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## Proteomic and metabolomic profiling underlines the stage- and time-dependent effects of high temperature on grape berry metabolism

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1 **Running Title:** Heat stress impacts grape berry proteome and metabolism

2

3 **Proteomic and metabolomic profiling underlines the stage- and time-**  
4 **dependent effects of high temperature on grape berry metabolism**

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25

26 **ABSTRACT**

27 Climate change scenarios predict an increase in mean air temperatures and in the frequency, intensity,  
28 and length of extreme temperature events in many wine-growing regions worldwide. Because elevated  
29 temperature has detrimental effects on the berry growth and composition, it threatens the economic  
30 and environmental sustainability of wine production. Using Cabernet Sauvignon fruit-bearing cuttings,  
31 we investigated the effects of high temperature (HT) on grapevine berries through a label-free shotgun  
32 proteomic analysis coupled to a complementary metabolomic study. Among the 2279 proteins  
33 identified, 592 differentially abundant proteins were found in berries exposed to HT. The gene  
34 ontology categories “Stress”, “Protein”, “Secondary metabolism” and “Cell wall” were predominantly  
35 altered under HT. High temperatures strongly impaired carbohydrate and energy metabolism, and the  
36 effects depended on the stage of development and duration of treatment. Transcript amounts correlated  
37 poorly with protein expression levels in HT berries, highlighting the value of proteomic studies in the  
38 context of heat stress. Furthermore, this work reveals that HT alters key proteins driving berry  
39 development and ripening. Finally, we provide a list of differentially abundant proteins that can be  
40 considered as potential markers for developing or selecting grape varieties that are better adapted to  
41 warmer climates or extreme heat waves.

42

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44 **Keywords:** *Vitis vinifera*, berry, heat stress, proteomic, primary and secondary metabolisms

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## 48 INTRODUCTION

49 Grapevine is economically the most important fruit crop in the world, providing dried fruits, table  
50 grapes and the basis for wine making ([www.oiv.int](http://www.oiv.int); [www.fao.org](http://www.fao.org)). The combination of climate, soil  
51 and viticultural practices in a given region constitutes a highly interactive system known as “terroir”  
52 (Deloire et al. 2005). The interaction between “terroir” and the plant’s genotype (cultivar) significantly  
53 affects vine development and berry composition, and thereafter determines wine quality and character  
54 (Van Leeuwen and Seguin 2006). Grapevine requires suitable temperatures, radiation intensities and  
55 duration, and water availability during the growth cycle for optimal fruit production and quality (Kuhn  
56 et al. 2014). Because of on-going climate change, the environmental parameters in most wine  
57 producing regions around the world are expected to change, potentially lowering productivity and  
58 altering the traditional characteristics (“typicity”) of the wine from a given region (Schultz and Jones  
59 2010). While some of these climatic constraints can be overcome, at least partially, by viticultural  
60 practices, temperature remains more difficult to control under field conditions (Van Leeuwen et al.  
61 2013). Climate change scenarios predict an increase in global mean temperatures between 1.5 and  
62 5°C, during the 21<sup>st</sup> century (IPCC, 2014), in addition to an increase in the frequency, intensity, and  
63 duration of extreme temperature events (Lobell et al. 2008). Considering that air temperature is a key  
64 climate variable affecting grapevine phenology, berry metabolism and composition (Jones et al. 2005;  
65 Martínez-Lüscher et al. 2016), global warming represents a serious threat for wine production and/or  
66 typicity in many wine-growing regions worldwide (Fraga et al. 2016).

67 Temperatures above the optimum are sensed as heat stress by living organisms. Heat stress disturbs  
68 cellular homeostasis and can lead to severe retardation in growth and development and even death  
69 (Wahid et al. 2007). In grapevine, warming promotes vegetative growth and disturbs the plant’s  
70 carbon balance (Greer and Weedon 2012), adversely affecting flower set and young berry  
71 development (Greer and Weston 2010). HS also impacts fruit primary and secondary metabolism,  
72 desynchronizing sugar and organic acid metabolism and delaying sugar and polyphenol accumulation  
73 during ripening (Torregrosa et al. 2017; Gouot et al. 2018). As a consequence, heat waves occurring  
74 during key berry development stages can dramatically affect the organoleptic properties of wine even  
75 when more favourable weather conditions prevail for the remaining of the season.

76 In this context, the common viticultural practice of removing leaves around the fruit zone to  
77 enhance aroma development and avoid fungal infections (Alem et al. 2018), might be questioned since  
78 it affects light exposure and temperature of the berries. Because of the direct irradiative effects,  
79 temperature within the fruit may differ very significantly from that of the whole plant or surrounding  
80 air (Cola et al., 2009). The microclimate (mostly temperature and light conditions) strongly influences  
81 the metabolite composition of berries, affecting flavonols, anthocyanins, amino acids and aroma  
82 precursors (Downey et al. 2006; Pereira et al. 2006; Asproudi et al. 2016; Young et al. 2016; Reshef et  
83 al. 2017). These effects can be explained, at least in part, by the substantial remodelling of the fruit  
84 transcriptome observed under these conditions (du Plessis et al. 2017; Lecourieux et al. 2017).

85 In a previous report, we showed dramatic biochemical and transcriptomic changes in heat-exposed  
86 berries, depending on both the developmental stage and the stress duration (Lecourieux et al. 2017).  
87 Although transcriptomic analyses have offered comprehensive insights into the mechanisms  
88 underlying the HS responses in grape (Liu et al. 2012; Carbonell-Bejerano et al. 2013; Rienth et al.  
89 2014b; Jiang et al. 2017; Lecourieux et al. 2017), it is recognised that changes in transcript abundance  
90 do not always correlate with the levels of the encoded proteins (Keller and Simm 2018). Furthermore,  
91 proteins represent a more functional level of gene expression than transcripts because they have a  
92 direct impact on metabolism and other cellular processes. Therefore, state of the art mass spectrometry  
93 analysis of the proteome is a powerful tool to study molecular mechanisms and biological traits in  
94 plants, including responses to abiotic stresses (Kosova et al. 2018). Using shotgun proteomic tools  
95 such as iTRAQ (isobaric Tags for Relative and Absolute Quantitation) and label-free quantification  
96 techniques, several proteomic studies have recently investigated the heat responses of grapevine leaves  
97 (Liu et al. 2014; Jiang et al. 2017) or grape cell cultures (George et al. 2015).

98 To better understand the consequences of high temperature (HT), inherent to leaf removal, on berry  
99 development and to improve our knowledge about the molecular mechanisms involved in grapevine  
100 fruit response to HT, the present work combines transcriptomic, proteomic and metabolite analysis  
101 using Cabernet Sauvignon fruit-bearing cuttings. A label-free proteomic approach was used to  
102 investigate changes in the grape berry proteome resulting from direct exposure to elevated  
103 temperature. The collected proteomic data were compared with our previous transcriptomic analysis  
104 performed on the same berry samples (Lecourieux et al., 2017). These data showed a strong and  
105 negative effect of HT on proteins related to carbohydrate and energy metabolism, which led us to  
106 analyse the impact of HT on primary metabolites using tandem mass spectrometry (LC-MS/MS).  
107 Finally, we focused on proteins accumulating in berries exposed to HT that are potentially involved in  
108 thermotolerance. Altogether, this work helps to better understand the consequences of heat stress on  
109 developing grape berries and provides a list of potential heat-tolerance molecular markers that could  
110 be useful for breeders and viticulturists.

111

## 112 **RESULTS AND DISCUSSION**

113

### 114 **Quantitative analysis of heat stress responsive proteins in grape berries**

115 Experiments were performed on fruit-bearing cuttings of *V. vinifera* Cabernet Sauvignon as described  
116 in Lecourieux et al. (2017). In brief, berries at three different stages of development were exposed to  
117 elevated temperatures for 12 h (7:00 am to 7:00 pm) during each day of treatment and harvested for  
118 analysis after 1 and 7 days of treatment. The average pulp temperature of the heat-treated berries was  
119 about 8°C higher than in control berries. A label-free proteomic approach was performed to identify  
120 proteins with differential abundance between control and heat-treated berry samples.

121 Using the parameters and filters described in the Material and Methods, the conducted label-free

122 experiment led to the identification of 3972 protein groups (from here on referred to as proteins) in the  
123 combined dataset from the six conditions (three stages x two treatment durations), of which 2279  
124 showed two or more unique peptides (**Table 1**). Protein abundance changes between control and heat-  
125 exposed fruits were considered to be significant when a minimum fold change of 1.5 ( $\log_2FC > 0.58$  or  
126  $< -0.58$ ) was reached, with a significance threshold of  $p < 0.05$ . Only proteins identified in all three  
127 biological replicates were considered for quantification. Among the quantified proteins with a  
128 minimum of two unique peptides, 592 DAPs (differentially abundant proteins) displayed significant  
129 differences in abundance between control and HT berries in at least one of the six conditions. The  
130 entire dataset is available via ProteomeXchange (identifier PXD014693) and in Supplementary **Table**  
131 **S1**. Further information on the 592 DAPs is provided in Supplementary **Table S2**. Among these 592  
132 DAPs, 501 proteins were identified in only one condition (139 up-regulated and 362 down-regulated)  
133 while the 91 remaining proteins were retrieved in more than one condition (16 up-regulated, 61 down-  
134 regulated, and 14 proteins that were up-regulated in some conditions but down-regulated in others)  
135 (**Table 1 and Table S2**). More DAPs were identified at the ripening stage (304 DAPs) than at the  
136 green (146 DAPs) or veraison stages (222 DAPs). Whereas the numbers of up- and down-regulated  
137 proteins were similar at veraison (115 up, 107 down), more down-regulated proteins were found when  
138 the HT was applied at the green stage (24 up, 122 down) or ripening (49 up, 255 down) stages (**Figure**  
139 **1A**). The DAPs identified after short (1 day) treatments were mostly different from those observed  
140 after long (7 days) treatments. Indeed, only 1 (up), 9 (4 up, 5 down) and 18 (2 up, 16 down) common  
141 DAPs between 1D or 7D of HT were observed at the green, veraison and middle ripening stages,  
142 respectively (**Figure 1B and Tables S2, S3, S4 & S5**). Similarly, at all three stages, only one common  
143 down-regulated DAP was identified after 1 day of HT (VIT\_12s0057g01020, fasciclin-like protein),  
144 with none being detected after 7 days of treatment (**Table S2**). The number of common DAPs per  
145 stage increased when looking for proteins with modified abundance after 1 and/or 7 days treatment. In  
146 this case, 7 DAPs (3 up, 4 down) were found in common for the three stages, and up to 31 DAPs were  
147 obtained when comparing heat effects between green and veraison stages (**Figure 1C and**  
148 **Supplementary Table 2**).

149 To determine the functional categories affected by HT, the identified DAPs were functionally  
150 classified using MapMan BIN codes (Usadel et al. 2005) and a *V. vinifera* mapping file (Lecourieux et  
151 al., 2017). The predominant proteins changed in berries in response to HT were assigned to “protein  
152 metabolism” (114/592 DAPs, 19.3%), “stress” (54/592 DAPs, 9.1%), “secondary metabolism”  
153 (31/592 DAPs, 5.2%) and “cell wall” (25/592 DAPs, 4.2%) (**Figure 2**). Except for the categories  
154 “stress” (36 up, 18 down) and “RNA” (10 up, 9 down), all the others categories contained more down-  
155 regulated proteins than up-regulated ones, with particularly asymmetrical changes in some categories,  
156 such as “lipid metabolism” (0 up, 16 down) and “transport” (2 up, 17 down). No particular effect of  
157 HT according to the developmental stage was observed on these functional categories, whereas  
158 categories linked to carbohydrate and energy metabolism (glycolysis, tricarboxylic acid (TCA) cycle,

159 fermentation, gluconeogenesis, minor and major carbohydrate (CHO) metabolism) or related to  
160 “photosynthesis”, “cell wall” and “redox” were mostly impacted during veraison and/or ripening  
161 stages (**Supplementary Table 2**).

162

### 163 **Poor correlation between the transcriptome and proteome in heat-stressed berries**

164 Global analyses based on gene expression and protein profiles provide a powerful tool for  
165 understanding how organisms respond to environment changes. To integrate proteomic data with  
166 transcriptomic results, the present protein abundance data were compared to each corresponding  
167 differentially expressed gene (DEG) identified in the same grape berry samples exposed to HT  
168 (Lecourieux et al., 2017). Whereas 592 DAPs were retrieved in at least one of the six HT conditions  
169 (**Table 1, Supplementary Table S1**), 6848 DEGs were identified using the same berry samples  
170 (Lecourieux et al. 2017). A linear correlation between differentially expressed transcripts and the  
171 corresponding proteins was established for 64 out of 592 DAPs (**Figure 4, Supplementary Table**  
172 **S11**). Two types of correlation profiles were found. The first one corresponds to proteins and  
173 transcripts that exhibit profiles with the same tendency (up- or down-regulation in response to HT) and  
174 the second one includes proteins and transcripts that are inversely correlated for a same gene. The best  
175 relationship between mRNA and protein levels (39 DAPs) was observed when HT was applied for one  
176 day at veraison, and for proteins belonging to functional BINs associated to “heat stress” (25  
177 members), “hormone” (4), and “secondary metabolism” (5) (**Figure 4, Supplementary Table S11**).

178 Our results underline the poor correlation between the proteome and the transcriptome in HT  
179 berries, with only 11% (64/592 DAPs) of the differentially abundant proteins that were also impacted  
180 at the mRNA expression level. This is not unexpected since HT leads to denaturation and aggregation  
181 of proteins, and because translation, especially under elevated temperature, is governed by a variety of  
182 regulatory mechanisms independent of transcription rate (Bokszczanin and Fragkostefanakis 2013).  
183 These include the impacts of miRNAs on mRNA stability and translatability, mRNA half-life, global  
184 and transcript specific translational rates, as well as protein turnover. Any lag between the synthesis of  
185 a gene transcript and its translation into a protein could also give rise to an apparent discrepancy  
186 between transcript and protein abundance at a given sampling time (Keller and Simm 2018). Last but  
187 not least, the poor correlation between transcript and protein levels in heat-treated berry samples may  
188 also be due to alternative splicing (AS) events. Increasing evidence has shown that AS is a critical  
189 post-transcriptional event and plays an important role in plant stress responses (Baurle 2016).  
190 Particularly, many genes are under transcriptional regulation by the circadian clock, and several  
191 circadian clock genes show AS in response to even small changes in temperature (James et al. 2018).  
192 In grapevine, AS changes were observed for nearly 70% of the genes in leaves exposed to  
193 temperatures above 35°C. In the same study, only 64 out of the 808 identified DAPs were retrieved as  
194 differentially expressed at the transcript level (Jiang et al. 2017), that fits well with the weak  
195 transcript/protein correlation observed in our work. Jiang et al. (2017) suggested that AS changes



196 under high temperature could provide molecular plasticity allowing the plants to adapt to stress  
197 conditions. Using *Arabidopsis* (*Arabidopsis thaliana*) plants exposed or not to a non-lethal HT,  
198 referred to as priming, Ling et al. (2018) have recently demonstrated that AS functions as a novel  
199 component of heat-shock memory.

200 Despite the generally poor correlation between individual transcripts and proteins levels in berries  
201 exposed to HT, there was a much better correspondence at the functional category level, with  
202 transcripts and proteins assigned to the “stress”, “protein metabolism” and “secondary metabolism”  
203 MapMan BINs showing consistent responses as a group (**Supplementary Figure S1**). We also  
204 noticed that, at the transcript level, most of the functional categories were differentially affected  
205 according to the developmental stage and the stress duration whereas it was less obvious at the protein  
206 level. The best relationship between transcripts and proteins levels was observed with genes related to  
207 the category “stress”, and especially to the “heat” sub-category. Among the 30 HSPs whose protein  
208 abundance increased in response to HT, 25 were also up-regulated at the transcript level  
209 (**Supplementary Table S12**). Most proteins are inherently thermo-labile, with some becoming  
210 denatured at the temperatures that grapevines experience during a heat wave. In addition, high  
211 temperature is often associated with oxidative stress leading to production of reactive oxygen species  
212 that irreversibly damage proteins. Maintaining proteins in stable and functional conformations and  
213 preventing their aggregation are essential for survival of cells under high temperature. HSPs function  
214 as molecular chaperones in maintaining protein quality and folding, and are required for the  
215 acquisition of plant thermotolerance (Bokszczanin and Frangkostefanakis 2013). It is well established  
216 that transcription factors from the HSF family regulate the heat stress response in plants, through a  
217 massive up-regulation of *HSP* genes (Scharf et al. 2012). However, as transcription factors are low  
218 abundance proteins they largely escaped proteomic detection, and no HSF was retrieved in the present  
219 study despite the observed transcriptional up-regulation of six *VviHSF* genes in heat-exposed berries  
220 (Lecourieux et al. 2017).

221 In good agreement with our transcriptomic data, the protein abundance of the functional category  
222 “Protein metabolism” was strongly impacted with nearly 20% of the identified DAPs that belong to  
223 this category being affected, especially those belonging to the sub-categories “protein synthesis” (34  
224 DAPs) and “protein degradation” (55 DAPs) (**Supplementary Table S6**). However, the HT triggered  
225 a strong down-accumulation effect on the “protein metabolism” category (92 out of the 114 DAPs), in  
226 contrast with what was observed on transcripts (333 up- and 307 down-regulated genes). A number of  
227 studies demonstrated that different abiotic stresses, including heat, result in a general inhibition of  
228 protein translation (Yanguéz et al. 2013). Accordingly, we observed a significant decrease in  
229 abundance of many proteins related to protein synthesis including various ribosome structural proteins  
230 and several translation initiation factors (**Supplementary Table S6**). Whereas inhibiting translation  
231 under stress helps to reduce energy consumption, certain specific mRNAs are selectively translated to  
232 produce relevant proteins involved in the proper establishment of the stress adaptation process. By



233 studying the translome of Arabidopsis seedling exposed to HT, Yanguéz et al. (2013) observed that  
234 regulation of gene expression at the translational level is superimposed on regulation at the  
235 transcriptional level, and often reinforces the response of individual genes/proteins to heat stress.  
236 Protein translation is accomplished by the combined actions of the ribosomes and ancillary proteins  
237 such as initiation, elongation, and termination factors. Yanguéz et al. (2013) showed that translation  
238 could be regulated at the initiation phase in Arabidopsis seedlings exposed to HT, even if the  
239 mechanisms involved in this regulation remain unclear. To date, there is some evidence for the role of  
240 different plant translation initiation factors (eIFs) in various abiotic stresses responses (Dutt et al.  
241 2015). Our proteomic data highlighted the increase in abundance of four eIFs upon HS, namely eIF1A  
242 (VIT\_15s0046g01500), two isoforms of eIF3 (VIT\_14s0060g01630, VIT\_00s0880g00020) and eIF5  
243 (VIT\_14s0006g01990), whereas others eIF isoforms were decreased (**Supplementary Table S6**).  
244 eIF5 (VIT\_14s0006g01990) showed the highest accumulation score (Log<sub>2</sub>FC 5.22) within this  
245 functional category. The importance of eIF5 proteins in thermotolerance was highlighted in pea  
246 (Suragani et al. 2011) and Arabidopsis (Xu et al. 2011; Zhang et al. 2017).

247

## 248 **Heat stress alters key proteins driving berry development and ripening**

249

### 250 *Heat stress impacts the secondary metabolism in developing berry*

251 The strong effects of HT observed on transcripts assigned to the “secondary metabolism” functional  
252 category (291 DEGs across the six conditions, (Lecourieux et al. 2017)) were confirmed at the protein  
253 level (31 out of the 592 DAPs), albeit to a lesser extent (**Supplementary Table S8**). These 31 DAPs  
254 (11 up, 20 down) mainly belonged to the subcategories: “isoprenoids” (7 DAPs), “phenylpropanoid-  
255 lignin” (12) and “flavonoids” (7).

256 HT altered expression of numerous genes related to aromatic potential, including genes encoding  
257 biosynthetic enzymes for terpenes and methoxypyrazines (Lecourieux et al. 2017), but the proteomic  
258 data from the current study suggested that these changes in transcript abundance were not translated  
259 through to the protein level. A few proteins related to isoprenoid subfamily were down-accumulated  
260 under HT including a 3-ketoacyl-CoA synthase (KCS10, VIT\_04s0008g02250), which is involved in  
261 the synthesis of very long chain fatty acids that are incorporated into plant lipids (precursors of  
262 cuticular waxes), and several enzymes related to the synthesis of precursors of terpenoids  
263 (**Supplementary Table S8**). By contrast, a carotenoid cleavage dioxygenase (CCD1,  
264 VIT\_13s0064g00810) was found more abundant in stressed berries at veraison. Expression of various  
265 *VvCCD* genes was reported to increase towards berry ripening and enzymes in this family were  
266 demonstrated to promote desirable flavor and aroma compounds in grapes and wine (Mathieu et al.  
267 2005; Rienth et al. 2014a; Alem et al. 2018). The putative role of CCD proteins in heat responses is  
268 still unknown but these proteins were suggested to enhance tolerance to various abiotic stresses  
269 through their enzymatic products (e.g. apocarotenoids) acting as stress signals (Havaux 2014). For

270 instance, some members of the CCD family are involved in generating carotenoid-derived  
271 phytohormones, such as strigolactones known to be involved in various plant developmental and  
272 adaptation processes (Jia et al. 2018).

273 It is well known that extreme high temperatures significantly affect the phenylpropanoid content of  
274 grape berries, with the impact being dependent on the intensity and duration of the HS as well as the  
275 developmental stage of the fruit (Gouot et al. 2018). Consistent with changes observed at the transcript  
276 level (Lecourieux et al. 2017), HT triggered an enhanced abundance of proteins involved in lignin  
277 synthesis, including shikimate *O*-hydroxycinnamoyltransferase (HCT; VIT\_09s0018g01190),  
278 cinnamyl alcohol dehydrogenase (CAD; VIT\_03s0180g00260, VIT\_02s0025g03100,  
279 VIT\_00s0615g00030) and caffeic acid *O*-methyltransferase (CCoAOMT1; VIT\_16s0098g00850). A  
280 significant increase in lignin biosynthesis would confer additional strength to lignified secondary cell  
281 wall, helping berries to protect against shriveling mediated-HT. For instance, the activation of the  
282 lignin pathway is a reaction to advanced stages of dehydration in grape berry fruit (Zamboni et al.  
283 2010). Key enzymes of the general phenylpropanoid pathway (phenylalanine ammonia-lyase PAL,  
284 VIT\_06s0004g02620, VIT\_08s0040g01710, VIT\_13s0019g04460; cinnamate 4-hydroxylase C4H,  
285 VIT\_06s0004g08150; 4-coumarate-CoA ligase 4CL; VIT\_11s0052g01090), enzymes from the  
286 flavonoid biosynthesis pathway (dihydroflavonol 4-reductase DFR, VIT\_18s0001g12800; flavonoid  
287 3'-hydroxylase F3'H, VIT\_17s0000g07210; flavonoid 3',5'-hydroxylase F3'5'H,  
288 VIT\_06s0009g02860; flavanone 3'-hydroxylase F3H, VIT\_18s0001g14310; isoflavone reductase,  
289 VIT\_03s0038g04700) as well as enzymes mediating the glycosylation of flavonoids (UDP-glucosyl  
290 transferases, VIT\_12s0055g00290, VIT\_02s0033g00130, VIT\_17s0000g04750; UDP-rhamnosyl  
291 transferase, VIT\_00s0218g00150) were also affected at the protein level upon HT. Most of these  
292 proteins were less abundant in heated berries, suggesting a sharp decline of the corresponding  
293 enzymatic activities (**Supplementary Tables S2 & S8**). This lower amount of key enzymes probably  
294 contributes to the reduction of flavonoid synthesis in grape berries exposed to heat. However, other  
295 concomitant processes may explain the decrease of flavonoid contents in warmed berries, including  
296 reduced biosynthetic enzyme activities due to non-optimal temperature, enzymatic oxidation or  
297 chemical degradation after ROS scavenging (Gouot et al. 2018). Upon their biosynthesis in the  
298 cytosol, flavonoids are rapidly accumulated into vacuoles or other cellular compartments through  
299 vesicle trafficking, membrane transporters or glutathione S-transferase (GST)-mediated transport  
300 (Zhao 2015). In addition to transport of flavonoids into vacuoles mediated by ATP-binding cassette  
301 (ABC) transporters (Francisco et al. 2013), there is also H<sup>+</sup>-dependent transport mechanism in  
302 grapevine (Gomez et al. 2011; Kuang et al. 2019). Vacuolar transport of anthocyanins and other  
303 metabolites, such as malate, directly or indirectly depends on the transmembrane pH gradient  
304 generated by both tonoplast H<sup>+</sup>-ATPases and H<sup>+</sup>-pyrophosphatases (V-PPase) (Shiratake and  
305 Martinoia 2007). Three V-ATPase subunits (VIT\_03s0038g00790, VIT\_18s0001g01020,  
306 VIT\_04s0008g02460) and two V-PPases (VIT\_11s0118g00350, VIT\_09s0002g07880) showed strong

307 decrease in protein abundance in HT berries, and this may impair the efficient transport of  
308 anthocyanins into the vacuole. Finally, the translational repression of several GST proteins  
309 (VIT\_01s0026g01340, VIT\_01s0011g01900, VIT\_08s0040g03100, VIT\_19s0093g00310,  
310 VIT\_07s0104g01800) observed in heat-stressed berries during ripening could have an impact on fruit  
311 secondary metabolism as well (Perez-Diaz et al. 2016).

312

### 313 *Harmful effects of heat stress on carbohydrate and energy berry metabolism*

314 A drastic negative effect of HT was observed for proteins assigned to primary carbohydrate  
315 metabolism (**Supplementary Table S9**). Almost all the DAPs related to the following processes were  
316 under-accumulated after HT: major CHO metabolism (6 out of 8 DAPs), glycolysis (8 out of 9  
317 DAPs), fermentation (4 out of 5 DAPs), gluconeogenesis (3 out of 4 DAPs), oxidative pentose  
318 phosphate pathway (6 DAPs) and TCA cycle (13 out of 14 DAPs). To better understand the  
319 consequences of this HT repressive effect, and to expand beyond the previously reported metabolite  
320 changes (Lecourieux et al., 2017), we measured an additional 31 metabolites that are involved in sugar  
321 or organic acid metabolism, or respiration. To put the measured metabolites into a metabolic context, a  
322 schematic representation of plant central carbon metabolism was created to display the metabolite  
323 profiles under HT compared to control conditions, for each developmental stage and each stress  
324 duration (**Figure 3, Supplementary Table S10**). A significant effect of the developmental stage (two-  
325 way ANOVA,  $p < 0.05$ ) was observed for most quantified metabolites (23 out of 31), in agreement  
326 with the previously reported metabolite profiles in developing berries from Cabernet Sauvignon fruit-  
327 bearing cuttings (Dai et al. 2013). HT significantly influenced the content of 14 metabolites including  
328 shikimate, glycerate, glycerol-3-P (G-3-P), several glycolytic intermediates (fructose 6-phosphate  
329 (Fru6P), 3-phosphoglycerate (3-PGA) and pyruvate), fumarate, and several sugar phosphates (glucose  
330 6-P (G6P), trehalose 6-P (T6P), sucrose 6'-P (S6P) and mannose 6-P (M6P)). The well-known  
331 accelerated decrease of malate under elevated temperature was also observed although the changes  
332 were below the level of significance in an ANOVA test. However, when considered over the 14-day  
333 time frame of the experiments, the decrease in malate was statistically significant according to a t-test  
334 (**Figure 3**). This agrees with the significantly lower malate content detected previously in HT berries  
335 at harvest (Lecourieux et al., 2017).

336 Metabolism undergoes profound reprogramming during fleshy fruit development (Beauvoit et al.  
337 2018). The contents of primary and secondary metabolites have been investigated in developing grape  
338 berries (Dai et al. 2013; Degu et al. 2014; Cuadros-Inostroza et al. 2016; Wang et al. 2017). Using  
339 Cabernet Sauvignon fruit-bearing cuttings as in the present work, Dai et al. (2013) conducted a deep  
340 analysis of metabolites from central carbon metabolism using an LC-MS/MS approach. They reported  
341 the timing of important switches in primary carbohydrate metabolism during grape berry development  
342 and discussed the relationship between these metabolite changes and transcriptional and translational  
343 modifications within the fruit. Similarly, Wang et al. (2017) reported close correlations between the

344 metabolome and the proteome at the interface of primary and secondary metabolism in developing *V.*  
345 *vinifera* Early Campbell fruits. Down-regulation of carbohydrate production and protein synthesis is a  
346 conserved response of plants to abiotic stresses (Cramer et al. 2011). This may be a way for plants to  
347 save energy, and reflects a shift from plant growth to mechanisms of protection. However, with the  
348 exception of organic acids, the effects of high temperature on berry primary metabolism in general are  
349 still poorly described (Serrano et al. 2017). It is well known that warm climatic conditions enhance the  
350 degradation of malate in grapevine fruits, leading to a decrease in total acidity (Sweetman et al. 2014).  
351 Sugar metabolism is desynchronized from organic acid metabolism in ripening berries at high  
352 temperatures (Rienth et al. 2016). The micro-environment plays an active role in controlling the  
353 dynamics of primary metabolite accumulation that affect fruit composition. For instance, heating  
354 strongly alters the berry concentration of various amino acids, including phenylalanine and  $\gamma$ -  
355 aminobutyric acid, and decreases malate content (Lecourieux et al. 2017). Integrating microclimatic  
356 parameters and metabolomic analysis, Reshef et al. (2017) reported that solar irradiance affected the  
357 overall levels and patterns of accumulation of sugars, organic acids, amino acids and  
358 phenylpropanoids, across the grape cluster. The relationship between the diurnal microclimatic  
359 changes (radiation and temperature) and the berry metabolic dynamics was recently described (Reshef  
360 et al. 2019). The present study provides additional evidence about the harmful effects of heat on berry  
361 primary metabolism through the quantification of 31 metabolite intermediates related to sugar  
362 accumulation, glycolysis, and the TCA cycle, as well as through the identification of numerous  
363 proteins related to the corresponding metabolic pathways whose abundance is affected by HS. Among  
364 the 31 metabolites quantified, 14 were significantly impacted upon HT, with reduced contents in  
365 warmed berries for all of these (**Figure 3, Supplementary Table S10**). Some intermediates were  
366 more affected when the HT was applied during the green stage (glycerate, fumarate), and others  
367 during ripening (T6P and S6P), whereas the remaining ones were negatively impacted at all three  
368 developmental stages (pyruvate, shikimate, G3P, 3-PGA, G6P, Man6P, F6P). Reduced malate  
369 contents were also observed under HS, including the green stage (**Figure 3**). These primary metabolite  
370 profiles coincide with a dramatic down-regulation of numerous enzymes related to carbohydrate and  
371 energy metabolism in heat-treated berries (48 out of 56 DAPs, **Supplementary Table S9**), and more  
372 than 70% of these DAPs were repressed during the ripening period. Using a Cabernet Sauvignon cell  
373 suspension culture subjected to HS (42°C), George et al. (2015) observed a similar decline in proteins  
374 related to sugar metabolism. Our results highlighted changes in many metabolic pathways in grape  
375 berries exposed to heat, possibly due to the inhibitory effect of elevated temperature on metabolic  
376 enzyme activities from these pathways.

377 Several enzymes from the pathways of sugar and sugar-phosphate metabolism were less abundant  
378 in heated berries, including a fructokinase (FRK, VIT\_14s0006g01410) and two hexokinases (HXK,  
379 VIT\_18s0001g14230, VIT\_11s0016g03070). The decrease in these enzymes may contribute to the  
380 lower concentration in sugar phosphates (T6P, S6P, G6P and F6P) under heat, since the hexoses

381 (glucose and fructose) resulting from cleavage of imported sucrose can be converted to hexose  
382 phosphates by FRK and HXK in the cytosol (Stein and Granot 2018). In plants, trehalose 6-phosphate  
383 (T6P) acts as a signal of sucrose availability connecting plant growth and development to its metabolic  
384 status, and it was reported that T6P levels can exhibit dynamic responses to environmental cues  
385 (Figueroa and Lunn 2016). An increase in T6P content was observed in berries from veraison on, but  
386 this increase was lower under heat (**Figure 3, Supplementary Table S10**). In some plants, T6P  
387 inhibits the SnRK1 Ser/Thr protein kinase, in turn contributing to a reduction in anthocyanin content  
388 (Baena-Gonzalez et al. 2007). It is thus tempting to speculate that the increase in T6P concentration  
389 from veraison onwards contributes to anthocyanin accumulation through repression of SnRK1, and  
390 that this regulatory process is less effective under heat. However, the roles of T6P as a signalling  
391 molecule and the SnRK1/T6P pathway during the berry ripening process and in HT response remain  
392 to be demonstrated. In sink organs such as developing berries, sucrose cleavage is carried out either by  
393 invertase (INV) to yield glucose and fructose, or by sucrose synthase (SUS) to yield UDPG and  
394 fructose. Our proteomic analysis revealed a strong accumulation of a SUS isoform after HT at  
395 veraison (SUS2, VIT\_05s0077g01930). However, glucose, fructose and sucrose contents were not  
396 differentially altered by heat treatment (**Figure 3, Supplementary Table S10**), whereas a delay in the  
397 increase in total soluble solids (TSS) content was observed in developing berries after heat exposure  
398 during the green stage (Lecourieux et al. 2017). Accumulation of SUS may be a sign of hypoxia  
399 resulting from increased respiration rate under high temperature conditions, thus consuming oxygen  
400 faster than it can diffuse into the bulky grape berry tissue. Such as *AtSUS1* and *AtSUS4* in  
401 Arabidopsis, *SUS* genes are described as hypoxic responsive genes and a role for sucrose synthase as  
402 part of the acclimation mechanism to anoxia in dicots was proposed (Santaniello et al., 2014).

403 The present study also revealed the lower abundance of many glycolytic enzymes (9 DAPs) in  
404 warmed berries, mostly during ripening, together with the reduced content of some glycolytic  
405 intermediates (Fru6P, 3-PGA, pyruvate). A similar decrease in abundance of glycolytic enzymes or  
406 glycolytic intermediates was also reported for some grape varieties including Cabernet Sauvignon,  
407 when berries ripen under normal growth conditions (Giribaldi et al. 2007; Dai et al. 2013; Martinez-  
408 Estes et al. 2013). Thus, the consequence of heat stress on the glycolytic pathway remains unclear  
409 since the glycolytic flux might have been maintained or even increased at high temperature although  
410 the decrease of some glycolytic-related DAPs. Disturbing the glycolytic pathway may impair the  
411 generation of energy (ATP), reducing equivalents (NADH) and various intermediates required for the  
412 production of amino acid, lipids and secondary metabolites. For example, it has been reported that cell  
413 energy, reducing power and  $\alpha$ -ketoglutarate availability are potential drivers for flavonoid  
414 biosynthesis in grape cells (Soubeyrand et al. 2018).

415 The TCA cycle is a central metabolic hub since it generates energy, reducing power and carbon  
416 skeletons. Several enzymes from the TCA cycle were repressed in heated berries but consequences on  
417 the corresponding TCA intermediates were not obvious within the time frame of the analysis. The



418 transcript levels of MDH, which catalyzes a reversible reaction between oxaloacetate and malate, and  
419 ME which oxidizes malate to pyruvate, were reported to increase during grape berry ripening. This  
420 higher expression might contribute to the decline of malate concentration after veraison (Martinez-  
421 Estes et al., 2011; Wang et al., 2017). In our study, however, heat exposure reduced protein amount of  
422 both enzymes in ripening fruits (MDH: VIT\_10s0003g01000, VIT\_19s0014g01640,  
423 VIT\_00s0373g00040; ME: VIT\_15s0046g03670, VIT\_11s0016g03210), therefore questioning their  
424 precise role in hastening the malate decrease under heat. The amount of a phosphoenolpyruvate  
425 carboxylase (PEPC, VIT\_19s0014g01390) was also reduced by HT at the green stage. This may  
426 contribute to lower the amount of malate under heat before veraison (**Figure 3**), since the first step of  
427 anaplerotic malate synthesis involves the carboxylation of PEP by PEPC (Sweetman et al. 2009).  
428 However, the loss of malate during ripening has been attributed to increased degradation rather than  
429 decreased synthesis pre-veraison (Ruffner et al. 1976; Sweetman et al. 2014). Accordingly, PEPC  
430 activity decreases in pre-veraison berries with day heating, and malate content did not correlate with  
431 changes in PEPC activity (Sweetman et al. 2014). The PEPC activity is highly regulated by reversible  
432 protein phosphorylation and mono-ubiquitination, which affects its sensitivity to feedback inhibition  
433 by malate (Ruiz-Ballesta et al. 2016). Thus, changes in the phosphorylation and/or the ubiquitination  
434 status of PEPC might be more important than changes in total PEPC protein abundance. Energy-  
435 consuming processes such as gluconeogenesis may also be impacted in ripening berries exposed to  
436 HT, with three down-regulated enzymes (MDH, PEPC and isocitrate lyase). Lower MDH amounts  
437 would reduce the accumulation of oxaloacetate (OAA), required for PEP formation through  
438 phosphoenolpyruvate carboxykinase activity (PEPCK). In normal growth conditions, both PEPCK  
439 activity and transcript abundance are increased in berries after veraison (Walker et al. 2015). In  
440 ripening grapes, PEPCK may function in gluconeogenesis when malate released from the vacuole  
441 exceeds the demand from other malate-consuming processes (Walker et al. 2015).

442

#### 443 *Heat stress promotes changes in lipid metabolism and cell wall remodelling*

444 The functional category “Lipid metabolism” was particularly affected in heat-exposed berries since all  
445 the lipid-related proteins (20 DAPs) identified in our work were negatively regulated after HT  
446 (**Supplementary Table S2**). Moreover, we also observed that warmed berries contain lower amount  
447 of glycerol 3-phosphate, precursor of the membrane glycerolipids (**Figure 3**). These lipid-related  
448 DAPs are involved in fatty acid (FA) synthesis, FA elongation or FA degradation, among which were  
449 the following enzymes: acetyl-CoA carboxylase (ACC, VIT\_18s0001g04980, VIT\_11s0065g00360),  
450 glycerol-3-phosphate acyltransferase (GPAT, VIT\_15s0046g02400), beta-ketoacyl-CoA synthase  
451 (KCS, VIT\_13s0067g03890) and phosphoinositide phosphatase (SAC, VIT\_04s0044g00030). ACC  
452 proteins catalyse the carboxylation of acetyl-CoA to form malonyl-CoA during fatty acid synthesis  
453 (Sasaki and Nagano 2004). The cytosolic ACCase contributes to the synthesis of very-long chain fatty  
454 acids, with a role in membrane stability and biosynthesis of flavonoids. Its lower abundance in

455 warmed berries may negatively impact the flavonoid accumulation upon veraison. The heat-repressive  
456 effects observed at the protein level may also lead to a deep remodelling of the berry protection  
457 system. Particularly, the down-regulation of some proteins such as GPAT and KCS may disturb the  
458 berry protective barrier, namely the cuticle, since these two proteins belong to families involved in  
459 cutin and wax biosynthesis, respectively (Guo et al. 2016; Petit et al. 2016). The strongest translational  
460 repression was observed for a protein from the SAC (suppressor of actin) family ( $\log_2FC$ : -6.22) when  
461 the HS was applied during the green stage. SAC proteins are phosphatases that hydrolyze  
462 polyphosphoinositides, and roles in stress responses, cell wall formation, vacuolar trafficking and  
463 vacuolar morphology and function have been demonstrated in plants (Novakova et al. 2014). Among  
464 the known effects of high temperatures, one of the most relevant is the alteration of membrane-linked  
465 processes due to modifications in membrane fluidity and permeability (Niu and Xiang 2018). Heat  
466 stress decreases the total content of lipids and glycolipids (Escandon et al. 2018). To preserve  
467 membrane integrity under elevated temperatures, plants can regulate the ratio of bilayer to non-bilayer-  
468 forming lipids (Welti et al. 2007). Plant thermotolerance might also be improved by reducing the  
469 membrane content of unsaturated fatty acids or by accumulating more saturated fatty acids prior to  
470 HS, thus helping to maintain optimal membrane fluidity (Higashi et al. 2015; Escandon et al. 2018).  
471 Accordingly, we found that two desaturase proteins (VIT\_05s0094g00750, VIT\_00s0499g00030)  
472 were down-regulated in warmed berries. Membrane components may also be involved in initiating  
473 key signalling pathways that lead to the appropriate plant responses to heat (Niu and Xiang 2018).  
474 Consequences of the heat-induced lipid metabolism changes for berry development and quality remain  
475 to be determined.

476 The cell wall forms a dynamic boundary for plant cells and plays important roles during grape  
477 berry development, governing the potential for cell expansion during fruit growth and modulating the  
478 texture of mature berries through the depolymerisation and the solubilisation of cell wall polymers  
479 (Goulao et al. 2012b). Cell wall remodelling also constitutes an important component of plant  
480 responses to HS that maintain overall function and growth, although the cell wall factors contributing  
481 to the acquisition of plant thermotolerance remain largely unknown (Wu et al. 2018). Our previous  
482 transcriptomic analysis highlighted the strong effect of HT on genes involved in cell wall homeostasis,  
483 and especially on enzymes contributing to cell wall expansion and loosening such as xyloglucan endo-  
484 transglycosylase and expansins (Lecourieux et al. 2017). At the protein level, most of the 25 DAPs  
485 related to the category “cell wall” displayed lower amounts in heated berries, except for a few proteins  
486 among which were two pectin methylesterases (PME, VIT\_07s0005g00730, VIT\_11s0016g00290)  
487 and two polygalacturonases (PG, VIT\_02s0025g01330, VIT\_08s0007g08330) that accumulate in  
488 ripening berries exposed to heat. Both PME and PG are implicated in pectin disassembly and fruit  
489 softening process, but it is difficult to assess the impact of changes in these four proteins, since both  
490 enzymes belong to large families, with 36 PMEs and 60 PGs being annotated in the reference grape  
491 genome (Goulao et al. 2012a). However, there is increasing evidence that cell wall-modifying



492 enzymes are involved in plant responses to HS, and PMEs appear to be key players in the process (Wu  
493 et al. 2018). Similar observations were made in fruits of other species exposed to high temperature,  
494 such as strawberry (Langer et al. 2018).

495

#### 496 **Identification of proteins with a potential role in berry responses to heat**

497 Global warming represents a substantial risk for the sustainability of wine production in many regions.  
498 Plant breeding combined with molecular plant biotechnology has the potential to deliver stable yields  
499 and quality under warmer climate conditions, but this requires the identification of key traits of  
500 tolerance to heat (Torregrosa et al. 2017). Our proteomic study revealed 54 DAPs related to the  
501 functional category “Stress” and mainly belonging to the “abiotic/heat stress” cluster (**Supplementary**  
502 **Table S7**; 35 out of the 54 “stress” DAPs). Most of the proteins associated with the “heat stress”  
503 category were over-accumulated, mainly at veraison stage, and all were members of the heat shock  
504 protein (HSP) family. Looking for potential protective mechanisms activated in berries exposed to HT,  
505 we paid particular attention to up-regulated proteins previously described in the HS responses and  
506 thermotolerance in other plant species (**Table 2**; **Supplementary Tables S7 and S12**). We also listed  
507 some proteins that increased in abundance under HT and thus have a putative role in stress tolerance.  
508 These proteins (**Table 2**) are potential markers for developing or selecting grape varieties that are  
509 better adapted to warmer climate or the extreme heat waves that are expected due to climate change.

510 Cabernet Sauvignon berry can improve its HS response abilities by elevating the levels of  
511 particular proteins associated with the maintenance of protein homeostasis (synthesis, folding and  
512 degradation). HSP/chaperones are considered as powerful buffers against environmental stress  
513 including HS. Their chaperone functions include not only protein folding, but also signalling and  
514 protein targeting and degradation (Bokszczanin and Fragkostefanakis 2013). Data collected over the  
515 last decades suggest that members of the HSP/chaperone network are valuable targets for engineering  
516 multiple stress resistance in crops (Jacob et al. 2017). Among the 30 up-regulated HSPs in heat-  
517 exposed berries, four of these were increased irrespective of the fruit developmental stage (HSP70,  
518 HSP90, HSP101, HSP17.6II), and all of these were described as required for thermotolerance in  
519 various plant species (**Table 2**). Another up-regulated protein under HS corresponds to a Bcl-2-  
520 associated athanogene isoform (BAG). Plant BAGs are multifunctional proteins that regulate cell  
521 development and contribute to cytoprotection from pathogen attack and abiotic stress (Doukhanina et  
522 al. 2006). In particular, AtBAG7 was described to be a key player of the heat-induced unfolded protein  
523 response (UPR) pathway that alleviates the detrimental effects of accumulation of misfolded proteins  
524 (Li et al. 2017). VvROF1 abundance was also increased in HS conditions. In Arabidopsis, AtROF1  
525 encodes a peptidyl prolyl isomerase catalysing the *cis/trans* isomerization of peptide bonds N-terminal  
526 to proline residues within polypeptide chains. AtROF1 is transcriptionally induced by HT and is  
527 involved in acquired thermotolerance through the interaction with HSP90.1 and HSFA2 (Meiri and

528 Breiman 2009). We also observed that two thaumatin-like proteins (VvTLPs) from the osmotin sub-  
529 family strongly accumulated in heat stressed berries. The grape genome contains 33 VvTLPs (Yan et  
530 al. 2017), and this family is known to be involved in biotic and abiotic stress responses including  
531 grapevine (Liu et al. 2010). Osmotins have been shown to have multiple functions in enhancing stress  
532 tolerance in many plant species, although the mechanisms by which osmotins mediate plant response  
533 to abiotic stress are not well established (de Jesus-Pires et al. 2019). Finally, a strong increase in the  
534 translation initiation factor VveIF5 was detected in heated berries (**Table 2**). The role of eIF5 in  
535 thermotolerance was described in Arabidopsis and pea (Suragani et al., 2011; Zhang et al., 2017).

536 How plants sense and transduce the HT signal is still an important topic to be addressed. It was  
537 reported that phytochromes function as plant thermosensors (Jung et al. 2016). Transduction of the HT  
538 signal involves various players (Li et al. 2018), including members of the plasma membrane cyclic  
539 nucleotide gated calcium channel family (CNGC). In Arabidopsis, CNGC2 and CNGC6 were  
540 described as heat sensors, contributing to the Ca<sup>2+</sup> signalling induced by HT and leading to the onset  
541 of plant acquired thermotolerance (Finka et al. 2012). Whereas *CNGC6* gene is ubiquitously expressed  
542 in Arabidopsis (Gao et al. 2012), our results revealed that the protein abundance of the grape ortholog  
543 VvCNGC2 increased after berry exposure to heat, underlining the need for *de novo* synthesis of this  
544 protein to achieve its role in thermotolerance.

545 Integration of elevated temperature, signal transduction and the appropriate stress response is  
546 mediated, at least partially, by plant hormones (Ku et al. 2018). Several proteins related to hormone  
547 homeostasis were seen to be more abundant in heat-exposed berries (**Table 2**). Four abscisic acid  
548 (ABA)-related DAPs were up-regulated in the present study. The first DAP is a 9-cis-epoxycarotenoid  
549 dioxygenase (NCED). This enzyme catalyses the first committed step in ABA biosynthesis (Tan et al.  
550 1997) and NCED overexpression improves drought tolerance in grape (He et al. 2018). At the  
551 transcript level, a mixed picture has emerged for the *NCED* isogenes in HS grape berries (Carbonell-  
552 Bejerano et al. 2013; Rienth et al. 2014a; Lecourieux et al. 2017). The second ABA-related DAP was  
553 GER5/GRE5, a protein closely related to GEM (GLABRA2 Expression Modulator) and belonging to  
554 a subfamily of the GRAM domain-containing proteins (Mauri et al. 2016). In heat-stressed berries,  
555 GER5 was up-regulated at both mRNA and protein levels (**Supplementary Table S11**). GER5 and  
556 closely related GRAM domain genes (GER1, GEM) were proposed to be required in the reproductive  
557 development of Arabidopsis whereas a putative role in abiotic stress responses remains unclear  
558 (Bhatta et al. 2018). The third ABA-related DAP is an ABA-, stress-, and ripening- (ASR) induced  
559 protein known to play prominent roles in the protection of plants against abiotic stress (Gonzalez and  
560 Iusem 2014). For instance, the grape ASR VvMSA was reported to be involved in responses to water  
561 deficit (Medici et al. 2014). The last ABA-related DAP is RACK1 (receptor for activated C kinase 1).  
562 In rice, this protein positively regulates seed germination by controlling endogenous levels of ABA  
563 and H<sub>2</sub>O<sub>2</sub> (Zhang et al. 2014). More broadly, plant RACK1 proteins regulate various signalling  
564 pathways ranging from developmental processes such as seed germination, flowering and leaf

565 initiation, to immune and stress responses against pathogens and environmental stimuli (Islas-Flores et  
566 al. 2015). Another phytohormone-related DAP was a brassinosteroid-related protein phosphatase  
567 which accumulated in heated berries. In *Arabidopsis*, this protein is one of a four-member gene family  
568 (BSL, for BRI1-SUPPRESSOR1 Like) shown to participate in brassinosteroid signalling (Maselli et  
569 al. 2014), and potentially involved in stress tolerance (Gruszka 2018).

570 Heat stress is accompanied by oxidative stress, which may trigger adverse effects through oxidative  
571 damage of cell membranes and important macromolecules (Bokszczanin and Fragkostefanakis 2013).  
572 Therefore, redox regulation and signalling have been recognised as crucial mechanisms for acute  
573 responses to HT (Katano et al. 2018). Our proteomic analysis of HT berries highlighted several up-  
574 regulated DAPs with antioxidant properties, including two glutathione S-transferases (GSTs), a  
575 glutathione reductase (GR, VIT\_07s0141g0039), one catalase, a protein-disulfide isomerase (PDI), an  
576 aldo-keto reductase (AKR), two mannitol dehydrogenases (MTD) and an *S*-nitrosoglutathione  
577 reductase (GSNOR). Glutathione S-transferases belong to a well-characterized detoxification enzyme  
578 family, catalyzing the conjugation of the reduced glutathione tripeptide (GSH) to electrophilic  
579 substrates (Kumar and Trivedi 2018). GSTs quench reactive molecules through the addition of GSH  
580 and protect the cell from oxidative damage. Accordingly, plant GSTs play an important role in abiotic  
581 and biotic stress responses (Nianiou-Obeidat et al. 2017). The two up-regulated GSTs were observed  
582 at veraison but an opposite pattern was seen at ripening with five GST isoforms being strongly  
583 repressed at the protein level. This GST down-regulation observed under HT may negatively impact  
584 the accumulation efficiency of flavonoids into the vacuolar compartment of ripening berries. Indeed,  
585 GSTs function as non-enzymatic carriers in intracellular transport, allowing the accumulation of  
586 flavonoid-GSH conjugates into vacuoles (Petrucci et al. 2013). The GST-mediated transport of  
587 anthocyanins and proanthocyanidins has been established in grape as well (Perez-Diaz et al. 2016).  
588 Among the four other heat-affected proteins, GR catalyses the reduction of glutathione disulfide  
589 (GSSG) to GSH, and its role in heat tolerance was described in different plant species (Jegadeesan et  
590 al. 2018). The ROS detoxifying enzyme catalase is considered as an acclimation protein required for  
591 basal thermotolerance (Vanderauwera et al. 2011), whereas *PDI* encodes an oxidoreductase enzyme  
592 belonging to the thioredoxin superfamily. PDI can be considered as a folding enzyme since it catalyses  
593 the formation of correct disulfide bridges between the cysteine residues in proteins to maintain ER-  
594 homeostasis (Park and Seo 2015). In potato, StPDI1 was found to interact with a sucrose transporter  
595 (StSUT1) (Krugel et al. 2012), and a role in abiotic stress tolerance and H<sub>2</sub>O<sub>2</sub> detoxification was  
596 suggested (Eggert et al. 2016). Recently, the heterologous over-expression of a *Methanothermobacter*  
597 *thermautotrophicus* PDI with a chaperone function and disulfide isomerase activity could confer  
598 thermotolerance to transgenic rice (Wang et al. 2018). The aldo-keto reductase (AKR) superfamily  
599 contains proteins able to reduce a broad spectrum of substrates, ranging from simple sugars to  
600 potentially toxic aldehydes. Plant AKRs are involved in diverse metabolic reactions including reactive  
601 aldehyde detoxification, biosynthesis of osmolytes, secondary metabolism and membrane transport.

602 These proteins may also confer multiple stress tolerance (Sengupta et al. 2015), including heat stress  
603 tolerance as reported in rice (Turoczy et al. 2011). Two mannitol dehydrogenases (MTD) increased in  
604 abundance in grape berries exposed to HT. The role of mannitol and by extension, its catabolic  
605 enzyme MTD, is well established in resistance to both biotic and abiotic stresses. Several studies  
606 suggest that mannitol protects plants from various abiotic stresses by acting as an osmoprotectant and  
607 by quenching the damaging ROS (Patel and Williamson 2016). In grapevine, VvMTD1 was described  
608 as involved in water-deficit stress tolerance (Conde et al. 2015). Finally, GSNOR has an important  
609 function in the homeostasis of NO (nitric oxide), which is considered as a broad-spectrum anti-stress  
610 molecule when acting at low concentration in plants. Whereas NO was described to accumulate in  
611 tobacco exposed to HT (Gould et al. 2003), the key role of GSNOR1-mediated de-nitrosylation in  
612 thermotolerance was revealed by the characterization of *Arabidopsis hot5* (sensitive to hot  
613 temperatures) knock-out mutants (Lee et al. 2008). Taken together, these up-regulated and redox-  
614 related proteins may play a role in signalling as well as in preventing harmful effects of ROS and  
615 reactive nitrogen species in berries under HS conditions.

616 Finally, our proteomic work highlighted the heat-induced accumulation of two proteins related to  
617 vitamin metabolism, namely VvPDX1.2 and VvPANK (**Table 2**). The pseudoenzyme PDX1.2  
618 (Pyridoxine biosynthesis 1) is a noncatalytic homolog of the PDX1 subunit of the vitamin B<sub>6</sub>  
619 biosynthesis protein machinery. Transcriptionally regulated by HsfA1, PDX1.2 serves to stabilize the  
620 catalytic PDX1s under HT, thereby maintaining vitamin B<sub>6</sub> homeostasis in plants (Dell'Aglio et al.  
621 2017). AtPDX1.2 was reported as essential for plant growth under heat and oxidative stress conditions  
622 (Moccand et al. 2014). The second up-regulated protein corresponds to a pantothenate kinase (PANK).  
623 Pantothenate (known as vitamin B<sub>5</sub>) is the key precursor of CoA and acyl carrier protein, which are  
624 essential co-factors for many metabolic enzymes (Raman and Rathinasabapathi 2004). The PANK  
625 converts pantothenate to phosphopantothenate, using ATP as phosphate donor. PANK activity plays a  
626 critical role in regulating intracellular coenzyme A (CoA) levels in bacteria and animals but little is  
627 known about the role of this enzyme in plants (Ottenhof et al. 2004). It was suggested that an  
628 increased level of CoA, through an increased PANK activity, may be responsible for improved plant  
629 growth and stress resistance (Rubio et al. 2008).

630

## 631 **Conclusion**

632 Global warming represents a substantial risk for the sustainability of wine production in many regions.  
633 Plant breeding combined with molecular plant biotechnology have the potential to deliver stable yields  
634 and quality under warmer climate conditions, but this requires the identification of key traits of  
635 tolerance to heat. To this end, we combined omics approaches to decipher the molecular components  
636 and processes affected in developing Cabernet Sauvignon berries exposed to heat. In the present study,  
637 592 differentially abundant proteins were identified in heated berries among which some may play a

638 substantial role in heat tolerance. Our data also show that there is not a straightforward correlation  
639 between the heat-modulated berry transcriptome and proteome, indicating the major involvement of  
640 post-transcriptional regulation under heat stress. These pronounced changes contribute on the one  
641 hand to generate fruit damaging effects, including disruption of carbohydrate and secondary  
642 metabolism, and on the other hand to mobilize key heat-signalling players as well as protective  
643 molecules such as antioxidants and chaperones (**Figure 5**). In summary, these findings help elucidate  
644 heat-triggering effects on developing grape berries and identify genes and proteins that potentially  
645 control heat acclimation in grapevine. The functional characterization of some putative candidates is in  
646 progress.

647

## 648 **MATERIAL AND METHODS**

### 649 **Plant material, temperature and sampling**

650 Elevated temperature (+ 8°C) was directed on clusters of *V. vinifera* Cabernet Sauvignon fruiting  
651 cuttings. The HT was applied at three different berry developmental stages and maintained throughout  
652 the light period (7:00 am to 7:00 pm) until harvesting. To separate short- and long-term responses,  
653 berries were collected after 1 and 7 days of treatment. Details of the experimental protocol and  
654 sampling were provided in Lecourieux et al. (2017). The same pools of deseeded berries were used for  
655 both the transcriptomic analysis reported in Lecourieux et al. (2017) and the proteomic analysis  
656 reported here.

657

### 658 **Tissue preparation and total protein extraction**

659 Each biological replicate contained 25 pooled berries underwent independent protein extraction using  
660 a protocol adapted from Lücker et al. (2009) and Martinez-Estezo et al. (2011). Briefly, deseeded  
661 berries were ground to a fine powder in a mortar with liquid nitrogen and 3 g of frozen ground tissue  
662 were used to prepare a total protein extract. Pectins and pigments were removed after incubation of the  
663 frozen tissues in 25 mL of a cold (-20°C) ethyl acetate: ethanol (1 : 2 (v/v) for 30 min at -20°C with  
664 repeated vortexing. The protein extract was precipitated by centrifugation for 3 min at 21000 × g at  
665 4°C, and a second ethyl acetate: ethanol wash was performed in a similar way. The pellet was next  
666 extracted twice with cold (-20°C) 100% acetone by vortexing and centrifuging, as before. The pellet  
667 was transferred to a mortar and dried in a fume hood until complete acetone evaporation. The dried  
668 pellet was finely ground after addition of 1/3 vol of sand, and washed with 1.5 mL chilled 10% (w/v)  
669 trichloroacetic acid (TCA) in acetone. This washing step was repeated seven times. This was followed  
670 by two washes with aqueous 10% (v/v) TCA, two with chilled 80% (v/v) acetone, and drying at 4 °C.  
671 Each washing step included incubation at -20°C for 20 min followed by centrifugation for 3 min at  
672 21000 × g at 4°C. Subsequently the dried pellet was resuspended in 500 µL protein extraction buffer  
673 containing: 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl (pH 7.5), 50 mM EDTA, 1% PVPP, 1%



674 DTT and protease inhibitor cocktail (Roche, Germany), and incubated at room temperature (RT) for  
675 30 min with intermittent vortexing. Then, an equal volume of Tris-saturated phenol (pH 7.5) was  
676 added, the sample was incubated at RT for 30 min with vortexing every 5 min. After centrifugation at  
677  $15,000 \times g$  at RT for 40 min, the phenol phase in the upper layer was transferred into a new 2-mL  
678 Eppendorf tubes and the remaining aqueous lower phase was re-extracted with another 500  $\mu$ L Tris-  
679 saturated phenol (pH 7.5). The two phenol phases were combined and washed twice with an equal  
680 volume of phenol washing buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris (pH 7.0, 50 mM EDTA, 1%  
681 DTT, and protease inhibitor cocktail). Proteins contained in the organic phase were precipitated  
682 overnight at 4°C with 5 volumes of 0.1 M ammonium acetate in methanol. Precipitated proteins were  
683 washed three times in 0.1 M ammonium acetate in methanol and twice in ice-cold 80% (v/v) acetone.  
684 All these precipitating and washing steps included incubation for 30 min followed by centrifugation at  
685  $15,000 \times g$  at 4°C for 40 min. Finally, proteins were dissolved in 200  $\mu$ L freshly prepared  
686 solubilization solution (7 M urea, 70 mM SDS, 20 mM DTT) and quantified according to the Bradford  
687 method (Bradford, 1976) with bovine serum albumin as standard.

688

#### 689 **Sample preparation and protein digestion**

690 Thirty  $\mu$ g of each protein sample (three biological replicates for each condition; 12 conditions in total:  
691 three stages x two treatments x two treatment durations) were solubilized in Laemmli buffer and were  
692 separated by SDS-PAGE in a 10% (w/v) acrylamide gel. After colloidal blue staining, each lane was  
693 horizontally divided into four parts of equal length and each part was subsequently cut in 1 mm x 1  
694 mm gel pieces. Gel pieces were destained in 25 mM ammonium bicarbonate dissolved in 50% (v/v)  
695 acetonitrile (ACN), rinsed twice in ultrapure water and dehydrated in 100% ACN for 10 min. After  
696 ACN removal, gel pieces were dried at room temperature, covered with proteolysis solution  
697 (containing 10 ng/ $\mu$ l trypsin dissolved in 40 mM  $\text{NH}_4\text{HCO}_3$  and 10% (v/v) ACN), rehydrated at 4 °C  
698 for 10 min, and finally incubated overnight at 37 °C. The gel pieces were then incubated for 15 min in  
699 40 mM  $\text{NH}_4\text{HCO}_3$  and 10% (v/v) ACN at room temperature with rotary shaking. The supernatant was  
700 collected, and an  $\text{H}_2\text{O}/\text{ACN}/\text{formic acid}$  (47.5:47.5:5 by volume) extraction solution was added and  
701 gel slices incubated for 15 min. The extraction step was repeated twice. Supernatants were pooled and  
702 concentrated in a vacuum centrifuge to a final volume of 40  $\mu$ L. Digests were finally acidified by  
703 addition of 2.4  $\mu$ L of 5% (v/v) formic acid and the resulting peptide mixtures stored at -20 °C until  
704 analysis.

705

#### 706 **nLC-MS/MS analysis**

707 Peptide mixtures were analyzed on an Ultimate 3000 nanoLC system (Dionex, Amsterdam, The  
708 Netherlands) coupled to a nanospray LTQ-Orbitrap XL mass spectrometer (ThermoFinnigan, San  
709 Jose, USA). Ten microliters of peptide mixtures were loaded onto a 300- $\mu$ m-inner diameter x 5-mm  
710  $\text{C}_{18}$  PepMap<sup>TM</sup> trap column (LC Packings) at a flow rate of 30  $\mu$ L/min. The peptides were eluted from

711 the trap column onto an analytical 75-mm id x 15-cm C18 Pep-Map column (LC Packings) with a 5–  
712 40% linear gradient of solvent B in 105 min (solvent A was 0.1% formic acid in 5% ACN, and solvent  
713 B was 0.1% formic acid in 80% ACN). The separation flow rate was set at 200 nL/min. The mass  
714 spectrometer operated in positive ion mode at a 1.8-kV needle voltage and a 39-V capillary voltage.  
715 Data were acquired in a data-dependent mode alternating a Fourier transform mass spectrometry  
716 (FTMS) scan survey over the range  $m/z$  300–1700 and six ion trap MS/MS scans with CID (Collision  
717 Induced Dissociation) as activation mode. MS/MS spectra were acquired using a 3- $m/z$  unit ion  
718 isolation window and a normalized collision energy of 35%. Only +2 and +3 charge-state ions were  
719 selected for fragmentation. Dynamic exclusion duration was set to 30s.

720

### 721 **Database search and MS data processing**

722 Data were searched using the SEQUEST algorithm (supported by the Proteome Discoverer 1.4  
723 software; Thermo Fisher Scientific Inc.) against a subset of the 2012.01 version of UniProt database  
724 restricted to *Vitis vinifera* Reference Proteome Set (29,817 entries). Spectra from peptides higher than  
725 5000 Da or lower than 350 Da were rejected. The search parameters were as follows: mass accuracy of  
726 the monoisotopic peptide precursor and peptide fragments was set to 10 ppm and 0.8 Da respectively.  
727 Only *b*- and *g*-ions were considered for mass calculation. Oxidation of methionines (+16 Da) was  
728 considered as a variable modification. Two missed trypsin cleavages were allowed. Peptide validation  
729 was performed using the Percolator algorithm (Käll et al. 2007), and only “high confidence” peptides  
730 were retained corresponding to a 1% false positive rate at the peptide level.

731

### 732 **Label-Free Quantitative Data Analysis**

733 Raw LC-MS/MS data were imported into the Progenesis LC-MS 4.0 (Non Linear Dynamics)  
734 software. Data processing includes the following steps: (i) Features detection, (ii) Features alignment  
735 across the six samples for each part, (iii) Volume integration for 2-6 charge-state ions, (iv)  
736 Normalization on total protein abundance, (v) Import of sequence information, (vi) ANOVA test at the  
737 peptide level and filtering for features  $p < 0.05$ , (vii) Calculation of protein abundance (sum of the  
738 volume of corresponding peptides), (viii) ANOVA test at protein level and filtering for features  $p$   
739  $< 0.05$ . Only non-conflicting features and unique peptides were considered for calculation at the  
740 protein level. Quantitative data were considered for proteins quantified by a minimum of two peptides.  
741 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via  
742 the PRIDE (Perez-Riverol et al. 2019) partner repository with the dataset identifier PXD014693.

743 Protein abundance changes between control and heat-exposed fruits were considered to be  
744 significant when a minimum fold change of 1.5 ( $\log_2FC > 0.58$  or  $< -0.58$ ) was reached, with a  
745 significance threshold of  $p < 0.05$ . Only proteins identified in all three biological replicates were  
746 considered for quantification. Significantly affected protein categories based on the MapMan  
747 Ontology (Usadel et al., 2005) were identified using a Chi-square test. MapMan mappings for the



748 Cribi 12X grapevine genome were based on closest homologs regarding to the *Arabidopsis thaliana*  
749 genome. Protein annotations were taken from Grimplet et al. (2012).

750

### 751 **Primary metabolism quantification**

752 Deseeded berries were ground to a fine powder in liquid nitrogen and aliquots (15–20 mg) of frozen  
753 powder were extracted with chloroform/methanol as described by Lunn et al. (2006). Primary  
754 metabolites were measured by LC-MS/MS as described by Lunn et al. (2006), with modifications as  
755 described in Figueroa et al. (2016) and Dai et al. (2013).

756

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761

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765

### 766 **AUTHOR CONTRIBUTIONS**

767 DL, FL and PP designed the research; DL oversaw the research; PP and DL performed the greenhouse  
768 experiments; FL, JC, and DL carried out the protein extraction; CK performed the bioinformatics  
769 analysis; RF, DL and JEL did the metabolic analysis; SC and MB did the metabolic analysis, FL, CK,  
770 and DL analysed and interpreted the data; DL and FL drafted the manuscript; SD, JEL, LW and EG  
771 critically revised the manuscript. All authors read and approved the final manuscript.

772

### 773 **CONFLICTS OF INTEREST**

774 The authors declare no conflicts of interest.

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1176 **FIGURE LEGENDS**

1177

1178 **Figure 1. Venn diagrams displaying the numbers of DAPs in heat-treated berries, according to**  
1179 **the developmental stage (A) or the treatment duration (B).** DAPs were selected using a 1.5 fold  
1180 expression change and an adjusted  $p < 0.05$  (with FDR correction). Only proteins identified in all three  
1181 biological replicates and with a minimum of two unique peptides were considered for quantification.  
1182 The detailed information of the common DAPs upon HT for each stage or for each exposure time was  
1183 listed as in Supplementary Tables 1 and 2.

1184

1185 **Figure 2. Total number of differentially abundant proteins for each MapMan main category.**  
1186 Category description is given on the y-axis. Numbers of down- and up-regulated proteins across the 6  
1187 experimental conditions are provided on the x-axis for each category.

1188

1189 **Figure 3. Metabolite profiles of Cabernet Sauvignon developing berries exposed (red line) or not**  
1190 **(blue line) to heat stress.** Metabolites are shown in their respective metabolic pathways (sugar  
1191 metabolism, glycolysis, and the TCA cycle) and their temporal profiles during berry treatment  
1192 ( $\mu\text{mol.g}^{-1}$  FW for glucose, and fructose; and  $\text{nmol.g}^{-1}$  FW for the others) are presented alongside. For  
1193 each profile, the x-axis shows the developing stage (G: middle-green, V: véraison and R: middle-  
1194 ripening) and the stress duration (1, 7 and 14 days).

1195

1196 **Figure 4. Correlation between the expression of proteins and mRNAs in developing control and**  
1197 **HT berries.** DAPs and DEGs were selected using a 1.5 and 2 fold expression change, respectively,  
1198 and an adjusted  $p < 0.05$  (with FDR correction). Venn diagrams display the number of DAPs and  
1199 DEGs (red: up-regulated and blue: down-regulated) according to the developing stage (A: middle-  
1200 green, B; véraison and C: middle-ripening) and the stress duration (1 and 7 days).

1201

1202 **Figure 5. Schematic overview of heat stress effects on developing Cabernet Sauvignon berries.**  
1203 This illustration summarizes the main findings deduced from transcriptomic, proteomic and  
1204 metabolomics analyses (this work and (Lecourieux et al. 2017)). Up and down arrows reflect the  
1205 accumulation levels of the corresponding transcripts, proteins or metabolites in heated berries.

1206

1207 **SUPPORTING INFORMATION**

1208 **Figure S1:** MapMan GO categories impacted at the transcriptomic and proteomic levels in heated  
1209 grape berries

1210

1211 **Table S1:** All identified proteins with calculated differentials and corresponding statistics

1212

1213 **Table S2:** List and information about the differentially abundant proteins between control and heat  
1214 stress conditions

1215

1216 **Table S3:** Differentially abundant proteins between control and heat stress condition at green stage

1217

1218 **Table S4:** Differentially abundant proteins between control and heat stress condition at veraison stage

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1220 **Table S5:** Differentially abundant proteins between control and heat stress condition at ripening stage

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1222 **Table S6:** List of "Protein metabolism" related DAPs

1223

1224 **Table S7:** List of "Stress" related DAPs

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1226 **Table S8:** List of "Secondary metabolism" related DAPs

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1228 **Table S9:** List of "carbohydrate and energy metabolism" related DAPs

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1230 **Table S10:** Effects of the HT and developmental stages on individual metabolites,

1231

1232 **Table S11:** List of overlapping DEGs and DAPs in Cabernet Sauvignon berries exposed or not to HT

1233

1234 **Table S12:** DAPs with potential role in thermotolerance.

1235

1236 **Table 1: Amount of identified proteins and differentially abundant proteins in control**  
 1237 **and heat berries according to the developmental stage and the stress duration**

Samples	Total protein identified	Total protein quantified ( $\geq 2$ peptides)	Total DAPs	Number of DAPs up-regulated	Number of DAPs down-regulated	Number of DAPs up- or down-regulated
All 6 conditions	3972	2279	592			
			→ DAPs in 1 condition (501)	139	362	--
			→ DAPs in > 1 condition (91)	16	61	14
G1D		1633	39	11	28	
G7D		1263	108	14	94	
V1D		1580	148	70	78	
V7D		1054	83	49	34	
R1D		1244	238	38	200	
R7D		857	84	13	71	

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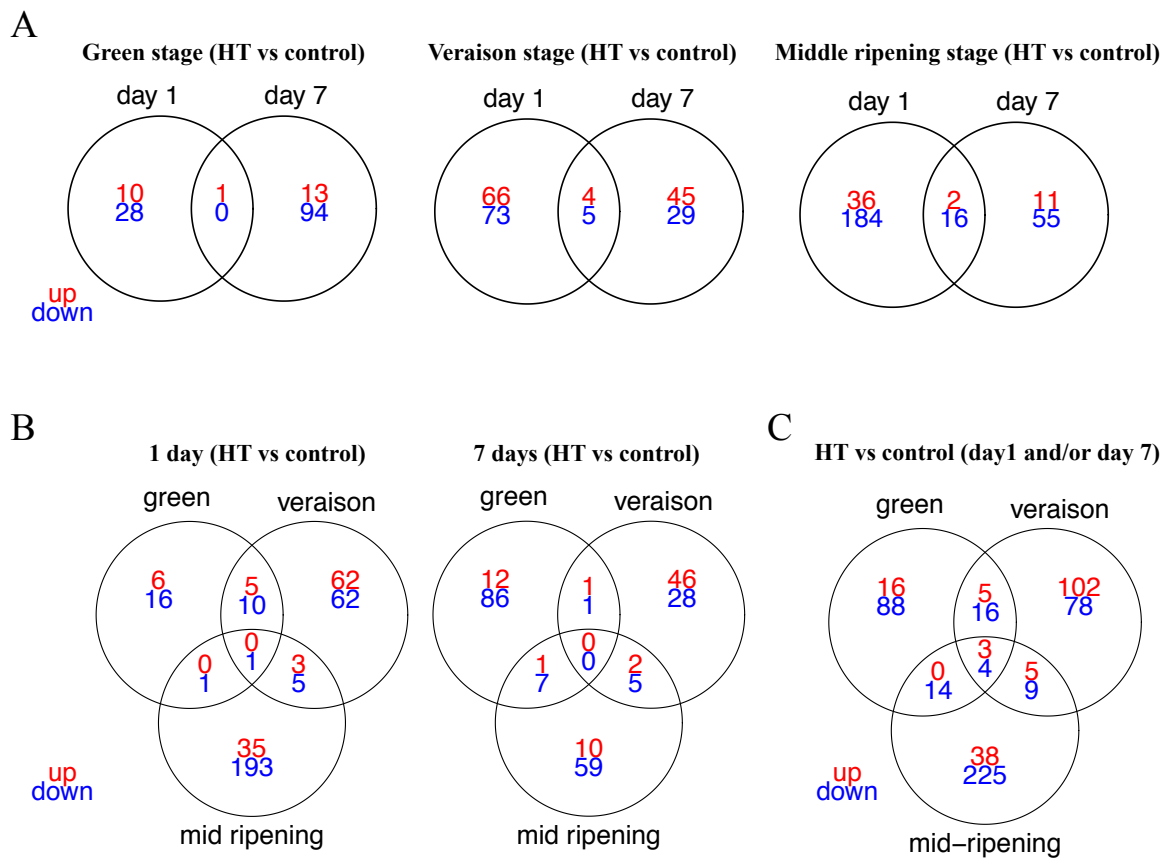
**Table 2. Deregulated proteins with potential role in heat-induced berry responses**

Functional category	Grape accession number	Putative Name	Function	Related proteins - role described in the literature	References			
<b>Stress</b>	VIT_16s0050g01150	<b>VvHSP90</b>	Chaperone	AtHSP90	Role in thermotolerance	Hong & Vierling, 2001; Meiri and Breiman, 2009; Jacob et al., 2017		
	VIT_17s0000g07190	<b>VvHSP101</b>		AtHSP101				
	VIT_18s0041g01230	<b>VvHSP70</b>		AtHSP70				
	VIT_04s0008g01570	<b>VvHSP17.6II</b>		AtHSP17.6II				
<b>Protein synthesis</b>	VIT_02s0025g04330	<b>VvTLP</b>	Chaperone	Thaumatococcus-like proteins	Enhance abiotic stress tolerance in many plant species	Le et al., 2018; de Jesus-Pires et al., 2019		
	VIT_02s0025g04290	<b>VvBAG</b>		AtBAG7 (BCL-2-ASSOCIATED ATHANOGENE 7)			Role in the unfolded protein response (UPR) to mitigate heat stress	Williams et al., 2010; Li et al., 2017
	VIT_00s0769g00010	<b>VvROF1</b>		Peptidyl propyl isomerase			AtROF1 (AtFKBP62)	Role in thermotolerance
<b>Transport</b>	VIT_14s0006g01990	<b>VveIF5</b>	Translation initiation factor	AtelF5 PseIF5	Role in thermotolerance	Suragami et al., 2011; Zhang et al., 2017		
	VIT_14s0108g01420	<b>VvCNGC2</b>	Cyclic nucleotide gated calcium channel	AtCNGC2 AtCNGC6 PpCNGCb	Primary heat sensor Role in thermotolerance	Saidi et al., 2009; Finka et al., 2012; Gao et al., 2012		
<b>Hormone homeostasis</b>	VIT_13s0064g00810	<b>VvNCED</b>	9-cis-epoxycarotenoid dioxygenase		ABA biosynthesis Strigolactone biosynthesis Role in drought tolerance in grape	He et al., 2018; Jia et al., 2018		
	VIT_01s0010g01840	<b>VvGER5</b>		GEM (Glabra2 Expression Modulator)	ABA signalling pathway	Baron et al., 2014; Bhatta et al., 2018		
	VIT_18s0072g00380	<b>VvASR</b>		VvMSA	Responses to water deficit	Medici et al., 2014		
	VIT_17s0000g02750	<b>VvRACK1</b>	Receptor for activated kinase 1		Role in various signalling pathways including drought and salt stress	Islas-Flores et al., 2015		
	VIT_06s0009g00260	<b>VvBSL</b>	Protein phosphatase	BRI1-SUPPRESSOR1	Role in brassinosteroid signalling Role in stress tolerance	Maselli et al., 2014; Gruszka, 2018		
<b>Redox regulation</b>	VIT_05s0049g01090	<b>VvGST</b>	Glutathione S-transferase		Role in abiotic and biotic responses	Nianiou-Obeidat et al., 2017		
	VIT_19s0015g02610	<b>VvGR</b>		Glutathione reductase			Role in heat tolerance	Khanna-Chopra & Chauhan, 2015; Jegadeesan et al., 2018
	VIT_07s0141g00039							
	VIT_18s0122g01320	<b>VvCAT</b>	Catalase		Role in basal thermotolerance	Vanderauwera et al., 2011		
	VIT_00s0615g00030	<b>VvMTD</b>	Mannitol dehydrogenase		Resistance to abiotic and biotic stress	Patel & Williamson, 2016		
	VIT_00s0371g00050			VvMTD1	Role in water-deficit tolerance	Conde et al., 2015		
	VIT_06s0004g02890	<b>VvPDI</b>	Protein-disulfide isomerase		Role in abiotic stress tolerance and H <sub>2</sub> O <sub>2</sub> detoxification	Eggert et al., 2016		
	VIT_07s0005g04600	<b>VvGSNOR</b>	Chaperone S-nitrosogluthathione reductase	AtHOT5	Role in thermotolerance	Gould et al., 2003; Lee et al., 2008		
<b>Co-factor and vitamin metabolism</b>	VIT_09s0002g04490	<b>VvPDX1.2</b>	Pseudoenzyme	AtPDX1.2	Role under heat and oxidative stress conditions	Mocceand et al., 2014; Dell'Aglio et al., 2017		
	VIT_01s0127g00490	<b>VvPANK</b>	Pantothenate kinase		Induced in response to salt stress	Liu et al., 2013; Aloisi et al., 2016		

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1246 **Figures:**  
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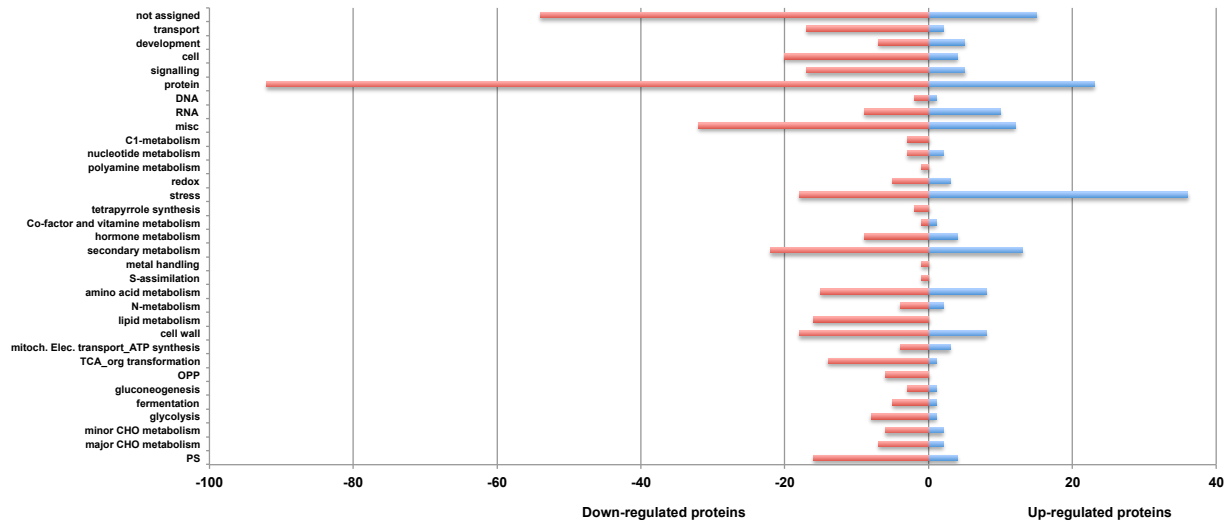


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1250 **Figure 1. Venn diagrams displaying the numbers of DAPs in heat-treated berries, according to**  
1251 **the developmental stage (A) or the treatment duration (B).** DAPs were selected using a 1.5 fold  
1252 expression change and an adjusted  $p < 0.05$  (with FDR correction). Only proteins identified in all three  
1253 biological replicates and with a minimum of two unique peptides were considered for quantification.  
1254 The detailed information of the common DAPs upon HT for each stage or for each exposure time was  
1255 listed as in Supplementary Tables 1 and 2.

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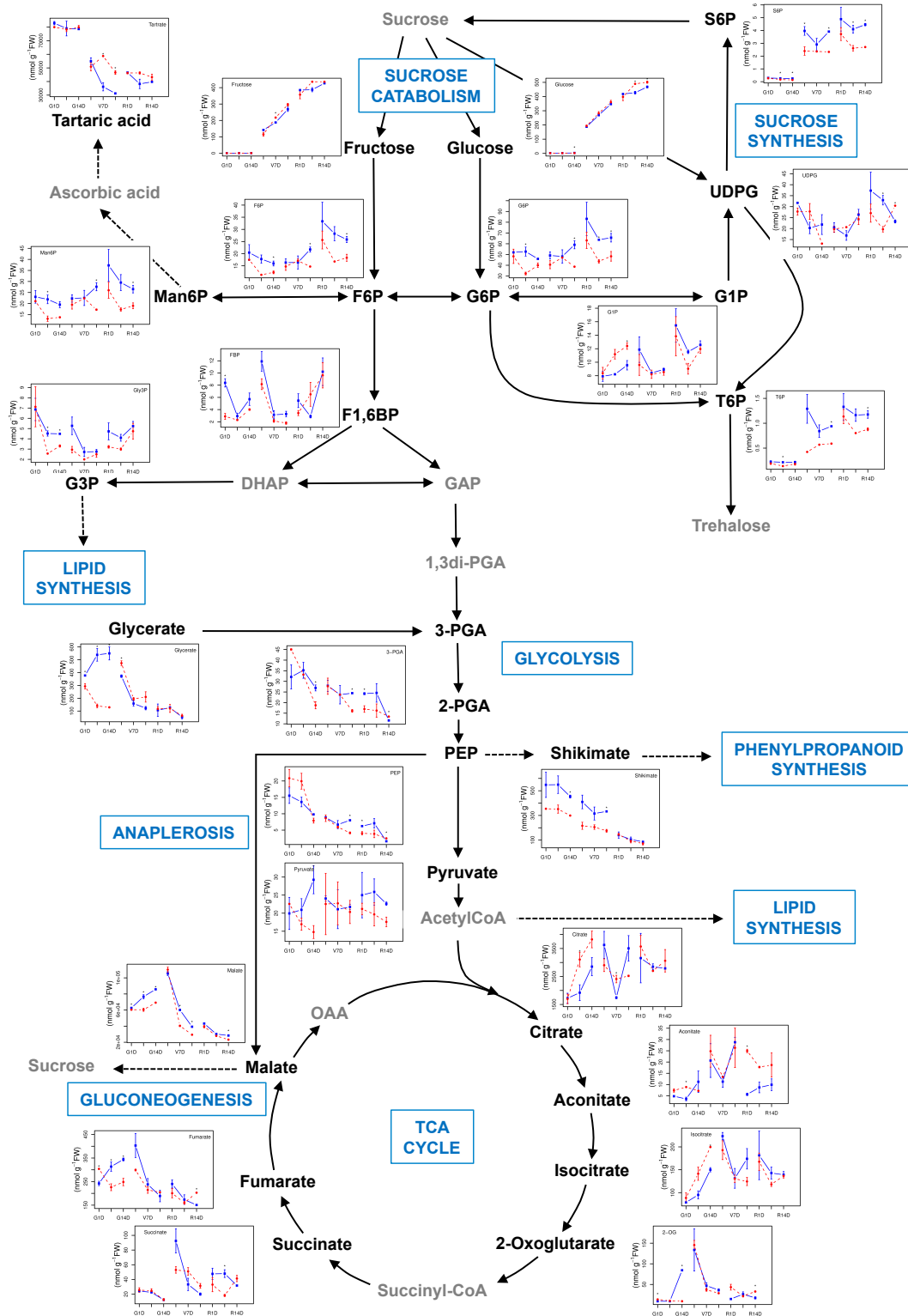
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1259 **Figure 2. Total number of differentially abundant proteins for each MapMan main category.**

1260 Category description is given on the y-axis. Numbers of down- and up-regulated proteins across the 6

1261 experimental conditions are provided on the x-axis for each category.

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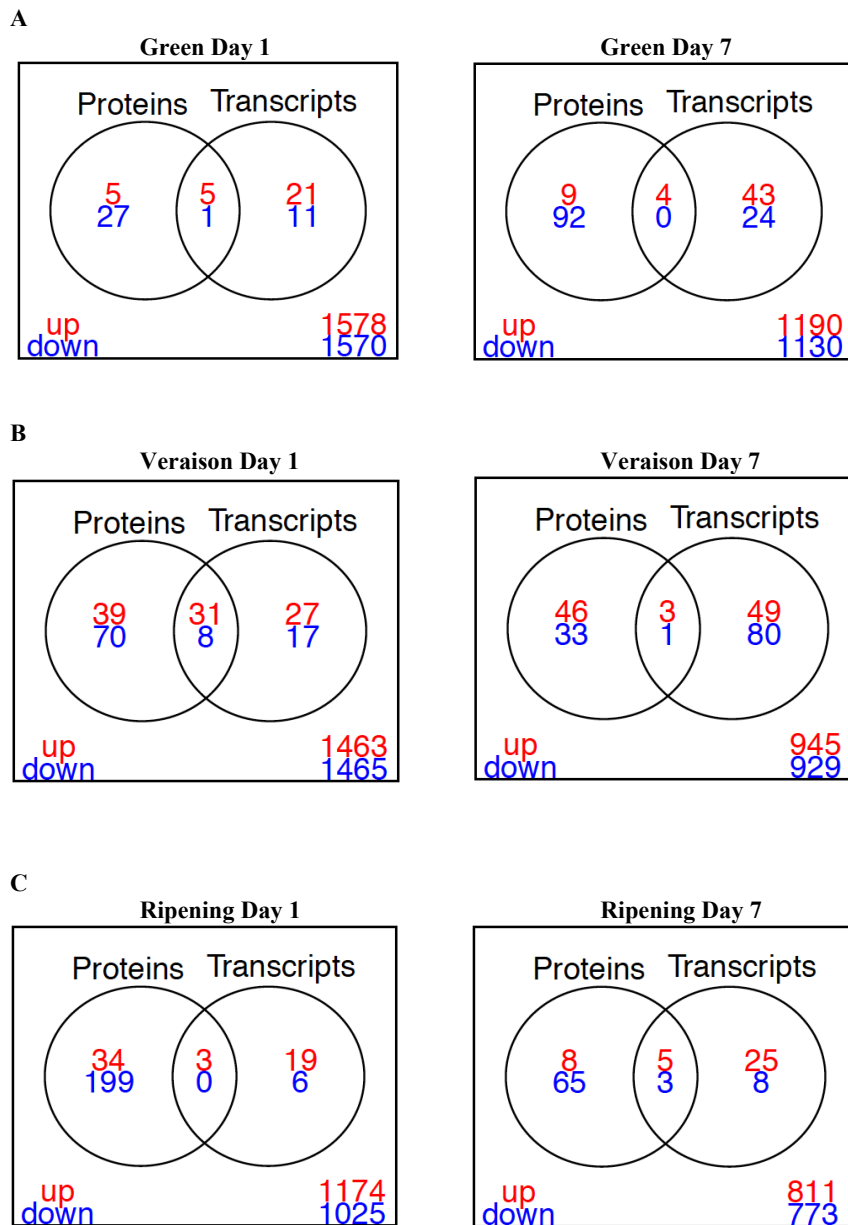
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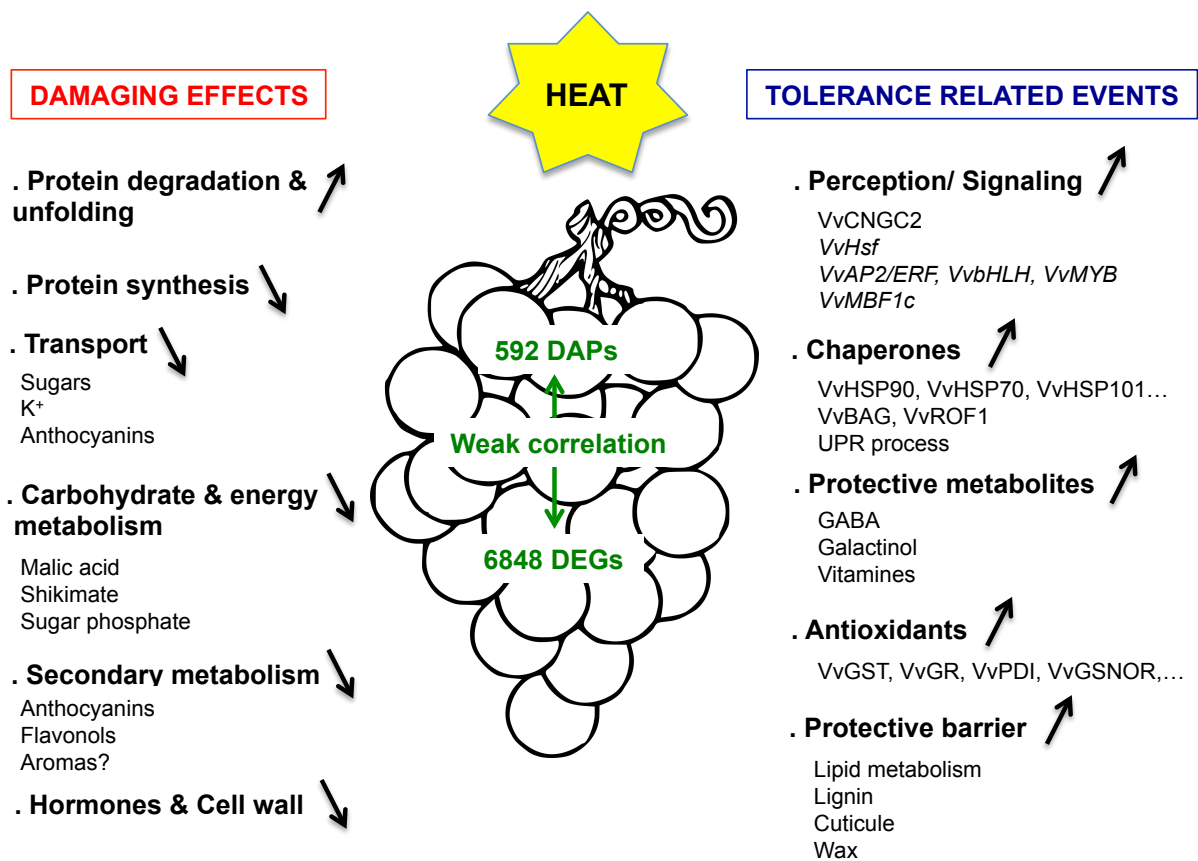
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**Figure 3. Metabolite profiles of Cabernet Sauvignon developing berries exposed (red line) or not (blue line) to heat stress.** Metabolites are shown in their respective metabolic pathways (sugar metabolism, glycolysis, and the TCA cycle) and their temporal profiles during berry treatment ( $\mu\text{mol.g}^{-1}$  FW for glucose, and fructose; and  $\text{nmol.g}^{-1}$  FW for the others) are presented alongside. For each profile, the x-axis shows the developing stage (G: middle-green, V: véraison and R: middle-ripening) and the stress duration (1, 7 and 14 days).



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**Figure 4. Correlation between the expression of proteins and mRNAs in developing control and HT berries.** DAPs and DEGs were selected using a 1.5 and 2 fold expression change, respectively, and an adjusted  $p < 0.05$  (with FDR correction). Venn diagrams display the number of DAPs and DEGs (red: up-regulated and blue: down-regulated) according to the developing stage (A: middle-green, B; véraison and C: middle-ripening) and the stress duration (1 and 7 days).



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1280 **Figure 5. Schematic overview of heat stress effects on developing Cabernet Sauvignon berries.**

1281 This illustration summarizes the main findings deduced from transcriptomic, proteomic and  
 1282 metabolomics analyses (this work and (Lecourieux et al. 2017)). Up and down arrows reflect the  
 1283 accumulation levels of the corresponding transcripts, proteins or metabolites in heated berries.

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