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Thermotolerance Responses in Ripening Berries of *Vitis vinifera* L. cv Muscat Hamburg

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Berry organoleptic properties are highly influenced by ripening environmental conditions. In this study, we used grapevine fruiting cuttings to follow berry ripening under different controlled conditions of temperature and irradiation intensity. Berries ripened at higher temperatures showed reduced anthocyanin accumulation and hastened ripening, leading to a characteristic drop in malic acid and total acidity. The GrapeGen GeneChip® combined with a newly developed GrapeGen 12Xv1 MapMan version were utilized for the functional analysis of berry transcriptomic differences after 2 week treatments from veraison onset. These analyses revealed the establishment of a thermotolerance response in berries under high temperatures marked by the induction of heat shock protein (HSP) chaperones and the repression of transmembrane transporter-encoding transcripts. The thermotolerance response was coincident with up-regulation of ERF subfamily transcription factors and increased ABA levels, suggesting their participation in the maintenance of the acclimation response. Lower expression of amino acid transporter-encoding transcripts at high temperature correlated with balanced amino acid content, suggesting a transcriptional compensation of temperature effects on protein and membrane stability to allow for completion of berry ripening. In contrast, the lower accumulation of anthocyanins and higher malate metabolization measured under high temperature might partly result from imbalance in the expression and function of their specific transmembrane transporters and expression changes in genes involved in their metabolic pathways. These results open up new views to improve our understanding of berry ripening under high temperatures.

Keywords: ABA • Berry ripening • Light • Temperature • Transcriptomics • *Vitis vinifera*.

Abbreviations: AM, anthoMATE; ANOVA, analysis of variance; AP2/ERF, apetala 2/ethylene response factor; DAT, days after treatment; DREB, dehydration responsive element binding; ERF, ethylene response factor; FDR, false discovey rate; GOLS, galactinol synthase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; HSF, heat shock factor; HSP, heat shock protein; MBF, transcriptional coactivator multiprotein bridging factor; NCED, 9-*cis*-epoxycarotenoid dioxygenase; PC, principal component; PCA, principal component analysis; PPFD, photosynthetic photon flux density; qRT–PCR, quantitative real-time reverse transcription–PCR; TSS, total soluble solids

Introduction

Fruit development is an essential process for seed dissemination and plant propagation. Ripening of the grape berry, like that of other fleshy fruits, is directed to these goals, favoring animal attraction for seed dispersal. To ensure its function, the ripening process should have evolved adaptive responses to a range of environmental conditions. Grapevine reproductive development including berry ripening is influenced by temperature (Carmona et al. 2008), which by the end of this century is expected to display a global average rise of between 1.8 and 4.0°C depending on the gas emission scenario (IPCC 2007). Although adaptive responses of grapevine berry ripening can be assumed since this developmental process is completed under different environmental conditions, high temperatures during berry growth and ripening can alter its final size and composition,

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compromising traits that are relevant for either fresh grape consumption or wine production (Ruffner et al. 1976, Buttrose et al. 1971, Jones and Davis 2000, Spayd et al. 2002, Mori et al. 2007, Cohen et al. 2008, Tarara et al. 2008, Greer and Weston 2010, Keller 2010, Azuma et al. 2012, Cohen et al. 2012). Characteristic high temperature effects on berry composition include the reduction of anthocyanin content (Kliewer and Torres 1972, Mori et al. 2007), which has consequences on the visual aspect of the fruit, relevant for table grape marketing, and on the final color achieved in red wines (Waterhouse 2002, Downey et al. 2006). Another consequence of elevated temperatures is a hastened fall of acidity and malate content (Kliewer 1968, Ruffner et al. 1976), which can become a problem for the final alcohol–acidity balance required in the production of quality wines (Yang 1955, Conde et al. 2007, Keller 2010).

A major effect of temperature at the molecular level results from its thermodynamic influence on protein structure and consequently on the activity of cellular proteins (Bischof and He 2005). High and low temperatures have direct effects on protein stability and may indirectly affect the activity of transmembrane transporters via protein conformational changes and alterations in membrane fluidity (Clarkson et al. 1988, Delrot et al. 2000). Essential fruit ripening processes, including import of assimilates and nutrients as well as accumulation of sugars and polyphenols inside grapevine mesocarp and skin cell vacuoles, respectively, require the activity of transmembrane transporters in order to be completed (Moskowitz and Hrazdina 1981, Delrot et al. 2000, Conde et al. 2007, Conn et al. 2008, Tegeder and Rentsch 2010, Gomez et al. 2011), and can be unbalanced by the effect of temperature.

In this work, berry ripening at two different temperatures and irradiation conditions integrating composition evolution and transcriptional changes has been followed. With this purpose, the fruiting cutting model system for grapevine (fruiting plants developed from rooted cuttings), which allows for generation of reproducible conditions independently from environmental fluctuations as well as overcoming experimental difficulties derived from the grapevine perennial cycle and large size, has been used, providing a much more feasible system to study environmental effects on ripening than the use of vines under field or greenhouse conditions. Reproductive physiology, berry ripening and berry metabolism in fruiting cuttings are comparable with those in vineyard vines (Geny et al. 1998, Lebon et al. 2005, Guillaumie et al. 2011, Dai et al. 2013). Indeed this system has previously been useful in the evaluation of berry ripening interactions with environmental or developmental factors (Geny and Broquedis 2002, Lund et al. 2008, Giribaldi et al. 2010, Salazar-Parra et al. 2010, Pillet et al. 2012, Dai et al. 2013, Niculcea et al. 2013). In that way, the existence of a thermotolerance response in ripening berries probably focused on protein folding and transmembrane transport homeostasis has been identified in this study. These results also provide new clues on putative causes of high temperature-induced alterations in ripening berries such as low contents of anthocyanins and organic acids.

Results

Berry ripening in fruiting cuttings under different temperature and light intensity conditions

Berry physicochemical parameters were determined throughout ripening in fruiting cuttings grown under experimental combinations of temperature and irradiation conditions. According to berry diameter measurements, little berry growth took place from veraison onset, the time when temperature and light intensity treatments on the fruiting cuttings started (**Fig. 1A**). Nonetheless, despite the berry size was smaller than usual as previously reported for fruiting cuttings (Geny et al. 1998), the berry relative growth rate was similar to that observed in 'Muscat Hamburg' berries ripened in field-grown grapevines (Lijavetzky et al. 2012). Furthermore, no significant growth differences were found among treatments until ripeness [45 days after treatment (DAT)]. The sugar accumulation rate during the first 2 weeks of treatment was slightly slower under high temperature 30/25°C (day/night) conditions, although similar total soluble solids (TSS; approximately 18°Brix) were reached in all four treatments at 45 DAT (**Fig. 1B**). On the other hand, the well-known fall in berry acidity paralleled by decreased malic acid levels and increased pH was clearly hastened under high temperature, and significant differences were obvious from 7 DAT until the end of ripening (**Fig. 1C–E**). Malic acid content at 45 DAT was also significantly affected by light under a 20/15°C temperature regime as it was slightly higher under high irradiation intensity (**Fig. 1E**). Conversely, the anthocyanin accumulation rate was more coupled to sugar gain since it was lower under high temperature from 14 DAT. In this case, differences were maintained until 45 DAT, mainly under low irradiation intensity conditions that reduced anthocyanin accumulation at ripeness (**Fig. 1F**). The total polyphenol content was not significantly affected by temperature. However, irradiation intensity produced statistically significant differences at 7 DAT under the 20/15°C regime, when the 400 μmol $m^{-2} s^{-1}$ photosynthetic photon flux density (PPFD) condition induced a higher polyphenol content than the 200 μmol $m^{-2} s^{-1}$ condition (**Fig. 1G**). It is worth mentioning that the chief ripening parameters measured in fruiting cuttings were comparable with those present at commercial ripeness in 'Muscat Hamburg' berries originating from conventional plants grown in a field in a hot climate (Fenoll et al. 2009, Lijavetzky et al. 2012). This parallelism included total acidity and pH reached at 45 DAT under high temperature, as well as TSS reached in all studied conditions. Such ripening reproducibility in fruiting cutting berries provides reliability for the use of this system for berry ripening studies.

Temperature and light intensity effects on the transcriptome of ripening berries

The GrapeGen GeneChip® to compare global pericarp gene expression among all four applied growing conditions was used. This transcriptomic analysis was carried out in 14 DAT

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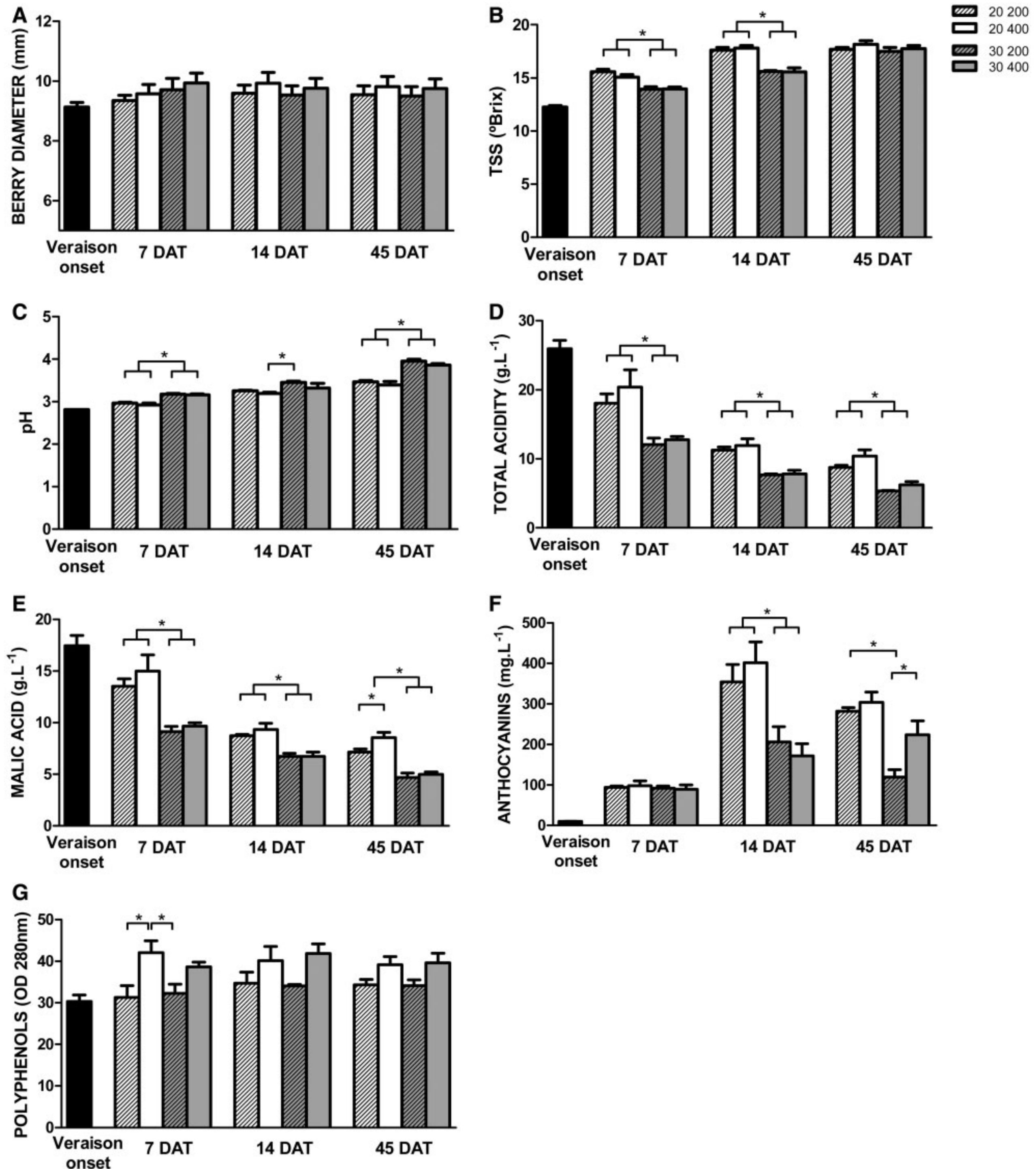

Fig. 1 Growth and ripening parameters determined throughout Muscat Hamburg berry ripening under different temperature and irradiation intensity conditions. (A) Berry diameter, (B) TSS, (C) pH, (D) total acidity expressed in $g\,l^{-1}$ of tartaric acid, (E) content of malic acid, (F) content of anthocyanins and (G) content of total polyphenols. Treatments for day and night temperatures and irradiation intensity (PPFD) conditions were: 20/15°C and 200 $\mu mol\,m^{-2}\,s^{-1}$ (20 200); 20/15°C and 400 $\mu mol\,m^{-2}\,s^{-1}$ (20 400); 30/25°C and 200 $\mu mol\,m^{-2}\,s^{-1}$ (30 200); and 30/25°C and 400 $\mu mol\,m^{-2}\,s^{-1}$ (30 400). Sampling points were: veraison onset (prior to treatments), 1 week after start of treatment (7 DAT), 2 weeks after start of treatment (14 DAT) and 45 d after start of treatment (45 DAT). Columns represent means and bars show the standard error of three independent biological repetitions. Asterisks indicate significant differences ($P < 0.05$) among treatments at the same sampling point according to Duncan's post-hoc multiple comparisons test.

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berries to identify stable responses to temperature and PPFD selected conditions instead of quick transient responses to environmental changes. Moreover, grapes of the same density from all four treatments were compared in order to ensure the study of treatment effects other than ripening rate shifts generated by the treatments, at least in terms of saccharimetric ripening (Stein et al. 1983).

The growth temperature regime was the most determinant component for transcriptome variation according to sample plotting in a principal component analysis (PCA). Principal component 1 (PC1) explained >45% of the expression variation and separated berry samples grown under different temperature conditions, indicating a consistent temperature effect (**Supplementary Fig. S1A**). In contrast, light condition effects were only detected by PC6 explaining <5% of total gene expression variation (**Supplementary Fig. S1B**). In fact, no significant differentially accumulated transcript was identified between both light conditions in a two-class *limma* comparison (**Supplementary Table S1**). The limited light intensity effects on the transcriptome were in agreement with the little ripening parameter differences observed between 200 and 400 μmol m^{-2} s^{-1} PPFD regimes (**Fig. 1**).

Temperature expression differences were specifically analyzed by comparing all 30/25°C vs. all 20/15°C berry samples (six biological replicates per temperature condition). A total of 852 non-redundant differentially accumulated transcripts were identified [<1% false discovery rate (FDR) in a two-class *limma* comparison and >1.6 fold change; **Supplementary Table S2**]. Among them, 59% were down-regulated and 41% up-regulated by the high temperature regime.

The GrapeGen GeneChip® 12Xv1 functional classification implemented in the MapMan software was used to understand the biological significance underlying the 852 differentially accumulated transcripts (see the Materials and Methods). In this way, several significantly altered functional categories were identified and most of them were high temperature up-regulated (**Table 1**, **Fig. 2**). Reasonably, the linked categories 'HSP-mediated protein folding' and 'temperature stress response' (see 'Folding' and 'Temperature' boxes in **Fig. 2B** and **C**, respectively) were significantly activated, as was the 'transcription factor' category (**Fig. 2A**). Temperature-responsive transcripts were almost the only abiotic stress-responsive transcripts up-regulated by high temperatures (**Fig. 2C**), most of them coding for heat shock protein (HSP). Among 24 differentially expressed *HSP* transcripts, 21 were significantly up-regulated under high temperature (**Table 1**; **Supplementary Table S2**). The 'temperature stress response' functional category also included a *HSF* (heat shock factor) probe set up-regulated by high temperature (*VVTU3420_at*; **Supplementary Table S2**). This probe set sequence matched (99.6% similarity) with the *VIT_04s0008g01110* gene locus in a blast alignment vs. the 12× genomic sequence. This gene locus was recently named as *VvHsfA2* (Pillet et al. 2012). Probably, the probe set *VVTU3420_at* did not pass the Grapegen GeneChip® annotation filters (Lijavetzky et al. 2012) to be assigned to the *VIT_04s0008g01110* locus because the automatically predicted structure for this gene (Jaillon et al. 2007) is not completely accurate (Pillet et al. 2012). Interestingly, heat stress response activation correlated with that of the 'ERF subfamily transcription factor' category (**Fig. 2A**), with seven *ERF* (ethylene response factor) transcripts [*VvERF104* (*GSVIVT00014242001*), *VvERF079* (*VIT_16s0013g00900*), *VvERF080* (*VIT_16s0013g00950*), *VvERF083*, (*VIT_16s0013g00970*), *VvERF108* (*VIT_16s0013g01060*), *VvERF088* (*VIT_16s0013g01110*) and *VVTU27635_x_at*] and one DREB (dehydration responsive element binding) subfamily AP2/ERF transcription factor [apetala 2/ethylene response factor; *VvERF008* (*VIT_05s0029g00140*)] up-regulated by high temperature.

Transcripts coding for macromolecule and ion membrane transporters were strongly represented among transcripts down-regulated under high temperature (**Table 1**, **Fig. 2D**). They included amino acid, carbohydrate, lipid, oligopeptide and anion transporter-encoding transcripts (**Supplementary Table S2**). Eight transcripts coding for putative amino acid transporters were found among high temperature-repressed transporters: five of them encoding putative amino acid permeases (*VIT_01s0011g04210*, *VIT_06s0004g00790*, *VIT_06s0004g00800*, *VIT_14s0083g00740* and *VIT_18s0001g08270*); two encoding putative cationic amino acid transporters (*VIT_01s0011g01140* and *VIT_10s0003g04540*); and one coding for a putative lysine- and histidine-specific transporter (*VIT_01s0011g03180*; **Supplementary Table S2**).

The main ripening-related cellular processes were not significantly altered in terms of gene expression despite the fact that they included a few differentially expressed transcripts. This was the case of the 'generation of metabolite precursors and energy' pathway, potentially involved in sugar accumulation and fall in acidity during grape ripening, which only showed differential expression in several sugar transport- and sucrose and starch metabolism-related transcripts down-regulated by high temperatures (**Fig. 3A**). Similarly, amino acid metabolism showed few genes altered by temperature (generally repressed by high temperature) that did not specifically affect any particular amino acid biosynthetic pathway (**Fig. 3B**). Regarding secondary metabolism, some phenylpropanoid metabolism-related transcripts were differentially expressed (**Fig. 3C**), including the *AM1* gene (*VIT_16s0050g00930*; **Supplementary Table S2**), encoding an anthocyanin transporter that was down-regulated in 30/25°C ripening berries (**Fig. 3C**).

In addition and concerning hormone metabolism and signaling, it is relevant to mention the high temperature up-regulation of two *NCED* transcripts (*VIT_02s0087g00930* and *VIT_10s0003g03750*), encoding 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in the biosynthesis of the abiotic stress-related phytohormone ABA (**Fig. 3D**; **Supplementary Table S2**).

Transcriptomic data validation by quantitative real-time reverse transcription–PCR (qRT–PCR)

In order to validate the microarray data, expression levels in up to 16 transcripts (**Supplementary Table S3**) were analyzed by

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Table 1 Functional categories significantly altered by temperature in 14 DAT berries

Bin	30/25°C regime up-regulated category	Up/differential transcripts	Total transcripts	*P*-value
4.2.10	Protein metabolism and modification	43/72	1601	2.23E-02
4.2.10.1	Protein folding	23/28	326	1.06E-03
4.2.10.1.1	Chaperone-mediated protein folding	21/25	263	1.06E-03
4.2.10.1.1.2	**HSP-mediated protein folding**	**21/24**	**165**	**4.09E-04**
5	Regulation overview	33/58	1396	1.52E-02
5.2	Regulation of gene expression	33/57	1270	1.52E-02
5.2.2	Regulation of transcription	33/56	1200	1.33E-02
5.2.2.1	Transcription factor	31/54	1178	2.02E-02
5.2.2.1.3	AP2 family transcription factor	8/8	60	1.73E-03
5.2.2.1.3.2	**ERF subfamily transcription factor**	**7/7**	**34**	**5.00E-03**
6.2.1.8	**Temperature stress response**	**7/7**	**47**	**2.16E-02**
BIN	30/25°C regime down-regulated category	Down/differential transcripts	Total transcripts	*P*-value
8	Transport overview	54/67	917	1.33E-02
8.14	**Macromolecule transport**	**29/35**	**459**	**2.16E-02**

GrapeGen 12Xv1 MapMan BINs showing significantly different behavior in a Wilcoxon rank sum test (Benjamini–Hocheberg adjusted *P*-value < 0.05), BIN category name, transcripts up- or down-regulated, differential transcripts, total number of transcripts within the category in the GeneChip® and significant *P*-value are shown. The most specific levels of non-redundant significant categories are highlighted in bold.

qRT–PCR in four biological replicates for each temperature condition. Four housekeeping genes were analyzed by qRT–PCR as well for among-sample normalization (**Supplementary Table S3**). Similar results were obtained when data were normalized to each of them. Data normalized to glycerol-3-phosphate dehydrogenase (GPDH) *VIT_17s0000g10430* transcript levels are shown in **Fig. 4** because this was the control gene most stably expressed among treatments based on microarray data (**Supplementary Fig. S2**).

A reliable R^2 correlation coefficient of 0.77 was obtained between temperature expression differences measured by both methods (**Fig. 4A**). In fact all transcripts analyzed showed the same expression trend in both types of analysis, with the exception of *VvNECD1* (*VIT_19s0093g00550*), recently renamed as *VvNCED3* (Young et al. 2012), which showed a flat expression profile in the GeneChip results (**Supplementary Table S1**) and treatment differences not consistent between replicates in the qRT–PCR (**Fig. 4B**). Probe set cross-hybridization with transcripts coming from other family members may have occurred since qRT–PCR primers were designed to be gene specific.

Concerning *ERF* genes up-regulated in 30/25°C ripening berries, GeneChip probe set *VVTU15419_x_at* could hybridize with transcripts coming from three different genes (according to a blat alignment of the probe set sequence to the 8× grapevine genomic sequence). These genes were *GSVIVT00014238001*, *GSVIVT00014244001* and *GSVIVP00014245001*; named *VvERF081*, *VvERF083* and *VvERF084*, respectively (Licausi et al. 2010). Gene-specific primers were used in a qRT–PCR analysis that indicated probe set differential expression to be the result of *VvERF083* transcription, whereas expression was not detected for the other two *ERF* genes (**Fig. 4B**). All three grapevine members of the anthoMATE (AM) subfamily (Gomez et al. 2009) were also analyzed by qRT–PCR using gene-specific primers (**Supplementary Table S3**), confirming the high temperature-induced down-regulation of *AM1*, more slightly for *AM3*, and showing the absence of *AM2* expression (**Fig. 4B**).

Temperature regime effects on amino acid and ABA accumulation in the pericarp throughout ripening

The generalized down-regulation of different macromolecule- and ion membrane transporter-encoding transcripts caused by high temperature (**Fig. 2D**) raised questions on its impact on the accumulation of the putatively transported metabolites. Since eight amino acid transporter-encoding transcripts were down-regulated under high temperature, pericarp amino acid levels were compared between both temperature regimes. Four biological replicates per temperature regime (both light intensity conditions in repetitions 1 and 3) were analyzed for their amino acid composition at 14 and 45 DAT. These were the most diverse temperature regime replicates, at least at the transcriptome level, according to PCA results (**Supplementary Fig. S1**). Therefore, in the case of significant differences, they could be considered highly reliable.

Levels of 19 proteinogenic amino acids and of the non-proteinogenic γ-aminobutyric acid and ornithine were compared between both temperature regimes. Significant differences were only identified for tyrosine, valine, methionine and ornithine levels. In all four amino acids, the significant difference took place only at 14 DAT and involved higher levels under the 30/25°C condition (**Table 2**). The absence of significant differences at 45 DAT may indicate that they were somehow equilibrated after 14 DAT for these four amino acids. On the other hand, levels of most amino acids did not significantly change

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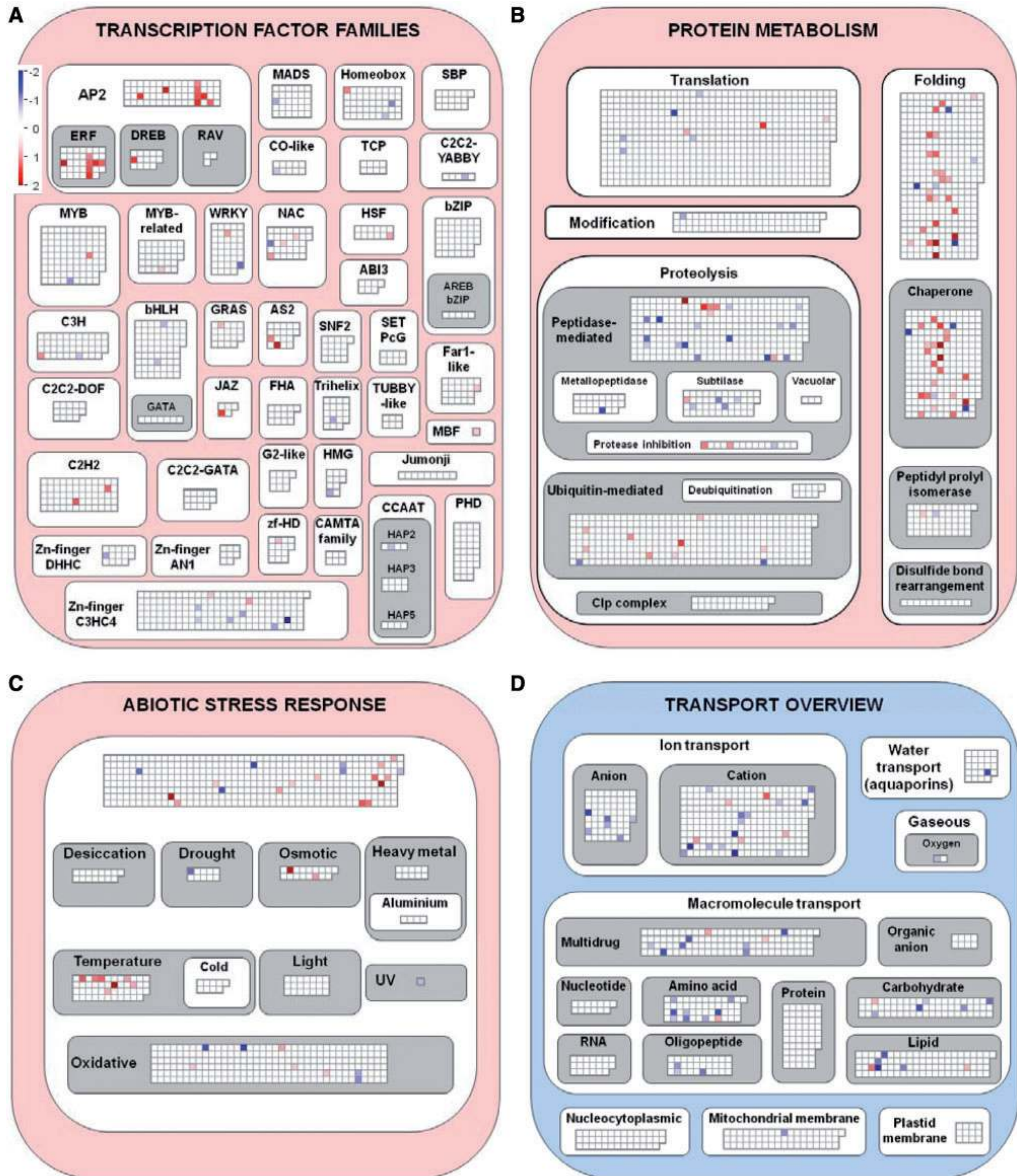

Fig. 2 MapMan display of temperature differentially expressed genes in berries 14 DAT in significantly altered pathways. The following categories are represented: (A) transcription factor families; (B) protein metabolism; (C) abiotic stress response; (D) transport overview. The expression ratio [Log_2 (30–25°C/20–15°C)] of genes assigned to each category is represented by a colored square following the color scale shown in A. Red, expression up-regulated under high temperature; blue, expression down-regulated under high temperature; white, no significant differential expression.

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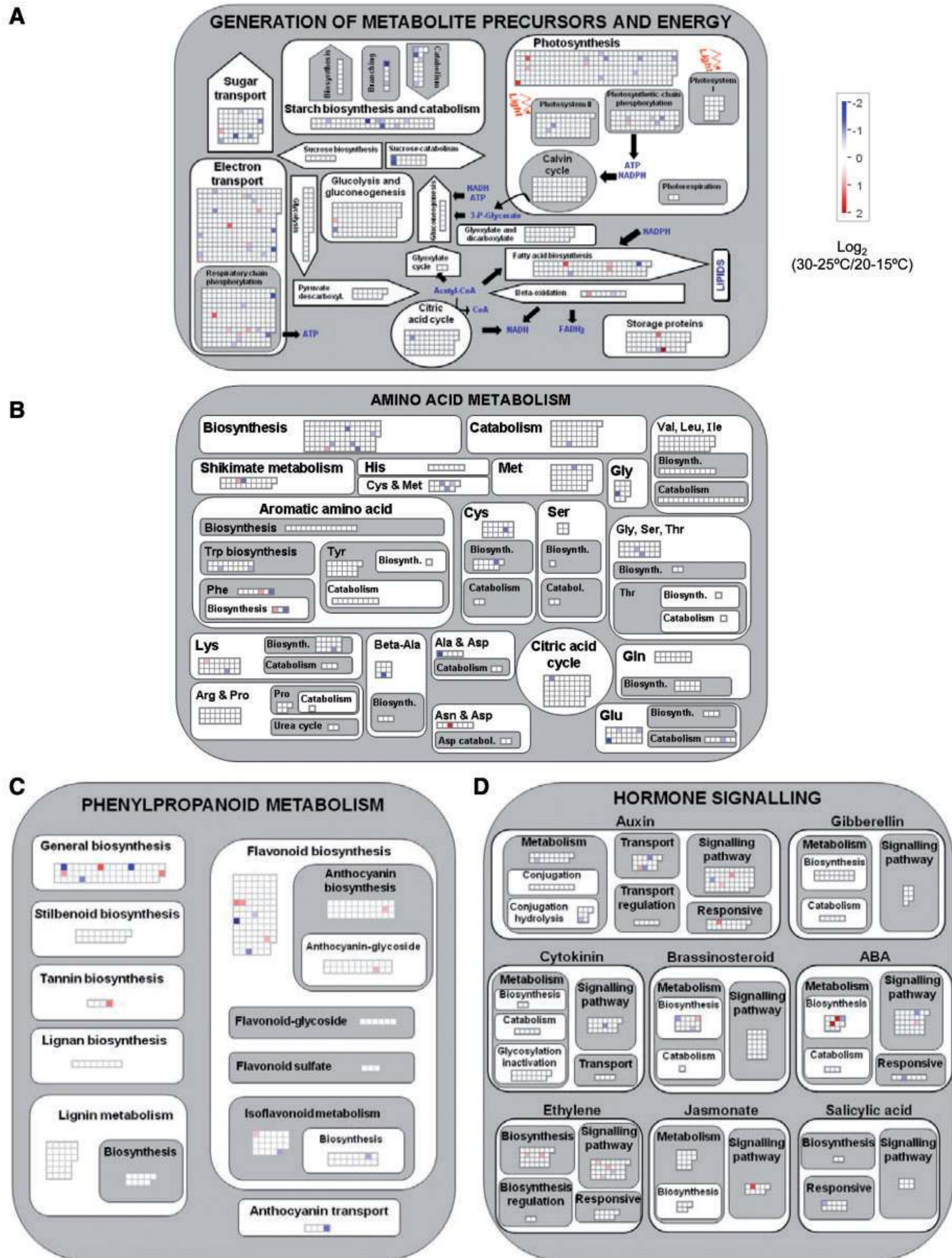

Fig. 3 MapMan display of temperature differentially expressed genes in 14 berries DAT in selected pathways. The following categories are represented: (A) generation of metabolite precursors and energy; (B) amino acid metabolism; (C) phenylpropanoid metabolism; (D) hormone signaling. The expression ratio [Log_2 (30–25°C/20–15°C)] of genes assigned to each category is represented by a colored square following the color scale shown in A. Red, expression up-regulated under high temperature; blue, expression down-regulated under high temperature; white, no significant differential expression.

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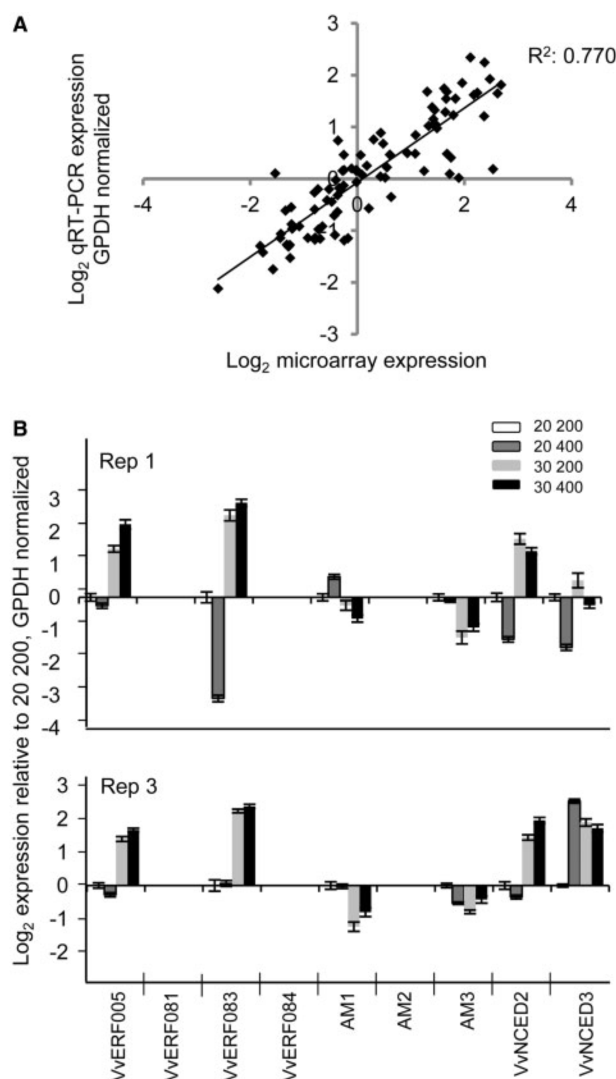

Fig. 4 Validation of microarray expression data by qRT–PCR. (A) Correlation of Log^2 expression relative to 20/15°C–200 µmol $m^{-2}s^{-1}$ is shown for replicates 1 and 3. A total of 16 genes were considered (see **Supplementary Table S3**). (B) qRT–PCR expression analyses of selected genes in berries at 14 DAT. Expression values, normalized to GPDH, are shown as Log^2 relative to 20/15°C–200 µmol $m^{-2}s^{-1}$. White, 20/15°C–200 µmol $m^{-2}s^{-1}$; dark gray, 20/15°C–400 µmol $m^{-2}s^{-1}$; light gray, 30/25°C–200 µmol $m^{-2}s^{-1}$; black, 30/25°C–400 µmol $m^{-2}s^{-1}$. Error bars represent the SD of three technical replicas.

throughout ripening. Threonine was one of the most abundant amino acids and the only one whose levels significantly decreased from 14 to 45 DAT. Conversely, phenylalanine was the only one whose levels rose with ripening, although only significantly under the low temperature regime.

ABA was quantified in the same samples as amino acids levels. Although the ABA level decreased throughout ripening, it was significantly higher under high temperature at full ripeness. A similar temperature effect was observed at 14 DAT, although the differences were not significant due to high variability under the 30/25°C regime (**Table 2**).

Table 2 Amino acid and hormone levels in 14 and 45 DAT berries at both applied temperature regimes (means ± SE; $n = 4$)

Compound	30/25°C 14 DAT	20/15°C 14 DAT	30/25°C 45 DAT	20/15°C 45 DAT
ALA	62.2 ± 10.9	93.5 ± 7.1	69.7 ± 5.2	89.2 ± 15.8
ASN	18.2 ± 1.4	22.4 ± 3.1	22.1 ± 4.4	53.4 ± 10.4
ASP	33.8 ± 5.6	41.5 ± 4.2	31.8 ± 2.9	31.9 ± 2.9
CYS	1 ± 0.3	2.2 ± 0.7	0.8 ± 0.2	1.5 ± 0.6
GLN	296.4 ± 47.8	343.3 ± 62.3	141.3 ± 26.7	183.5 ± 38.9
GLU	75.9 ± 6.4	74.2 ± 7.4	70.2 ± 3.9	68.8 ± 13.6
GLY	5.1 ± 0.2	8.8 ± 1.4	6.5 ± 0.4	6.7 ± 0.9
HIS	28.3 ± 1.8	25.7 ± 11.7	29.6 ± 1.4	52.4 ± 6
ILE	10.6 ± 0.6	15.9 ± 1.7	15.5 ± 2.1	16.2 ± 1.6
LEU	18 ± 1.6	20.6 ± 1.2	32.3 ± 3.8	30.9 ± 3.5
LYS	7.4 ± 1.7	10.9 ± 3.5	6.2 ± 0.9	8.9 ± 1.6
MET[a]	3.9 ± 0.1	8.4 ± 0.9	5.7 ± 0.6	8.6 ± 0.9
PHE[c]	17.8 ± 0.7	19.3 ± 1.2	23.4 ± 0.8	24.2 ± 1.8
PRO	218 ± 1.9	210.4 ± 52.5	174.8 ± 11.5	193.6 ± 58.9
SER	87.8 ± 6.9	59.7 ± 3.4	64.7 ± 4.1	48 ± 8.1
THR[c]	543.3 ± 28.9	441 ± 49.9	182.4 ± 24	245.5 ± 40.3
TYR[a]	40.1 ± 3.1	57.2 ± 2.2	42.2 ± 2.3	67.1 ± 9.1
VAL[a]	17.1 ± 0.6	26.8 ± 1.8	25.3 ± 2.4	27 ± 2.9
GABA	36.7 ± 5.8	45.7 ± 7.6	32.4 ± 2.1	44.9 ± 5.3
ORN[a]	16.5 ± 2.8	40.6 ± 5.3	15.5 ± 2.7	30.3 ± 6
ABA[b]	119.9 ± 60.6	38.9 ± 7.6	41.2 ± 5.5	13.46 ± 2.95

Amino acid concentration is expressed as mg l^{-1} and ABA concentration as ng g^{-1} FW.
Compound content showed significant differences ($P < 0.05$) for: [a]temperature at 14 DAT; [b]temperature at 45 DAT; and [c]sampling time under 20°C.

Discussion

The effect of different temperature and irradiation intensity regimes on the process of grape berry ripening from veraison to full ripeness using fruiting cuttings was analyzed. Ripening in 'Muscat Hamburg' fruiting cuttings was comparable with that observed in plants of this cultivar growing under field conditions (Fenoll et al. 2009, Lijavetzky et al. 2012). More importantly, fruiting cuttings berries ripened under high temperature showed a hastened fall in acidity and pH increase together with strong reduction in anthocyanin accumulation, reproducing the major effects of high temperatures on ripening observed in conventional vines (Buttrose et al. 1971) and, therefore, confirming the reliability of this experimental system. Despite the fact that heat at late ripening stages has been related to higher sugar concentration, this might be an indirect consequence of increased berry desiccation (Jones et al. 2005). Indeed, the reduction of the sugar accumulation rate under high temperature reported here (**Fig. 4B**) has also been previously described in conventional vines (Kliewer 1977, Sepúlveda and Kliewer 1986, Greer and Weston 2010). Down-regulated *GIN2* expression by high temperatures (*VIT_02s0154g00090*; **Supplementary Table S2**) could be related to the lower sugar accumulation rate since

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this vacuolar invertase-encoding gene is putatively involved in the import and accumulation of hexoses into vacuoles (Davies and Robinson 1996). All observed effects on berry composition were generally more extreme after 7 or 14 d of treatment and were later reduced during ripening.

Thermotolerance response of ripening berries to high temperature

Transcriptome analyses of the berry pericarp identified expression differences dominated by the up-regulation of HSP- and other chaperone-encoding transcripts under high temperatures (**Table 1**, **Fig. 2B**), which is probably directed to maintain protein folding homeostasis (Wang et al. 2004). The requirement of *HSP* genes for plant thermotolerance has been consistently reported (Lee and Schoffl 1996, Queitsch et al. 2000, Sung and Guy 2003, Yang et al. 2006, Larkindale and Vierling 2008, Chauhan et al. 2012), and grapevine *HSP* genes have also been shown to confer thermotolerance when expressed in *Arabidopsis thaliana* L. (Kobayashi et al. 2010). However, the widespread repression of expression of metabolism-related transcripts observed in heat-stressed plants (Larkindale and Vierling 2008, Mangelsen et al. 2011) and in grapevine leaves (Wang et al. 2012) was not observed in ripening berries 2 weeks after the onset of the high temperature treatment (**Figs. 2B**, **3**). Additionally, the prominent transcriptional activation of antioxidant systems observed in heat-shocked grapevine leaves (Wang et al. 2012) was not detected in berries ripened under the high temperature regime. In the present study, only one lactoylglutathione lyase and one glutathione *S*-transferase (GST)-encoding transcript (*VIT_10s0116g01660* and *VIT_18s0001g00690*, respectively) were up-regulated, whereas another lactoylglutathione lyase (*VIT_04s0023g03040*) and two *GST* (*VIT_07s0104g01800* and *VIT_19s0093g00350*) transcripts were down-regulated under high temperature (**Supplementary Table S2**). Hence, different isogenes seem to be activated at each temperature for a balanced redox homeostasis instead of the activation of a general oxidative stress response to elevated temperatures. This is consistent with results reported for 'Cabernet Sauvignon' where even a higher temperature (35°C) did not induced the expression of *GST* transcripts in berry skins (Mori et al. 2007). Thus, the lack of generalized stress response activation in grapevine berries (**Fig. 2C**) supports the idea that a thermotolerance transcriptional response (Saidi et al. 2011), rather than a heat stress response, becomes established in grape berries to overcome long-term maintained high temperature conditions.

Furthermore, transcriptional hints of osmoprotection activation by elevated temperature were observed. A galactinol synthase- (GOLS; *VIT_05s0020g00330*), an osmotin- (*VIT_02s0025g04340*) and a betaine-aldehyde dehydrogenase- (*VIT_14s0066g02410*) encoding transcript were up-regulated by high temperature (**Supplementary Table S2**). GOLS is a key enzyme in the synthesis of galactinol and other raffinose family oligosaccharides that accumulate as osmoprotectants in plant cells after heat stress (Panikulangara et al. 2004). In fact, galactinol levels were increased in ripening 'Cabernet Sauvignon' berries exposed to high temperatures over a period of 3 weeks (Pillet et al. 2012).

Possible regulation of the thermotolerance response in the grapevine berry

Considering that the observed transcriptional thermotolerance response is established in ripening berries independently of other abiotic stress responses (**Table 1**, **Fig. 2C**), most high temperature-up-regulated transcription factors could be involved in the maintenance of this state. Among them, several AP2/ERF transcription factors were significantly up-regulated in high temperature ripening berries (**Table 1**, **Fig. 2A**). Members of this gene family participate in the activation of thermotolerance transcriptional cascades in plants, acting upstream of HSFs (Sakuma et al. 2006, Lim et al. 2007, Qin et al. 2007, Yoshida et al. 2008, Almoguera et al. 2009, Chen et al. 2010). The closest *Solanum lycopersicum* L. homolog to most grapevine *ERF* genes up-regulated by high temperatures in 14 DAT berries is *LeERF5*. This gene is also heat-inducible in tomato and confers abiotic stress tolerance (Chuang et al. 2010, Pan et al. 2012). These data suggest that elevated expression of these AP2/ERFs could trigger the maintenance of the thermotolerance response in the grapevine berry. Transcriptional cofactors such as *MBF1a* (*VIT_19s0014g01260*) and *MBF1c* (*VIT_11s0016g04080*), which were also up-regulated by high temperature (**Supplementary Table S2**), might be even upstream of AP2/ERF factors in the thermotolerance cascade. They are central regulators of the establishment of heat tolerance in Arabidopsis and both AP2/ERFs and HSFs are included within the AtMBF1c heat-dependent regulon (Kim et al. 2007, Suzuki et al. 2008, Suzuki et al. 2011).

Out of 12 HSF genes present in the GrapeGen GeneChip®, *VvHsfA2* was the only one induced by high temperatures at 14 DAT (**Fig. 2A**). VvHsfA2 has been shown to participate in the regulation of heat responses in heated berries (Pillet et al. 2012) and, similarly to the role of its Arabidopsis homolog AtHsfA2 (Charng et al. 2007, Li et al. 2010), the data suggest this is the *HSF* gene specifically activated for maintenance of grapevine berry thermotolerance.

High temperature-up-regulated ABA biosynthetic *NCED* genes (**Supplementary Table S2**) are probably related to the higher ABA level measured in the pericarp of berries ripening under that condition (**Table 2**). This suggests the participation of ABA in the high temperature berry acclimation responses. In a parallel experiment in tomato, ABA treatment up-regulated the above-cited *LeERF5* (Pan et al. 2012). Furthermore, a peak of ABA at the onset of grape berry ripening has been proposed to accelerate the process, which could be consistent with a possible role for ABA in the acceleration of the fall in malate and acidity caused by high temperatures, although it would not explain the reduction in anthocyanin content (**Fig. 1D, E**) (Jeong et al. 2004, Yamane et al. 2006, Wheeler et al. 2009,

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Koyama et al. 2010). Perhaps, relative to the effect of ABA in promoting anthocyanin accumulation, the observed heat-induced increase in ABA content in 'Muscat Hamburg' berries may result from increased ABA accumulation in the pulp, given that the ABA level has been shown to diminish in the skin of berries from interspecific hybrids subjected to high temperatures (Yamane et al. 2006, Azuma et al. 2012). Alternatively, an increased ABA level in response to high temperature at late ripening stages could modulate thermotolerance responses in the pericarp without altering ripening parameters. Additional measurements of ABA content at earlier ripening stages after temperature treatments and separately in berry pulp and skin would be required to clarify the role of ABA in the berry response to heat.

Completion of berry ripening at different temperatures could require transcriptional regulation of transmembrane transport

Membrane fluidity is temperature sensitive and can affect many cellular functions including those related to transmembrane transport. This could be a reason why maintained high temperature regimes involve changes in the expression of a large number of transmembrane transporters (**Table 1**). Amino acid transporter-encoding transcripts stood out among the transmembrane transporters down-regulated by high temperature (**Fig. 2D**; **Supplementary Table S2**). Most down-regulated amino acid transporters (**Supplementary Table S2**) are predicted to be localized in the plasma membrane (Grimplet et al. 2012) and might be involved in the cellular import of amino acids (Ortiz-Lopez et al. 2000). Regardless, down-regulation of such expression did not result in lower amino acid levels in ripe berries of the high temperature condition. Rather, it could have contributed to balancing tyrosine, valine, methionine and ornithine levels that were significantly lower in 20/15°C ripening berries at 14 DAT but not at 45 DAT (**Table 2**). Valine and methionine levels also increased in the pulp and the skin of sun-exposed berries as compared with shaded ones (Pereira et al. 2006), suggesting a consistent effect of temperature, and perhaps light, on the accumulation of these amino acids. These data could suggest that expression of transmembrane transporters is down-regulated by high temperatures to reduce their protein density within the membrane and thus compensate a likely higher transport activity related to higher membrane fluidity. This possibility should be confirmed at the protein density and transport activity levels.

Like amino acid transporters, ion, sugar, lipid and even anthocyanin transporter-encoding genes might have shown increased expression under low temperatures to compensate lower transporter activity. Such a general response also included four zinc zipper ZIP transporter-encoding transcripts (**Supplementary Table S2**) whose Arabidopsis homologs were shown to reduce their activity drastically at low temperatures (Grotz et al. 1998). Overall, tuning of cellular transport homeostasis to temperature conditions could be a requirement for completion of fruit ripening.

Berry transcriptional responses related to decreased organic acid and anthocyanin levels under high temperatures

In the experiments, concurrently with the described thermotolerance response and coincident with effects described in vines from vineyards, high temperature reduced berry total acidity, and malate and anthocyanin content at ripeness, which affect fruit commercial quality. There is controversy on the mechanisms affected by heat that accelerate malate loss (Sweetman et al. 2009). It has been proposed that malate concentration in the fruit could be mostly dependent on the thermodynamics of its tonoplastic transport (Lobit et al. 2006). In this sense and in contrast to other metabolite transporter-encoding genes, high temperature-induced down-regulation of dicarboxylate transporters in 'Muscat Hamburg' berries (**Supplementary Table S2**) could not be detected. Therefore, unless there were other regulatory mechanisms, a higher malate export rate from the storage pools in the vacuole to the cytoplasm might be expected at high temperature, which could accelerate its catabolism (Conde et al. 2007, Iyer et al. 2008).

Regarding the accumulation of anthocyanins under our conditions of fruiting cuttings (**Fig. 1F**), low temperature rather than irradiation intensity might be the main driver for their accumulation, as described for conventional vines (Bergqvist et al. 2001, Spayd et al. 2002, Tarara et al. 2008). Nonetheless, anthocyanin biosynthetic genes do not seem to be greatly down-regulated by high temperatures in 'Muscat Hamburg' berries ripening in fruiting cuttings (**Fig. 3C**; **Supplementary Table S2**), in agreement with a previous report in 'Cabernet Sauvignon' conventional plants (Mori et al. 2007). Expression of four transcripts coding for putative flavonoid-modifying enzymes [two flavonoid hydroxylases (*VIT_03s0063g01690* and *VIT_18s0001g11430*) and two *O*-methyltransfeses (*VIT_01s0010g03510* and *VIT_15s0048g02480*)] was down-regulated by high temperature (**Supplementary Table S2**), which could contribute to variation in the composition of anthocyanins and other flavonoids (Azuma et al. 2012), but probably not to the total flavonoid content. *MYB5a*, encoding a general inducer of the phenylpropanoid pathway (Deluc et al. 2006, Deluc et al. 2008, Czemmel et al. 2012), was also down-regulated by high temperatures (*VIT_08s0007g07230*; **Supplementary Table S2**), contrasting with a previous work showing *MYB5a* temperature-independent expression in the skin of detached berries (Azuma et al. 2012). Total anthocyanin content was not affected by temperature at 7 DAT (**Fig. 1F**), in agreement with results reported in berries of conventional plants (Mori et al. 2007). This suggests that later anthocyanin accumulation-related processes could be affected by high temperature instead of a more prominent alteration of gene expression taking place before the transcriptomic analysis stage. In this sense, anthocyanin

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degradation enhancement by high temperature has been proposed as a mechanism participating in the lower anthocyanin accumulation under such conditions (Mori et al. 2007). Furthermore, the possibility that high temperature could reduce the anthocyanin content by affecting its subcellular transport has only rarely been considered (Azuma et al. 2012). This hypothesis could be supported by the observed high temperature-induced down-regulation of *AM1* and *AM3* transcripts (**Fig. 4B**) encoding transmembrane transporters involved in the import of acylated anthocyanins into the vacuole (Gomez et al. 2009, Gomez et al. 2011). Indeed, acylated anthocyanins fraction has been shown to be greatly reduced by exposure to sunlight and heat (Mori et al. 2007, Tarara et al. 2008), and *AM* expression was also found to be repressed by high temperature in the skin of detached berries (Azuma et al. 2012). The only putative flavonoid transporter-encoding transcript significantly up-regulated by high temperature was a *TRANSPARENT TESTA12-like* MATE transporter (*VIT_18s0001g06790*). In contrast, similarly to *AM1* and *AM3*, several other MATE efflux protein- (*VIT_08s0007g08200* and *VIT_17s0000g02970*) and ABC multidrug transporter- (*VIT_09s0002g05490*, *VIT_09s0002g05570*, *VIT_16s0050g01620* and *GSVIVT00012684001*) encoding genes were down-regulated under high temperature (**Supplementary Table S2**). The expression patterns of some of these genes during 'Muscat Hamburg' berry skin ripening correlate with accumulation of polyphenols and anthocyanins (Lijavetzky et al. 2012) and they might also contribute to the final content of non-acylated anthocyanins in the berry skin. If these hypotheses were true, regulation of expression of anthocyanin transporters would not result in balanced anthocyanin accumulation among berries ripening at different temperatures, as proposed here for other metabolites, but rather in a lower content under high temperatures. The peculiar anthocyanin import mechanism involving vesicle-mediated trafficking to the vacuole (Gomez et al. 2011) might be behind this differential consequence as intracellular trafficking pathways can be selectively, rather than globally, slowed down by low temperatures (Shibasaki et al. 2009).

In contrast to anthocyanin content reduction, proanthocyanidin and flavonol content can be higher in the skin of heated berries (Cohen et al. 2008, Cohen et al. 2012). Indeed two transcripts, annotated as *VvMYBPA1* (*VIT_15s0046g00170*) and flavonol synthase (*VIT_10s0003g02450*), the former encoding a transcription factor that activates proanthocyanidin biosynthesis (Bogs et al. 2007, Terrier et al. 2009) and the latter encoding an enzyme that catalyzes the committed step toward flavonol biosynthesis (Czemmel et al. 2012), were similarly up-regulated by high temperatures (**Supplementary Table S2**). A putative flavonol 3′-methyltransferase-encoding transcript was also up-regulated (*VIT_02s0025g02920*; **Supplementary Table S2**). Thus, up-regulation of genes in the proanthocyanidin and flavonol pathways could consume phenylpropanoid precursors, which could also contribute to reduction of skin anthocyanin levels under high temperature. Conversely, down-regulation of *VvMYBPA1* expression by high temperatures has been reported in the skin of heat-treated detached berries from the interspecific hybrid 'Pione' (Azuma et al. 2012). Differences in genotype, tissue, experimental conditions and assay system could be behind these contrasting results.

In conclusion, fruiting cuttings reproduced the effects of high temperature on berry ripening described in vines, providing a suitable system to study them. The results of transcriptional analyses of berry ripening under controlled temperature conditions identify the existence of a thermotolerance response in berries ripened under high temperature, which could probably allow for the completion of ripening, through the stabilization of protein function and transmembrane transporter density. Our analysis also suggests that this thermotolerance response could be regulated by a transcriptional cascade responsive to ABA and other signal transducers that would include MBF1, VvHsfA2 and ERF transcription factors. In addition, specific events affecting tonoplastic transporters as well as other temperature-regulated processes could participate in the observed alteration of berry anthocyanin and malate content by high temperatures. Further analyses will be required to confirm all these suggested possibilities.

Materials and Methods

Plant material and growth conditions

Dormant cuttings of *Vitis vinifera* cv. 'Muscat Hamburg' were obtained in December 2006 from an experimental vineyard of IFAPA Rancho de la Merced (Jerez de la Frontera, Cádiz, Spain). Cuttings were selected to generate fruiting cuttings according to the protocol of Mullins (1966) later modified by Ollat et al. (1998) and Santa María (2004). Rooting was induced in a heat-bed (27°C) kept in a cold room (5°C). One month later, rooted cuttings were planted in 3 liter plastic pots containing a mixture of perlite : vermiculite : sand (1 : 1 : 1) and transferred to the greenhouse for vegetative development. Growth conditions in the greenhouse were 26/15°C and 50% relative humidity, and a photoperiod of 14 h with natural daylight supplemented with high-pressure sodium lamps (SON-T Agro Phillips), providing a minimum PPFD of 350 $\mu mol\ m^{-2} s^{-1}$ at inflorescence level. Plants were irrigated with the nutrient solution proposed by Ollat et al. (1998). Only a single flowering stem was permitted to develop on each plant during growth. Vegetative development was controlled by pruning, which was used to maintain in all treatments a leaf area to grape mass ratio optimal for berry ripening (between 10 and 15 leaves per plant) (Kliewer and Weaver 1971, Jackson and Lombard 1993).

Experimental design and berry sampling

At the onset of ripening (veraison), plants growing from cuttings were exposed to four different environmental regimes in two growth chamber set-ups: 20/15°C (day/night) and 30/25°C (day/night) and with two different irradiation conditions (200 and 400 $\mu mol\ m^{-2} s^{-1}$ PPFD) at grape bunch level during a 14 h

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photoperiod. Fruiting cuttings were selected to have similar grape bunch size, avoiding changes in berry quality. At least nine plants were transferred to each treatment. These environmental conditions were maintained until maturity (about 45 d after veraison). Berry samples were collected during treatments at veraison (just before treatment), 1 week after treatment (7 DAT), 2 weeks after treatment (14 DAT) and at ripeness (45 DAT). Three independent and consecutive experimental repetitions were carried out.

Berry physicochemical analyses

For each sampling, berries from different fruiting cuttings under the same treatment were collected and pooled together. Among them, the most representative ripening state in the sample was selected based on berry density as an indicator of accumulated sugar levels (Stein et al. 1983). Density was estimated by berry flotation into a range of NaCl solutions, each having a decrease in salinity of 10 g NaCl l^{-1} (from 160 to 70 g NaCl l^{-1}). Samples were frozen in liquid nitrogen and stored at −80°C until analysis. Sample collection and freezing were carried out approximately 2h after the light period onset.

Three samples of 50 berries pooled from plants of each treatment and harvest time (three technical replicates per biological replicate) were crushed without skins and seeds. Then extracts were centrifuged and the supernatants used for the following determinations: TSS measured by a refractometer (Zuzi model no. 315, Digital ABBE); pH (by pH meter); and total acidity determined by titrating 10 ml of extract against 0.1 N NaOH, and converted to a weighed quantity of tartaric acid and malic acid measured by an enzymatic method (Enzytec L-Malic acid, Boehringer Mannheim/R-biopharm). In parallel, samples of 50 de-seeded berries, from each treatment and sampling time, were ground in a blender to determine phenolic maturity according to the method of Glories and Augustin (1993). Phenolic content was determined after sample grinding and maceration during 4 h with a buffer at pH 3.2 (tartaric acid) in a 1 : 1 (w/w) mixture. Subsequently, the macerated samples were centrifuged, and the supernatants were used for the following determinations. Phenolic content was measured in the supernatant obtained after maceration (diluted 100 times with distilled water) at 280 nm (OD_{280}). The content of anthocyanins was measured by the sodium bisulfite discoloration method (Ribéreau-Gayon and Stonestreet 1965). All absorbance readings were made in a Hitachi spectrophotometer (model U-2001, Hitachi Instruments Inc.).

Amino acid and ABA determination

About 10–15 de-seeded berries from 14 and 45 DAT with a density of 100–110 and 120–130 g NaCl l^{-1}, respectively (the most representative density intervals of the corresponding sampling stages), were used for amino acid and ABA extraction. For each sampling point and temperature regime, samples from both light regimes of repetitions 1 and 3 were selected as biological replicates to analyze the effect of the temperature regime. All the proteinogenic amino acids, except tryptophan and arginine, as well as ornithine and γ-aminobutyric acid, were determined in grape juice following a modified 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate (AQC) method (Hernández-Orte et al. 2003). Additionally, ABA extraction and analysis were carried out essentially as described in Durgbanshi et al. (2005) with slight modifications. Briefly, frozen berry pericarps were extracted in distilled water using a tissue homogenizer. Before extraction, samples were spiked with deuterated standards of ABA (d_6-ABA) as an internal standard. After extraction and centrifugation, the pH of the supernatant was adjusted to 3.0 and partitioned twice against diethyl ether. The organic layers were combined and evaporated in a centrifuge vacuum evaporator. The dry residue was thereafter resuspended in a water : methanol (9 : 1) solution, filtered, and injected in a HPLC system (Alliance 2695, Waters Corp.). Hormones were separated in a reversed-phase C18 column using methanol and 0.01% acetic acid in water as solvents. The mass spectrometer, a triple quadrupole (Quattro LC, Micromass Ltd.), was operated in negative ionization electro spray mode and ABA was detected according to its specific transition using a multiresidue mass spectrometric method.

Statistical treatment of physicochemical data

Statistical analyses for measured parameters were carried out with the SPSS 15.0 statistical package for Microsoft Windows (SPSS Inc.). Data were first tested using a two-way analyis of variance (ANOVA) to determine the effects of the treatments and their possible interactions. When effects of treatments were statistically significant, differences among groups were tested with Duncan's post-hoc test. Results were considered statistically significant at a P-value <0.05 for both tests. Data are presented as means ± SE.

RNA isolation

For each treatment and experiment repetition, 14 DAT de-seeded berry samples were used. Berry density of 100–110 g NaCl l^{-1} was selected as the most representative of this sampling stage. Total RNA was extracted according the procedures described by Reid et al. (2006). DNase digestion of contaminating DNA in the RNA samples was carried out with the RNase-Free DNase Set (QIAGEN). Final RNA purification was performed using the RNeasy Mini Kit (QIAGEN) according to standard protocols.

Microarray expression analysis

GeneChip® hybridization. RNA integrity analysis was performed with an Agilent Bioanalyzer 2100. The custom GrapeGen Affymetrix GeneChip® (A-AFFY-162 and GPL11004 ArrayExpress and GEO accession numbers, respectively), which contains 23,096 probe sets corresponding to 18,711 non-redundant grapevine transcripts (Grimplet et al. 2012), was used as previously published (Lijavetzky et al. 2012).

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Expression data preprocessing and PCA plot. The full GeneChip® raw expression data set is available on PlexDB (Dash et al. 2012) under the accession number VV46. Probe set signal values from all the microarray hybridizations were normalized together using Robust Microarray Average (RMA) (Irizarry et al. 2003) by RMAExpress (http://rmaexpress.bmbolstad.com). After the averaging of normalized expression values for redundant probe sets using Babelomics pre-processing tools (Medina et al. 2010), a PCA (Raychaudhuri et al. 2000) was directed over the full data set. The six first PCs were analyzed using Acuity 4.0 (Axon Molecular Devices, http://www.moleculardevices.com).

Identification of differentially accumulated transcripts. Two-class *limma* comparisons (Smyth 2004) were conducted in MeV (Saeed et al. 2006) to detect differential expression between both temperature conditions and both light conditions in ripening pericarps. All probe set *limma* FDR values, normalized expression data and annotations can be found in **Supplementary Table S1**. A stringent *limma* significance *P*-value <0.01 after Bonferroni–Hochberg multiple test correction was applied. Furthermore, only probe sets showing at least a 1.6-fold change between conditions were considered as significant to take into account only those genes more likely to be involved in biological changes. Finally, differentially expressed non-redundant transcripts were obtained after the average of expression values from redundant significant probe sets were determined by using Babelomics pre-processing tools (Medina et al. 2010). Probe set redundancy was reduced considering the GrapeGen GeneChip® 12Xv1 annotations version (http://bioinfogp.cnb.csic.es/tools/grapegendb).

New GrapeGen GeneChip® MapMan version development and use in functional analysis. The 941 functional categories from the GrapeGen GeneChip® 12Xv1 annotations version (Lijavetzky et al. 2012) were used as MapMan BINs (Thimm et al. 2004) to build a mapping file including all the 18,711 GrapeGen GeneChip® represented unique transcripts. A total of 34 pathway pictures were constructed, altogether including 489 GrapeGen functional categories where, apart from the 7,923 classified in the 'unknown' functional category, 10,014 unique transcripts can be mapped. Developed mapping and pathway files, useful to perform MapMan analysis from GrapeGen GeneChip® data, are available for the research community in the updated GrapeGenDB database (http://bioinfogp.cnb.csic.es/tools/grapegendb). The described MapMan BINs were used to search for significantly altered functions from the list of temperature differentially expressed transcripts. Functional categories showing a Bonferroni–Hochberg adjusted *P*-value <0.05 in the Wilcoxon rank sum test implemented in MapMan were considered as significantly altered. Finally, MapMan display of expression ratios for some selected pathways was depicted and the direction of the expression differences within the significantly altered categories was checked for groups up-regulated or down-regulated.

Quantitative RT–PCR

The DNase-digested purified RNA of repetitions 1 and 3 used for GeneChip® analysis was also reverse transcribed in a 20 μl reaction mixture containing 1 μg of total RNA 1× PCR buffer II (Applied Biosystems), 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, 20 U of RNase inhibitor, 50 U of murine leukemia virus reverse transcriptase (Applied Biosystems), 2.5 μM oligo$(dT)_{18}$ and diethyl pyrocarbonate-treated water.

Transcript levels were determined by qRT–PCR using a 7300 Real-Time PCR System (Applied Biosystems) and SYBR Green dye (Applied Biosystems). Reactions were performed in a final volume of 20 μl containing 10 μl of 2× Power SYBR Green PCR Master Mix (including AmpliTaq Gold DNA Polymerase-LD, deoxynucleoside triphosphates and SYBR Green dye), 333 nM of forward and reverse primers, and a 1 : 50 dilution of cDNA. After enzyme activation at 95°C for 10 min, amplification was carried out in a two-step PCR procedure with 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing/extension. Gene-specific primers were designed using the Oligo Explorer 1.2 software (Gene Link) and the gene sequences from the grapevine 8× genomic sequence assembly (Jaillon et al. 2007) as templates. Blat analysis was run against the 8× genomic sequence to check the gene specificity of designed primer pairs (http://www.genoscope.cns.fr/blat-server/cgi-bin/vitis/webBlat). No-template controls were included for each primer pair, and each PCR was performed in triplicate. Amplification data were analyzed using the 7300 SDS software 1.3 (Applied Biosystems). Dissociation curves for each amplicon were obtained by heating the amplicons from 60 to 95°C, and were analyzed to verify the specificity of each amplification reaction. The amplification efficiency for each primer pair was calculated from a standard curve and resulted in all cases between 89% and 111%. Transcript relative levels were calculated after normalization to the grapevine *GPDH* gene (*VIT_17s0000g10430*) using the ΔΔCt method. Graphical representations of transcript relative levels with standard deviation and regression analysis were performed in the Microsoft Excel software.

Supplementary data

Supplementary data are available at PCP online.

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