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Impact of high CO_2 levels on heat shock proteins during postharvest storage of table grapes at low temperature. Functional *in vitro* characterization of VVIHSP18.1

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ABSTRACT

The role played by heat shock proteins (HSPs) in improving fruit quality during postharvest treatments has mainly been studied regarding heat treatments, while little is known about the effect of CO_2 treatments. In this study, we have analyzed the gene expression of five heat shock proteins (*HSPs*) and one heat shock factor (*HSF*) in the skin of red table grapes (*Vitis vinifera* cv. Cardinal) to determine whether a pretreatment with high CO_2 levels (20 kPa) modulated their expression and how the length of the treatment (1 or 3 d) could influence this change. The 3-d high CO_2-treatment was effective in reducing total decay and induced the accumulation of three small HSPs (*VviHSP18.1*, *VviHSP18.2* and *VviHSP22.0*), whereas *VviHSP70.0* and *VviHSF4-a* gene expression were induced by both treatments. To shed light on the putative physiological role of a small HSP (VviHSP18.1) acting as a chaperone, the recombinant protein was overexpressed in *Escherichia coli*. It was then purified and mass spectrometry confirmed that the isolated protein was VviHSP18.1, belonging to class I sHSP. Although the purified protein was stable at different high temperatures, when temperature was above 70 °C, a weaker and smaller protein band appeared which was identified by mass spectrometry as VviHSP18.1 with a C-terminal truncation. The recombinant VviHSP18.1 protein displayed chaperone activity which protects citrate synthase (CS) and malate dehydrogenase (MDH) from thermal aggregation at 45 °C, and also displayed the protection of alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) activities at 55 °C and 65 °C, respectively. By contrast, VviHSP18.1 did not protect LDH from freezing-induced inactivation. Taken together, these results support the hypothesis that a high CO_2 treatment is an active process where HSPs could participate in preventing the denaturation and dysfunction of different proteins.

1. Introduction

Plants are sessile organisms which have developed complex mechanisms for perceiving environmental biotic and abiotic stresses, and respond by altering the expression of defense-signaling molecules. Since exposure to different stresses normally causes protein dysfunction, it is particularly important for plants to maintain their functional conformations and prevent the aggregation of non-native proteins in order to maintain cellular homeostasis under stressful conditions. Plants have specific proteins for this purpose, among which is one particular family that comprises heat shock proteins (HSPs) (reviewed by Aghdam et al., 2015). While HSPs were first described in relation to high temperatures, they are also induced in plants in response to different abiotic stresses, such as salinity, osmotic, cold and water stress (Wang et al., 2004). Five main HSP families have been identified in plants and grouped according to their average molecular weights: Hsp100s, Hsp90s, Hsp70s, the chaperonins Hsp60s and the small HSPs (sHsp) family. Transcription of HSP genes is regulated by heat shock transcription factors (HSFs) through binding to *cis*-acting regulatory elements called heat shock element (HSEs), located in the promoter region of HSP genes (Scharf et al., 2012).

It has previously been suggested that HSPs exert their protection during stress mainly due to their biological function as molecular chaperones which prevent protein aggregation and promote the proper refolding of denatured proteins, as well as assisting in the assembly and disassembly of protein complexes and in protein degradation (Hartl and

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Hayer-Hartl, 2002; Kim et al., 2007; Ezemaduka et al., 2017). Among the molecular chaperone families, sHSP are the only known ATP-independent chaperones (Jakob et al., 1993) and as "holdase" chaperones (though incapable of folding polypeptides *per se*), they protect cells from protein aggregation. However, they must release their aggregation-prone clients to other downstream chaperones, which then ensure the correct folding (Eyles and Gierasch, 2010).

Sanchez-Bel et al. (2012) suggested that the accumulation of sHSP is key as the first response of tomato fruit to chilling injury. Recently, Ré et al. (2017) reported the increased expression of *sHSPs* in Micro-Tom fruit stored at 4 °C, substantiating the hypothesis that sHPSs may participate in the mechanisms involved in the protection against cold stress. It is known that HSPs not only protect against the stress that causes their accumulation but also against any subsequent stressful situation (Lin et al., 1984; Chen et al., 1988). The induction of *HSPs* gene expression after a moderately high temperature (6 h–72 h at 38 °C) in both avocado (Woolf et al., 1995) and tomato (Sabehat et al., 1998) fruit has been shown to protect them during subsequent prolonged low temperature storage and was correlated with protection against chilling injury. Likewise, in a recent work Salazar-Salas et al. (2017) indicated that hot water at 42 °C for 5 min induced chilling injury tolerance in tomato fruit and up-regulation and greater protein accumulation of sHSP during cold storage and after ripening. These findings are associated with cross-tolerance phenomena in which plants exposed to one type of stress condition may gain protection against other biotic or abiotic stresses (Bowler and Fluhr, 2000; Pastori and Foyer, 2002; Wang et al., 2003). Aghdam et al. (2013) indicated that various postharvest treatments such as chemical ones (treatments with salicylate and derivatives) and physical ones (hot water/hot air pretreatments, preconditioning at medium-low temperatures, UV-C light) applied to reduce chilling injury during storage of different fruit triggered *HSP* gene expression and protein accumulation.

In previous studies, we observed that applying a short-term high $-CO_2$ treatment (20 kPa CO_2) for 3 d at low temperature maintained the quality of different table grape cultivars during postharvest storage and improved the tolerance to temperature shift at 0 °C (Sanchez-Ballesta et al., 2007; Rosales et al., 2016; Vazquez-Hernandez et al., 2017). In a recent transcriptional analysis, we found that genes coding for HSPs were strongly represented among those up-regulated at 0 °C in the skin of non-treated Cardinal table grapes (Rosales et al., 2016). However, it is interesting to note that a small number of *HSPs* and *HSFs*, which are different from those activated in non-treated grapes, were induced by high CO_2 levels. To date, information about modulation of HSPs gene expression in *Vitis vinifera* is restricted to high temperatures (Liu et al., 2012; Carbonell-Bejerano et al., 2013; Wu et al., 2015), high light (Carvalho et al., 2011) or UV-C irradiation (Xi et al., 2014), whereas the effect of high CO_2 levels remains unknown. To our knowledge, the only study in *V. vinifera* with high CO_2 levels was carried out using detached wine grapes (cv. Trebbian) treated with 30 kPa of CO_2 for 3 d at 20 °C, which was effective in altering the general metabolism and inducing the expression of a *sHSP* (Becatti et al., 2010). In order to find out whether the application of high CO_2 levels modulate the expression of *HSPs* and *HSFs* in table grapes and also whether the length of the treatment affects this modulation, we have analyzed the gene expression of five *HSPs* (*VviHSP18.1*, *VviHSP18.2*, *VviHSP22.0*, *VviHSP70.0* and *VviHSP90.1*) and *VviHSF4-a* in the skin of Cardinal table grapes treated with high CO_2 for 1 d and 3 d during postharvest storage at 0 °C. Furthermore, to investigate the behavior of *V. vinifera* sHSPs, we produced the recombinant VviHSP18.1 protein in *Escherichia coli*. The purified recombinant VviHSP18.1 was stable at high temperature and remained soluble at 45 °C, although temperatures above 70 °C affected the stability since a putative truncation at the C-terminal seems to occur. Likewise, our results showed that the recombinant VviHSP18.1 behaves, *in vitro,* as a molecular chaperone which protects enzyme from thermal aggregation and thermal inactivation at high temperatures. The results obtained in this study could lead to a more thorough characterization of the molecular basis implicated in the beneficial effect of high CO_2 levels in maintaining table grape quality.

2. Materials and methods

2.1. Plant material

Table grapes (*Vitis vinifera* cv. Cardinal) were randomly collected in Camas (Sevilla, Spain) at the early commercial stage (15.73% soluble solids, 0.38% tartaric acid). The clusters were transferred to the ICTAN laboratory in Madrid (Spain) on the same day of harvesting and fruit were immediately forced-air precooled for 14 h at −1 °C. Thereafter, those that did not present mechanical or pathological defects were randomly divided into three lots and stored at 0 ± 0.5 °C with a relative humidity of 95% in three sealed neoprene containers of 1 m^3 capacity. Ten boxes with a content of around 3 kg of table grapes per box were stored in each container. One lot was stored under normal atmospheric conditions for a total of 13 d (non-treated fruit). The other lot was stored with a gas mixture containing 20 kPa CO_2 + 20 kPa O_2 + 60 kPa N_2 for 3 d (3-d CO_2-treated fruit) and then transferred to air under the same conditions as the non-treated bunches until the end of the storage period. In order to analyze the effectiveness of the treatment with high CO_2 levels during short time, a third lot of bunches was stored with high CO_2 levels for 1 d (1-d CO_2-treated fruit), then transferring the clusters to normal atmospheric conditions until the end of the storage period. At time 0 (after precooling at −1 °C) and after 1, 3, 6 and 13 d of storage under air or CO_2 conditions, berries from three biological replicates (each replicate consisting of two bunches) were peeled and the skin was collected, frozen in liquid nitrogen, grounded to a fine powder, and stored at −80 °C until analysis.

2.2. Quality assessments

SSC (expressed in %) was determined using a digital refractometer (Atago PR-101, Japan) at 20 °C. TA (expressed in % tartaric acid) was determined by titration with 0.1 N NaOH up to pH 8.1. Total decay was assessed as the percentage of decayed berries with respect to the original cluster weight.

2.3. RNA extraction, cDNA synthesis

Total RNA extraction and cDNA synthesis was performed according to Romero et al. (2016).

2.4. Isolation and purification of partial HSPs

The oligonucleotides used to determine the expression pattern of the different *VviHSPs* (*VviHSP18.1*, *VviHSP18.2*, *VviHSP22.0*, *VviHSP70.0* and *VviHSP90.1*) and the transcription factor *VviHSFA4-a* were designed using the program Primer 3 (Untergasser et al., 2012) from the information of the sequences used to design the GeneChip® oligonucleotide array of Affymetrix used in previous studies (Rosales et al., 2016) (see Table S1). The specificity of the oligonucleotides was checked by PCR (95 °C 4 min, 91 °C 1 min, 57 °C 1 min, 72 °C 1 min, 72 °C 10 min) using a mixture of cDNAs from treated and non-treated samples with CO_2 based on the results of the transcriptomic analysis of the mechanisms of adaptation to high concentrations of CO_2 applied during 1 or 3 d in table grape (data not shown). The PCR fragments were analyzed on a 2% agarose gel and the fragments were purified using the "Clean-Easy Agarose Purification" kit (Canvax) following the manufacturer's instructions. The sequence of the partial *HSPs* and *HSF* was confirmed by Sanger sequencing at the Genomics Department of the Biological Research Center (CIB-CSIC, Madrid, Spain).

2.5. Relative gene expression by quantitative real-time RT-PCR

The relative expression of *HSPs* and *HSF* in the skin of Cardinal 1-d and 3-d CO_2-treated and non-treated clusters stored at 0 °C was assayed by quantitative PCR with reverse transcription (qRT-PCR). The amplifications were carried out in 96-well plates in an iCycler IQ thermocycler (Bio-Rad) and the quantification was made with the respective associated software iCycler IQTM (Real Time Detection System Software, version 2.0). The amplification reactions were carried out in triplicate in a final volume of 20 µL containing 10 µL of SYBR® Green (2x), 1 µL of each primer (2 µM) and 10 ng of the cDNA. The PCR profile used was 2 min at 50 °C, 95 °C for 10 min, followed by 40 cycles of 20 s at 95 °C and 1 min at 58 °C. Two technical replicas were made from each of the genes studied. *Actin-1* (ACT1: XM 002282480) from *V. vinifera* was used as an internal reference gene to normalize the amplification values. Relative expression levels were estimated following the $2^{-\Delta\Delta Ct}$ method, and denoted as the fold difference from the expression present at the calibrator sample (time 0). The specificity of products was validated by dissociation curve analysis and by agarose gel; and its sequences confirmed at the Genomic Department of the CIB-CSIC (Madrid, Spain).

2.6. Heterologous expression of VviHSP18.1 in Escherichia coli

To characterize the *in vitro* functionality of the recombinant protein VviHSP18.1, the full-length cDNA was subcloned into the pET28a expression vector (KanR, T7 promoter, Novagen). Specific oligonucleotides were designed to amplify the full-length cDNA using the program Primer 3 (Koressaar and Remm, 2007) and the application of PCR Primer Stats, from the Sequence Manipulation Suite page, (http://www.bioinformatics.org/sms2/pcr_primer_stats.html) based on the sequence of *V. vinifera* (VviHSP18.1: XM_002280899) deposited in the NCBI database. The sense oligonucleotide of the gene (HSP18.1_Fw_EcoRI GTGGAATTCATGGCACTCATTTCAAGTGTTCTTGG) contained the target for the EcoRI restriction enzyme (underlined), while the antisense (HSP18.1_Rv_XhoI ACCCTCGAGTTAACCAGATATCTCGATCGCC TTC) contained the target for XhoI restriction enzyme (underlined). The PCR conditions were a first cycle of 95 °C for 5 min, followed by 30 cycles: 95 °C 1 min, 58 °C 1.5 min, 72 °C 1.5 min and a final elongation cycle of 72 °C for 10 min. The amplified DNA fragments were digested with EcoRI and XhoI and ligated into the multicloning site of the pET28a previously digested with the same enzymes. The vector pET28a-VviHSP18.1 was transformed into *Escherichia coli* BL21 (D3) cells. A single recombinant colony was grown overnight at 37 °C in 5 mL of lysogeny broth (LB) with Kanamycin (15 mg L^{-1}) with shaking (200 rpm). Then the overnight culture was transferred into 200 mL on LB-medium and grown with vigorous shaking until the optical density at 600 nm reached 0.6 and then induced with 1 mM isopropyl-*s*-D-thiogalactoside (IPTG). After 5 h of induction at 37 °C, bacteria were harvested by centrifugation at 12,000 × *g* for 5 min at 4 °C. In parallel, a culture of *E. coli* BL21 (D3) transformed with an empty pET28a plasmid was taken as a negative control.

Purification and concentration of VviHSP18.1 was then performed according to Romero et al. (2016). Protein analysis was performed on 13% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-Protean II Cell (Bio-Rad) equipment as described by Rosales et al. (2014). Western blots were probed with a mouse anti-6xHis monoclonal antibody (1:500) and a horse radish peroxidase-conjugated antimouse IgG secondary antibody (1:2000) (R&D Systems, Inc.). The immuno-complexes were visualized using the enhanced chemiluminescence detection system (Amersham,GE Healthcare, UK) and the equipment for analysis and documentation of ChemiDoc™ XRS gels with the associated software ImageLab ™ (Bio-Rad).

2.7. In vitro thermal stability of the recombinant VviHSP18.1 protein. Mass spectrometry analysis

The thermal stability of the recombinant protein VviHSP18.1 was analyzed by subjecting it to different high temperatures and boiling. Thus, samples of VviHSP18.1 (0.05 g L^{-1}) in 20 mM KPB were exposed to temperatures of 22, 45 and 70 °C for 45 min in a water bath. The samples were then centrifuged for 2 min at 12,000 × *g*. Another sample was boiled for 10 min and then centrifuged for 30 min at 12,000 × *g*. Then, samples were run in a 15% polyacrylamide SDS-PAGE gel in order to separate the bands. VviHSP18.1 protein bands were excised from the Coomassie-stained gel and were subjected to tryptic digestion and analyzed by MALDI-TOF MS for peptide mass fingerprint (PMF) identification and MS/MS sequencing analysis performed in a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) in positive reflector mode following the procedure described by Goñi et al. (2010), at the Proteomics Unit of Complutense University of Madrid (Spain). For protein identification, the non-redundant NCBI Data Base (17919084 sequences; 6150218869 residues) with taxonomy restriction to Viridiplantae (Green Plants) (1056156 sequences) and a home-made Data base with the VviHSP18.1 recombinant protein sequence were searched using a local license of MASCOT 2.3 server (www.matrixscience.com) through the Global Protein Server v 3.6 (ABSciex), using the same search parameters specified by Goñi et al. (2010). In the identified protein, the probability scores were greater than the score fixed by mascot as significant with a *p*-value minor than 0.05.

2.8. In vitro protection capacity of VviHSP18.1 against the thermal aggregation of the enzymes citrate synthase (CS) and malate dehydrogenase (MDH)

The aggregation of CS (EC 4.1.3.7, Sigma-Aldrich, USA) and MDH (EC 1.1.1.37, Sigma-Aldrich, USA), both from pig heart, upon thermal denaturation was determined by measuring the absorption due to increased turbidity from light scattering at 320 nm (Lee et al., 1995). In separate trials, CS at 0.15 µM or MDH at 0.3 µM were incubated alone or in the presence of VviHSP18.1 [0.5 µM (CS); 2 µM (MDH)] or BSA (control) [0.5 µM (CS); 3 µM (MDH)] in 100 mM Tris-HCl, pH 7.5 in a water bath at 45 °C for 1 h. The experiments were performed with recombinant protein from two independent inductions with two technical replicates of each sample.

2.9. In vitro protection capacity of VviHSP18.1 against thermal inactivation of alcohol dehydrogenase (ADH)

The thermal stability of the ADH of *Saccharomyces cerevisiae* (EC 1.1.1.1, Sigma-Aldrich, USA) was determined by monitoring the residual enzyme activity after 60 min of incubation at 50 °C in 10 mM NaH_2PO_4 (pH 7.5) and 100 mM NaCl. Mixtures of ADH (2.5 µM) were used with VviHSP18.1 (2 µM) or bovine serum albumin (BSA) (2 µM) (control), in a final volume of 200 µL. The samples were incubated on ice for 5 min and transferred to a 50 °C water bath for 1 h. The residual ADH activity was determined every 10 min, adding 5 µL of the above samples to the assay buffer (10 mM $Na_4P_2O_7$, pH 8.8, 4.15 mM NAD^+, 350 mM ethanol) in a final volume of 250 µL. The formation of NADH was measured spectrophotometrically at 340 nm ($\varepsilon 340 = 6.2\ mM^{-1}\ cm^{-1}$). The results are shown as the percentage of residual activity compared to non-heated controls. The experiments were performed with recombinant VviHSP18.1 protein from two independent inductions, with two technical replicates of each sample.

2.10. In vitro lactate dehydrogenase (LDH) protection assay

The protective effect of the recombinant VviHSP18.1 protein on LDH activity after prolonged exposure to high temperature or after two

freeze-thaw cycles was analyzed following the protocol described by Romero et al. (2008) with slight modifications. A stock solution of rabbit muscle LDH (EC 1.1.1.27, Sigma-Aldrich, USA) was prepared (2.85 μM) in 20 mM potassium phosphate buffer, pH 7.5. Mixtures were prepared with 8 μL of LDH of the stock solution and different amounts of VviHSP18.1 or BSA (control) with a molar ratio [protein] / [enzyme] that ranged between 2 and 4, in a final volume of 100 μL of potassium phosphate buffer 20 mM pH 7.5. The resulting solutions were incubated at 65 °C for up to 45 min, at 37 °C during 16 h or were subjected to two cycles of 30 s of freezing in liquid nitrogen and thawing at room temperature. Residual LDH enzyme activity was determined by adding 5 μL of previous samples to the assay buffer (80 mM Tris-HCl, pH 7.5, 2 mM pyruvic acid, 100 mM KCl, 0.3 mM NADH) in a final volume of 250 μL. The formation of NADH was measured spectrophotometrically at 340 nm (ε340 = 6.2 mM^{-1} cm^{-1}). The data are presented as the percentage of the activity compared to non-treated controls. The experiments were performed with recombinant VviHSP18.1 protein from two independent inductions with two technical replicates of each sample.

2.11. Statistical analyses

The data were analyzed by ANOVA (one-way analysis of variance) and Duncan's multiple range test was used (IBM Corp. SPSS Statistics version 22.0. Armonk, NY). Statistical significance was assessed at the level $P \leq 0.05$.

3. Results

3.1. Effect of high CO_2 levels on HSPs and HSF gene expression in the skin of Cardinal table grapes during storage et 0 °C. The importance of CO_2 treatment duration

To determine whether high CO_2 levels applied to maintain table grape quality modulate *HSPs* and *HSFs* transcript accumulation, and how the length of the treatment could affect these changes, we have analyzed the expression of three *sHSPs* (*VviHSP18.1*, *VviHSP18.2*, *VviHSP22.0*), *VviHSP70.0, VviHSP90.1* and a *VviHSF4-a* by qRT-PCR in the skin of non-treated and 1- or 3-d CO_2-treated table grapes. It is important to highlight that the duration of the gaseous treatment influenced its effect on maintaining table grape quality at 0 °C. When high CO_2 levels were applied for 1 or 3 d at 0 °C, there was a significant reduction in the total decay in Cardinal table grapes stored for up to 27 d at 0 °C in comparison to non-treated grapes (Table 1). Although at this point no significant differences were observed between both treatments, bunches treated for 3 d showed similar values to freshly-harvested ones.

The application of high CO_2 levels for 1 d at 0 °C did not modify the accumulation of *sHSPs* transcripts in comparison to non-treated berries (Fig. 1A). Nevertheless, the levels of *VviHSP18.1* and *VviHSP22.0* increased when 1-d CO_2-treated table grapes were transferred to air after 6 d of storage. This increase was transitory in the case of *VviHSP22.0,* whereas the expression of *VviHSP18.1* increased along with the duration of storage under normal atmosphere at 0 °C. By contrast, the application of CO_2 for 3 d significantly increased *VviHSP18.1* and *VviHSP22.0* gene expression but when bunches were transferred to air the only transcript accumulation that continued increasing was *VviHSP18.1*. In non-treated bunches, only the expression of *VviHSP18.1* was induced concomitant with the time of storage at 0 °C, although after 13 d the levels of the transcript were significantly lower than those observed in 1- and 3-d CO_2 treated bunches. The accumulation of *VviHSP18.2* showed almost no variation under any of the conditions assayed.

The transcript abundance of *VviHSP70.0* increased in response to 1 d and 3 d of gaseous treatment (Fig. 1B). Although this increase was significantly higher after 1 d, when these treated bunches were transferred to air there was a sharp decrease in gene expression after 6 ds of storage at 0 °C. By contrast, the transfer of 3-d CO_2-treated bunches did not modify the accumulation of *HSP70.0* until day 13, where the gene expression levels reached were similar to those observed in non-treated and 1-d CO_2-treated fruit. On the other hand, the expression levels of *HSP90.1* showed no significant changes throughout storage at 0 °C in any of the postharvest conditions analyzed.

Regarding the transcription factor, we observed that the application of high levels of CO_2 for 1 d induced the accumulation of *HSF4-a*, decreasing when bunches were transferred to air (Fig. 1C). Although the application of high CO_2 levels for 3 d also induced *HSF4-a* transcript accumulation, no differences were observed in comparison to non-treated samples. In this case, gene accumulation was maintained when 3-d treated bunches were transferred to air, decreasing after 13 d. By contrast, a sharp increase in *HSF4*-a gene expression was observed in non-treated samples after 6 d, decreasing thereafter.

3.2. Isolation and sequence analysis of VviHSP18.1

The full-length sequence of the *VviHSP18.1* gene (Accession number MH049481) isolated from Cardinal table grapes was 482 bp and encoded a protein of 160 amino acids with a predicted molecular mass of 18.1 kDa and a p*I* of 6.78. Sequence comparison analyses against the grapevine genome 12 × .v2 (CRIBI V2) showed that *VviHSP18.1* was 100% identical with *VIT_213s0019g02930*. A phylogenetic analysis revealed that the deduced VviHSP18.1 protein had the most similar amino acid sequence to class I cytosolic sHSPs (Fig. S1 A). The sequence alignments with the deduced VviHSP18.1 protein sequence and those of other class I cytosolic sHSPs indicated that VviHSP18.1 contains an N-terminal region characteristic of class I cytosolic proteins, and a conserved 90-amino-acid C-terminal α-crystallin domain, which can be further divided into two homologous regions, namely consensus region II and I (Fig. S1B).

3.3. Expression and purification of HSP18.1

To investigate the *in vitro* protective role of the heat shock protein VviHSP18.1 in *V. vinifera*, the gene was expressed in *E. coli* BL21 (DE3) as a fusion protein with a 6x His-tag. After purifying the recombinant protein by Ni-NTA affinity columns, a band of approximately 24 kDa, correlating with the molecular weight expected for the recombinant protein linked to His-tag, was identified by SDS-PAGE (Fig. 2A). We performed western blot analysis with the anti-6xHis monoclonal antibody and the results confirmed that the band observed in SDS-PAGE corresponded to the VviHSP18.1 protein (Fig. 2B).

Table 1
Quality parameters of table grapes cv. Cardinal treated with 20 kPa CO_2 for 1- and 3-d and stored up to 27 d at 0 °C.

	At harvest	27 d air	1 d CO_2 + 26 d air	3 d CO_2 + 24 d air
SSC (%)	15.73 ± 0.33 a	16.08 ± 0.95 a	16.90 ± 1.16 a	16.42 ± 0.38 a
TA (%)	0.383 ± 0.011 b	0.357 ± 0.006 a	0.392 ± 0.007 bc	0.402 ± 0.005 c
Total decay	0.0 a	21.45 ± 8.83 c	11.30 ± 2.87 b	5.41 ± 1.94 ab

Values are the mean of three replicate samples ± SE. Different letters within each row indicate that means are statistically different using Duncan's test ($P \leq 0.05$).

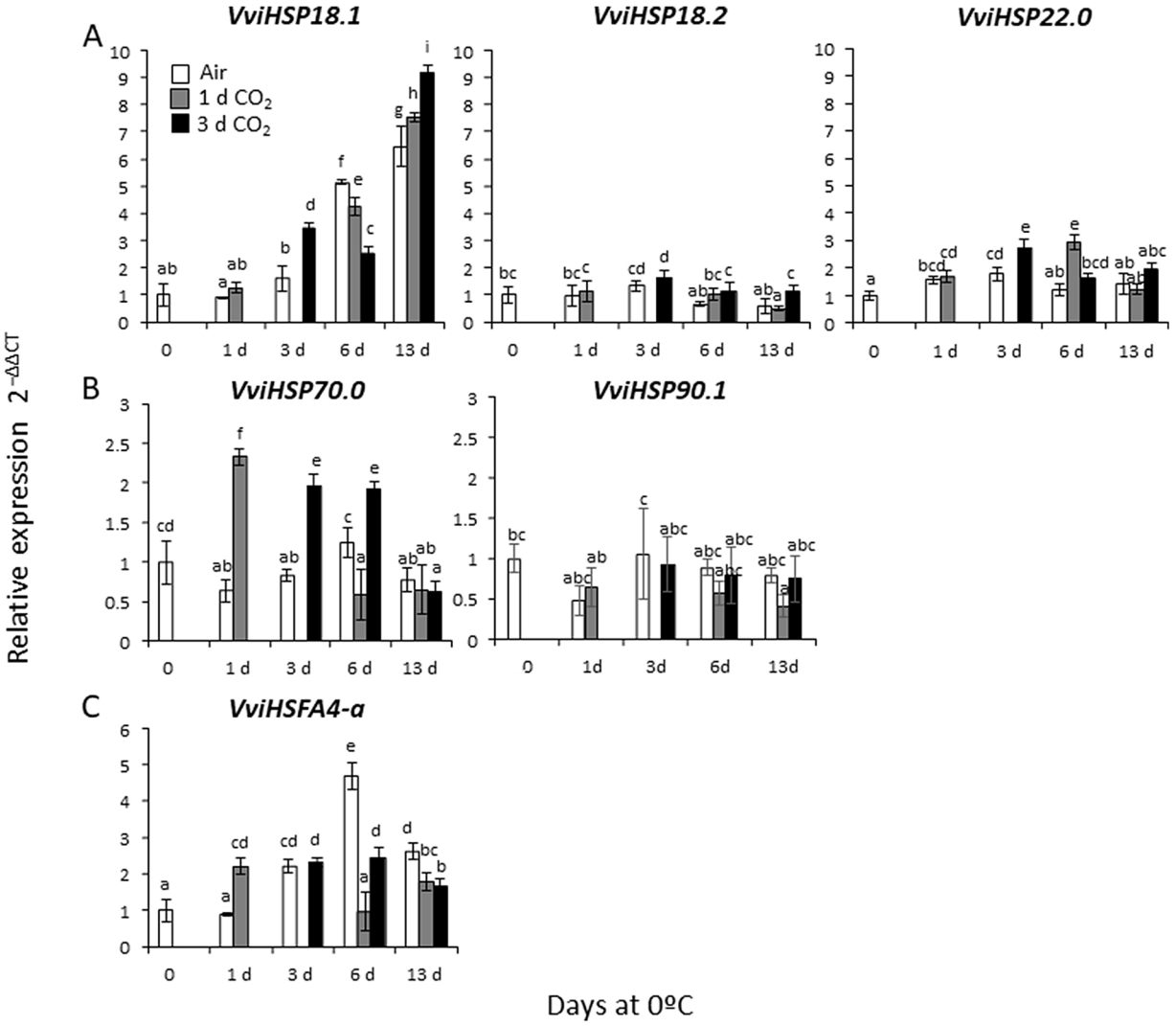

Fig. 1. Effect of the 1- and 3-d CO_2 treatment on gene expression of five *VviHSPs* and *VviHSF4-a* in the skin of table grapes cv. Cardinal stored at 0 °C. Transcript levels of each gene were assessed by RT-qPCR and normalized using actin (*ACT1*) as a reference gene. Results were calculated relative to a calibrator sample (time 0) using the formula $2^{-\Delta\Delta Ct}$. Values are the mean ± SD, n = 6. Different letters on bars indicate that the means are statistically different using the Duncan´s test ($P \leq 0.05$).

3.4. Heat stability of recombinant VviHSP18.1 protein

The heat stability of VviHSP18.1 was assessed in terms of its ability to remain soluble after heating at 45 °C and 70 °C for 45 min, or boiling for up to 10 min. SDS-PAGE analysis of the supernatant obtained after centrifugation of the heated samples together with VviHSP18.1 exposed at ambient temperature (22 °C), used as a control, showed that the intensity of the band corresponding to VviHSP18.1 slightly decreased after heating at 70 °C or boiling, and another weaker and smaller band appeared close to it, whose intensity increased with the severity of the heat (Fig. 3A). To identify the upper- and down-proteins, both isolated Coomassie stained bands were subjected to tryptic digestion and were analyzed by MALDI-TOF MS coupled with tandem mass spectrometry. The NCBI nr database search based on the tryptic peptide mass fingerprint (PMF) obtained and the MS/MS spectra of two of the peptide ions of each protein, identified the upper- (42% sequence coverage, 178 MASCOT score) and the down-protein (22% sequence coverage, 76 MASCOT score) with the XP_002280935 18.1 kDa class I heat shock protein from *V. vinifera* (GI: 225449280; GeneBank: XM_002280899) (Data not shown). This protein corresponds to VIT_213s0019g02930 in the V2.1 grape genome database hosted at CRIBI. To identify the difference between the upper- and down-protein, MS/MS spectra was searched against the predicted sequence of the recombinant VviHSP18.1 protein using the MASCOT search engine. Results showed that nine (upper-protein) and seven (down-protein) independent peptide fragments matched the predicted recombinant VviHSP18.1, the sequence coverage being of 50% and 42%, respectively (Fig. 3B). These results confirmed that the enzymatic hydrolysis of peptides of both protein bands belonged to the recombinant VviHSP18.1. Likewise, the slight difference in the size of both proteins could be explained by a truncation at the C-terminal, since the peptide (ATMENGVLTVRVPK) was not identified in the smaller protein.

3.5. VviHSP18.1 as chaperone in vitro

3.5.1. Recombinant VviHSP18.1 protects CS and MDH from thermal aggregation

A well-established assay for sHSPs chaperone activity was used to analyze its ability to prevent heat-induced aggregation of CS and MDH as measured by light scattering (Basha et al., 2004; Lee and Vierling, 1998). The heat of CS and MDH at 45 °C began to form insoluble aggregates, measured by an increase in relative light scattering (Fig. 4A and B). However, the presence of VviHSP18.1 almost completely and significantly suppressed the aggregation of CS and MDH at a molar ratio of 3.33:1 and 6.66:1, respectively (Fig. 4A and B). By contrast, the addition of BSA (used as a control) did not have any effect on MDH

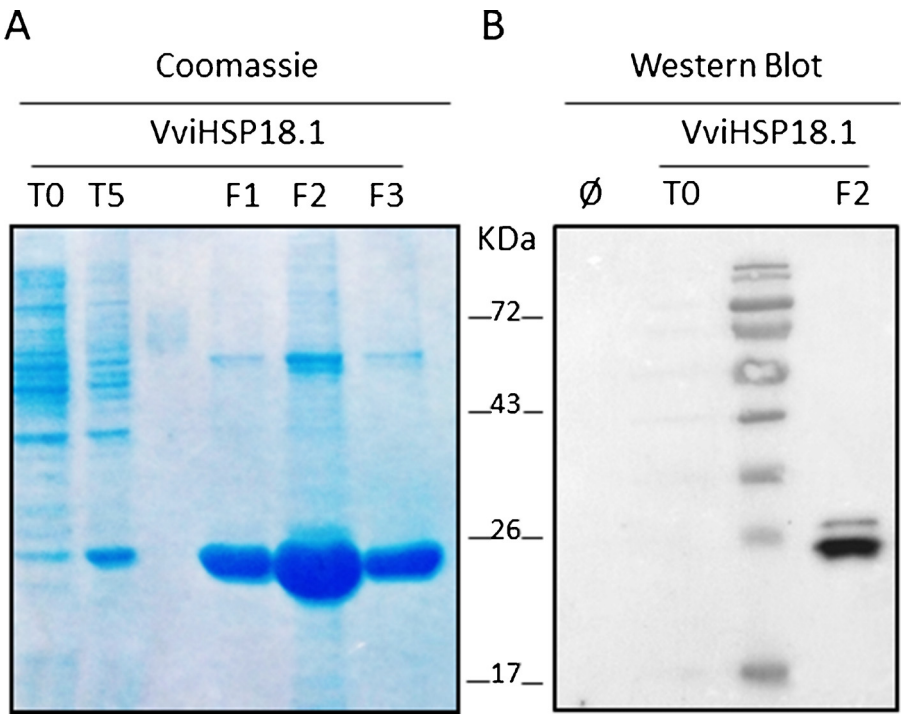

Fig. 2. Overexpression of VviHSP18.1 protein in *E. coli*. A: Coomassie blue staining of protein extracts obtained before induction with IPTG (T0), after 5 h of induction with IPTG (T5) and after Ni-NTA agarose purification (F1, F2 and F3). B: The empty pET28a plasmid (Φ), T0 and F2 extract was observed by Western Blot using the anti-6xHis antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aggregation (molar ratio of 6.66:1) (Fig. 4B), and in the case of CS (molar ratio of 3.33:1) (Fig. 4A), the effect was slight.

3.5.2. Recombinant VviHSP18.1 protects ADH and LDH from thermal inactivation

VviHSP18.1 showed a slight protective effect on the thermal inactivation of ADH. The incubation of ADH alone (negative control, C-) resulted in a loss of about half of its activity after 45 min at 50 °C, reaching 65% of loss after 90 min. The addition of VviHSP18.1 at a molar ratio of 0.8 had a slightly positive effect on ADH inactivation through heat, recovering 9% of the original ADH activity (Fig. 5). However, at the same molecular ratio, the addition of BSA recovered approximately 27% of ADH activity.

Recombinant VviHSP18.1 was assayed for its role in protecting LDH, which loses its activity by freezing-thawing (Reyes et al., 2008) and enduring prolonged high temperature (Halder et al., 2016). The enzyme LDH lost about 57% of its activity upon incubation at 65 °C for 40 min (Fig. 6A). The addition of recombinant VviHSP18.1 protected LDH activity by recovering 35% and 43% of its activity, at a protein:enzyme molar ratio of 2:1 and 4:1, respectively. BSA was shown to be more efficient than VviHSP18.1 at protecting LDH while maintaining 100% of the initial LDH activity at a molar ratio of 2:1 and 4:1. The incubation at 37 °C for 16 h affected LDH by reducing 40% of its activity, whereas in the presence of recombinant VviHSP18.1 or BSA at a molecular ratio of 4:1, its activity remained unperturbed (Fig. 6B). However, the protective effect of VviHSP18.1 on the inactivation of LDH was not observed upon extreme cold shock. LDH lost 90% of its activity when the enzyme was exposed to two freeze-thaw cycles. By adding BSA at a molecular ratio of 4:1, the LDH activity was recovered by about 80% (Fig. 6C). However, the presence of recombinant VviHSP18.1 at the same molecular ratio did not modify LDH activity.

4. Discussion

In an attempt to go one step further and discover the molecular bases implicated in the beneficial effect of high CO_2 levels to maintain table grape quality during postharvest storage at 0 °C, we have analyzed the role of *HSPs* and an *HSF*. The study of *HSPs* in *V. vinifera* has mainly focused on the role they play under high temperature, with only the study by Becatti et al. (2010) showing that the application of high levels of CO_2 for 3 d at 20 °C induced the transcript accumulation of a small *HSP* in wine grapes. In the case of strawberries, when a heterologous

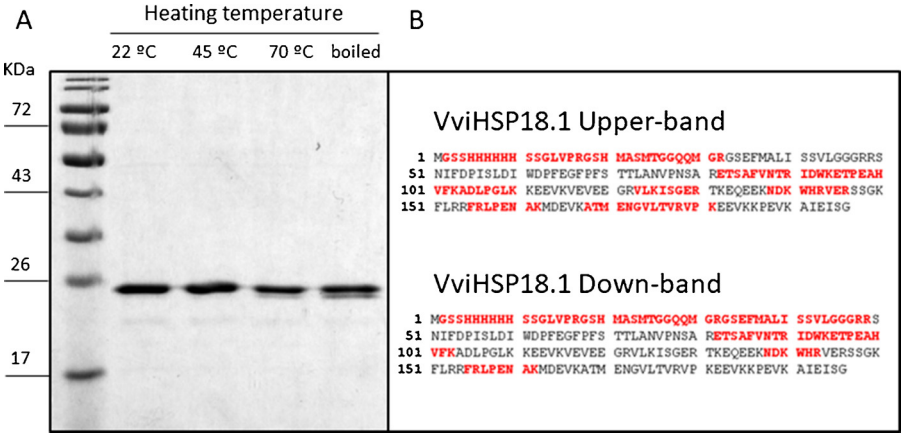

Fig. 3. (A) Coomassie blue staining showing the thermal stability of the recombinant protein. VviHSP18.1 recombinant protein was incubated for 45 min at 22, 45 and 70 °C. A fourth sample was boiled for 10 min. (B) Mascot search results of upper- and down-recombinant proteins indicating matched peptides from the MS/MS analyses after trypsin digestion was sown in bold red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

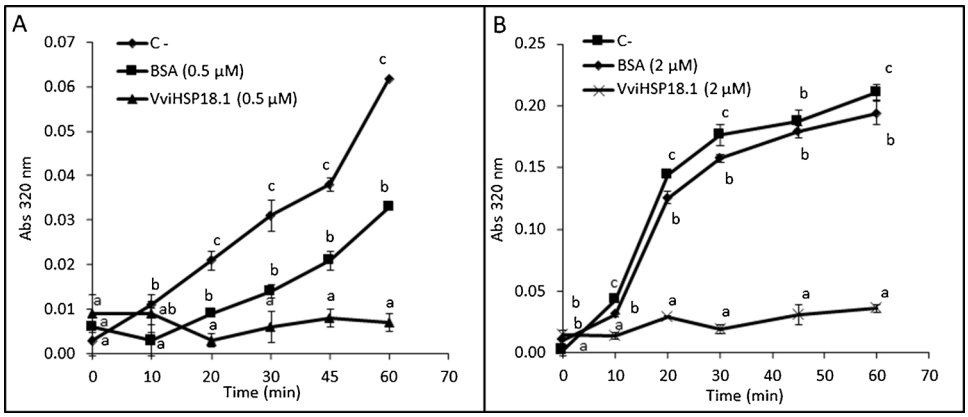

Fig. 4. Effect of VviHSP18.1 recombinant protein on thermal aggregation of CS (A) and MDH (B) at 45 °C. The recombinant protein VviHSP18.1 (0.5 µM (A), 2 µM (B)) and BSA (0.5 µM (A), 2 µM (B)) were evaluated in the protection of the aggregation of the enzyme CS (0.15 µM) and MDH (0.3 µM) at 45 °C for 1 h. The negative control (C-) represents the aggregation of the enzyme in the absence of protein. Each value corresponds to the average of two independent replicates ± SD, n = 4. Different letters on bars indicate that the means are statistically different using the Duncan´s test ($P \leq 0.05$).

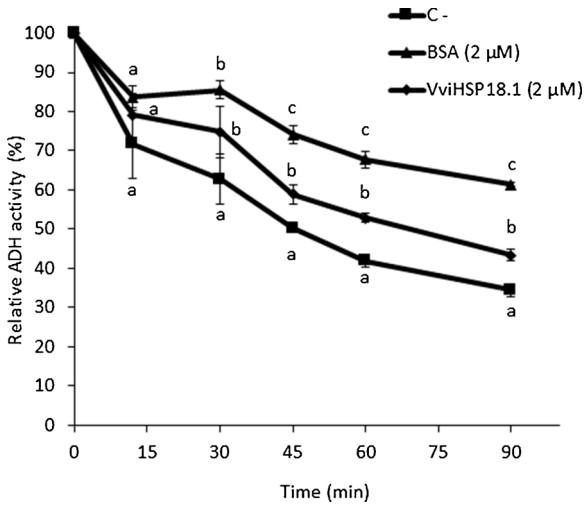

Fig. 5. Effect of VviHSP18.1 recombinant protein on the protection of ADH activity. The enzyme ADH (2.5 µM) was heated at 50 °C for 90 min alone or in the presence of 2 µM VviHSP18.1 or BSA. The percentage of residual activity was calculated in relation to the absorbance of the non-heated control. Each value corresponds to the average of two independent replicates ± SD, n = 4. Different letters on bars indicate that the means are statistically different using the Duncan´s test ($P \leq 0.05$).

cDNA microarray from tomato (TOM1) was used to analyze gene expression in two cultivars treated with 20 kPa CO_2 for 48 h, it was observed that the expression of several *HSPs* was induced in both cultivars in response to CO_2 (Ponce-Valadez et al., 2009). Likewise, in the case of tomatoes, a treatment with 20% CO_2 for 3 d at 20 °C delayed ripening and induced the expression of a *sHSP* (Rothan et al., 1997). In our study, we have observed that applying high CO_2 levels at 0 °C modulated the expression of different *HSPs* and *HSF4 A,* and also found that the length of the treatment affected this regulation. Among the different *HSPs* analyzed, only *VviHSP90.1* did not show any change in gene expression in response to high CO_2 levels and to low temperature. It is important to note that the expression of the three small *VviHPSs* was induced significantly by high CO_2 levels but only after 3 d of treatment at 0 °C. However, the accumulation of *HSP70.0* and *HSF4-a* transcripts increased after 1 d and 3 d of CO_2-treatment at low temperature. Although both treatments were effective in reducing total decay in comparison to non-treated bunches, the increase observed in 3-d CO_2-treated table grapes did not show any significant differences in comparison to freshly-harvested fruit, whereas in the case of 1-d treated bunches it was indeed significant. Likewise, it is important to note that when treated bunches were transferred to air in all cases, except for the *HSF*, the accumulation of transcripts induced by CO_2 were also higher than in non-treated table grapes. Cold storage of Kyoho grape berries increased the expression abundance of several stress related proteins including three HSP70 s, suggesting that a physiological adaptation to

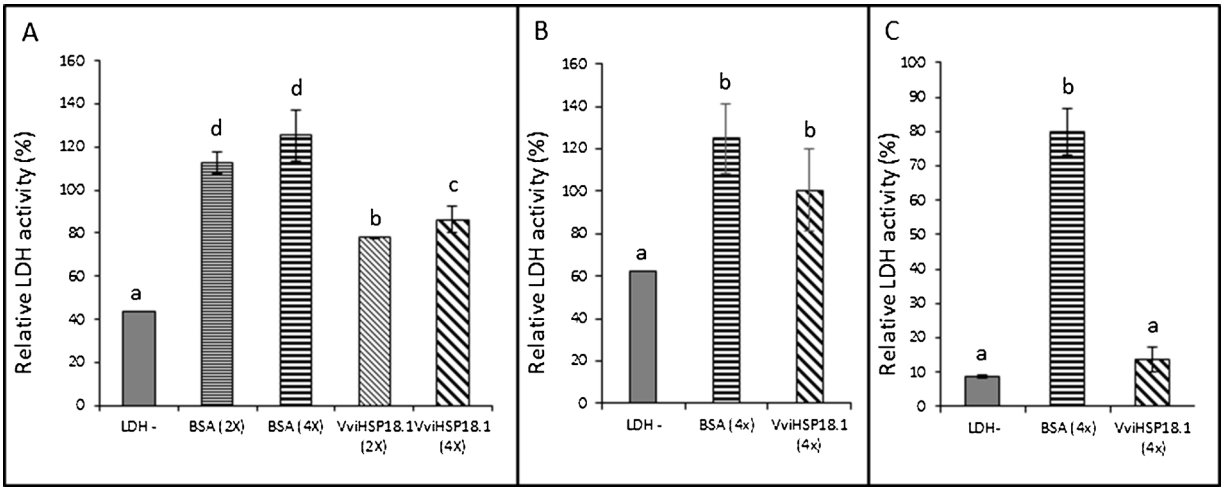

Fig. 6. Effect of VviHSP18.1 recombinant protein on the protection of LDH activity under different stress conditions. (A) Heat shock treatment of LDH at 65 °C for 40 min in the presence of BSA or VviHSP18.1 protein in two different molar ratios (2X and 4X). (B) LDH enzyme was incubated at 37 °C 16 h in the presence of BSA or VviHSP18.1 protein in a molar ratio 4X. (C) Cold shock treatment of LDH enzyme after two freeze-thaw cycles in the presence of BSA or VviHSP18.1 in a molar ratio 4X. The percentage of residual activity was relative to the absorbance of the no-treated controls. Each value corresponds to the average of two independent replicates ± SD, n = 4. Different letters on bars indicate that the means are statistically different using the Duncan's test ($P \leq 0.05$).

low temperature was induced in grape berries (Yuan et al., 2014). Our results match those obtained in previous studies where the protective effect of postharvest heat treatments mitigating the development of chilling injury in many commodities was associated with the prolonged presence of HSPs and the protective effect they exert (reviewed by Lurie and Pedreschi, 2014; Salazar-Salas et al., 2017). Likewise, in the cases of peach and tomato, treatments with methyl derivatives of jasmonic acid and/or salicylic acid before low-temperature storage induced HSP biosynthesis and tolerance to chilling injury (Ding et al., 2001; Wang et al., 2006). In the case of table grapes, although they are classified as chilling tolerant, it should be noted that during postharvest storage at low temperature they are indeed highly susceptible to fungal decay and water loss. Thus, we have observed that the application of high CO_2 levels maintained table grape quality and improved their tolerance to temperature shift at 0 °C, controlling the cold- and antifungal-defense response through phenylpropanoid and PRs (pathogenesis-related proteins) genes expression (Romero et al., 2006; Sanchez-Ballesta et al., 2007). High levels of CO_2 also activated the expression of transcription factors (Rosales et al., 2016; Romero et al., 2016; Vazquez-Hernandez et al., 2017) and the retention of introns in the transcripts of the dehydrins (Vazquez-Hernandez et al., 2017). The results obtained in our study support the idea that the treatment with high CO_2 levels is an active process in which HSPs could play a role in preventing the denaturation and dysfunction of many proteins.

Although there are many studies focusing on the function of sHSPs, little is known about their biological function in fruit-producing crops. To this end, we have *in vitro* characterized VviHSP18.1, since its gene expression was induced by a 3-d high CO_2 treatment and also when 1- and 3-d CO_2-treated bunches were transferred to air, which means it could play a role in the beneficial effect of the gaseous treatment. Thus, the full-length *VviHSP18.1* cDNA was isolated, and the analyses of homology and phylogenetic relationships suggested that VviHSP18.1 is a Class I sHSP. The VviHSP18.1 protein was produced by the pET28a expression system in *E. coli* and its expression was successful, yielding a highly pure and soluble protein which was tested for possible chaperone activity. First, heat stability tests were performed, showing that the recombinant VviSHP18.1 was stable for 45 min at 45 °C, 70 °C and also after boiling for 10 min. However, when the heat conditions were more restrictive (70 °C and boiling), the intensity of the VviHSP18.1 protein band decreased, and a second band appeared whose intensity increased with temperature, although the upper-protein was predominant even after boiling. The identity of both proteins, as determined by mass spectrometry, was indeed 18.1 kDa class I heat shock protein from *V. vinifera*, although the down-protein seems to present a C-terminal truncation of about 3 kDa. It is important to note that the highest temperature used for the chaperone activities was 65 °C, and the elevated temperatures that give rise to the truncated protein exceed the heat stress temperatures generally experienced by fruit under field conditions and during postharvest storage. Until now, a truncation of a recombinant sHSP has only been observed when sHSP18 from *Mycobacterium leprae* was overexpressed in *E. coli* since a fraction underwent cleavage resulting in a truncated protein with a mass of 16.7 kDa, in addition to the 19.3 kDa full-length his-tagged protein (Lini et al., 2008). Subsequently, Maheshwari and Dharmalingam (2013) observed a decrease in the level of the 16.7 kDa form in *E. coli* cells exposed to 50 °C with a concomitant increase in the 19.3 kDa form of sHsp18.

It is important to note that the recombinant VviHSP18.1 displayed chaperone activity *in vitro*, protecting the heat-labile proteins CS and MDH from thermal aggregation and avoiding the heat-induced loss of ADH and LDH enzyme activity. It has already been shown that sHSPs from different species can act *in vitro* as molecular chaperones by binding denaturing proteins and preventing them from irreversible aggregation (Lee et al., 1995; Waters et al., 1996; Basha et al., 2004; Wang et al., 2017), but this is the first time that it has been described in a sHSP from *V. vinifera*. When thermal aggregation of CS and MDH was analyzed, the protection effect of VviHSP18.1 was higher than BSA, whereas in the case of ADH and LDH it was similar or lower. This less efficient protection given by VviHSP18.1 to ADH at 55 °C and to LDH at 65 °C could indicate that these temperatures may exceed the functional temperature range for this sHSP. In this regard, we also have observed that after heating VviHSP18.1 protein samples above 70 °C, the recombinant protein was functional in protecting CS from thermal aggregation. Chaperone activity, however, was lower than those found in samples without heating (data not shown). Our studies indicated that VviHSP18.1 did not prevent freeze-induced inactivation of LDH, while Lopez-Matas et al. (2004) reported that CsHSP17.5 (a cytosolic class I sHSP from sweet chestnut) acts as cryoprotectant *in vitro* protecting LDH against freeze-inactivation at −20 °C for 18 h and thawed at 25 °C for 15 min. Nevertheless, it is important to indicate that in our case, cryoprotectant assays have been performed by freezing the mixtures twice in liquid nitrogen for 30 s and then thawed at room temperature for five min. Therefore, further research is needed to elucidate whether these differences in the experimental procedure could explain the different results obtained.

In conclusion, our results indicated that the beneficial effect of high CO_2 levels in maintaining table grape quality during postharvest storage at 0 °C seems to be linked to the activation of *sVviHSPs*, *VviHSP70.0* and *VviHSF4-a* gene expression. Furthermore, the recombinant VviHSP18.1 showed molecular chaperone activities *in vitro*. Another remarkable aspect of this work is that heating the protein above 70 °C resulted in the appearance of a truncated protein at the C-terminal. Overall, the findings presented in this work could help shed light on the complex mechanisms and roles of HSPs not only during postharvest but also, and more specifically, in table grapes where little information is available to date.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2018.06.006.

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