

1 **Differences in berry primary and secondary metabolisms identified by**  
2 **transcriptomic and metabolic profiling of two table grape color somatic**  
3 **variants**

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27

28 **ABSTRACT**

29

30 Anthocyanins are flavonoids responsible for the color of berries in skin-pigmented grapevine  
31 (*Vitis vinifera* L.). Due to the widely adopted vegetative propagation of this species, somatic  
32 mutations occurring in meristematic cell layers can be fixed and passed into the rest of the plant  
33 when cloned. In this study we focused on the transcriptomic and metabolic differences between  
34 two color somatic variants. Using microscopic, metabolic and mRNA profiling analyses we  
35 compared the table grape cultivar (cv.) ‘Red Globe’ (RG, with purplish berry skin) and cv.  
36 ‘Chimenti Globe’ (CG, with a contrasting reddish berry skin color). As expected, significant  
37 differences were found in the composition of flavonoids and other phenylpropanoids, but also  
38 in their upstream precursors’ shikimate and phenylalanine. Among primary metabolites, sugar  
39 phosphates related with sucrose biosynthesis were less accumulated in cv. ‘CG’. The red-  
40 skinned cv. ‘CG’ only contained di-hydroxylated anthocyanins (i.e. peonidin and cyanidin)  
41 while the tri-hydroxylated derivatives malvidin, delphinidin and petunidin were absent, in  
42 correlation to the reddish cv. ‘CG’ skin coloration. Transcriptomic analysis showed alteration  
43 in flavonoid metabolism and terpenoid pathways and in primary metabolism such as sugar  
44 content. Eleven *flavonoid 3’5’-hydroxylase* gene copies were down-regulated in cv. ‘CG’. This  
45 family of cytochrome P450 oxidoreductases are key in the biosynthesis of tri-hydroxylated  
46 anthocyanins. Many transcription factors appeared down-regulated in cv. ‘CG’ in correlation to  
47 the metabolic and transcriptomic changes observed. The use of molecular markers and its  
48 confirmation with our RNA-seq data showed the exclusive presence of the null *MYBA2* white  
49 allele (i.e. homozygous in both L1 and L2 layers) in the two somatic variants. Therefore, the  
50 differences in *MYBA1* expression seem sufficient for the skin pigmentation differences and the  
51 changes in MYBA target gene expression in cv. ‘Chimenti Globe’.

## 52 INTRODUCTION

53 Table grapes are produced for their fresh and dried (i.e. raisins) consumption, with their  
54 markets continuously demanding better fruit quality but also searching for the generation of new  
55 cultivars with appealing features. The berry developmental process displays a double sigmoid  
56 curve with two growth phases separated by a lag phase, at the end of which veraison takes place  
57 (COOMBE, 1995). The accumulation of pigments (i.e. secondary metabolites known as  
58 anthocyanins) occurs at this stage in red and black-skinned cultivars, concomitant with sugars  
59 (primary metabolites) and other secondary metabolites (e.g. aroma volatile compounds). All  
60 these accumulate until the fruit exhibits its desirable quality traits and it is ready for harvest.

61 Berry skin color is an important trait for the grapevine industry, used as a criterion for  
62 selection within breeding programs. Berry skin colors ranges from black to blue, red or pink and  
63 also yellowish-green tones and hues, as a consequence of natural hybridization and human  
64 selection processes (Azuma, 2018; Azuma et al., 2008; Frédérique Pelsy, Dumas, Bévilacqua,  
65 Hocquigny, & Merdinoglu, 2015). Clonal polymorphism affecting berry color is a common  
66 event that occurs in grapevines thanks to vegetative propagation. Under these circumstances  
67 somatic mutations that occur in the meristematic cells within buds (in the entire meristem -bud  
68 sports- or only a portion -chimeras-) are maintained and propagated, leading to somatic color  
69 variants (Pelsy et al., 2015; D'Amato, 1997). In most cases, somatic mutations affect only one  
70 cell layer of the meristem, leading to periclinal chimeras. This phenomenon engenders a  
71 heritable variation source creating new grape cultivars.

72 The main somatic polymorphisms accounting for color variation include insertions,  
73 duplications or SNPs, some caused by the activity of transposable elements disturbing  
74 regulatory regions (Carrier et al., 2012). Some well-known examples of clonal polymorphism  
75 affecting berry color correspond to the case of cultivars (cv.) 'Pinot Noir' and 'Pinot Blanc',  
76 with the later presenting a large deletion (over 260 kb-long) that removes the functional *MYBA1*  
77 and *MYBA2* genes that are the main regulators of anthocyanin synthesis (Walker et al., 2006;  
78 Yakushiji et al., 2006). A different somatic mutation was found in cv. 'Pinot Gris' but which  
79 affected the same two genes, producing a grey-skinned phenotype. In this second variant a  
80 chimeric structure was identified, with berries composed of a colored L1-derived epidermis  
81 (heterozygous for the functional and null alleles) while the L2 cells possessed a homozygous  
82 mutation in both *MYBA1* and *MYBA2* (Vezzulli et al., 2012).

83           The *MYBA1* and *MYBA2* genes form part of the berry color locus found in chromosome  
84 2 (Hocquigny et al., 2004). These factors are known to form ternary complexes with bHLH  
85 (basic helix–loop–helix) and WDR (tryptophan–aspartic acid repeat) proteins (Hichri et al.,  
86 2010; Matus et al., 2010) being all of them important for the activation of the structural genes  
87 of the flavonoid branch in the phenylpropanoid pathway. Additional factors have been identified  
88 to date in this species (Matus et al., 2017), demonstrating a complex hierarchical regulation of  
89 pigment accumulation according to organ and in response to the environment.

90           In the last decade several genome-wide studies have demonstrated that a large  
91 transcriptomic shift drives the transition from the green berry to the ripening stages (Fasoli et  
92 al., 2012; Massonnet et al., 2017; Palumbo et al., 2014). The availability of a high quality  
93 grapevine genomic sequence and of large amount of transcriptomics and metabolomics data has  
94 allowed to conduct integrative analyses to decipher the key transcriptomic reprogramming  
95 events occurring at the onset and very late stages of berry ripening (reviewed by (Serrano et al.,  
96 2017; Wong & Matus, 2017). However, most of the transcriptomic comparisons conducted so  
97 far have considered very distant cultivars (Ghan et al., 2015; Massonnet et al., 2017; Zenoni et  
98 al., 2016) while very few have compared closely related cultivars at the genome-wide level  
99 (Carbonell-Bejerano et al., 2017). Here, we performed a microscopic, metabolic and  
100 transcriptomic characterization of berry development and ripening in two almost isogenic  
101 cultivars with different berry skin pigmentation. Berries from the cultivar ‘Red Globe’ (RG),  
102 characterized by a large size, thick skin and dark purple skin color, were compared to those of  
103 its somatic variant cv. ‘Chimenti Globe’ (CG) with similar characteristics but differing in its  
104 skin color. The phenotype of ‘CG’ was initially observed in Talagante (Metropolitan Region,  
105 Chile) during grape harvest in 2005, within a cv. ‘RG’ field. ‘Chimenti Globe’ has reddish grape  
106 clusters and was selected by its producer for propagation via cuttings ([www.chgchile.cl](http://www.chgchile.cl)).

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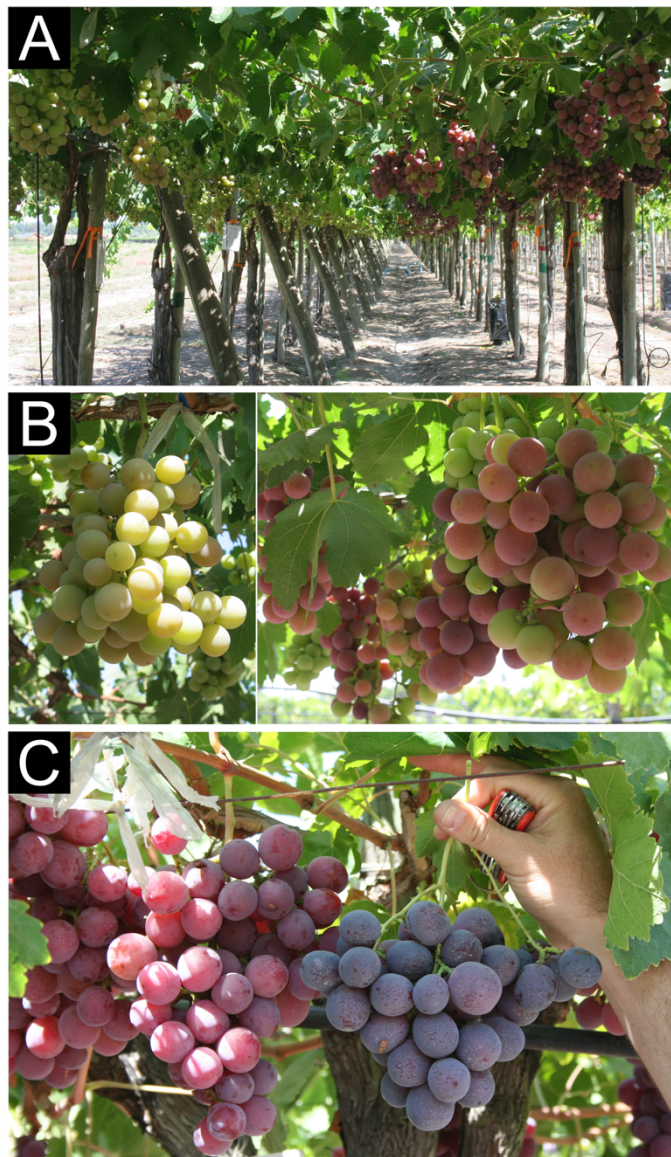
## 108 **MATERIALS AND METHODS**

109

### 110 **Plant material and experimental design**

111           Plant material used in this study corresponds to six plants of commercial table grape cv.  
112 ‘Red Globe’ (RG) and six plants of cv. ‘Chimenti Globe’ (CG), positioned in Camino Loreto,

113 Parcela 8-9, Talagante, (Región Metropolitana, Santiago of Chile; 33°38'26''S; 70°51'52'' W)  
114 (FIGURE 1A). Sampling was performed during seasons 2013 and 2014. Veraison stage was set  
115 as the period at which clusters were 30–50% colored and 5° Brix (5% w/w soluble solids)  
116 (FIGURE 1B), and the late ripening stage when berry sugar content reached 22–23°Brix  
117 (FIGURE 1C). One cluster from each plant (experimental unit) was randomly sampled in both  
118 cultivars, in the two stages and seasons. Berries were peeled, and berry skins were frozen in  
119 liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until required.



120

121 **FIGURE 1: Pigmentation differences at field of the color somatic variants under study.**

122 **(A-B)** Cultivar (cv.) ‘Chimenti Globe’ (CG) and cv. ‘Red Globe’ (RG) at the onset of

123 ripening/veraison (EL-35) and (C) at a late ripening stage (EL-38). Clusters of CG plants are  
124 shown at the left side of each photograph in all cases. Plants from both cultivars used in this  
125 study are situated immediately adjacent to each other in the same field.

126

### 127 **Microscopy**

128 Oblique hand sections of ripening berries were cut with a scalpel blade and floated on a  
129 glycerol 50% droplet and mounted on slides. Samples were examined under spectral confocal  
130 optical microscope *Eclipse C2si*, Nikon Instruments Inc. (Minato-ku, Tokyo, Japan).

131

### 132 **Metabolite extractions and quantification analyses**

133 For metabolite analyses, berry skins from both seasons were used and processed  
134 independently (24 total samples per cultivar), except for the case of anthocyanin analysis, for  
135 which five out of six samples were considered for each cultivar (20 samples per cultivar).  
136 Primary metabolites such as Trehalose-6-Phosphate (T6P) together with glycolytic and  
137 tricarboxylic acid (TCA) cycle intermediates were extracted from 25 mg of frozen powder with  
138 chloroform/methanol solution, and measured by high-performance anion-exchange liquid  
139 chromatography coupled to tandem mass spectrometry (LC-MS/MS) as described by (John E.  
140 Lunn et al., 2006). Amino acids were extracted and analyzed by HPLC as described by  
141 (Martínez-Lüscher et al., 2014). For sugars and organic acids, an aliquot of 150-200 mg of skin  
142 fine powder was extracted sequentially with ethanol 80% and 50%, dried in a SpeedVac and re-  
143 dissolved in 1 ml of sterile ultrapure water. Hexose content (glucose and fructose) was measured  
144 enzymatically with an automated micro-plate reader (Elx800UV, Biotek Instruments Inc.,  
145 Winooski, VT, USA) according to (L. Gomez, Bancel, Rubio, & Vercambre, 2007). Tartaric  
146 acid content was measured by using the colorimetric method based on ammonium vanadate  
147 reactions (Pereira et al., 2006). Malic acid content was determined using an enzyme-coupled  
148 spectrophotometric method that measures the change in absorbance at 340 nm from the  
149 reduction of NAD<sup>+</sup> to NADH (Pereira et al., 2006).

150 Anthocyanins were extracted from 300 mg freeze-dried ground powder from skin of cv.  
151 'RG' and 'CG' using 1 ml methanol containing 0.1% HCl (v/v). Extracts were filtered across  
152 0.45 µm polypropylene syringe filter (Pall Gelman Corp., Ann Harbor, MI, USA) for high

153 performance liquid chromatography (HPLC) analysis. Each individual anthocyanin was  
154 analyzed as described in (Dai et al., 2013). For anthocyanin quantification was integrated peak  
155 area at 520 nm and using Malvidin 3-glucoside as standard (Extrasynthèse, Lyon, France).

156

### 157 **RNA extraction**

158 Total RNA were isolated from berry skins according to the procedure of (Reid, Olsson,  
159 Schlosser, Peng, & Lund, 2006), using a CTAB-spermidine extraction buffer. This material was  
160 used for transcriptome sequencing by RNA-seq technology and validation of data by  
161 quantitative real-time RT-PCR (qRT-PCR). For RNA sequencing, berry skins from 2013 season  
162 were used and pooled in 3 samples per cultivar (cv. 'CG' and 'RG') and stage (veraison and  
163 ripening), reaching a total number of 12 samples to be deep sequenced. Each pool was generated  
164 by mixing 2 individual RNA extractions (1 µg each one) and then mixing them to obtain a  
165 concentration of 2 µg per pool (TABLE S1). Total RNA was sent to Macrogen (Macrogen Inc.  
166 Seoul, Korea) after ethanol precipitation (0.1 volume of 3 M Sodium Acetate pH 7-8 and 2  
167 volumes of 100% ethanol).

168

### 169 **RNA Sequencing and Read Mapping**

170 Ten micrograms of total RNA were fragmented, converted to cDNA, and amplified by  
171 PCR to Illumina® TruSeq™ RNA Sample Preparation Kit (Illumina, Inc., USA). Pair-end  
172 100bp sequence reads were generated using the Illumina Genome Analyzer II (Illumina) and  
173 Illumina HiSeq 2000 (Illumina) at Macrogen Inc. according to the manufacturer's  
174 recommendations. Trimmomatic (Bolger, Lohse, & Usadel, 2014) was used to trim and clip  
175 reads prior to mapping, removing the adapter sequences as well as the low-quality sequences  
176 from the ends of the reads. All the distinct clean reads were aligned to the Grape Genome  
177 Database hosted at CRIBI V2 (<http://genomes.cribi.unipd.it/grape/>) (Vitulo et al., 2014).  
178 Uniquely mapped reads were counted by HITSAT2 software (Kim, Langmead, & Salzberg,  
179 2015) and featureCounts in the Rsubread package (Liao, Smyth, & Shi, 2013).

180

### 181 **Analysis of differentially expressed genes (DEGs)**

182 DESeq2 was used for determining differentially expressed genes (DEGs) using a false

183 discovery rate (*FDR*) threshold of 0.05 and an absolute value  $\log_2$  ratio  $\geq 1$  (Love, Huber, &  
184 Anders, 2014). MultiExperiment Viewer (MeV) was used for gene clustering analysis that was  
185 performed by the k-means method with Euclidean distance. MeV also was used for graphical  
186 representation of DEGs in a heatmap using Fold Change  $\geq 1$  (Howe, Sinha, Schlauch, &  
187 Quackenbush, 2011). Additionally, we performed a new DESeq2 analysis with the same  
188 parameters described previously but removing the filter from the Fold Change; the DEGs  
189 obtained with this method were annotated using Mercator web tool and then loaded into  
190 MapMan software (Lohse et al., 2014; Usadel et al., 2009).

191

### 192 **Validation of RNA-seq data by quantitative real-time RT-PCR (qRT-PCR)**

193 Two micrograms ( $\mu\text{g}$ ) of total RNA were treated with TURBO DNA-free™ DNase  
194 (Ambion®) and subsequently reverse transcribed with random hexamer primers and  
195 SuperScript II RT (Invitrogen™ Co., Carlsbad, CA, USA) as in (Dauelsberg et al., 2011).  
196 Relative transcript quantification of differentially expressed genes (DEGs) was performed by  
197 real-time RT-PCR (qRT-PCR) using the BRILLIANT II SYBR® GREEN QPCR Master Mix  
198 and the Mx3000P qPCR system (Stratagene, Agilent Technologies Inc., Santa Clara, CA, USA)  
199 according to the manufacturer's instructions. Expression levels of all evaluated genes were  
200 calculated from six biological replicates, relative to *Vvi60SRP* housekeeping control gene. We  
201 used the  $2^{-\Delta\Delta\text{Ct}}$  method for the statistical analysis (Scheffe, Lehmann, Buschmann, Unger, &  
202 Funke-Kaiser, 2006). Primers are listed in Supplementary (TABLE S2).

203

### 204 **Statistical data analyses**

205 Data were analyzed with multivariate analysis methods using the R statistics  
206 environment (R Core Team, 2010). In order to evaluate alterations in metabolite levels during  
207 stages of grapevine development, principal component analysis (PCA) was performed on mean-  
208 centered and scaled data using the *ade4* package in R (Dray & Dufour, 2007). Differences  
209 between developmental stages and seasons were analyzed by a two-way ANOVA followed with  
210 a Tukey multiple comparison post-hoc test at  $P < 0.05$ , for example, in the case of PCA analysis,  
211 component 1. For differences between cultivars were analyzed using unpaired T-test, for  
212 example in the case of PCA analysis, component 2 and qRT-PCR analysis. GraphPad Prism  
213 version 6.00 for Windows (GraphPad Software, La Jolla California USA) was used for graphics



214 representation and analysis. For RNA-seq analysis, Trimmomatic (Bolger et al., 2014), HISAT2  
215 (Kim et al., 2015) and Rsubread package (Liao et al., 2013) were used with default parameters.  
216 DEGs analysis were performed with DESeq2 using *FDR* with a threshold of 0.05 and absolute  
217 value  $\log_2$  ratio  $\geq 1$  (Love et al., 2014). (MeV) was used for cluster analysis by the k-means  
218 method with Pearson's correlation distance and graphical representation of DEGs with a  
219 heatmap using  $FC \geq 1$  (Howe et al., 2011).

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## 222 **RESULTS**

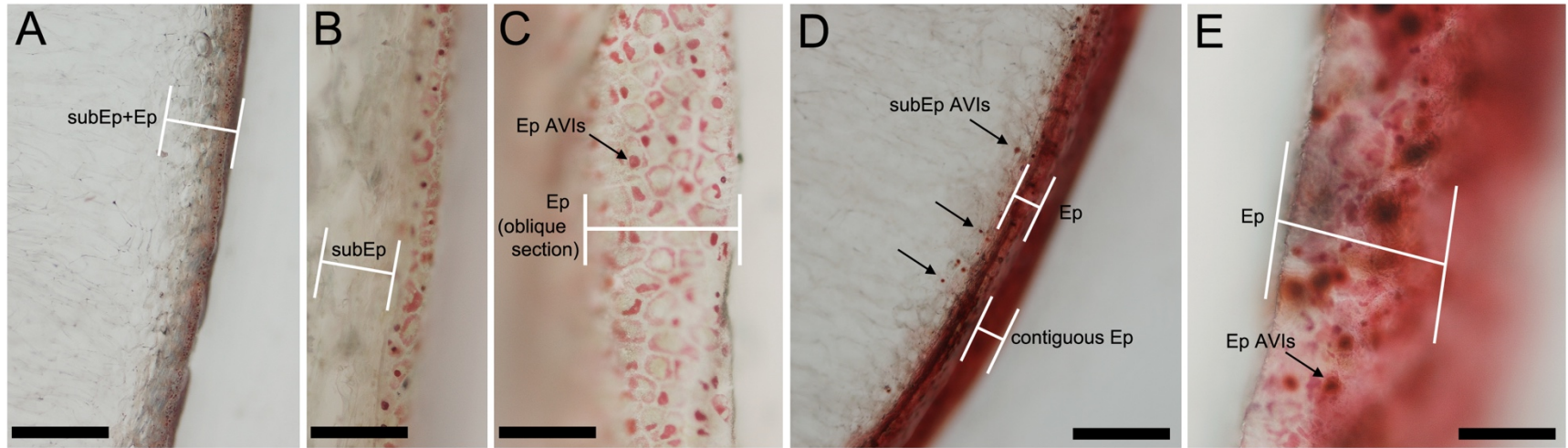
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### 224 **Characterization of cv. 'Red Globe' and its color somatic variant cv. 'Chimenti Globe'.**

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#### 226 ***Microscopy study***

227 Grape berry skin is composed of several cell layers: the epidermal cells comprising only  
228 a single layer (L1-derived) and the large underlying subepidermal cells (L2-derived) that also  
229 compromise the flesh. We observed in cv. 'Chimenti Globe' that anthocyanins only  
230 accumulated in the outermost single layer of the Epidermis (Ep) and not in the subEpidermis  
231 (subEp) (FIGURE 2A-B-C). A clear accumulation of anthocyanins was observed within the  
232 vacuoles of the epidermal cells, in Anthocyanin Vacuolar Inclusions (AVIs) (FIGURE 2C). This  
233 observation was similar to the previously characterized cv. 'Malian' (a bud sport of cv.  
234 'Cabernet Sauvignon'; Walker et al., 2006) and cv. 'Pinot Gris' (a periclinal chimera of cv.  
235 'Pinot Noir'; Vezzulli et al., 2012). In contrast, cv. 'Red Globe' showed colored cells in the Ep  
236 layer (FIGURE 2E), with diffuse edge, and some specific AVIs inside some subEp cells that  
237 form part of the skin (FIGURE 2D).



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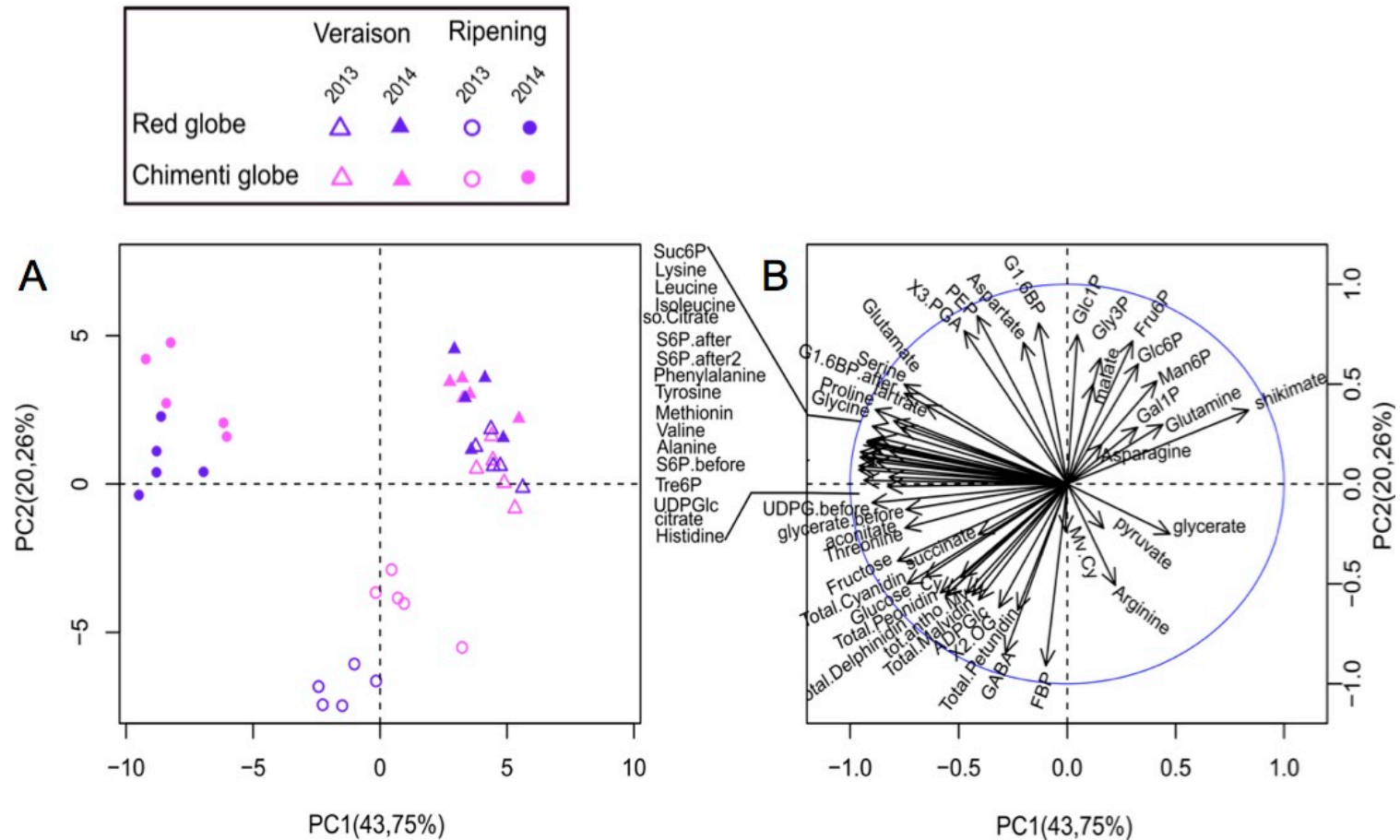
240 **FIGURE 2: Differential accumulation of anthocyanins in epidermal and subepidermal cell layers of CG and RG.** Light  
 241 microscopy sections showing cells from the epidermis (Ep; L1 ontology) and subepidermis (subEp; L2 ontology) in cv. 'Chimenti  
 242 Globe' (A-C) and cv. 'Red Globe' (D-E). Anthocyanin vacuolar inclusions (AVIs) are depicted with arrows. Oblique angle hand  
 243 sections permitted observe contiguous cells from the epidermis as in Walker et al. (2006). In both cultivars the mesocarp/flesh (L2  
 244 ontology) is devoid of anthocyanins. Bar in A and D is 500  $\mu\text{m}$ ; B, C and E is 100  $\mu\text{m}$

245 ***Primary metabolite profiling identifies several compounds affected in the skin of ‘CG’.***

246 The composition of the main primary metabolites was assessed by different methods  
247 depending of each metabolite (described in Methodology). Quantifications were analyzed with  
248 Principal Component Analysis (PCA) to obtain a comprehensive view of main metabolites  
249 showing differences between both somatic variants in veraison and ripening. The first two  
250 principal components (PC<sub>1</sub> and PC<sub>2</sub>) explained about 64% of the total variance and allowed to  
251 discriminate developmental stages between CG and RG (FIGURE 3A). PC<sub>1</sub> (43,75%) was  
252 inferred to capture predominantly variation according to developmental stage and also to the  
253 effect of season but the latter exclusively for the ripening stage samples (no variation was  
254 observed for season at the veraison stage). Metabolites contributing to these differences were  
255 related to phenylpropanoid metabolism such as shikimate, UDP-glucose and phenylalanine but  
256 in addition the molecular regulator trehalose-6-phosphate (T6P) and TCA cycle intermediates  
257 such as citrate, isocitrate and several amino acids also contributed to differentiate veraison from  
258 ripening (FIGURE 3B and FIGURE S1). PC<sub>2</sub> variation (20,26%) was associated to cultivar type,  
259 but this discrimination was much more evident for the ripening stage samples. These results are  
260 explained in changes observed in metabolites related with biosynthesis of sucrose:  
261 glyceraldehyde 3-phosphate, fructose 6-phosphate, glucose 6-phosphate, fructose 1,6-  
262 biophosphate, glucose 1,6-biophosphate, glycerol 3-phosphate, fructose, glucose and  
263 phosphoenolpyruvate; and also, with anthocyanin compounds (FIGURE 3B and FIGURE S2).  
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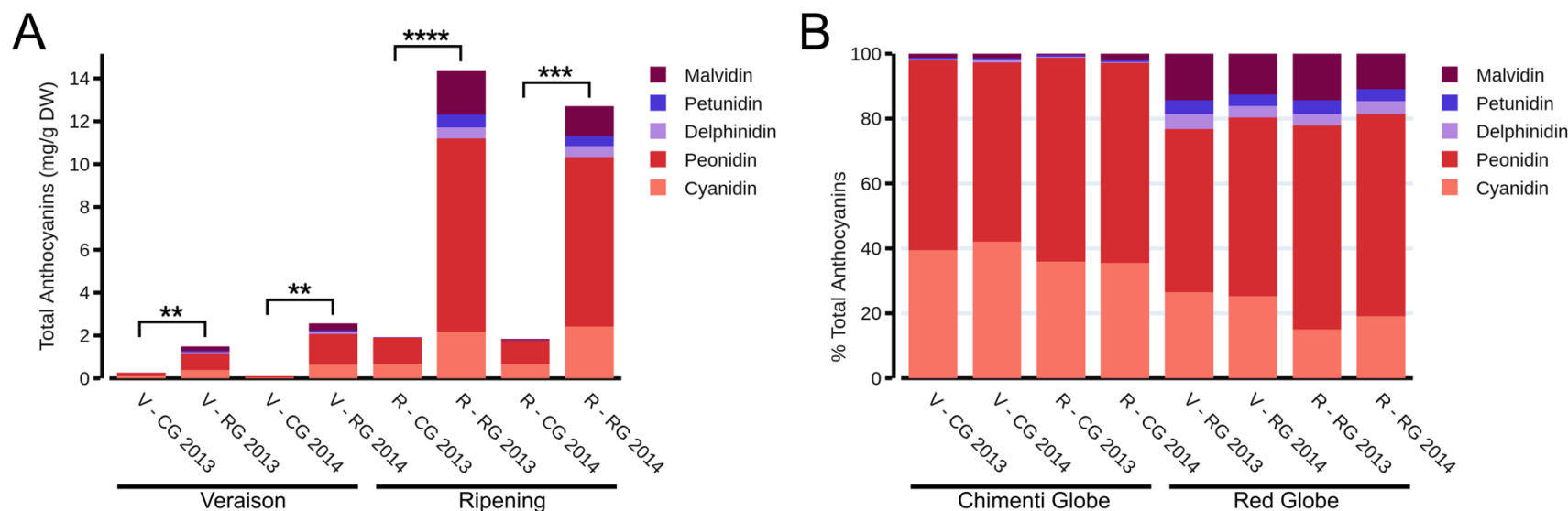
265 ***Anthocyanin profiles in berry.***

266 We analyzed anthocyanin levels present in berry skins of cv. ‘RG’ and its somatic variant  
267 ‘CG’ at the same developmental stages and seasons described previously. During the veraison  
268 stage, ‘CG’ showed trace amounts of some anthocyanin types (Table S3). At ripening, we  
269 observed the most significant differences in total anthocyanin abundances between cv. ‘CG’ and  
270 ‘RG’, with RG containing more than seven times higher amount (FIGURE 4A).



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**FIGURE 3: Differential metabolomes in berries of color somatic variants influenced by developmental stage and vintage.** (A) Principal component analysis (PCA) of primary metabolites present in berry skins of RG and CG, discriminating by cultivar depending on the developmental stage. The variance explained by season (2013/2014) is implied at both stages but is more evident at the ripening stage. (B) Loading plots of metabolites analyzed for the first two components (PC1 and PC2). Main metabolites that explained PC1 correspond to those close to the ordinate axis such as tre6P, UDPGlc, methionine, phenylalanine and shikimate; and for PC2 correspond to those close to the abscissa axis such as phosphorylate intermediates and anthocyanins.



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279

**FIGURE 4: The decreased anthocyanin content in cv. 'Chimenti Globe' is accentuated by the insufficiency in accumulating tri-hydroxylated derivatives.** (A) Content of total anthocyanins ( $\text{mg g}^{-1}$  DW, all derivatives) in both somatic variants at veraison and ripening stages in 2013 and 2014 seasons. Asterisks indicate statistical significance as the result of an unpaired T-test. \*\*,  $P < 0.0081$ ; \*\*\*,  $P = 0.0001$ ; \*\*\*\*,  $P < 0.0001$ . (B) Relative abundance of each anthocyanin type for each sample. Di-hydroxylated derivatives found were cyanidin 3-glucoside, cyanidin 3-(*p*-coumaryl)-glucoside, peonidin-3-glucoside and peonidin-3-(*p*-coumaryl)-glucoside. Tri-hydroxylated derivatives found were delphinidin 3-glucoside, delphinidin 3-(acetyl)-glucoside, petunidin 3-glucoside and malvidin-3-glucoside. Values in (A) and (B) represent the mean of five biological replicates. Anthocyanin derivative content for each replicate can be found in Supplementary Table S3). DW: Dry Weight.

286

287 Detailed analysis of anthocyanin derivatives revealed that ‘CG’ mainly contains di-  
288 hydroxylated types, namely peonidin (in the form of peonidin 3-glucoside and peonidin 3-(*p*-  
289 coumaryl)-glucoside) and cyanidin (in the form of cyanidin 3-glucoside and cyanidin 3-(*p*-  
290 coumaryl)-glucoside), which confer more reddish tones (FIGURE 4B) compared to tri-  
291 hydroxylated forms. Tri-hydroxylated anthocyanins (the most commonly and abundantly found  
292 in black-skinned *V. vinifera* cultivars and that confer purplish and bluish colors), were present  
293 in very small amounts or even non-detectable in cv. ‘CG’, while they were found increasing  
294 towards ripening in cv. ‘RG’. The proportion of total tri-hydroxylated derivatives with respect  
295 to that of di-hydroxylated forms was maintained relatively constant between veraison and  
296 ripening (between 20-35% of the total amount of anthocyanins), being Malvidin the most  
297 abundant of all three (TABLE S3).

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299 **Genome-wide characterization of cv. ‘Chimenti Globe’ and ‘Red Globe’ transcriptomes.**

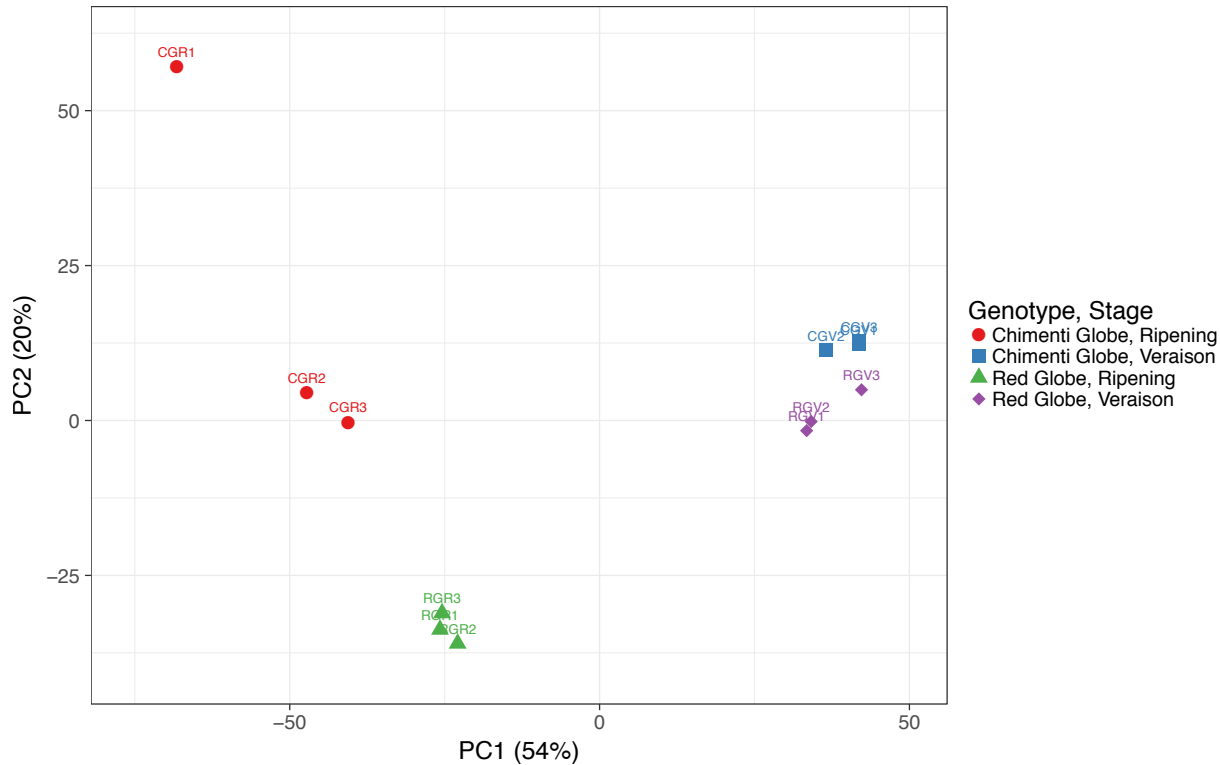
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301 ***RNA-Seq data mapping to the grapevine genome and category enrichment analysis.***

302 We originally hypothesized that the genes responsible for changes in berry skin color in  
303 cv. ‘CG’ should be differentially altered in expression when compared to cv. ‘RG’. We  
304 performed mRNA-Seq profiling using twelve RNA samples corresponding to three independent  
305 pools of biological replicates for ‘CG’ and ‘RG’ for each developmental stage in the 2013 season  
306 (TABLE S1). From a total range of ~4.8 – 8.1 million sequencing raw read pairs, from each  
307 pool sample, only ~4.5 – 7.6 million read pairs corresponded to both kept reads that passed the  
308 quality analysis, representing a 93% of the total read pairs (TABLE S4). Approximately ~3.3 –  
309 5.1 million reads aligned uniquely to the annotated transcriptome, while ~0.5 – 1.5 million reads  
310 aligned 0 times and ~0.7 – 1.2 million reads aligned multiple times (72%, 11-20% and 15-17%  
311 of the total reads after quality analysis, respectively) (TABLE S5). Only the reads that aligned  
312 uniquely and where mapped to the genome were further considered in the analysis (TABLE S5,  
313 S6).

314 To explore the differences between both color somatic variants, all clean reads were  
315 aligned to the grape genome database V2 (Vitulo et al., 2014) and then a comparative  
316 transcriptomic analysis was performed to screen for differentially expressed genes (DEGs) using

317 DEseq2 package (Love et al., 2014). The first inspection of the complete RNA-seq dataset  
318 showed that the major differences between both somatic variants are observed at ripening  
319 (FIGURE 5). The PC1, accounting for approximately 50% of the total variance, was able to  
320 discriminate 'CV' and 'RG' only at ripening while the PC2 accounted for differences mostly  
321 attributable to 'RG' at ripening compared to the three other samples (with the exception one  
322 replicate in CG ripening).



323  
324 **FIGURE 5: PCA of transcriptomics data.** X and Y axis show principal component 1 and  
325 principal component 2 that explain 54% and 20% of the total variance, respectively. Original  
326 FPKM values are  $\ln(x + 1)$ -transformed (NAs removed). No scaling was applied to individual  
327 genes and SVD with imputation was used to calculate principal components. N = 12 data points.

328  
329 A total of 438 genes showed differential expression ( $\log_2FC > 1$  and p adjusted  
330 value  $< 0.05$ ) in at least one stage. We performed a Gene Ontology (GO) enrichment analysis to  
331 enlighten functional categories represented in the 438 DEGs (Supplemental Dataset 1), by using  
332 the Mercator web tool and then loaded into MapMan software version 3.5.1R2 (Lohse et al.,

333 2014; Usadel et al., 2009). Three major types of secondary metabolic pathways were  
334 differentially expressed between cultivars at veraison and ripening: terpenoids and the  
335 phenylpropanoid-related lignin/lignans and flavonoids (FIGURE S3). Additional categories  
336 found enriched were those related to enzymes families (e.g. cytochrome P450, UDP-  
337 glycosyltransferases, glutathione-S-transferases, glucosidases, o-methyltransferases,  
338 peroxidases and beta 1,3 glucan hydrolases, FIGURE S4), and those related to biotic stresses  
339 (FIGURE S5) including genes involved in the synthesis of stress-related hormones,  
340 pathogenesis related (PR) proteins and transcription factors, among others.

341

342 ***The cultivar ‘Chimenti Globe’ exhibits an altered expression of the entire flavonoid pathway***  
343 ***compared to cv. ‘Red Globe’.***

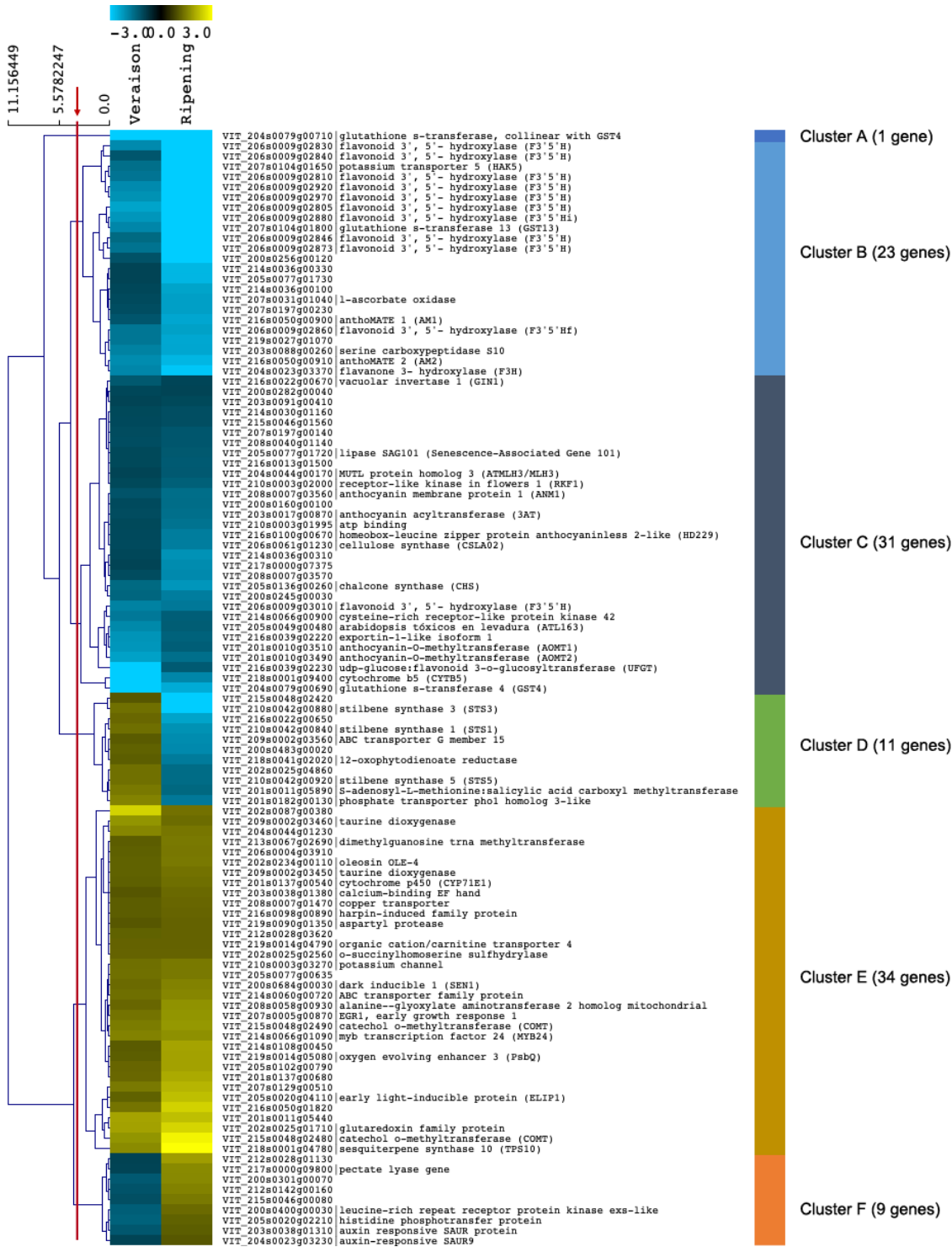
344 From the 438 DEGs, a total of 109 genes were differentially expressed at both veraison  
345 and ripening stages. These were grouped in 6 clusters according to a hierarchical clustering of  
346 their expression profiles (FIGURE 6). From the 109 DEGs, many were related to flavonoid  
347 metabolism. Cluster A consisted in only one gene: a *glutathione-S-transferase* collinear with  
348 GST4 (VIT\_204s0079g00710) that is involved in anthocyanin transport in grapes. Cluster B (23  
349 genes) and cluster C (31 genes), where genes are down-regulated in cv. ‘CG’ at both  
350 developmental stages, contained many flavonoid/anthocyanin-related genes such as GST13  
351 (VIT\_207s0104g01800), GST4 (VIT\_204s0079g00690), *flavonoid 3,5-hydroxylases* (F3’5’-H,  
352 VIT\_206s0009g02805 – 2810 – 2830 – 2840 – 2846 – 2860 – 2873 – 2880 – 2970 – 2920 –  
353 3010), *anthoMATEs* (AM1, VIT\_216s0050g00900 and AM2, VIT\_216s0050g00910),  
354 *flavanone 3-hydroxylase* (F3-H, VIT\_204s0023g03370), *anthocyanin membrane protein 1*  
355 (ANM1, VIT\_208s0007g03560), *anthocyanin acyltransferase* (3AT, VIT\_203s0017g00870)  
356 *chalcone synthase* (CHS, VIT\_205s0136g00260), anthocyanin-o-methyltransferases (AOMT1,  
357 VIT\_201s0010g03510 and AOMT2, VIT\_201s0010g03490) and *UDP-glucose:flavonoid 3-O-*  
358 *glucosyltransferase* (UFGT, VIT\_216s0039g02230). These clusters also included the genes  
359 *vacuolar invertase 1* (GIN1, VIT\_216s0022g00670) involved in sugar transport, *cellulose*  
360 *synthase* (CSLA02, VIT\_206s0061g01230) and an uncharacterized *cytochrome b5* (CYTB5,  
361 VIT\_218s0001g09400) (FIGURE 6). Another interesting gene repressed in cv. ‘CG’ in both  
362 developmental stages encodes a *homeobox-leucine zipper* (HD-Zip) transcription factor  
363 (VIT\_216s0100g00670) homologue to *anthocyaninless 2*, a homeobox gene involved in



364 anthocyanin distribution and root development in Arabidopsis (Kubo, Peeters, Aarts, Pereira, &  
365 Koornneef, 1999) (FIGURE 6). Cluster E (34 genes) encompassed genes that were induced at  
366 both developmental stages in cv. 'CG', some of which may be related to phenylpropanoid  
367 metabolism, such as *cytochrome p450* (CYP71E1, VIT\_201s0137g00540), *ABC transporter*  
368 *family protein* (VIT\_214s0060g00720) and a *catechol-O-methyltransferases*  
369 (VIT\_215s0048g02480-2490). The transcription factor *MYB24* (VIT\_214s0066g01090) and a  
370 *sesquiterpene synthase* (TPS10, VIT\_218s0001g04780) were also listed in this group. Cluster  
371 D (11 genes) and Cluster F (9 genes) combined genes with contrasting expressions at veraison  
372 and ripening. Genes falling in these categories included three (VIT\_210s0042g00840,  
373 VIT\_210s0042g00880 and VIT\_210s0042g00920) and *ABC transporter G member 15*  
374 (VIT\_209s0002g03560) that were induced at ripening but repressed in veraison (FIGURE 6).

375 We performed a less stringent analysis excluding the Fold Change filtering step to search  
376 for additional pathways altered by color somatic variation. Within transcription factors, we  
377 found that *MYBA1* (VIT\_202s0033g00410), *MYBA8* (VIT\_202s0033g00380), *MYB30B*  
378 (VIT\_214s0108g00830), *MYB141* (VIT\_214s0108g01080) and *MYB147*  
379 (VIT\_217s0000g09080) were repressed at veraison in cv. 'CG', while the stilbene regulator  
380 *MYB15* (VIT\_205s0049g01020), *MYB24* (VIT\_214s0066g01090) and *MYB162*  
381 (VIT\_219s0090g00590) were induced at this stage. The truncated and non-functional *MYBA3*  
382 gene (VIT\_202s0033g00450, collinear with *MYBA1* and *MYBA2*), and *MYB24* were also  
383 induced at ripening. In contrast to what was observed at veraison, *MYB30B* was induced during  
384 ripening in cv. 'CG'. Furthermore, the flavonoid regulator *MYBPA1* (VIT\_215s0046g00170)  
385 and *MYB145* (VIT\_219s0014g03820) were repressed during the ripening stage in cv. 'CG'.  
386 Additional TF genes were differentially expressed in ripening cv. 'CG' berries, such as bHLH  
387 145-like gene (VIT\_218s0001g07210) and an uncharacterized WD repeat-containing protein  
388 C16727 isoform 1 (VIT\_208s0007g00730), both being induced (Supplemental Dataset 2).

389  
390



392 **FIGURE 6 (previous page): Differentially expressed genes (DEGs) associated to somatic**  
393 **color variation at both veraison and ripening as surveyed by RNA-seq.** Heatmap of 109  
394 DEGs in CG compared to RG, where differential expression is defined by  $\log_{2}FC \geq 1$  of the  
395 CG/RG ratio and  $FDR \leq 0.05$ . Hierarchical clustering shows the presence of six major clusters  
396 outlined on the right panel (these were defined by cutting the dendrogram at the site shown by  
397 the red line). 12X.v1 (CRIBIv2 annotation) gene IDs were included for those genes with either  
398 an annotation or those previously characterized in grape with relevant functions to our study.

399

#### 400 ***Genes related to ‘Primary metabolism’.***

401 Inspection of the less stringent list of DEGs allowed to identify several genes related to  
402 primary metabolism between cv. ‘CG’ and cv. ‘RG’. At veraison, we identified genes related to  
403 sucrose and trehalose 6-phosphate biosynthesis and also to sugar transport, pathways that have  
404 been related in *Arabidopsis thaliana* to a homeostatic mechanism of maintaining sucrose levels  
405 within a range that is appropriate for each cell type and developmental stage of the plant  
406 (Tre6P:sucrose ratio; Yadav et al., 2014). The bidirectional sugar transporters *VviSWEET1*  
407 (VIT\_218s0001g15330), *VviSWEET10* (VIT\_217s0000g00830) and *VviSWEET15*  
408 (VIT\_201s0146g00260), three hexose transporters (VIT\_205s0020g03140,  
409 VIT\_211s0149g00050 and VIT\_214s0006g02720) and two genes encoding a fructose-  
410 bisphosphate aldolase (VIT\_203s0038g00670 and VIT\_204s0023g03010) were induced in cv.  
411 ‘CG’ in the veraison stage. At ripening, we observed genes related to sucrose and trehalose  
412 biosynthesis and sugar transportation such as *VviSWEET11* (VIT\_207s0104g01340, induced in  
413 cv. ‘CG’), two genes encoding a sucrose-phosphate synthase (VIT\_218s0075g00330 and  
414 VIT\_218s0089g00490, induced and repressed at ripening, respectively), a sucrose transporter  
415 and sucrose synthase 1 (VIT\_201s0026g01960 and VIT\_207s0005g00750) both being  
416 repressed. A fructose-1,6-bisphosphatase (VIT\_208s0007g01570) and two genes putatively  
417 encoding a fructose-bisphosphate aldolase (VIT\_204s0023g03010 and VIT\_203s0038g00670)  
418 were induced. Finally, four genes putatively encoding a trehalose-phosphate synthase  
419 (VIT\_206s0009g01650, VIT\_217s0000g08010, VIT\_203s0063g01510 and  
420 VIT\_212s0028g01670) were induced in cv. ‘CG’ during ripening (Supplemental Dataset 3).

421

422

423 **Genes from other secondary metabolic pathways.**

424 Other genes found in the less-strict list were related to other metabolic pathways such as  
425 isoprenoid biosynthesis: two isoprene synthase genes up-regulated in cv. ‘CG’ at veraison  
426 (VIT\_212s0134g00020, VIT\_212s0134g00030) and ripening (VIT\_212s0134g00030); and the  
427 carotenoid pathway: a probable carotenoid cleavage dioxygenase 4 gene down-regulated in cv.  
428 ‘CG’ during veraison (VIT\_202s0087g00930). Amongst DEGs in cv. ‘CG’, the terpenoid  
429 biosynthesis pathway genes TPS35 (VIT\_212s0134g00030) and TPS10  
430 (VIT\_218s0001g04780) were up-regulated both at veraison and ripening.

431

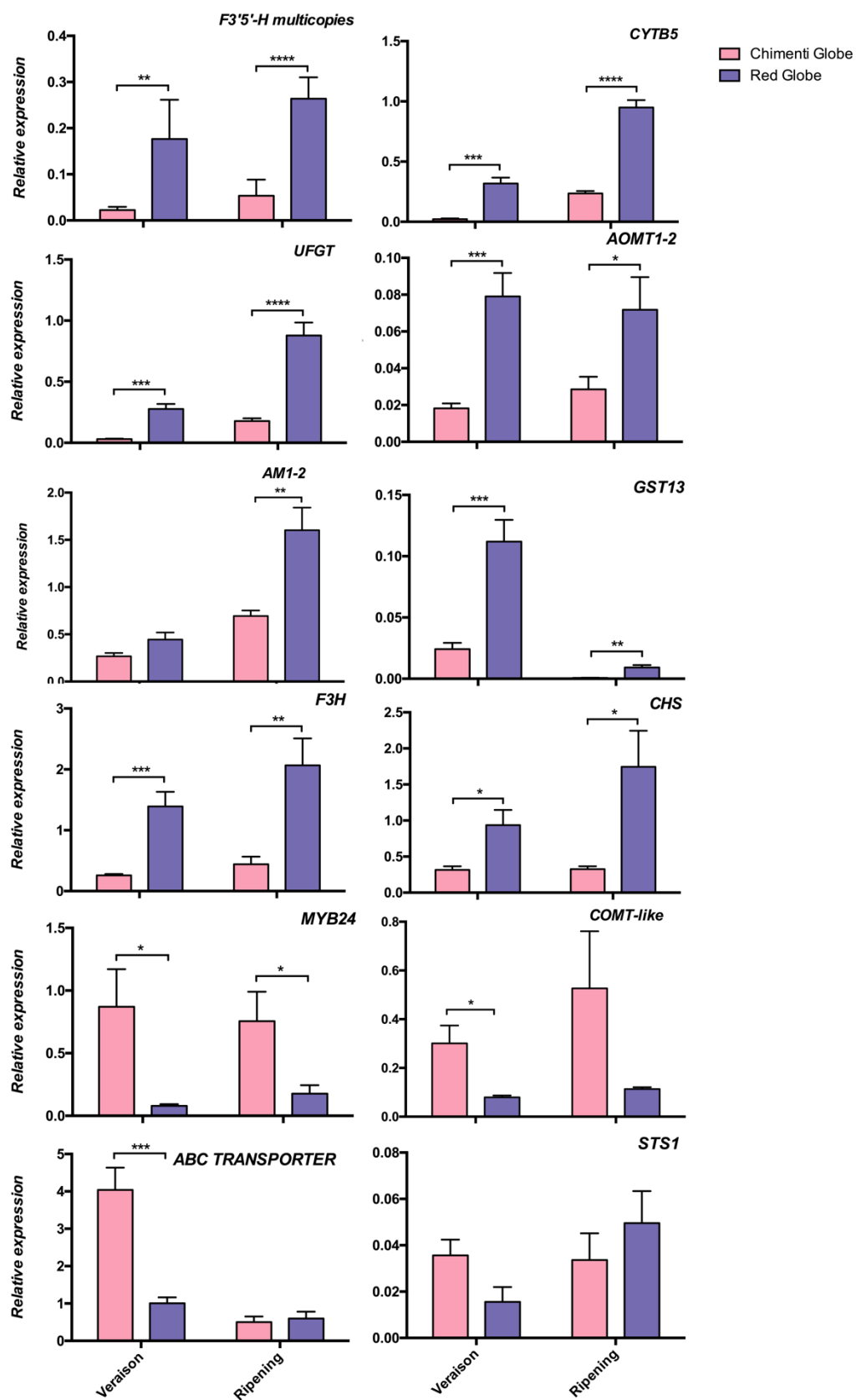
432 **Validation of the RNA-seq gene expression profiles.**

433 Twelve DEGs were selected for qRT-PCR validation (FIGURE 7). From clusters of  
434 down-regulated genes (clusters A, B and C) nine genes were selected: nine isoforms of *F3’5’-*  
435 *H*, *CYTB5*, *UFGT*, *AOMT1-2*, *AMI-2*, *GST13*, *F3H* and *CHS*. Seven of these genes (*F3’5’-H*,  
436 *CYTB5*, *UFGT*, *AOMT1-2*, *GST13*, *F3-H* and *CHS*) showed significant differences between cv.  
437 ‘CG’ and cv. ‘RG’ at veraison and ripening, fully supporting the RNA-seq results, while *AMI-*  
438 *2* only showed differences at the ripening stage (FIGURE 7).

439

440

441 **FIGURE 7 (next page): Real-time quantitative PCR (qRT-PCR) validation of selected**  
442 **DEGs in berry skins of CG and RG.** Samples were collected at veraison and ripening stages  
443 in 2013. Genes were selected based in their role or position in the repression, induction or  
444 combined repression/induction clusters. Gene expression in berries is shown relative to *SRP60*  
445 (VIT\_205s0077g02060) housekeeping gene expression. Values represent the mean of six  
446 biological replicates, and bars represent the standard error of mean. These were derived from  
447 one qPCR run with duplicate PCR reactions on each of the biological replicates. Gene  
448 expressions were normalized together for both developmental stages. Asterisks indicate  
449 statistical significance as the result of unpaired T-test between both somatic variants,  
450 independently taken at each developmental stage (\*,  $P < 0.0465$ ; \*\*,  $P < 0.0053$ ; \*\*\*,  $P <$   
451  $0.0009$ ; \*\*\*\*,  $P < 0.0001$ ). FW, Fresh Weight.



453 To gain more insight in gene expression of the nine isoforms of *F3'5'-H* we were able  
454 to design specific primers for six of them. In general, we observed significant differences in  
455 each isoform (i.e. induction in cv. 'RG' compared to cv. 'CG') at both stages (FIGURE S6B).  
456 We confirmed this observation by inspecting the specifically-assigned reads in the RNA-seq  
457 analysis (TABLE S7). From the Cluster of induced genes (Cluster E), three were further  
458 selected, validating *MYB24*'s significant induction in both veraison and ripening stages, while  
459 an *ABC transporter family protein* (VIT\_214s0060g00720) and two *COMT-like* genes  
460 (designed primers recognized both VIT\_215s0048g02480 and VIT\_215s0048g02490) were  
461 induced in cv. 'CG' only at veraison (FIGURE 6). The *STS1* gene, that codified a Stilbene  
462 synthase was selected from clusters D, exhibiting a higher expression at ripening and a lower  
463 one at veraison in cv. 'CG' compared to cv. 'RG', but without significant differences.

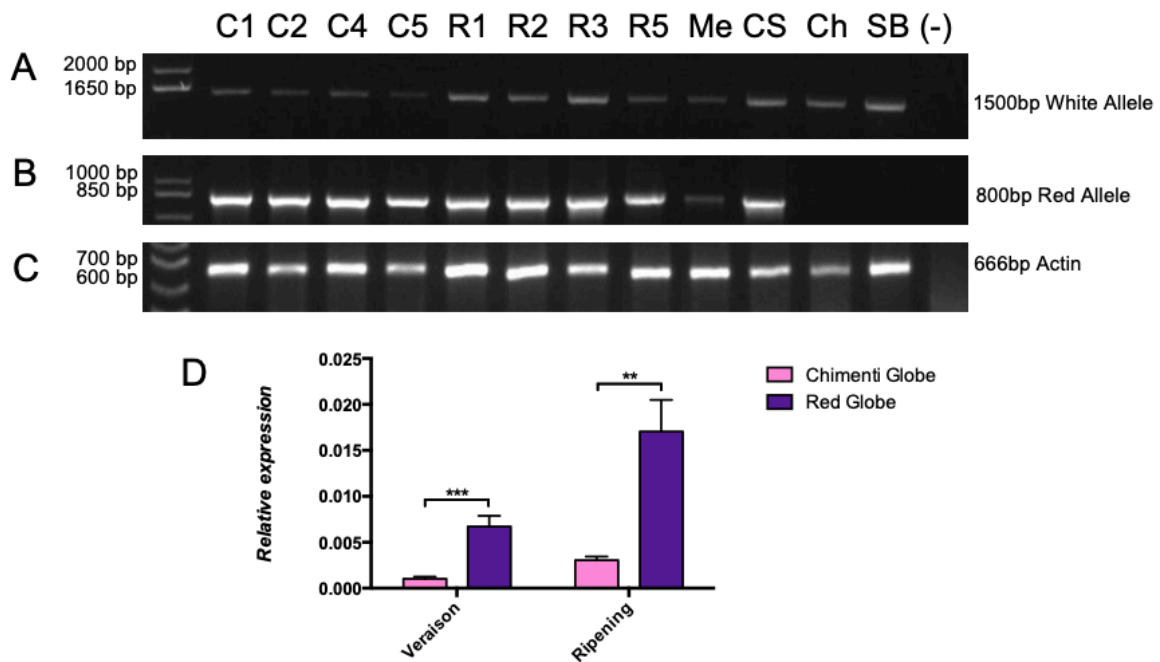
464 We observed that within the list of genes belonging to the repression cluster on the  
465 heatmap generated (FIGURE 6) there were 11 copies of the gene *F3'5'-H* not induced in cv.  
466 'CG': VIT\_206s0009g02805, VIT\_206s0009g02810, VIT\_206s0009g02840,  
467 VIT\_206s0009g02860, VIT\_206s0009g02873, VIT\_206s0009g02880, VIT\_206s0009g02920,  
468 VIT\_206s0009g02970 and VIT\_206s0009g03010. These genes were located closely in the  
469 same region of chromosome 6 (FIGURE S6A). This corresponded to a segment of the  
470 chromosome that span from approximately 15,660 to 16,060 MB.

471

### 472 **Exploration of the MYBA color locus identifies MYBA1 as the only gene responsible for** 473 **the skin color differences.**

474 In order to try to elucidate the genetic origin of the 'Chimenti Globe' phenotype, we  
475 checked the presence of MYBA1 white and red alleles in cv. 'CG' and cv. 'RG' using the same  
476 set of primers used in (Shimazaki et al., 2011), while also studied *MYBA1* expression levels by  
477 qPCR. We observed the presence of white and red alleles of *MYBA1* in all cv. 'CG' and cv.  
478 'RG' samples tested (FIGURE 8), demonstrating that both cultivars are heterozygous for this  
479 gene, at least in the L2 cell layer. These results were validated with molecular marker analysis  
480 (TABLE S8), which in addition revealed a homozygous null allele configuration of MYBA2, in  
481 both L1 and L2 cell layers, in the two somatic variants. We further confirmed the null allele of  
482 MYBA2 in >99% samples (both cultivars) by inspecting all the reads matching the G/T  
483 positions related to MYBA2 mutation (i.e. variant calling analysis; TABLE S9). As MYBA2 is

484 non-functional in both cultivars, only the differences in *MYBA1* expression could account for  
485 the skin pigmentation differences of ‘Chimenti Globe’. We tested *MYBA1* expression by qRT-  
486 PCR, observing that it was down-regulated at veraison (RNA-seq log<sub>2</sub>FC -0,947) and ripening  
487 (confirmed only by qRT-PCR) in cv. ‘CG’ (FIGURE 8).  
488



489  
490

491 **FIGURE 8: Heterozygous *VviMYBA1* gene allelic configuration in both somatic variants**  
492 **and reduced *MYBA1* expression in cv. ‘Chimenti Globe’ compared to cv. ‘Red Globe’.** (A-  
493 B) PCR identification of the (A) non-functional white allele and (B) functional red allele in cv.  
494 ‘Chimenti Globe’ (C1,2,3), cv. ‘Red Globe’ (R1,2,3), and other cultivars used as controls (cv.  
495 ‘Merlot’ -Me, cv. ‘Cabernet Sauvignon’ -CS, cv. ‘Chardonnay’ -Ch and cv. ‘Sauvignon Blanc’  
496 -SB); (C) *ACTIN* gene amplification was used as an internal PCR control check for gDNA  
497 integrity. Numbers on the left side of each electrophoresis gel photograph indicate the band size  
498 according to the DNA marker ladder, while numbers on the right show the exact size of the  
499 white and red alleles according to (Shimazaki, Fujita, Kobayashi, & Suzuki, 2011). (D) qRT-  
500 PCR analysis of *VviMYBA1* in berry skins. Relative gene expression calculation as the mean of  
501 six biological replicates, with error bars representing the standard error of the mean. Asterisks  
502 indicate the result of unpaired T-test. \*\*,  $P = 0.0023$ ; \*\*\*,  $P = 0.0007$ .

503 **DISCUSSION**

504 **Anthocyanin accumulation in the berry skin of cv. ‘Chimenti Globe’ is diminished in**  
505 **correlation to the repression of both *MYBA1* and the flavonoid/anthocyanin pathways.**

506 Grape somatic variants have been well described in the literature and specifically those  
507 affecting skin pigmentation have become a source of new commercial genotypes (Azuma, 2018;  
508 Pelsy, 2010; Torregrosa et al., 2011; Vezzulli et al., 2012; Walker et al., 2006; Carbonell-  
509 Bejerano et al., 2017). In this work, we studied cv. ‘Chimenti Globe’ (‘CG’) that was originated  
510 as a somatic variation of cv. ‘Red Globe’ (‘RG’). Microscopic analysis showed in ripe berries  
511 that cv. ‘CG’ had a particular pattern of anthocyanin accumulation, exclusive to the epidermis.  
512 In contrast, ‘RG’ also presented pigmentation in the sub-epidermis. This pattern was similarly  
513 described in cv. ‘Malian’ (Walker et al., 2006) and cv. ‘Pinot Gris’ (Vezzulli et al., 2012). The  
514 microscopy analysis allowed to suggest initially that cv. ‘CG’ was a periclinal chimera with  
515 only the L1 cell layer capable of producing anthocyanins. However, the molecular marker  
516 analysis showed that ‘CG’ was heterozygous for the functional *MYBA1* allele in the L2. Under  
517 this configuration, *MYBA1* is still active and transcriptionally regulates the accumulation of  
518 anthocyanins in vacuoles (Ford, Boss, & Hoj, 1998). In the case of pink-skinned cultivars of  
519 oriental origin, Shimazaki et al. (2011) reported a 33bp insertion in the second intron of  
520 *VviMYBA1* as a consequence of low expression levels of *VviMYBA1*. Thus, it would be  
521 necessary to sequence *MYBA1*’s genomic region in both L1 and L2 derived tissues and see if  
522 there is any change that may account for *MYBA1* lower expression in ‘CG’.

523 The lower *VviMYBA1* expression, together with the particular pattern of anthocyanin  
524 accumulation in cv. ‘CG’ observed by microscopy i.e. absence of anthocyanin vacuolar  
525 inclusions in subepidermal cells, allows to suggest anthocyanin transport as a possible process



526 being largely affected and responsible for diminished anthocyanin accumulation. In plant cells,  
527 anthocyanins are synthesized in the cytoplasmatic face of the endoplasmic reticulum (Winkel-  
528 Shirley, 2001; Winkel, 2004) and then are transported to the vacuole for storage. Three different  
529 mechanisms have been described for flavonoid transport: membrane transporters, vesicle  
530 trafficking and glutathione S-transferase (GST)- mediated transport (Zhao, 2015). In grapevine,  
531 orthologs of TT12 have been identified, an Arabidopsis gene encoding a MATE protein required  
532 for anthocyanin and proanthocyanidin accumulation in the vacuole (Marinova et al., 2007). In  
533 addition, AM1 and AM3 transport acylated anthocyanins in the presence of Mg ATP (C. Gomez  
534 et al., 2009); and *VviABCCI* transports malvidin 3-O-glucoside into vacuoles (Francisco et al.,  
535 2013). The glutathione-S-transferases *VviGST1*, *VviGST3* and *VviGST4*, expressed in grape  
536 fruits, have the ability to bind different flavonoid ligands (Pérez-Díaz, Madrid-Espinoza,  
537 Salinas-Cornejo, González-Villanueva, & Ruiz-Lara, 2016). From the 109 differentially  
538 expressed genes from our study, we observed several genes related to anthocyanin vacuolar  
539 transportation being repressed in cv. ‘CG’: *GST* and two *anthoMATE* (AM1 and AM2) genes.  
540 Interestingly, *VviGST4* and *VviAM2* are specifically highly expressed at berry maturity (Sun et  
541 al., 2016). Other genes down-regulated in cv. ‘CG’ were *ANMI*, encoding a putative  
542 anthocyanin membrane protein 1, and *3AT* related with anthocyanin acylation, a process known  
543 to improve anthocyanin intensity and color stabilization (Yonekura-Sakakibara, Nakayama,  
544 Yamazaki, & Saito, 2008). All these genes are directly regulated by MYBA1 and in less  
545 intensity by its homologues MYBA6 and MYBA7 (Matus et al., 2017)  
546  
547

548 **Metabolic changes during veraison and ripening stages in cv. ‘Red Globe’ and cv.**  
549 **‘Chimenti Globe’.**

550 Berry ripening is an active process that involves regulation of global gene expression  
551 (Fasoli et al., 2012) and changes in the accumulation of primary and secondary metabolites  
552 (Boss, Davies, & Robinson, 1996; Conde et al., 2007). Primary metabolites and in particular  
553 sugars are indeed elicitors of anthocyanin synthesis, reason why we decided to analyze them in  
554 this study. Metabolic comparison between the two somatic variants showed that the  
555 developmental stage was the primary discriminant parameter, despite the use of different  
556 cultivars. This observation is concordant with previous published studies (D.-L. Guo et al.,  
557 2016; Massonnet et al., 2017; D. C. J. Wong, Lopez Gutierrez, Dimopoulos, Gambetta, &  
558 Castellarin, 2016; Wu et al., 2014) in which, despite the use of different cultivars, the importance  
559 of developmental stages appeared to overrule cultivar origin. T6P seems a main influential  
560 metabolite (as seen in PC1, FIGURE 3A). T6P is a sugar phosphate considered as a metabolic  
561 signaling molecule in plants that regulates developmental growth (John Edward Lunn, Delorge,  
562 Figueroa, Van Dijck, & Stitt, 2014) and was demonstrated to be involved in sugar signaling in  
563 *Arabidopsis thaliana* (John E. Lunn et al., 2006). In *Vitis vinifera* there is no clear evidence of  
564 control of T6P during berry development, although several transcriptomic studies have shown  
565 that orthologue genes encoding T6P synthase, T6P synthase/phosphatase and SnRK1 (Sucrose-  
566 non-fermentative Related Kinase 1, i.e. another sugar signaling pathway component) are tightly  
567 regulated during berry development (Deluc et al., 2007; Gambetta, Matthews, Shaghasi,  
568 McElrone, & Castellarin, 2010). Although no significant differences were observed at the level  
569 of quantification of T6P, the analysis obtained from the RNA-seq analysis data (DEG analysis

570 without Fold Change filter) showed the down-regulation of four genes encoding trehalose -  
571 phosphate synthase in cv. ‘CG’ during ripening).

572 Due to the fact that T6P modulates sugar signaling, we searched for DEGs between cv.  
573 ‘RG’ and cv. ‘CG’ related with sugar, fructose, glucose and sucrose biosynthesis in our RNA-  
574 seq analysis. We identified three genes at veraison, *VviSWEET1* (VIT\_218s0001g15330),  
575 *VviSWEET10* (VIT\_217s0000g00830) and *VviSWEET15* (VIT\_201s0146g00260) and in  
576 ripening *VviSWEET11* (VIT\_207s0104g01340), as induced in cv. ‘CG’. These constitute a  
577 protein family of sugar uniporters involved in sugar export (Chen et al., 2010; Chong et al.,  
578 2014; W.-J. Guo et al., 2014). Indeed, it has been described that several SWEET proteins in *V.*  
579 *vinifera* increase from veraison onwards in cv. ‘Syrah’ and cv. ‘Muscatel Ottonel’ (Chong et al.,  
580 2014). Despite these genes are less expressed in green berries, *VviSWEET1* is the mainly  
581 expressed in young and adult leaves; *VviSWEET10* and *VviSWEET11* in flowers; *VviSWEET10*,  
582 *VviSWEET11* and *VviSWEET15* in berries after veraison (Chong et al., 2014).

583 We found other genes related directly with sucrose biosynthesis induced in cv. ‘CG’: two  
584 fructose-bisphosphate aldolases (VIT\_203s0038g00670 and VIT\_204s0023g03010) were  
585 induced during veraison and ripening stage. This enzyme catalyzes the conversion of  
586 glyceraldehyde 3-phosphate (PGAL) to fructose 1,6- bisphosphate (F1,6-BP). Concordantly, we  
587 also found that these metabolites were decreased in cv. ‘CG’ during ripening stage in 2013, and  
588 in fact was one of the metabolites that explained the differences observed in cultivars (PC2 ,  
589 FIGURE 3A). A similar pattern was observed with fructose-1,6-bisphosphatase  
590 (VIT\_208s0007g01570), induced in cv. ‘CG’. This enzyme catalyzes the conversion of F1,6-  
591 BP to fructose 6-phosphate (F6-P), which was found decreased in cv. ‘CG’ at ripening. The  
592 sucrose-phosphate synthases encoded by VIT\_218s0075g00330 and VIT\_218s0089g00490

593 catalyze the conversion of uridine diphosphate-glucose (UDP-glucose) to glucose-fructose-  
594 phosphate (GF-P), leading to the last step of sucrose biosynthesis (Ruan, 2014).

595 Taking all these observations together, it is possible to suggest an alteration in cv. ‘CG’  
596 occurring at the level of sugar export and in sucrose biosynthesis. This process could be related  
597 to the anthocyanin biosynthesis pathway in cv. ‘CG’ as anthocyanin accumulation in plants can  
598 be induced by sugar (Das, Shin, Choi, & Park, 2012). Specifically in grapevines, fructose,  
599 glucose and sucrose induce anthocyanin accumulation (Dai et al., 2013; Larronde et al., 1998;  
600 Zheng et al., 2009).

601 Among primary metabolites occurring in the intersection with secondary metabolism, we  
602 found shikimate as significant. This is an intermediary of the shikimate pathway that mediates  
603 the carbon flow from carbohydrate metabolism to phenylpropanoid and aromatic compound  
604 biosynthesis (Maeda & Dudareva, 2012; Zhang et al., 2012). This strongly suggest the  
605 importance of this metabolite in species such as grapevine, in which the berry consumes  
606 phenylalanine for the biosynthesis of flavonoid compounds such as anthocyanins.

607 Anthocyanins explained major differences in the PC analysis of metabolites. We observed  
608 that only traces of malvidin, petunidin and delphinidin were detected in cv. ‘CG’, indicating a  
609 block in the tri-hydroxylated branch of the anthocyanin biosynthesis pathway, an observation  
610 latter confirmed by the RNA-seq analysis. Accordingly, an increase in F3’5’-H activity has been  
611 previously reported to explain higher concentration of tri-hydroxylated anthocyanins in  
612 Cabernet Sauvignon and Shiraz, pinpointing F3’5’-Hs as key molecular players driving the  
613 phenylpropanoid metabolic flux towards bluish anthocyanin production (Degu et al., 2014).

614

615

## 616 **Transcriptomic changes during veraison and ripening in cv. ‘CG’ and cv. ‘RG’**

617 In our study, besides metabolic studies, the skin transcriptomes of both somatic variants  
618 were analyzed using Illumina Hiseq2000. We report that several genes related to flavonoid  
619 pathway, grouped in three main clusters, were differentially expressed in cv. ‘CG’ compared to  
620 cv. ‘RG’. Among these, we identified eleven *F3’5’-H* isoforms repressed in cv. ‘CG’, consistent  
621 with the depletion of delphinidin-type anthocyanins. The adjacent location of each one of these  
622 copies in a single cluster within chromosome 6 (FIGURE S6A; Castellarin et al., 2006;  
623 Falginella et al., 2010) allows to suggest a common regulation for all of them, presumably by  
624 MYBA1 and other TFs. *CYTB5* was down-regulated in cv. ‘CG’ at both stages. *CYBT5* has been  
625 previously involved in F3’5’-H activity modulation in Petunia flowers, catalyzing the transfer  
626 of electrons to their prosthetic heme group (de Vetten et al., 1999). In 2006, (Jochen Bogs,  
627 Ebadi, McDavid, & Robinson, 2006) demonstrated that a putative *CYBT5* from grapevine cv.  
628 ‘Shiraz’ modulated both F3’5’-H and F3’-H activities even though the exact mechanism remains  
629 to be deciphered. This is of interest because this suggests a possible link regarding the drastic  
630 down-regulation of eleven gene copies of F3’5’-H.

631 Three *Stilbene synthase* genes (*STS1*, *STS3* and *STS5*), involved in resveratrol  
632 biosynthesis (Dubrovina & Kiselev, 2017; Kiselev, Aleynova, Grigorchuk, & Dubrovina, 2016)  
633 were induced in cv. ‘CG’ at veraison and down-regulated during ripening stage. MYB15 is  
634 involved in the transcriptional regulation of stilbene synthases in grapevine (Holl et al., 2013)  
635 and was also induced in cv. ‘CG’. This suggests a possible compensation point in flavonoid  
636 metabolism: CHS utilizes the same substrates as STS but, is responsible for flavonoid-type  
637 compound formation. Chalcone synthase (*CHS*) was indeed down-regulated in cv. ‘CG’, in  
638 correlation with less total anthocyanins. Thus, STS could act as an escape valve to reflux the

639 exceeded carbon after the decrease in CHS activity. Taken together, this would suggest a  
640 multilevel alteration of the flavonoid pathway, and not only of its anthocyanin tri-hydroxylated  
641 branch in cv. ‘CG’.

642

643 **The berry color locus shows similar allelic configurations in both somatic variants.**

644 White skinned grape cultivars that do not produce anthocyanins contain a *MYBA1* null  
645 allele in homozygosity, in which the presence of the retrotransposon *Gret1* in the  
646 promoter/5’UTR region interrupts the normal transcription (Kobayashi et al., 2004). Pigmented  
647 cultivars possess at least one functional ‘red berry’ allele either without *Gret1* or with the  
648 excised retrotransposon (This et al., 2007). We observed the presence of white and red alleles  
649 of *MYBA1* in all cv. ‘CG’ and cv. ‘RG’ samples tested, thus, it seems that the allelic  
650 configuration of *MYBA1* is not responsible for the phenotype differences. Despite this, the  
651 down-regulation of flavonoid and anthocyanin structural genes in cv. ‘CG’ is only possibly  
652 related to MYBA1, as MYBA2 appears non-functional in all meristematic cell layers and  
653 cultivars. The significant differences of *MYBA1* expression between both cultivars at veraison  
654 and ripening should be the responsible of the changes in anthocyanin abundance (including the  
655 changes in di/tri-hydroxylated anthocyanin profiles). The expression of *MYBA1* in ‘Chimenti’  
656 is decreased by a different process not identified here.

657 **AUTHOR CONTRIBUTIONS**

658 EG, PAJ, SD and JTM designed experiments. CS performed material sampling, RNA  
659 extraction, for deep sequencing and qRT-PCR, gathered all the results and wrote the manuscript.  
660 TM and CM conducted the bioinformatics analyses and data mining. JL and RF carried out the  
661 LC-MS/MS metabolic analysis. GH and CR assisted in hexose, anthocyanin and organic acids  
662 quantifications. LM assisted in RNA extraction and material sampling collection. RA and DC  
663 performed molecular marker analysis. JTM reanalyzed RNA-seq data, produced PCA plots,  
664 edited figures and together with FMN, EG, PAJ and SD revised the manuscript.

665

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