesis Abundant) proteins, are among the most commonly observed proteins which accumulate in plants in response to cold and any other environmental factors, causing the dehydration of cells. In previous studies, we isolated a YSK_2-type VvcDHN1a gene from table grapes (*Vitis vinifera* cv. Cardinal) which presented two spliced variants (the spliced, *DHN1a_s* and the unspliced, *DHN1a_u*). Their expression was induced by low temperature storage and CO_2, although with different accumulation patterns. DHN1a_u codifies for a truncated YS protein lacking Φ- and K-segments, which might affect its functionality. In this work, we expressed both DHN1a_s and DHN1a_u recombinant proteins in *Escherichia coli*. We carried out a number of *in vitro* assays to analyze the implications that Φ- and K-segments have in the protective role of VvcDHN1 against different abiotic stresses and their antifungal activity against the fungal pathogen *Botrytis cinerea*. Our results showed that unlike DHN1a_u, DHN1a_s has a potent cryoprotective effect on lactate dehydrogenase activity, protects malate dehydrogenase against dehydration and partially inhibits *B. cinerea* growth. Moreover, the DHN1a promoter presented *cis*-regulatory elements related to cold and drought, as well as biotic stress-related elements. We also observed that both spliced variants interact weakly with DNA, suggesting that K-segments are not involved in DNA binding. Overall, this work highlights the crucial role of Φ- and K-segments in DHNs function in plant response to abiotic stress showing for the first time, the potential role of the *V. vinifera* DHN1a_s in the protection against freezing and dehydration as well as inhibiting *B. cinerea* growth.

1. Introduction

In order to cope with abiotic stress such as cold, drought or salinity, plants have developed defensive strategies to prevent loss of intracellular water that leads to dehydration. Dehydrins (DHNs), a subgroup of late embryogenesis abundant (LEA) proteins also known as LEAD11 or LEA type 2, are major proteins expressed in response to water-limited environments (Ingram and Bartels, 1996; Close, 1997). DHNs are characterized by a highly conserved lysine-rich 15-amino-acid motif (consensus EKKGIMDKIKEKLPG) called K-segment (Close, 1997). It has been proposed that K-segments may form amphipathic helices, although NMR studies showed that the protein is only very weakly helical in solution (Hughes and Graether, 2011). Other structural domains of most DHNs include the S-segment (a tract of serine residues) and the Y-segment (T/VDEYGNP), which is usually found in 1–3 copies in the N-terminus and is similar to the nucleotide-site of plants and bacterial chaperones (Close, 1997). Although not specifically included in the YSK naming system, DHNs also contain Φ-segments, which are rich in polar amino acids and either glycine or a combination of proline and alanine. Overall, DHN sequences are rich in charged and polar amino acids, making them highly hydrophilic and boiling stable. Furthermore, the high-polar residue content of DHNs causes them to be intrinsically disordered proteins (IDPs; Hughes and Graether, 2011), which do not adopt a well-defined folded structure but stay flexible even in their native environment (Tompa, 2002).

Low temperature is the most important condition for maintaining postharvest fruit quality. Although table grapes (*Vitis vinifera*) are classified as chilling tolerant, their storage life at low temperature is limited by their high susceptibility to fungal decay and

Abbreviations: BSA, bovine serum albumin; DHN, dehydrin; DRE, dehydration-responsive elements; EMSA, Electrophoresis Mobility Shift Assay; IDPs, intrinsically disordered proteins; IPTG, isopropyl-β-D-thiogalactopyranoside; LDH, lactate dehydrogenase; LEA, late embryogenesis abundant; MDH, malate dehydrogenase; MeJA-RE, MeJA-responsive elements; LTR, low temperature-responsive elements; PD_{50}, protein dosage that renders 50% of cryoprotection; TCA, salicylic acid-responsive elements.

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sensitivity to serious water loss after harvest. Exposure of plants to moderate temperature stress not only induces resistance to severe stress of this kind, but can also improve tolerance to other stresses. In previous studies, we have shown that a 3-day CO_2 pretreatment is effective in maintaining table grape quality during the storage at low temperature (Sanchez-Ballesta et al., 2006) and reported the isolation of a Y_2K-type *VvcDHN1a* gene from table grapes (Fernandez-Caballero et al., 2012). This gene presented an alternative splicing, consisting of the retention of a 84-bp intron resulting in a truncated YS protein. We have observed that low temperature storage and CO_2 induced spliced and unspliced *VvcDHN1a* transcripts but with a different temporal accumulation pattern (Fernandez-Caballero et al., 2012). Alternative splicing in response to environmental stress enables cells to synthesize different proteins from a single gene. The shortened polypeptides formed following an alternative splicing are not necessarily functionless forms of the full length protein. In *Arabidopsis*, cold and heat stresses regulate the alternative splicing of pre-mRNAs of many Serine/Arginine-rich proteins, a conserved family of splicing regulators in eukaryotes (Palusa et al., 2007).

The overexpression of genes encoding DHN proteins can improve abiotic stress in plants (Brini et al., 2007; reviewed by Kosová et al., 2007; Xu et al., 2008). Furthermore, it should be noted that DHNs are also responsive to biotic stress (Liu et al., 2004). In *V. vinifera DHN1* was found to be up-regulated following inoculation with *Erysiphe necator*, which is the causative agent of grapevine powdery mildew (Yang et al., 2012). Functional analyses of DHN proteins have also been carried out *in vitro*. These include the following: stabilization of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) during freezing and/or drying (Lin and Thomashow, 1992; Sanchez-Ballesta et al., 2004; Hughes and Graether, 2011); prevention of water loss (Tompa et al., 2006); capability of binding ions (Alsheikh et al., 2003) and nucleic acids (Hara et al., 2009); chaperone activity against the heat-induced inactivation and aggregation of various proteins (Kovacs et al., 2008); and prevention of ice crystal growth in a manner similar to antifreeze proteins (Wisniewski et al., 1999). The functions of DHNs are intimately related to their structure. For instance, it has been suggested that the phosphorylation state of serine residues in the S-segment enhances nuclear localization (Goday et al., 1994) and may play a role in activating ion binding (Alsheikh et al., 2003). On the other hand, the K-segment is necessary and sufficient for the binding of DHNs to anionic phospholipid vesicles (Koag et al., 2009). Likewise, a recent work on wheat DHN-5 suggested that K-segments are indispensable for the protective functions of DHNs (Drira et al., 2013). Hughes et al. (2013) indicated that disorder and length are important for DHNs to function as effective molecular shields. Furthermore, it has been documented that a flexible Φ-region together with the presence of K segments are required to ensure that the DHN is large enough to prevent enzyme denaturation (Hughes and Graether, 2011). However, to our knowledge, the possible physiological role of DHN proteins in *V. vinifera* has yet to be analyzed. Our hypothesis is that the lack of Φ- and K-segments in the YS-truncated form of DHN1a from *V. vinifera* might affect its functionality. In order to prove this and to further characterize VvDHN1a we expressed both spliced (DHN1a_s) and unspliced (DHN1a_u) recombinant proteins in *Escherichia coli*. After purification, we tested their protective role on enzymatic activities against different abiotic stresses such as cryoprotection or dehydration. We also investigated whether both recombinant proteins participate in table grape response to biotic stress by studying the antifungal activity against the fungal pathogen *Botrytis cinerea*.

2. Results

2.1. DHN1a_s and DHN1a_u sequence analysis

The full length of *DHN1a_s* and *DHN1a_u* was previously isolated from the pulp of table grapes by RT-PCR (Fernandez-Caballero et al., 2012). The predicted protein for DHN1a_s codifies a YSK_2 dehydrin, while DHN1a_u corresponds to an isoform with the retained intron that coded for a truncated YS protein (Fig. 1A). Despite the fact that DHN1a_u does not have Φ- and K-segments, it shares some structural characteristics with DHN1a_s, such as a high content of polar amino acids (59% and 54%, respectively) (http://www.alphalyse.com/gpmaw_lite.html). More specifically, they are enriched in glutamic acid, lysine, glycine, glutamine, serine, proline and alanine and depleted in cysteine, isoleucine, leucine, tryptophan, phenylalanine and tyrosine. This feature is typical of disordered proteins compared to sequence of ordered proteins (Lise and Jones, 2005), albeit with some differences between DHN1a_s and DHN1a_u (Supplementary Table 1). Moreover, *in silico* predictions with disorder predictor tools such

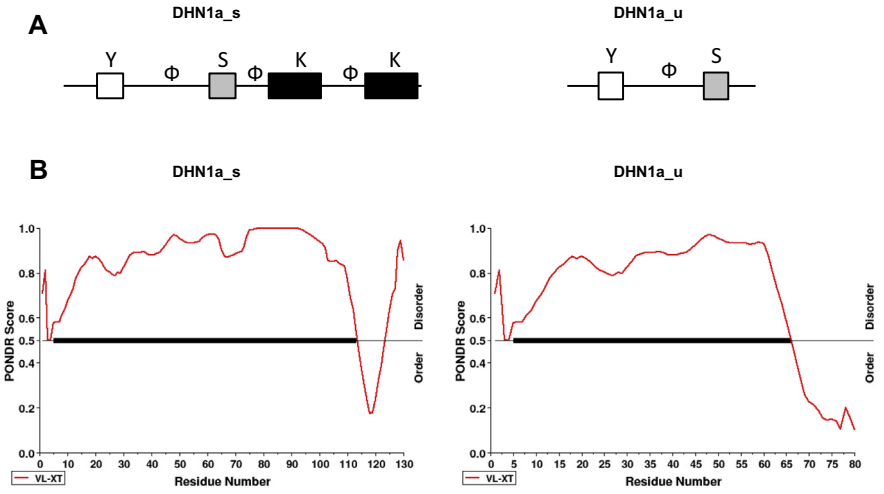

Fig. 1. Schematic representation indicating the position of the Y, S, K and Φ domains from DHNs (A) and *in silico* prediction of protein disorder (B) of spliced (DHN1a_s) and unspliced (DHN1a_u) sequences. Predictions were performed with PONDR VSL1 algorithm with the amino acid sequences of DHN1a_s (JN689936) and its alternative ORF, DHN1a_u.