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

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3   # Proteomic and metabolomic profiling underlines the stage- and time-

4   # dependent effects of high temperature on grape berry metabolism

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ABSTRACT

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

INTRODUCTION

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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; 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 21th 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).

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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**

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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

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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 ($\log_2$FC > 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 that 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**).

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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 Fragkostefanakis 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 (Yanguez 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 translatome of Arabidopsis seedling exposed to HT, Yanguez 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. Yanguez 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$_2$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).

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

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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^+-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^+-ATPases and H^+-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

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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

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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 γ-
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

11

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 Esteso et al. 2013). Thus, the consequence of heat stress on the glycolytic pathway remains unclear
409 since the glycolytic flux might have been maintained of 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 α-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 Esteso 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, PEPCK 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^{2+} 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_2O_2 (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

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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 (Petrussa 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_2O_2 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.

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

Finally, our proteomic work highlighted the heat-induced accumulation of two proteins related to vitamin metabolism, namely VvPDX1.2 and VvPANK (**Table 2**). The pseudoenzyme PDX1.2 (Pyridoxine biosynthesis 1) is a noncatalytic homolog of the PDX1 subunit of the vitamin B_6 biosynthesis protein machinery. Transcriptionally regulated by HsfA1, PDX1.2 serves to stabilize the catalytic PDX1s under HT, thereby maintaining vitamin B_6 homeostasis in plants (Dell'Aglio et al. 2017). AtPDX1.2 was reported as essential for plant growth under heat and oxidative stress conditions (Moccand et al. 2014). The second up-regulated protein corresponds to a pantothenate kinase (PANK). Pantothenate (known as vitamin B5) is the key precursor of CoA and acyl carrier protein, which are essential co-factors for many metabolic enzymes (Raman and Rathinasabapathi 2004). The PANK converts pantothenate to phosphopantothenate, using ATP as phosphate donor. PANK activity plays a critical role in regulating intracellular coenzyme A (CoA) levels in bacteria and animals but little is known about the role of this enzyme in plants (Ottenhof et al. 2004). It was suggested that an increased level of CoA, through an increased PANK activity, may be responsible for improved plant growth and stress resistance (Rubio et al. 2008).

Conclusion

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

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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 × 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 μL 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 × g at 4°C for 40 min. Finally, proteins were dissolved in 200 μL 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 μg 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/μl trypsin dissolved in 40 mM NH_4HCO_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 NH_4HCO_3 and 10% (v/v) ACN at room temperature with rotary shaking. The supernatant was

700   collected, and an H_2O/ACN/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 μL. Digests were finally acidified by

703   addition of 2.4 μL 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-μm-inner diameter x 5-mm

710   C_{18} PepMap^{TM} trap column (LC Packings) at a flow rate of 30 μL/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 ($\log_2 FC > 0.58$ 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

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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FIGURE LEGENDS

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

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

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 (μmol.g^{-1} FW for glucose, and fructose; and 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).

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).

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

SUPPORTING INFORMATION

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

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

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

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

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

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

Table S6: List of "Protein metabolism" related DAPs

Table S7: List of "Stress" related DAPs

Table S8: List of "Secondary metabolism" related DAPs

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

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

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

Table S12: DAPs with potential role in thermotolerance.

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 (≥ 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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1242 **Table 2. Deregulated proteins with potential role in heat-induced berry responses**

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Functional category	Grape accession number	Putative Name	Function	Related proteins - role described in the literature		References
Stress						
	VIT_16s0050g01150	**VvHSP90**	Chaperone	AtHSP90	Role in thermotolerance	Hong & Vierling, 2001; Meiri and Breiman, 2009; Jacob et al., 2017
	VIT_17s0000g07190	**VvHSP101**		AtHSP101		
	VIT_18s0041g01230	**VvHSP70**		AtHSP70		
	VIT_04s0008g01570	**VvHSP17.6II**		AtHSP17.6II		
	VIT_02s0025g04330	**VvTLP**	Osmotin	Thaumatin-like proteins	Enhance abiotic stress tolerance in many plant species	Le et al., 2018 de Jesus-Pires et al., 2019
	VIT_02s0025g04290					
	VIT_15s0046g01560	**VvBAG**	Chaperone	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	**VvROF1**	Peptidyl propyl isomerase	AtROF1 (AtFKBP62)	Role in thermotolerance	Meiri & Breiman, 2009
Protein synthesis						
	VIT_14s0006g01990	**VveIF5**	Translation initiation factor	AteIF5 PseIF5	Role in thermotolerance	Suragami et al., 2011; Zhang et al., 2017
Transport						
	VIT_14s0108g01420	**VvCNGC2**	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
Hormone homeostasis						
	VIT_13s0064g00810	**VvNCED**	9-cis-epoxycarotenoid dioxygenase		ABA biosynthesis Strigolactone biosynthesis Role in drought tolerance in grape	He et al., 2018 Jia et al., 2018
	VIT_01s0010g01840	**VvGER5**		GEM (Glabra2 Expression Modulator)	ABA signalling pathway	Baron et al., 2014; Bhatta et al., 2018
	VIT_18s0072g00380	**VvASR**		VvMSA	Responses to water deficit	Medici et al., 2014
	VIT_17s0000g02750	**VvRACK1**	Receptor for activated kinase 1		Role in various signalling pathways including drought and salt stress	Islas-Flores et al., 2015
	VIT_06s0009g00260	**VvBSL**	Protein phosphatase	BRI1-SUPPRESOR1	Role in brassinosteroid signalling Role in stress tolerance	Maselli et al., 2014; Gruszka, 2018
Redox regulation						
	VIT_05s0049g01090	**VvGST**	Glutathione S-transferase		Role in abiotic and biotic responses	Nianiou-Obeidat et al., 2017
	VIT_19s0015g02610					
	VIT_07s0141g0039	**VvGR**	Glutathione reductase		Role in heat tolerance	Khanna-Chopra & Chauhan, 2015; Jegadeesan et al., 2018
	VIT_18s0122g01320	**VvCAT**	Catalase		Role in basal thermotolerance	Vanderauwera et al., 2011
	VIT_00s0615g00030	**VvMTD**	Mannitol dehydrogenase	VvMTD1	Resistance to abiotic and biotic stress Role in water-deficit tolerance	Patel & Williamson, 2016 Conde et al., 2015
	VIT_00s0371g00050					
	VIT_06s0004g02890	**VvPDI**	Protein-disulfide isomerase Chaperone		Role in abiotic stress tolerance and H_2O_2 detoxification	Eggert et al., 2016
	VIT_07s0005g04600	**VvGSNOR**	S-nitrosoglutathione reductase	AtHOT5	Role in thermotolerance	Gould et al., 2003; Lee et al., 2008
Co-factor and vitamin metabolism						
	VIT_09s0002g04490	**VvPDX1.2**	Pseudoenzyme	AtPDX1.2	Role under heat and oxidative stress conditions	Moccand et al., 2014; Dell'Aglio et al., 2017
	VIT_01s0127g00490	**VvPANK**	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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A

Green Day 1

Proteins Transcripts

5 / 5 / 21
27 / 1 / 11

up 1578
down 1570

Green Day 7

Proteins Transcripts

9 / 4 / 43
92 / 0 / 24

up 1190
down 1130

B

Veraison Day 1

Proteins Transcripts

39 / 31 / 27
70 / 8 / 17

up 1463
down 1465

Veraison Day 7

Proteins Transcripts

46 / 3 / 49
33 / 1 / 80

up 945
down 929

C

Ripening Day 1

Proteins Transcripts

34 / 3 / 19
199 / 0 / 6

up 1174
down 1025

Ripening Day 7

Proteins Transcripts

8 / 5 / 25
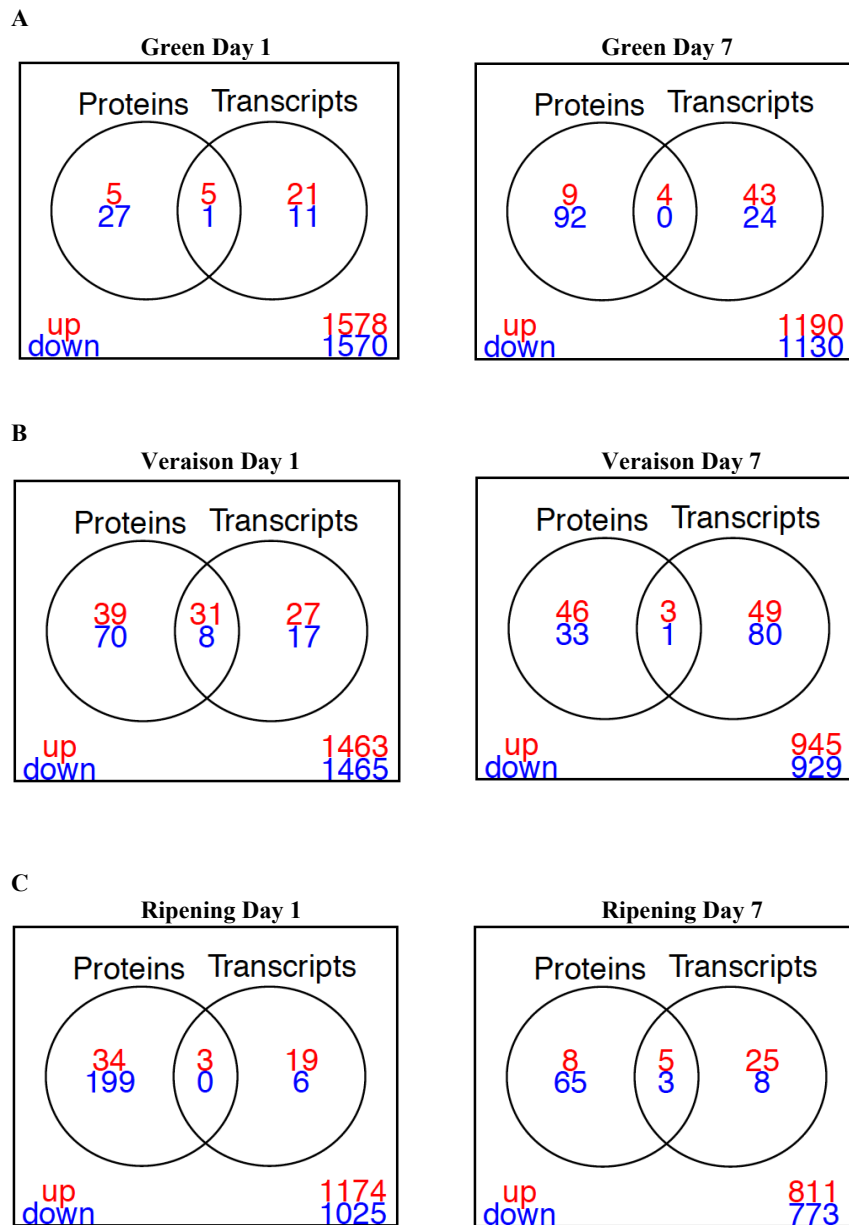65 / 3 / 8

up 811
down 773

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).
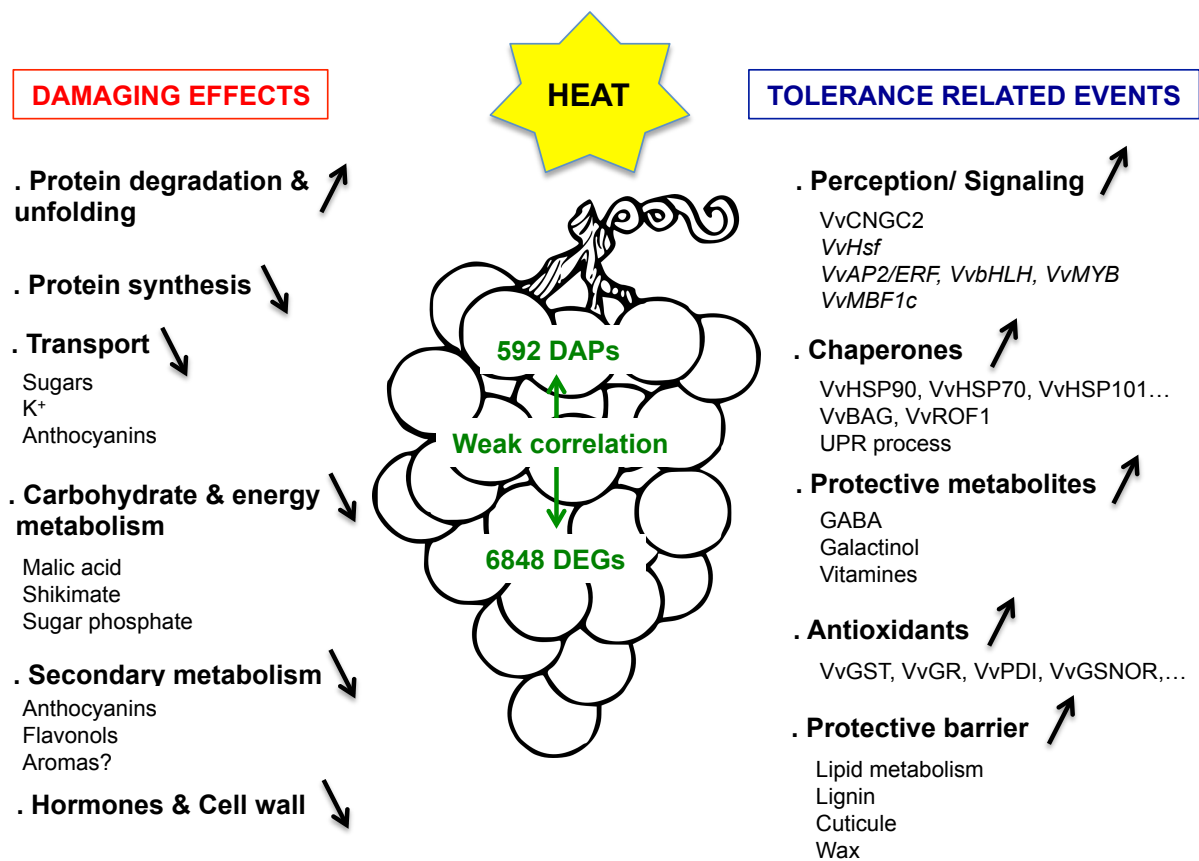
DAMAGING EFFECTS

. Protein degradation & unfolding

. Protein synthesis

. Transport
Sugars
K+
Anthocyanins

. Carbohydrate & energy metabolism
Malic acid
Shikimate
Sugar phosphate

. Secondary metabolism
Anthocyanins
Flavonols
Aromas?

. Hormones & Cell wall

HEAT

592 DAPs
Weak correlation
6848 DEGs

TOLERANCE RELATED EVENTS

. Perception/ Signaling
VvCNGC2
VvHsf
VvAP2/ERF, VvbHLH, VvMYB
VvMBF1c

. Chaperones
VvHSP90, VvHSP70, VvHSP101…
VvBAG, VvROF1
UPR process

. Protective metabolites
GABA
Galactinol
Vitamines

. Antioxidants
VvGST, VvGR, VvPDI, VvGSNOR,…

. Protective barrier
Lipid metabolism
Lignin
Cuticule
Wax

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