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VvGOLS1 and VvHsfA2 are Involved in the Heat Stress Responses in Grapevine Berries

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Among various environmental factors, temperature is a major regulator affecting plant growth, development and fruit composition. Grapevine is the most cultivated fruit plant throughout the world, and grapes are used for wine production and human consumption. The molecular mechanisms involved in grapevine tolerance to high temperature, especially at the fruit level, are poorly understood. To better characterize the sensitivity of berries to the microenvironment, high temperature conditions were locally applied to *Vitis vinifera* Cabernet Sauvignon clusters. Two genes, *VvGOLS1* and *VvHsfA2*, up-regulated by this treatment, were identified and further characterized. The expression profile of *VvGOLS1* correlated positively with galactinol accumulation in heat-stressed berries. However, no galactinol derivatives, such as raffinose and stachyose, accumulated upon heat stress. Heterologous expression of *VvGOLS1* in *Escherichia coli* showed that it encodes a functional galactinol synthase. Transient expression assays showed that the heat stress factor VvHsfA2 transactivates the promoter of *VvGOLS1* in a heat stress-dependent manner. Taken together, our results highlight the intrinsic capacity of grape berries to perceive heat stress and to initiate adaptive responses, suggesting that galactinol may play a signaling role in these responses.

Keywords: Climate change • Galactinol • Heat stress factor • Microenvironment • *Vitis vinifera* cv. Cabernet Sauvignon.

Abbreviations: CaMV, *Cauliflower mosaic virus*; DBD, DNA-binding domain; α-GAL, α-galactosidase; GOLS, galactinol synthase; β-GUS, β-glucuronidase; HSE, heat stress element; Hsf, heat stress factor; IPTG, isopropyl-β-D-thiogalactopyranoside; MU, methyl-umbelliferone; MUG, 4-methylumbelliferyl-α-D-glucuronide; NES, nuclear export motif; NLS, nuclear localization signal; PAD, pulsed amperometric detector; RAFS, raffinose synthase; RFO, raffinose family oligosaccharides; RFU, relative fluorescence unit; STAS, stachyose synthase; Suc, sucrose; WSC, water-soluble carbohydrate.

Introduction

Temperature, which is a major parameter controlling plant growth and development, is expected to increase significantly because of climate change. Grapevine is economically the most important fruit crop in the world, providing dried fruits, table grapes and the basis for wine making. Climate and viticultural practices greatly affect the internal temperature of the clusters, which significantly influences the organoleptic quality of berries. In this context, it is relevant to investigate the effect of high temperatures on berry metabolism and composition.

The impact of environmental factors on the development and composition of grape berries has been widely investigated in various wine-producing regions (Downey et al. 2006). According to the Intergovernmental Panel on Climate Change (IPCC), global warming may result in an increase in air and land temperatures of between 1.8 and 4.0°C by the end of this century (IPCC, 2007). Grapevine physiology is expected to respond to climate change during the whole period of growth, influencing the composition and the yield in vineyard regions throughout the world (Schultz 2000, Jones et al. 2005). Accordingly, Salazar-Parra et al. (2010) demonstrated that climate change (elevated CO_2, high temperature and moderate drought) affects phenology and berry composition. In particular, exposure to high temperatures during flowering can significantly inhibit berry set (Greer and Weston 2010), thus reducing yield. After fruit set, high temperatures are generally not favorable for the organoleptic quality of the wine, due to the decreased accumulation of compounds produced by both primary metabolism, such as organic acids

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(Champagnol 1984), and secondary metabolism, including phenolic compounds (Spayd et al. 2002, Mori et al. 2007) or aromas (Schultz 2000). Moreover, high temperatures stimulate sugar accumulation at the expense of other qualitative compounds (Greer and Weston 2010), resulting in the production of wines with higher alcohol concentrations.

Water imported by the berries controls their size and final concentration of berry solutes (Esteban et al. 1999, Conde et al. 2007). Sugars are imported from source organs to the clusters, following sink–source partitioning rules. In contrast, secondary metabolites, which are the most prominent discriminating compounds for berry quality, are synthesized in situ under the direct influence of the local microenvironment (mostly temperature and light conditions). Because of the direct irradiative effects, the temperature of reproductive organs can vary considerably compared with the whole plant or surrounding air (Spayd et al. 2002, Pieri and Fermaud 2005, Cola et al. 2009). Along with carbon and water supplies, the microenvironment of berries is therefore essential for their quality. For example, local temperature variations affect berry composition (flavonols, anthocyanins and amino acids) (Spayd et al. 2002, Pereira et al. 2006, Tarara et al. 2008, Cohen et al. 2012).

To overcome the effects of abiotic stresses, plants have developed complex and dynamic systems involving a wide range of biochemical and physiological processes (Ahuja et al. 2010, Cramer et al. 2011, Saidi et al. 2011, Walbot et al. 2011). Among these, the accumulation of compatible solutes consisting of non-toxic organic molecules protects the cells against deleterious osmotic and metabolic imbalances caused by stress (Hare et al. 1998). Compatible solutes include raffinose family oligosaccharides (RFOs), which are involved in the desiccation tolerance of seeds (Castillo et al. 1990, Downie et al. 2003) and the protection of plant cells against freezing (Pennycooke et al. 2003, Peters and Keller, 2009), oxidative damage (Nishizawa et al. 2008), drought, cold and heat stress (Taji et al. 2002, Panikulangara et al. 2004). Recently, it was also hypothesized that galactinol and RFOs may act as signals that mediate stress responses (Kim et al. 2008, Valluru and Van den Ende 2011).

RFOs are synthesized from sucrose (Suc) by subsequent additions of activated galactose moieties donated by galactinol. Galactinol synthase (GOLS; EC 2.4.1.123) catalyzes the first committed step in the RFO biosynthetic pathway, synthesizing galactinol from UDP-D-galactose and *myo*-inositol. Then, galactinol serves as a galactosyl donor to form raffinose and stachyose, through the action of raffinose synthase (RAFS; EC 2.4.1.82) and stachyose synthase (STAS; EC 2.4.1.67), respectively. The genes encoding these enzymes are transcriptionally up-regulated upon abiotic stress, leading to RFO accumulation (Taji et al. 2002, Downie et al. 2003, Zuther et al. 2004, Peters et al. 2007, Nishizawa et al. 2008, dos Santos et al. 2011). For instance, a positive correlation was established between the expression of Arabidopsis *GOLS* genes and the concentration of galactinol and raffinose under heat stress conditions. Furthermore, *GOLS* transcript accumulation depends on the activities of the two heat shock transcription factors (Hsfs), HsfA1a/1b and HsfA2 (Panikulangara et al. 2004, Busch et al. 2005, Nishizawa et al. 2006, Schramm et al. 2006).

The basic signaling events involved in tolerance to high temperatures are poorly understood in many species, and little is known about the accumulation of RFOs in grapevine. The presence of raffinose and stachyose in grapevine was reported several decades ago by Kliewer (1966), and their accumulation in response to cold hardiness was demonstrated (Hamman et al. 1996). Furthermore, although Hsfs are well-conserved final signaling components mediating the activation of genes responsive to both heat stress and a large number of chemical stressors (Baniwal et al. 2004), no Hsf has been characterized in grapevine so far. Recently, Kobayashi et al. (2010) reported the higher expression of a few heat stress-induced genes in grapevine leaves and berry skins. These genes encode small heat shock proteins that might be related to the acquisition of thermotolerance in grapevine.

The economic importance of grape berry composition, its sensitivity to the microenvironment, and particularly to heat stress, led us to investigate the RFO biosynthetic pathway under heat stress conditions. Our data show that the transcription of *VvGOLS1*, a *GOLS1* gene from *Vitis vinifera* Cabernet Sauvignon, is strongly induced in berries exposed to heat stress, positively correlating with galactinol accumulation in stressed berries, but raffinose and stachyose concentrations did not increase after heat stress. The functional expression of *VvGOLS1* demonstrated that it encodes a genuine GOLS enzyme, with properties similar to other known GOLSs. A grapevine *Hsf* gene strongly expressed in heat-stressed berries was also identified and corresponds to *VvHsfA2*. In transient expression assays, VvHsfA2 activated the promoter of *VvGOLS1* in a heat stress-dependent manner.

Results

Heat stress regimes applied to clusters of Cabernet Sauvignon fruit cuttings

To control and manipulate environmental conditions around the clusters easily, experiments were conducted with fruit cuttings grown in small pots under controlled greenhouse conditions. Separate microclimate treatments were applied to the clusters of three sets of plants, one control (CT) and two submitted to highly contrasted temperatures (TEMP1 and TEMP2) (**Fig. 1**). Air heating was applied during 21 d, from 07:00 h to 19:00 h to mimic a diurnal temperature cycle of exposed berries (**Fig. 1A**). The internal temperatures in control and heat-stressed berries were monitored continuously (**Fig. 1B**). According to the experimental set-up, average temperatures of 30.2 ± 2.2, 36.5 ± 2.5 and 39.7 ± 2.3°C corresponded to CT, TEMP1 and TEMP2 clusters, respectively (**Fig. 1C**). The temperature differences between heat-stressed and control clusters were 6.3 ± 0.3°C (TEMP1 vs. CT) and 9.5 ± 0.4°C (TEMP2 vs. CT) (**Fig. 1D**).

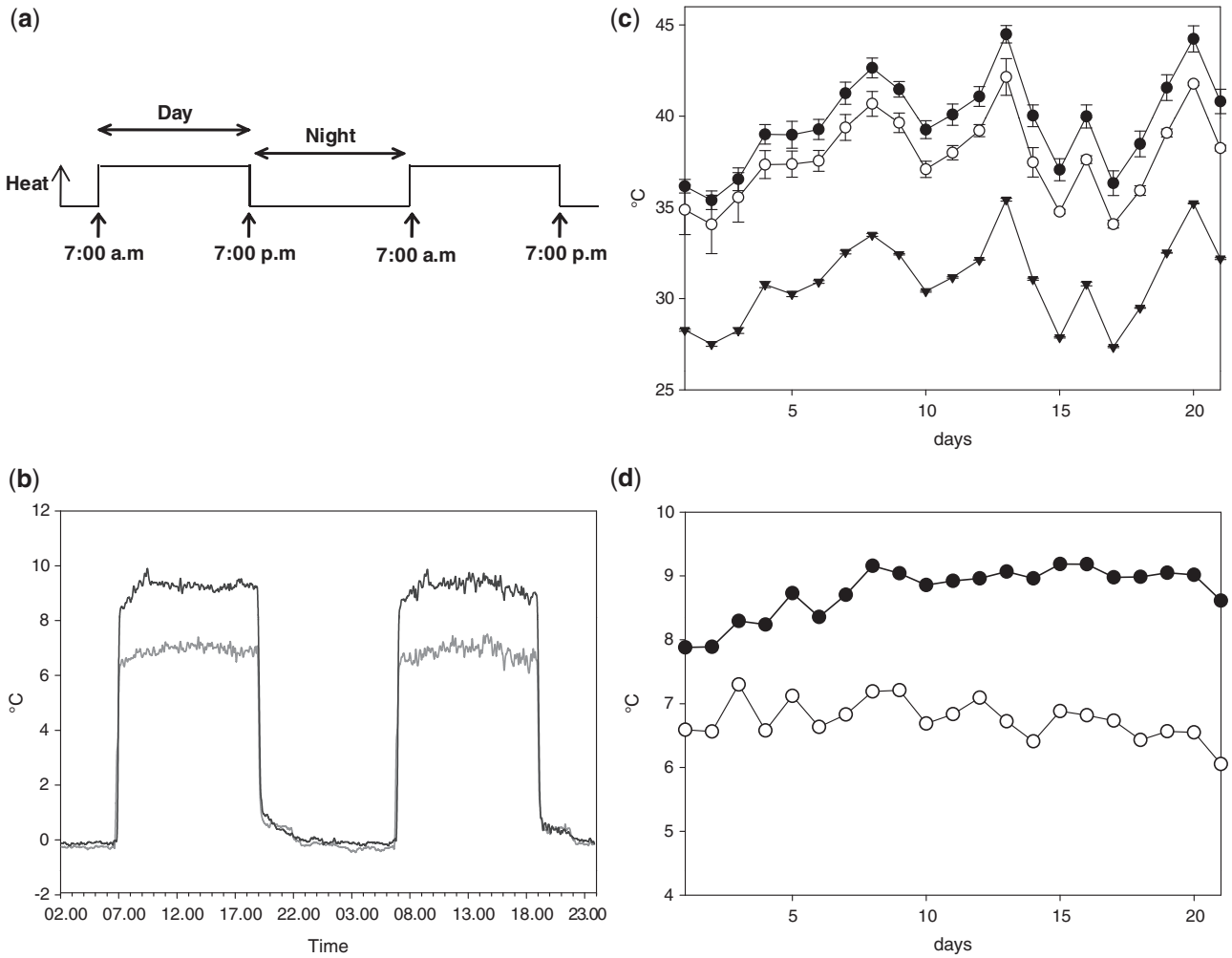

Fig. 1 Sample diurnal curves during heat experimentation for internal berry temperature. (A) Heat application protocol. (B) Typical temperature difference curves between TEMP1 and CT (gray) and between TEMP2 and CT (black) on two consecutive days. (C) Temperature means per day during the 21 d of treatment for TEMP2 (filled circle), TEMP1 (open circle) and CT (inverted triangle). Error bars represent temperature variations between 07:00 h and 19:00 h each day. (D) Mean temperature difference curves between TEMP1 and CT (open circle) and between TEMP2 and CT (filled circle).

Isolation of a candidate *GOLS* cDNA from grapevine

Total RNA extracted from Cabernet Sauvignon berries exposed to normal or heat stress conditions was hybridized with 70-mer oligoarrays bearing a set of 14,562 unigenes (Qiagen Operon Array-Ready Oligo Set for the Grape Genome version 1.0). Around 500 genes were affected by our heat stress regimes (J.P. and C.K, unpublished data). Among the genes that were significantly up-regulated by heat stress, one gene corresponding to the expressed sequence tag (EST) sequence TC62106 shared high similarity with a *GOLS* gene from soybean *Glycine max* (Q7XZ08) and was selected for further investigation. The corresponding full-length cDNA was amplified from heat-stressed berries by reverse transcription–PCR and was named *VvGOLS1* (VIT_07s0005g01970). The *VvGOLS1* cDNA comprised 1,029 nucleotides encoding a protein of 342 amino acids with a predicted mass of 39.3 kDa and a calculated pI of 5.39. A BLASTP search against the non-redundant (NR) GenBank protein database allowed the identification of well-matched GOLS sequences that were used for a multiple alignment (**Fig. 2**). VvGOLS1 shares a high amino acid sequence identity (between 77% and 82%) with others GOLS polypeptides in a wide variety of plant species. Common features of plant GOLSs were present in VvGOLS1, namely a domain of family 8 glycosyltransferases, a conserved putative serine phosphorylation site at position 264 and the hydrophobic APSAA pentapeptide at the C-terminal region (Sprenger and Keller 2000). Additionally, data mining of the 12X version of the grape genome (Jaillon et al. 2007; http://genomes.cribi.unipd.it/) yielded three additional *VvGOLS* members (numbered from 2 to 4) that share 69–71% amino acid sequence identity with the full-length VvGOLS1 (**Table 1**). Another gene sequence closely related to *VvGOLS1* was also identified and named

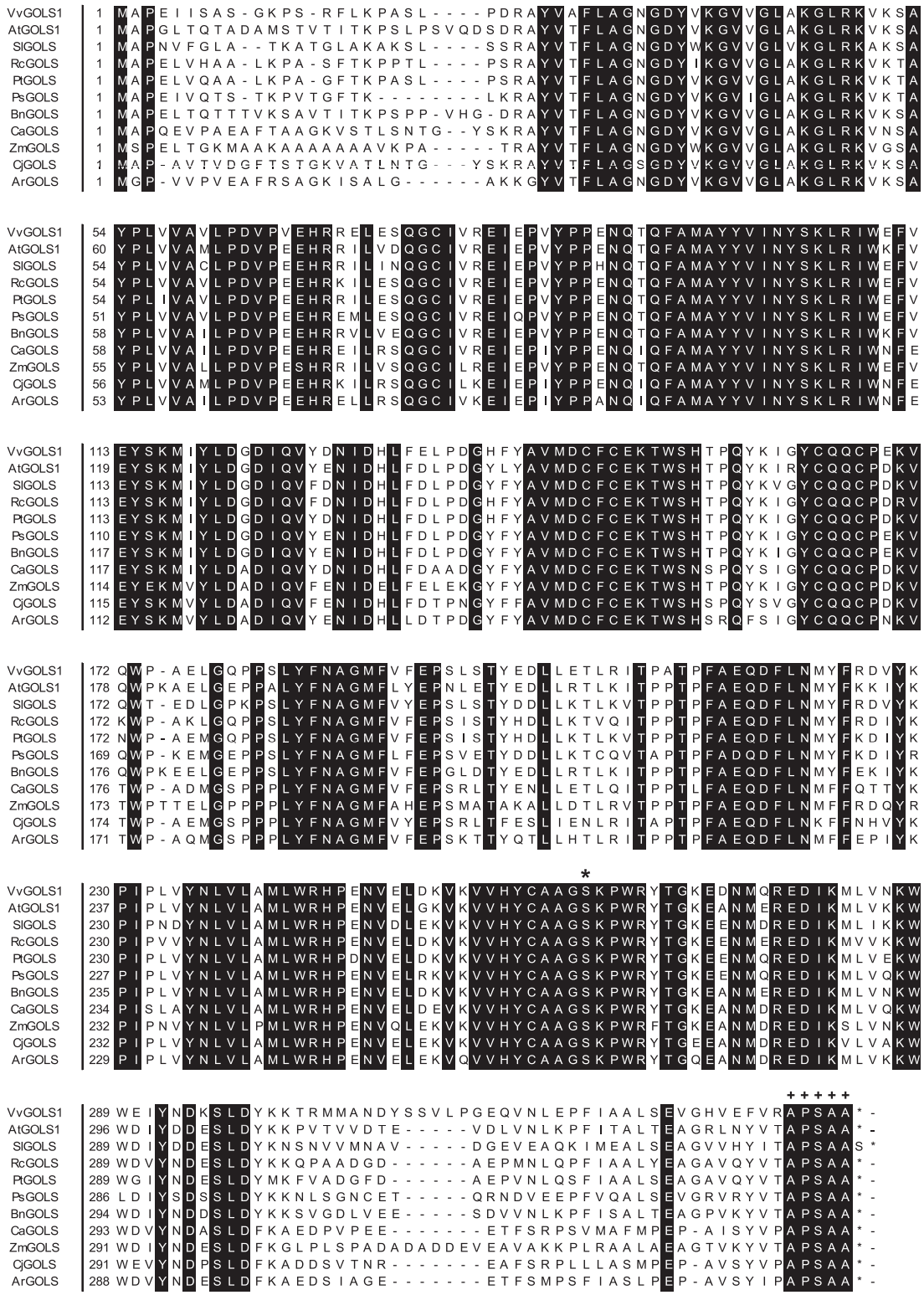

Fig. 2 Full-length sequence comparison of VvGOLS1 and its closest homologs. Alignments are made using the MUSCLE program. Conserved residues are shaded in black. Vv, *Vitis vinifera* (VIT_07s0005g01970); At, *Arabidopsis thaliana* (NP_182240); Sl, *Solanum lycopersicum* (BAH98060); Rc, *Ricinus citris* (XP_002515233); Pt, *Populus trichocarpa* (XP_002320958); Ps, *Pisum sativum* (CAB51130); Bn, *Brassica napus* (ADG03603); Ca, *Coffea arabica* (ADM92588); Zm, *Zea mays* (AF497507_1); Cj, *Coptis japonica* (BAF99254); Ar, *Ajuga reptans* (CAB51533). A conserved putative serine phosphorylation site (S) is indicated by an asterisk and the characteristic hydrophobic pentapeptide (APSAA) is labeled with the symbol +.

Table 1 Sequence identity among VvGOLS proteins

Accession Genoscope	Accession Cribi	Protein	Length[a]	Identity (%)			
				VvGOLS1	VvGOLS2	VvGOLS3	VvGOLS4
GSVIVT01028174001	VIT_07s0005g01970	VvGOLS1	340	100	68.6	69.1	70.7
GSVIVT01034938001	VIT_05s0077g00430	VvGOLS2	319		100	73.3	67.6
GSVIVT01031274001	VIT_14s0060g00810	VvGOLS3	325			100	67.4
GSVIVT01013763001	VIT_01s0127g00470	VvGOLS4	337				100

[a] Amino acids.

VvGOLS1-like (VIT_07s0005g01980). *VvGOLS1* and *VvGOLS1-like* were both located in the same scaffold (chromosome 7, scaffold 5) of the Genoscope genome browser, and the corresponding proteins exhibited 97% identity to each other (data not shown), suggesting that *VvGOLS1-like* may be a tandem repeat of *VvGOLS1*. Our attempts to clone *VvGOLS1-like* failed, suggesting that *VvGOLS1-like* is not expressed in control and heat-stressed berries. In comparison, the Arabidopsis genome contains seven *GOLS* genes (*AtGOLS1–AtGOLS7*) and three putative *GOLS* genes (Nishizawa et al. 2008). A phylogenetic analysis of selected members from the plant GOLS family showed that the GOLS proteins from grape can be divided into four different clusters (**Fig. 3**). This phylogenetic tree revealed that VvGOLS1 is closely related to PsGOLS from *Pisum sativum* (85% similarity) and belongs to a clade including AtGOLS1 (Panikulangara et al. 2004) and BnGOLS1 (Li et al. 2011).

Heat stress-induced expression of *VvGOLS1* in grape berries

To gain insight into the expression profiles of *VvGOLS1* transcripts under heat stress, real-time PCR was conducted with RNA extracted from either control grape berries or grape berries exposed to a temperature that was 6 or 9°C higher than the greenhouse ambient temperature (TEMP1 or TEMP2). *VvGOLS1* expression was determined for short (up to 8 h) and long (up to 3 weeks) periods of heat exposure. Under both conditions, *VvGOLS1* transcripts accumulated rapidly and dramatically within the first hour of treatment (**Fig. 4A**). Interestingly, significant transcript levels of *VvGOLS1* were detected when the heat stress was applied repeatedly for 3 weeks (**Fig. 4B**). Transcript levels of *VvGOLS1* were also quantified in the different berry tissues (skin, pulp and seeds) after heat stress exposure (**Fig 4C**). *VvGOLS1* transcripts were detected in all tissues and their accumulation was strongly increased by heat stress. Finally, similar temperature effects were observed for *VvGOLS1* expression when the stress was applied either at veraison or 3 weeks after veraison (**Fig. 4D**). In contrast, heat stress exposure reduced the transcripts of *VvGOLS3* and *VvGOLS4* in berries (**Supplementary Fig. S1**) whereas *VvGOLS2* was weakly expressed under both control conditions and heat stress (data not shown).

To extend the study to other grapevine genes involved in RFO biosynthesis, the grape genome sequence (Jaillon et al. 2007) was screened in silico with sequences corresponding to various plant RAFS and STAS sequences according to Tapernoux-Lüthi et al. (2004). We identified two grape genes encoding putative proteins with high sequence similarity to RAFS proteins, and one gene encoding a protein closely related to the STAS family, that were named *VvRAFS1*, *VvRAFS2* and *VvSTAS1*, respectively (**Supplementary Fig. S2**). Expression analysis comparing heat-stressed berries with control berries did not reveal any difference in transcript levels for any of these genes (**Supplementary Fig. S3**). Furthermore, the three genes were hardly detectable irrespective of the experimental conditions, indicating their low expression level in grape fruit organs (data not shown).

Heterologous expression and functional characterization of *VvGOLS1* in *Escherichia coli*

To confirm the identity of *VvGOLS1*, the cDNA was cloned into the pPROEX HTa expression vector and VvGOLS1 was heterologously expressed as an hexa-His-tagged recombinant protein in *E. coli*. The corresponding His-tagged protein could be detected by Western blots in isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cultures (data not shown). Moreover, crude extracts of induced *E. coli* colonies were able to synthesize galactinol from UDP-galactose and *myo*-inositol in vitro, in contrast to the empty vector control (**Fig. 5A**). The pH optimum of this reaction was about 8. The recombinant VvGOLS1 activity showed Michaelis–Menten-type kinetics, with apparent K_m and V_{max} values of 7.5 ± 1.1 mM and 3.7 ± 0.2 nkat mg^{-1} total protein for UDP-galactose, and apparent K_m and V_{max} values of 9.9 ± 1.2 mM and 1.88 ± 0.1 nkat mg^{-1} total protein for *myo*-inositol, respectively (data not shown). Using Suc and galactinol or raffinose as substrates, recombinant VvGOLS1 exhibited no ability to produce raffinose or to degrade raffinose, respectively (**Fig. 5B, C**). These results clearly identify VvGOLS1 as a galactinol synthase and exclude the enzyme as a RAFS or an alkaline-α-galactosidase (α-GAL).

Water-soluble carbohydrate (WSC) concentrations in grape berries exposed to heat stress

To determine whether increased *VvGOLS1* expression levels were coupled to increased galactinol concentrations, different berry tissues were analyzed by HPLC with a pulsed amperometric detector (HPLC-PAD). Under control conditions, no galactinol was detected either in skin, pulp or seeds (**Fig. 6D**).

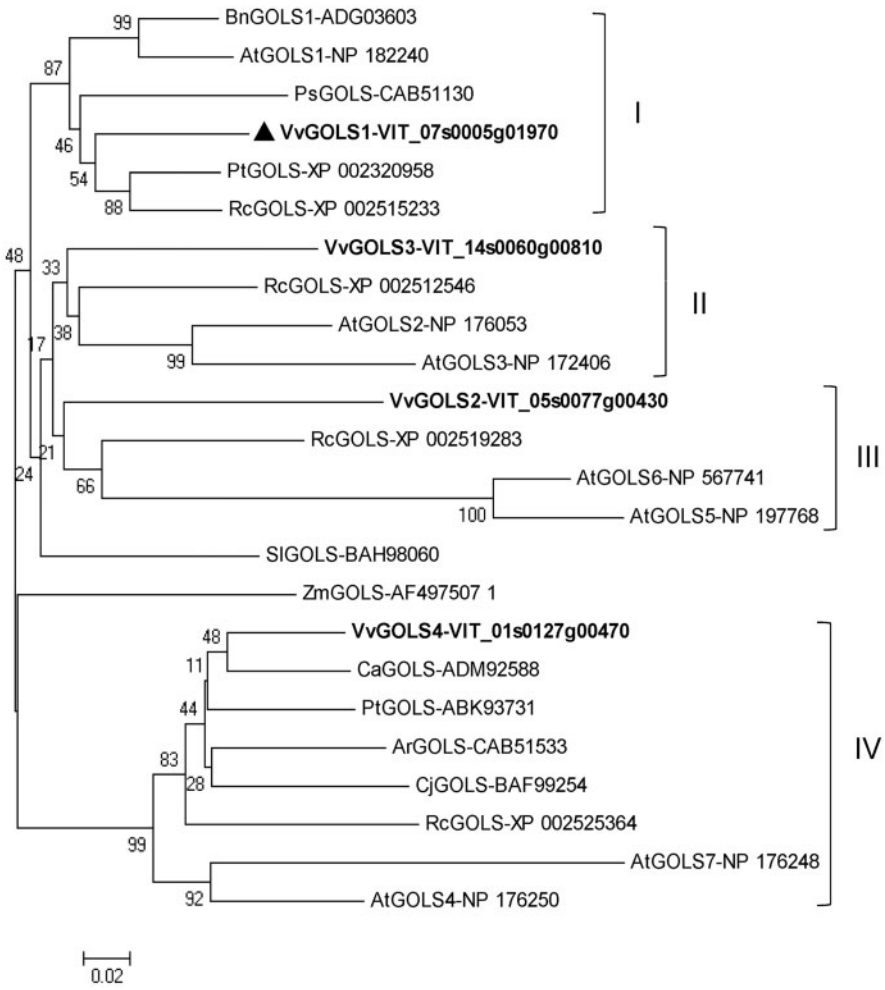

Fig. 3 Phylogenetic analysis using the Neighbor–Joining method and based on predicted amino acid sequences of confirmed and putative GOLS proteins from different plant species. GOLS protein from *Vitis vinifera* is grouped in four clades (I–IV). Vv, *Vitis vinifera*; At, *Arabidopsis thaliana*; Sl, *Solanum lycopersicum*; Rc, *Ricinus citris*; Pt, *Populus trichocarpa*; Ps, *Pisum sativum*; Bn, *Brassica napus*; Ca, *Coffea arabica*; Zm, *Zea mays*; Cj, *Coptis japonica*; Ar, *Ajuga reptans*. Bootstrap values are based on 1,000 replicates. Bars = 0.02 amino acid substitutions per site.

In contrast, heat stress exposure induced galactinol accumulation in all three tested tissues, with higher concentrations in skin and pulp than in seeds. The major WSCs detected were Suc, glucose and fructose (**Fig. 6**). In pulp and skin, their concentrations were not altered after heat stress. In contrast, in seeds, even though Suc content was not affected (**Fig. 6A**), glucose and fructose concentrations were significantly reduced upon heat stress (**Fig. 6B, C**). Remarkably, no raffinose or stachyose was detected under the experimental conditions used (data not shown).

Identification and characterization of VvHsfA2 in grape berries exposed to heat stress

The transcriptional regulation of the heat stress responses by Hsfs has been largely documented in plants (Baniwal et al. 2004, Scharf et al. 2012). Among these, AtHsfA2 from Arabidopsis was described as a major inducer of defense responses to environmental stresses, such as heat stress (Nishizawa et al. 2006). Interestingly, overexpression of *AtHsfA2* up-regulates the expression of various genes including *AtGOLS1* (Panikulangara et al. 2004, Busch et al. 2005, Schramm et al. 2006, Nishizawa et al. 2009). It was therefore tempting to study the potential involvement of such a mechanism in heat-stressed grape berries.

To identify VvHsf homologs within the grape genome, the conserved amino acid sequence of the Hsf-type DNA-binding domain (DBD; Pfam: PF00447) was used to conduct a BlastP search against the PN40024 grapevine sequences (Jaillon et al. 2007). We identified 15 putative and non-redundant VvHsf proteins. A phylogenetic tree was produced by alignment of the full-length amino acid sequences of all identified VvHsfs and the sequences of all Arabidopsis Hsfs described (von Koskull-Döring et al. 2007). To complete the tree, HsfA2-like proteins from other plant species were also included (**Fig. 7**). The phylogenetic tree clearly indicated that among the grapevine amino acid sequences, one belongs to the cluster

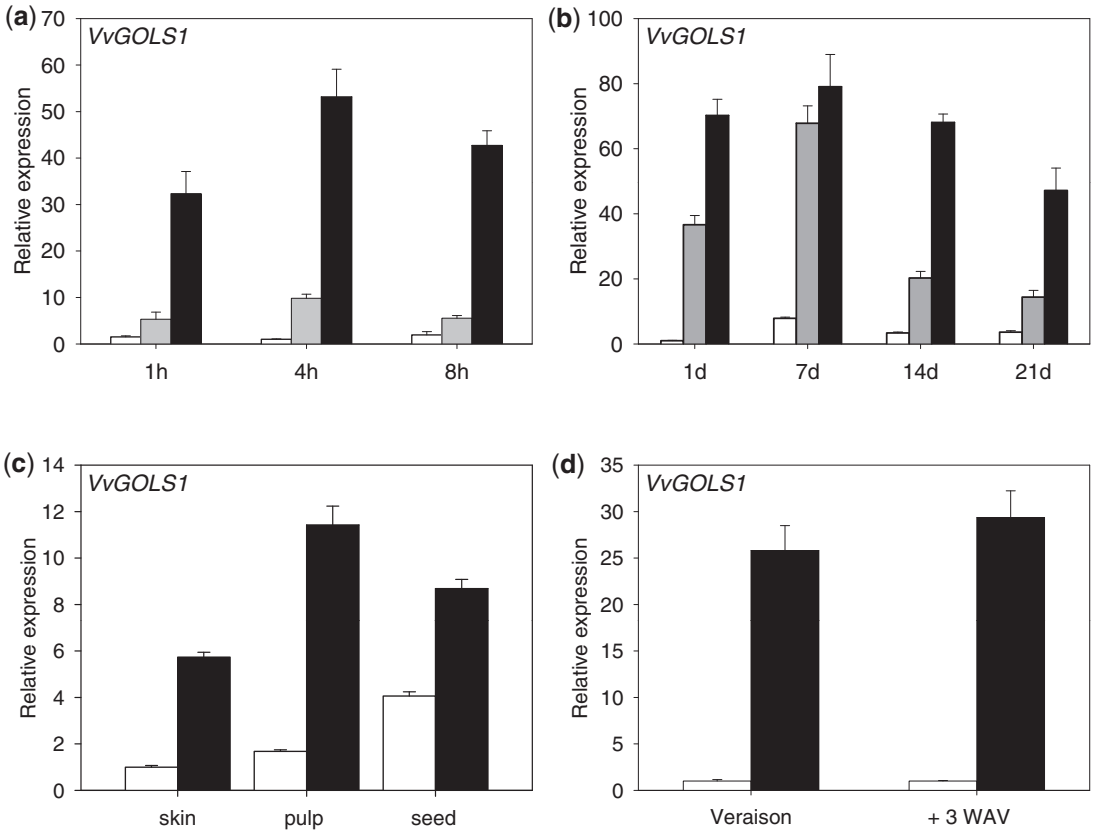

Fig. 4 Relative expression level of *VvGOLS1* transcripts in *Vitis vinifera* cv. Cabernet Sauvignon berries. The *VvGOLS1* transcript level was quantified by quantitative real-time PCR in control (white) and heat-exposed berries (gray, +6°C; and black, +9°C) after 1 d (A) or 3 weeks of heat treatment (B). (C) *VvGOLS1* expression in different compartments of a mature berry after 1 d of heat treatment. (D) *VvGOLS1* expression at different stages of berry development. WAV, weeks after veraison. Gene expression was normalized with *VvEF1γ* and *VvGAPDH*. Relative transcript abundance values represent the expression values obtained after scaling against the minimum expression value. Error bars were calculated as the SD for three independent experiments.

containing AtHsfA2 (**Fig. 7**). The corresponding 1,134 bp full-length cDNA (377 amino acids) was cloned by PCR using cDNAs from Cabernet Sauvignon heat-stressed berries, and was designated *VvHsfA2* (GenBank accession No. JQ801738). The deduced amino acid sequence analysis (**Supplementary Fig. S4**) indicated that VvHsfA2 contained typical Hsf domains and motifs according to Nover et al. (2001). These include one conserved DBD (amino acids 41–134), one oligomerization domain (HR-A/B; amino acids 148–214) and three motifs for nuclear import [the nuclear localization signal (NLS); amino acids 227–240] and transcriptional activation (AHA1; amino acids 311–320 and AHA2; amino acids 358–364). Surprisingly, a nuclear export motif (NES) was not clearly predicted in the VvHsfA2 sequence using the domain analysis program NetNES 1.1 (la Cour et al. 2004). This hydrophobic and leucine-rich motif that is frequently found at the C-terminus of Hsfs is required for the receptor-mediated nuclear export in complex with the NES receptor (Heerklotz et al. 2001). In the PN40024 grapevine genome sequence, *VvHsfA2* is located on chromosome 4 (Jaillon et al. 2007). In this region, gene VIT_04s0008g01110 was predicted (also referred to in the Heatster database as GenBank XM_002278673; Scharf et al. 2012). However, the in silico predicted coding region of VIT_04s0008g01110 is 11 amino acids longer than the VvHsfA2 experimentally observed (**Supplementary Fig. S4**). This difference may be due to a failure in the automatic detection procedure of intron/exon boundaries used for the annotation of the whole genome.

In grape berries, *VvHsfA2* expression was induced as early as after 1 h of heat stress and kept on accumulating during the first 8 h of treatment (**Fig. 8A**). Furthermore, transcript levels of *VvHsfA2* remained high when berries were exposed to heat stress in a repetitive manner during 3 weeks, peaking at 7 d (**Fig. 8B**). Interestingly, the *VvHsfA2* expression profile resembles that of *VvGOLS1* in heat-stressed berries (**Fig. 4A, B**). Together, these data suggest that VvHsfA2 may play a role as a transcriptional activator during the heat stress response and that *VvGOLS1* may be one of its target genes.

VvHsfA2 transactivates *VvGOLS1* expression upon heat stress

To test if *VvGOLS1* expression is regulated by VvHsfA2, transient expression experiments were conducted using tobacco

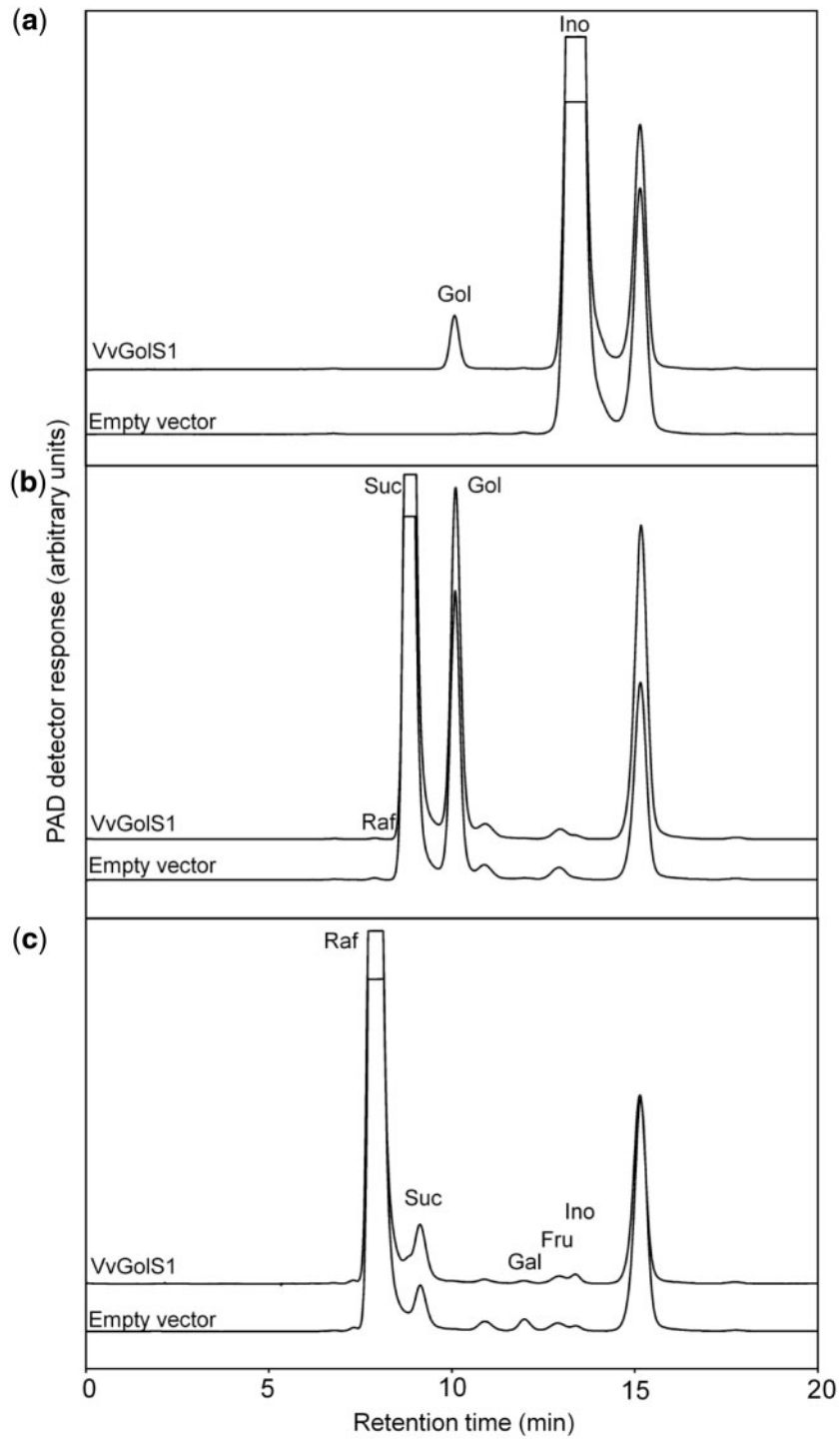

Fig. 5 HPLC-PAD chromatograms showing enzymatic activities of transfected *E. coli* cell lysates. (A) Chromatogram representing an in vitro galactinol synthesis reaction conducted in the presence of 50 mM UDP-galactose and 5 mM *myo*-inositol (Ino). (B) Chromatogram representing an in vitro RAF synthesis reaction conducted in the presence of 50 mM sucrose (Suc) and 5 mM galactinol (Gol). (C) Chromatogram representing alkaline α-GAL activities, with a reaction conducted in the presence of 50 mM raffinose. All the enzymatic assays were performed using crude *E. coli* cell lysates containing recombinant VvGOLS1 or the empty vector, in 50 mM HEPES-KOH buffer pH 7.5, and incubated for 1 h at 30°C. The reactions were boiled, desalted and analyzed by HPLC-PAD. Independent experiments were conducted using two individual *E. coli* colonies for inductions, and enzyme activities were measured in triplicate for each experiment.

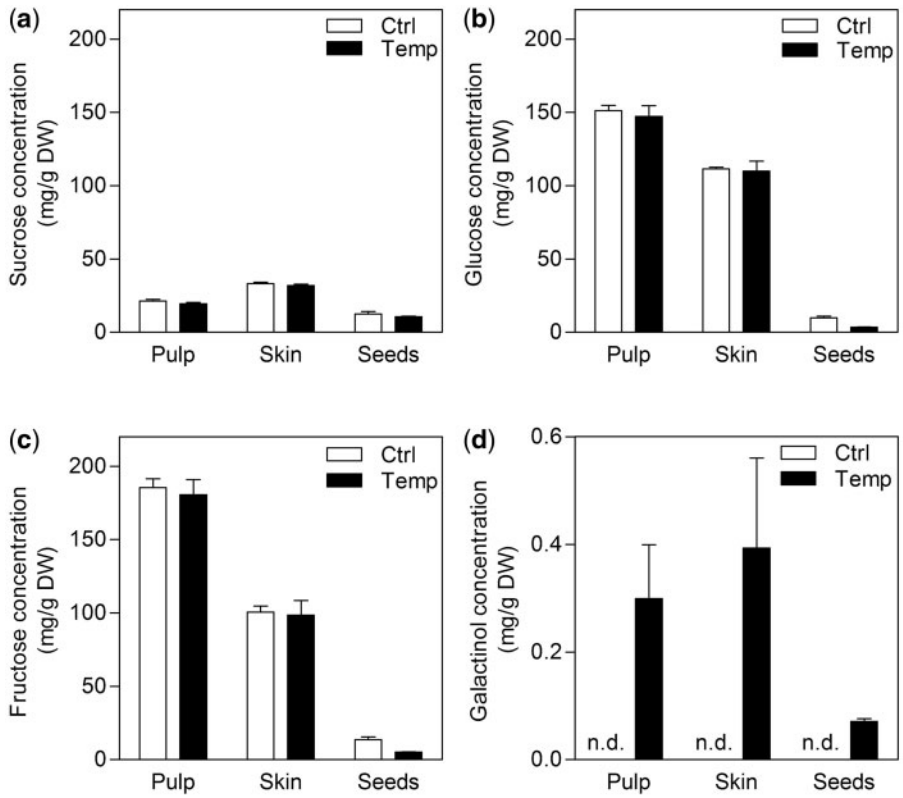

Fig. 6 Changes in the carbohydrate concentrations in different tissues of grapevine berries exposed to heat stress over a period of 3 weeks. Berry samples were collected on the last day of the experiment, from a control cluster (Ctrl) or from heat-stressed (Temp2 condition) clusters (Temp). Concentrations in sucrose, glucose, fructose and galactinol in pulp, skin and seeds tissues were determined by HPLC-PAD. Error bars indicate the standard error between the mean of six replicates. The double asterisks over bars indicate differences between control and heat stress conditions, with statistical significance set at $P < 0.01$ (*t*-test). nd, not detected.

protoplasts and the β-glucuronidase (GUS) reporter gene. To this end, VvHsfA2 was cloned into the pRT vector for expression under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. The inspection of the putative promoter region of *VvGOLS1* using MATinspector (Cartharius et al. 2005) predicted several perfect HSE (heat stress element) sequences upstream of a putative TATA box. These HSEs that are recognized by Hsfs are composed of repetitive palindromic binding motifs and are conserved in promoter sequences of heat stress-inducible genes of all eukaryotes (Nover et al. 2001). A fragment spanning 1,000 bp upstream from the start codon, including the HSE-containing regions, was inserted in front of the uidA reporter of the vector pAM35 (Guerineau et al. 2003). Following transient expression in tobacco protoplasts, the transactivation activity of VvHsfA2 on the *VvGOLS1* promoter was analyzed by measuring GUS activity. At room temperature (23°C), VvHsfA2 did not significantly activate the *VvGOLS1* promoter (**Fig. 9**). In contrast, GUS activity was much higher when the co-transformed protoplasts were incubated at 35°C for 2 h followed by a 4 h recovery period, prior to GUS assays. These results indicated that VvHsfA2 can transactivate the *VvGOLS1* promoter upon heat stimulation.

Discussion

RFOs act as compatible solutes and accumulate in various plants and plant parts during abiotic stresses such as drought, low and high temperature, high levels of reactive oxygen species or high salinity (Taji et al. 2002, Panikulangara et al. 2004, Peters et al. 2007, Peters and Keller 2009). As such, RFOs may protect cellular structures and sustain the osmotic balance in plant cells. RFOs may also act as hydroxyl radical scavengers, thus participating in the protection of the photosynthetic apparatus against oxidative damage caused by drought, salinity and low temperatures (Nishizawa et al. 2008). In grapevine, the protective role of RFOs has not been documented, although these oligosaccharides were detected in different organs of the plant (Hamman et al. 1996, Kliewer 1966).

The present study demonstrated that high temperature locally applied to grapevine clusters induces the accumulation of galactinol in berry skin and pulp, and, to a lesser extent, in seeds (**Fig. 6D**). The production of galactinol is the first committed step in RFO biosynthesis, catalyzed by a GOLS (Keller and Pharr 1996). Surprisingly, no raffinose and stachyose were detected in berries subjected to long-term heat exposure. One hypothesis is that RFOs accumulated during the early stage of heat exposure

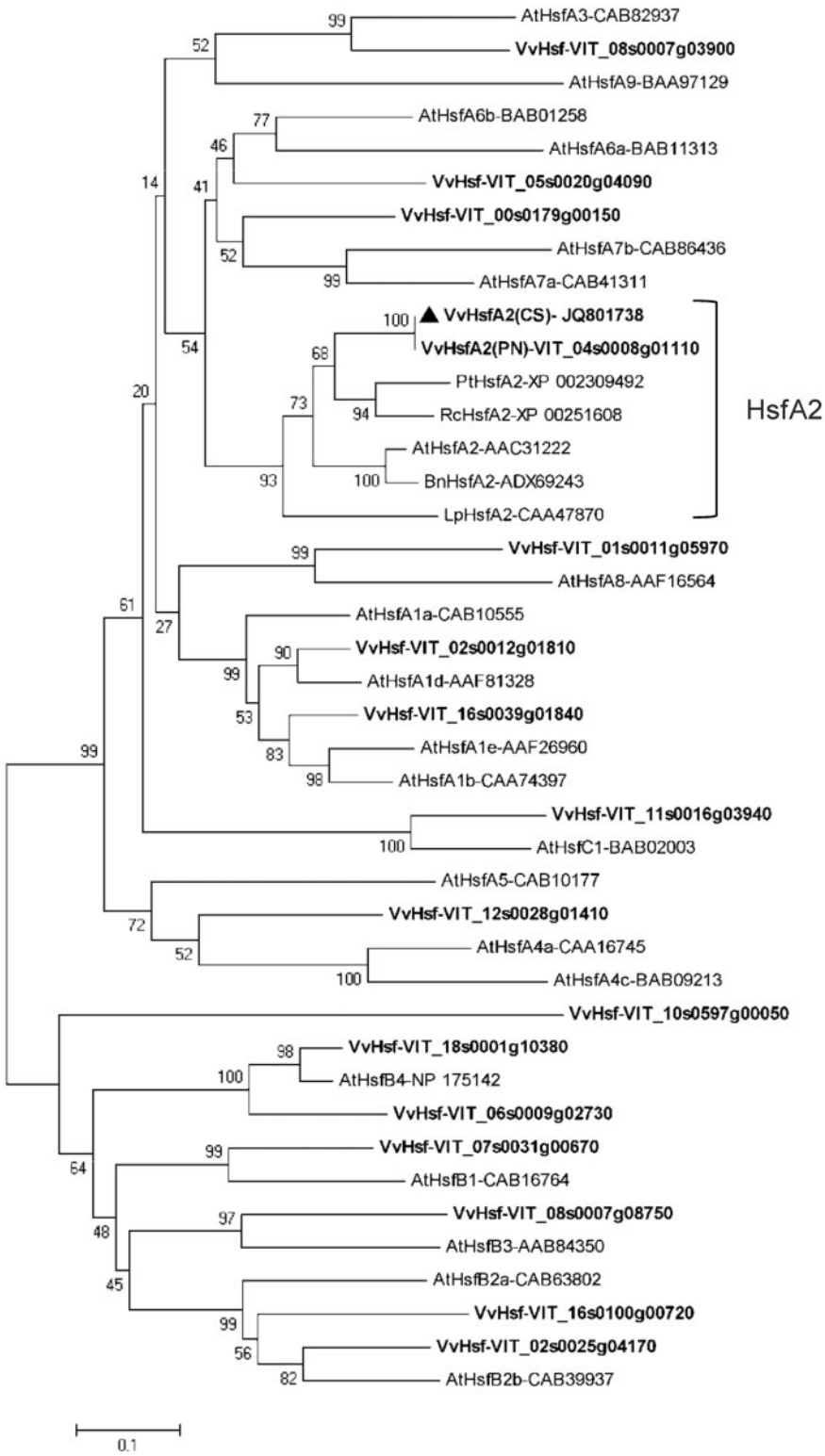

Fig. 7 Phylogenetic analysis using the Neighbor–Joining method and based on predicted amino acid sequences of confirmed and putative Hsf proteins from different plant species. Vv, *Vitis vinifera*, CS cultivar Cabernet Sauvignon, PN, cultivar Pinot Noir, At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Pt, *Populus trichocarpa*; Rc, *Ricinus citris*; Lp, *Lycopersicon peruvianum*. Bootstrap values are based on 1,000 replicates. Bars = 0.1 amino acid substitutions per site.

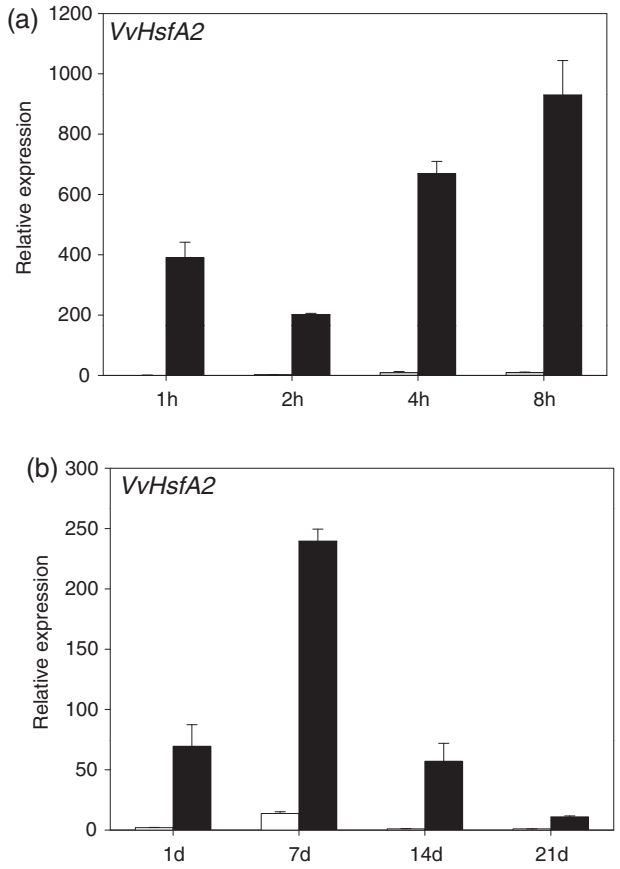

Fig. 8 Relative expression level of *VvHsfA2* transcripts in grapevine cv. Cabernet Sauvignon berries. The *VvHsfA2* transcript level was quantified by quantitative real-time PCR in control (white) and heat-exposed berries (dark, +9°C) after 1 d (A) or 3 weeks of heat treatment (B). Gene expression was normalized with *VvEF1γ* and *VvGAPDH*. Relative transcript abundance values represent the expression values obtained after scaling against the minimum expression value. Data are means of three independent experiments indicating the SD.

are rapidly catabolized to provide metabolizable energy and carbon skeletons. Indeed, the quantification of raffinose and stachyose was performed with berries collected at the end of the stress period of 21 d. Fleshy fruits such as melon actively synthesize raffinose and stachyose but contain very low concentrations of both sugars, suggesting that RFOs are rapidly catabolized (Carmi et al. 2003, Dai et al. 2006). Kang and Lee (2001) characterized an α-GAL from grape flesh (*V. vinifera* L. Muscat of Alexandria) which displayed a weak hydrolytic activity during the green stage, whereas it increased 15-fold during the ripening stage. The partially purified enzyme hydrolyzed the RFOs, raffinose and stachyose with high efficiency. Although this first hypothesis cannot be excluded, the lack of induction of *VvRAFS1*, *VvRAFS2* and *VvSTAS1* during the heat stress period provides a more direct explanation for the absence of RFOs in berries exposed to heat stress (**Supplementary Fig. S3**). Moreover, these three genes were scarcely expressed in control berries. These observations support the hypothesis that galactinol might not act as galactosyl donor and RFO synthesis regulator in heat-stressed berries, but rather as a signaling molecule. Indeed, it was recently demonstrated that galactinol can act as an endogenous molecular signal able to elicit defense responses against pathogen attack (Kim et al. 2008). In that context, the authors showed that the increase in galactinol concentrations in cucumber leaves triggered an induced systemic resistance against bacterial and fungal infections. The role of simple sugars (e.g. Suc, monosaccharides and trehalose) as signaling molecules is now well established in plants (Hanson and Smeekens 2009), and examples of sugar regulation of sugar transport have also been described in grapevine (Cakir et al. 2003, Lecourieux et al. 2010). Therefore, it is tempting to speculate that galactinol accumulation may signal heat stress to trigger adaptive responses in grape berries. Yet, the exact function of galactinol in stressed berries remains to be determined, as do the consequences of its accumulation on fruit development and quality.

GOLS gene expression is closely associated with GOLS activity and galactinol accumulation under abiotic stress conditions (Liu et al. 1998, Sprenger and Keller, 2000, Taji et al. 2002, Panikulangara et al. 2004, Nishizawa et al. 2008). In silico analysis of the 12X version of the *V. vinifera* genome revealed four full-length cDNA sequences encoding proteins with high similarity to GOLSs of other organisms (**Fig. 2**). Only *VvGOLS1* displayed higher expression levels in berries exposed to heat stress, and its induction was positively correlated with galactinol accumulation (**Figs. 4**, **6**). In contrast, *VvGOLS3* and *VvGOLS4* expression was down-regulated upon heat stress (**Supplementary Fig. S1**) whereas *VvGOLS2* transcripts were only weakly detectable in the fruit in both control and stress conditions (data not shown). Similarly, several *GOLS* isoforms were identified in other plants and showed differential transcriptional regulation. Among the 10 Arabidopsis *GOLS* isoforms, three were specifically up-regulated by abiotic stresses. Whereas *AtGOLS1* and *AtGOLS2* were induced by water deficit, heat and salt stress, *AtGOLS3* transcripts accumulated under low temperature stress (Panikulangara et al. 2004, Nishizawa et al. 2008). Similarly, the three *GOLS* isoforms from the *Coffea arabica* genome were differentially expressed in leaves of plants exposed to water deficit, high salt or heat stress (dos Santos et al. 2011). In grapevine, the different *GOLS* isoforms might also be involved in processes other than heat stress responses. Indeed, Tattersall et al. (2007) identified a *GOLS* gene that is differentially expressed in shoot tips of Cabernet Sauvignon plants when exposed to water deficit. The nucleotide sequence of this gene corresponds to *VvGOLS2*. Recently, another transcriptomic study indicated that the expression of *VvGOLS2* is clearly up-regulated in berries during the last stages of ripening, and the authors suggested that *VvGOLS2* could be considered as a late ripening indicator (Guillaumie et al. 2011).

The correlation between galactinol accumulation and *VvGOLS1* expression in heat-stressed berries is reinforced by the demonstration that *VvGOLS1* encodes a functional enzyme.

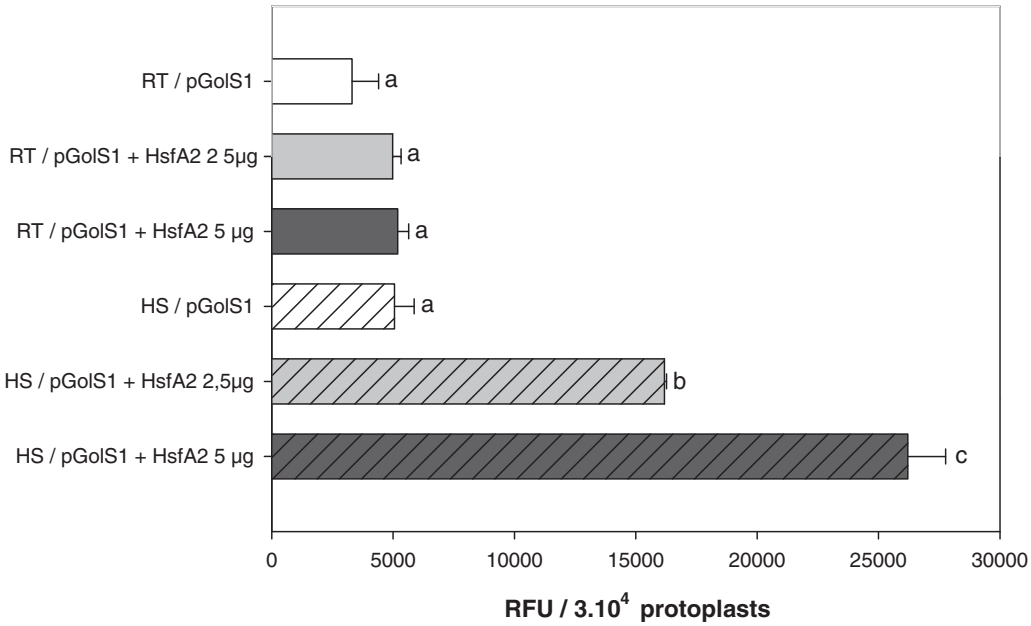

Fig. 9 Transactivation of the *VvGOLS1::GUS* promoter fusion by VvHsfA2 in tobacco protoplasts. The fusion contained a promoter sequence of 1 kb upstream of the transcription start site of the *VvGOLS1* gene (pGOLS1). Protoplasts were transformed with vector pRT101 containing 35S::*VvHsfA2* (JQ801738), or with the empty vector. Open bars indicate *VvGOLS1*::GUS activity without an additional construct; gray and black solid bars indicate GUS activity after co-transformation with the 35S::*VvHsfA2* construct (2.5 or 5 µg plasmid DNA used for protoplast transformation, respectively). Bars without diagonal stripes indicate that the transformed protoplasts were incubated at room temperature (23°C) for 6 h, prior to GUS assays (RT, room temperature). Diagonal stripes in the solid bars indicate that the transformed protoplasts were incubated at 35°C for 2 h followed by a 4 h recovery period, prior to GUS assays (HS, heat stress). The relative GUS activity of 10,000 relative fluorescence units (RFU) represents the cleavage of 5 pmol of MUG in 1 min by an aliquot of cell extract corresponding to 30,000 protoplasts. Data from three independent experiments were pooled and analyzed. Error bars indicate the SD. Differences were determined using one-way ANOVA. Statistical significance was evaluated using the Holm–Sidak method (P-value < 0.05).

When the *VvGOLS1* cDNA was expressed in *E. coli*, crude protein extracts from IPTG-induced cultures synthesize galactinol using *myo*-inositol and UDP-galactose as substrates (**Fig. 5A**). Conversely, they were not able to synthesize or degrade raffinose with the appropriate substrates (**Fig. 5B, C**). Crude protein extracts from empty vector control cultures lacked this synthetic capacity. The optimal pH, apparent K_m and V_{max} for the two GOLS substrates determined for recombinant VvGOLS1 were similar to those reported from other plants (Bachmann et al. 1994, Liu et al. 1995, Ribeiro et al. 2000, Wakiuchi et al. 2003, Li et al. 2011).

The mechanisms involved in the transcriptional regulation of *GOLS* genes are poorly known. In Arabidopsis, *AtGOLS3* expression is induced by cold stress through DREB/CBF transcription factor activities (Taji et al. 2002). The expression of *AtGOLS1* and *AtGOLS2* in Arabidopsis plants exposed to heat stress is regulated by an Hsf, such as HsfA1a, HsfA1b and/or HsfA2 (Panikulangara et al. 2004, Bush et al. 2005, Nishizawa et al. 2006, Schramm et al. 2006). Moreover, plants overexpressing AtHsfA2 showed higher galactinol and raffinose concentrations, which correlated with the increased tolerance of the transgenic plants to several abiotic stresses (Nishizawa et al. 2006, Nishizawa et al. 2008). The *AtGOLS1* promoter contains two modules of a TATA-proximal HSE that are essential for transcriptional activation by AtHsfA2 (Schramm et al. 2006, Nishizawa-Yokoi et al. 2009). The in silico analysis revealed that the promoter of *VvGOLS1* contains several HSE modules upstream and around the TATA box (data not shown), suggesting that a similar Hsf-dependent regulation may exist for *VvGOLS1* expression. Data mining of the 12X version of the grape genome allowed us to identify 15 putative *VvHsf* genes (**Fig. 7**). Very recently, Scharf et al. (2012) extracted and characterized the Hsf families from the genomes of numerous plant species. Their survey predicted 19 *VvHsf* genes in the *Vitis* genome, including that described in the present phylogenetic tree (**Fig. 7**). Therefore, the grape *Hsf* family is one of the smallest families observed so far in angiosperms, together with the *Ricinus* and *Prunus* Hsf families that both contain 18 predicted *Hsf* genes (Scharf et al. 2012). The number of Hsfs in other plants species is typically higher, with a current maximum of 52 *Hsf* genes identified in soybean (Scharf et al. 2012). Among the 19 VvHsfs, VvHsfA2 belonged to the HsfA2 cluster (**Fig. 7**), showing the highest similarities with HsfA2 from poplar (69%), tomato (66%) and Arabidopsis (61%). According to Nover et al. (2001), VvHsfA2 contains the conserved structural elements of the DBD, the oligomerization domain, the NLS domain and the C-terminal activation domain. Using the domain analysis program NetNES 1.1 (la Cour et al. 2004), no NES domain was

clearly identified in the C-terminal region of VvHsfA2, suggesting a constitutive nuclear localization of the protein. However, the Heaster database mentioned an NES domain (amino acid position 366–372) at the C-terminus of VvHsfA2 (Scharf et al. 2012). It was previously shown that the presence of both NLS and NES domains is crucial for the dynamic intracellular distribution of Hsfs between the nucleus and cytoplasm. The nucleocytoplasmic distribution of the tomato SlHsfA2 is markedly influenced by heterooligomerization with SlHsfA1 (Heerklotz et al. 2001). However, Arabidopsis HsfA2 protein can localize to the nucleus without interacting with the HsfA1 protein (Kotak et al. 2004).

The present work showed that *VvHsfA2* transcripts accumulate in grape berries exposed to heat stress (**Fig. 8**) as long as the stress is applied to the fruits. Transcriptional regulation of *VvHsfA2* fits well with data from the literature indicating that several plant Hsfs are heat stress-inducible genes, which among eukaryotic systems is a feature unique to plants (Scharf et al. 1998, Nover et al. 2001, Busch et al. 2005). The presence of HsfA2 exclusively after heat stress treatment suggests its role in the transcriptional regulation of *HSP* genes during prolonged heat stress or during the recovery period following heat stress (Baniwal et al. 2004). Among 21 Arabidopsis Hsfs, Hsfs from class A act as transactivators of stress-inducible genes (Kotak et al. 2004). Both AtHsfA1d and AtHsfA1e govern the regulation of *AtHsfA2* expression in response to environmental stress (Nishizawa-Yokoi et al. 2011). Two VvHsfA1 isoforms were found in the grapevine genome (**Fig. 7**; Scharf et al. 2012), but a similar transcriptional regulation of *VvHsfA2* expression remains to be determined. To the best of our knowledge, the results presented here are the first describing a role for a grapevine Hsf. To gain better insight into the function of this gene family in grape, and especially under heat conditions, we are currently extending our study to the expression analyses of the 18 additional *VvHsf* genes. The preliminary results (data not shown) are in agreement with previous studies indicating that, among Arabidopsis class A Hsfs, HsfA2 showed the highest expression level in response to different abiotic stresses (Nishizawa et al. 2006, Lin et al. 2011, Scharf et al. 2012). Thus, it is tempting to speculate that VvHsfA2 acts as a heat stress-induced enhancer of thermotolerance in grapevine, as described for other species (Scharf et al. 2012).

Using transient promoter assays, VvHsfA2 was found to transactivate the *VvGOLS1* promoter upon heat stress, suggesting its capacity to control galactinol synthesis in grape berries (**Fig. 9**). In Arabidopsis, the HsfA2 protein directly regulates the expression of various genes related to abiotic stress defense, including *GOLS1* (Panikulangara et al. 2004, Bush et al. 2005, Schramm et al. 2006, Nishizawa et al. 2009). As expected, plants overexpressing AtHsfA2 displayed high *AtGOLS1* expression and an increased galactinol concentration (Nishizawa et al. 2008). Our data indicate that the relationship between HsfA2 and GOLS1 is conserved in grapevine fruit under heat stress conditions. As described for others species (reviewed in Scharf et al. 2012), we hypothesize that VvHsfA2 has a broader role for the expression of general stress-related, non-chaperone-encoding genes, helping to protect grape cells against various abiotic stresses.

In summary, the present study demonstrates the accumulation of galactinol in grape berries exposed to high temperature. Because of the lack of RFO accumulation during this treatment, we suggest that galactinol may play a role in the heat signaling pathway. We also isolated and characterized two players in galactinol synthesis in this species, i.e. *VvGOLS1* and *VvHsfA2*. Both *VvGOLS1* and *VvHsfA2* expression was induced in heat-stressed berries, and VvHsfA2 transactivated the promoter of *VvGOLS1* in a transient assay. Additionally, VvGOLS1 was functionally characterized as a genuine GOLS. Taken together, this set of data highlights the intrinsic capacity of Cabernet Sauvignon berries to perceive heat stress and to create adaptive responses. This will prompt us to investigate the heat stress responses in grape berries from cooler or hotter varieties.

Materials and Methods

Plant material

Fruiting cuttings of *V. vinifera* L. cv Cabernet Sauvignon (Ollat and Gaudillere 1998) were grown in a greenhouse, in pots of 0.5 liter containing a mixture of perlite, sand and vermiculite (1 : 1 : 1). A drip irrigation system supplied water and a complete nutrient solution to the roots about five times a day throughout the experiment, avoiding any water or nutrient shortage. All fruiting cuttings bore only one single cluster and lateral shoots were removed as soon as they appeared during growth. Before the experiment, the tip of each shoot was removed as soon as 16 leaves per plant were produced in order to maintain about the same leaf area in all plants and a high leaf to fruit ratio (Ollat and Gaudillere 1998). Therefore, all bunches were assumed not to experience any water or assimilate limitation. In addition, the fruit cuttings were selected at the time of veraison, on the basis of similar vegetative growth and vigour as well as size and compactness of bunches.

Heat stress treatment, temperature measurements and sampling

During the experimental period, the maximal, minimal and mean air temperatures recorded in the greenhouse were 35.4, 15.5 and 23.3°C, respectively. Twenty-one fruiting cuttings were used as controls (CT); the temperature of their bunch closely matched ambient air temperature. Two sets of 21 bunches from other fruiting cuttings (TEMP1 and TEMP2) were submitted to an elevated temperature air flow produced by fan heaters (common domestic models, used at 1,000 W). Only bunches were heated, since shoots, leaves and roots were all protected from the heated air flow by extruded polystyrene foam deflectors. Air heating was applied repeatedly during 21 d, from 07:00 h to 19:00 h every day to mimic the usual diurnal

temperature course of exposed berries, although in a simpler way (Fig. 1A). To avoid any differential effect linked to air flow and possible mechanical stress, simple fan blowers were used to create a continuous air flow around the control clusters, which helped to maintain the temperature of the berries close to the ambient air temperature. The temperatures in control and heated berries (three replicates for each treatment) were monitored continuously by copper–constantan thermocouples inserted into the berries, connected to a Campbell datalogger (Campbell Scientific) (Fig. 1B). By adjusting the distance between the heaters and the clusters, an average temperature of 30.2 ± 2.2, 36.5 ± 2.5 and $39.7 \pm 2.3^{\circ}$C was obtained for CT, TEMP1 and TEMP2 clusters, respectively (Fig. 1C). This experimental set-up led to an average temperature difference of about $6.3 \pm 0.3^{\circ}$C (TEMP1 vs. CT) and $9.5 \pm 0.4^{\circ}$C (TEMP2 vs. CT) between heat-stressed and control clusters, and was maintained during the experiment (Fig. 1D).

Both control and heat stress treatments were applied at two phenological stages, veraison and mid-maturation, for 21 d periods beginning at veraison (50% of berries turning to a visible red color) and 3 weeks after veraison. In order to analyse short- and long-term responses, two control and two heat-stressed berries per cluster were sampled at seven different time points (1, 4 and 8 h, and 1, 7, 14 and 21 d), immediately frozen in liquid nitrogen and stored at -80°C. To obtain three biological replicates, the 21 fruiting cuttings corresponding to each condition (CT, TEMP1 and TEMP2) were divided into three groups of seven plants. The pool of deseeded berries from each group was intended as a biological replication and underwent an independent RNA extraction.

Water-soluble carbohydrate extraction

Control and heated berries were collected at 21 d of treatment. Ground, freeze-dried Cabernet Sauvignon berry tissues (20 mg of skin, pulp and seeds, respectively) were used to extract WSCs as previously described (Peters et al. 2007). Samples were desalted and analysed by HPLC-PAD as described below.

Desalting of extracts

Desalting of carbohydrate samples was conducted as described by Peters et al. (2007). Phenolic compounds and ions were removed by centrifuge-rinsing of the samples through pre-rinsed 1 ml Mobicol spin columns (MoBiTec), fitted with a 10 µm frit and filled with 150 µl of Bio-Rad AG 1-X8 resin (HCO_2 form, 200–400 mesh), 100 µl of Polyklar AT and 50 µl of Serdolit CS-2C (H^+ form), respectively.

HPLC-PAD analysis and quantification of WSCs

WSCs were identified and quantified from plant extracts by HPLC-PAD (Bachmann et al. 1994, Peters et al. 2007). A Ca^{2+}/Na^+-moderated ion partitioning carbohydrate column was used to separate carbohydrates (Benson BC-100 column, 7.8×300 mm; Benson Polymeric). WSCs were quantified using the Chromeleon software package, against a series of 5 nmol standard carbohydrates.

RNA and cDNA production

Berries collected from Cabernet Sauvignon fruit cuttings were quickly frozen in liquid nitrogen, ground to a fine powder with a Dangoumau blender, and stored at -80°C prior to use. Total RNA from grape organs and berries was extracted according to Lecourieux et al. (2010). RNA isolation was followed by DNase I treatment. For each sample, reverse transcription was performed from 2 µg of purified RNA using the Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. The cDNA obtained was diluted (1 : 10) in distilled water.

Gene expression analysis

Quantitative real-time PCR expression analysis was carried out using the CFX96 Real-Time PCR Detection system (Bio-Rad). Reaction mixes (10 µl) were prepared, which included 5 µl of iQ™ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer and 2 µl of diluted cDNA. Gene transcripts were quantified with normalization to *VvEF1γ* and *VvGAPDH* as internal standards. All biological samples were tested in triplicate, and SD values of the means were calculated using standard statistical methods. Specific oligonucleotide primer pairs were designed with Beacon Designer 7 software (Premier Biosoft International). Specific annealing of the oligonucleotides was controlled by dissociation kinetics performed at the end of each PCR run. The efficiency of each primer pair was measured on a PCR product serial dilution. Quantitative real-time PCR primer sequences are listed in Supplementary Table S1.

Enzyme expression and GOLS activities

Escherichia coli BL21 transformed with pPROEX HTa::*VvGOLS1* was used to express VvGOLS1. Crude extract preparation and GOLS assays were performed as previously described (Peters et al. 2007). Negative controls corresponding to *E. coli* BL21 transformed with empty pPROEX HTa vector were treated in the same manner as pPROEX HTa::*VvGOLS* extracts. Independent experiments were conducted using two individual *E. coli* colonies for inductions, and enzyme activities were measured in triplicate for each experiment. The pH optimum of the recombinant VvGOLS1 was determined using 50 mM *myo*-inositol and 5 mM UDP-galactose in the following buffers: 50 mM MES-KOH buffer (pH 5.0, 5.5, 6.0 and 6.5) and 50 mM HEPES-KOH buffer (pH 6.5, 7.0, 7.5, 8.0 and 8.5). The steady-state kinetics (apparent K_m and V_{max} values of both UDP-galactose and *myo*-inositol substrates) were determined at pH 7.5 in 50 mM HEPES-KOH and calculated using the Michaelis–Menten non-linear regression model of the GraphPad Prism 5.0 program (GraphPa). GOLS, RAFS and alkaline α-GAL activities were compared with the corresponding substrates (50 mM *myo*-inositol and 5 mM UDP-galactose for GOLS, 50 mM Suc and 5 mM galactinol for RAFS, and 50 mM

raffinose for alkaline α-GAL) in 50 mM HEPES-KOH buffer pH7.5 after 1 h incubation at 30°C. All the assays were desalted and analyzed by HPLC-PAD as described above.

Protoplasts isolation and transient expression assays

The coding sequence of *VvHsfA2* (VIT_04s0008g01110) was cloned into the pRT101 vector (Kiegerl et al. 2000) and expressed under the control of the CaMV promoter. The promoter region of *VvGOLS1* (VIT_07s0005g01970) was inserted upstream of the GUS reporter gene into pAM35 (Guerineau et al. 2003).

In vitro tobacco (*Nicotania tabacum*, SR1) plants used for protoplast preparation were cultivated in a growth chamber with a constant temperature of 25°C and a 14 h/10 h day/night photoperiod. Protoplasts were obtained from young leaves of a 15-day-old subcultured plant according to Hosy et al. (2005). A 10 μg aliquot of each plasmid DNA was used for polyethylene glycol (PEG)-mediated co-transformation. Transfected protoplasts were incubated for 16 h at 25°C before the heat stress experiments.

The fluorometric GUS assay was performed as followed. Protoplasts were centrifuged briefly for 30 s and vortexed with GUS extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM β-mercaptoethanol, 10 mM Na_2-EDTA (pH 8.0) and 0.1% Triton X-100. The extracts were centrifuged for 15 min in a microcentrifuge at 4°C, and the supernatants were incubated at 37°C for GUS assay using 1 mM 4-methylumbelliferyl-α-D-glucuronide (MUG) as substrate. The reaction was stopped with 0.2 M Na_2CO_3. The amount of methyl-umbelliferone (MU) production was determined using a fluorometer (Versafluor fluorometer, Bio-Rad). The relative GUS activity of 10,000 relative fluorescence units (RFU) represents the cleavage of 5 pmol of MUG in 1 min by an aliquot of cell extract corresponding to 30,000 protoplasts.

Gene annotation and sequence analysis

Except for *VvHsfA2*, all the grapevine genes presented were annotated according to the V1 version of the 12X draft annotation of the grapevine genome (http://genomes.cribi.unipd.it/DATA/). The *VvHsfA2* sequence from Cabernet Sauvignon determined in this study has been deposited in GenBank under accession number JQ801738. Full-length amino acid sequences of GOLS, RAFS, STAS and Hsf from several species were retrieved from public databases. Amino acid sequence alignments were performed using ClustalW2 (Thompson et al. 1994). The phylogenetic trees were constructed from the ClustalW alignments using MEGA version 4 (Tamura et al. 2007).

Statistical analyses

All the results presented here are means ± SEM of at least three independent experiments. Paired *t*-test was used to evaluate the significance of different WSC concentrations between heat stress and control conditions (P-value < 0.01). Fluorometric GUS assay was carried out with three replicates per sample and differences were determined using one-way analysis of variance (ANOVA). Statistical significances were evaluated using the Holm–Sidak method (P-value < 0.05).

Supplementary data

Supplementary data are available at PCP online.

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