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RESEARCH PAPER

Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*

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Abstract

Anthocyanins, flavan-3-ols, and flavonols are the three major classes of flavonoid compounds found in grape berry tissues. Several viticultural practices increase flavonoid content in the fruit, but the underlying genetic mechanisms responsible for these changes have not been completely deciphered. The impact of post-veraison sunlight exposure on anthocyanin and flavonol accumulation in grape berry skin and its relation to the expression of different transcriptional regulators known to be involved in flavonoid synthesis was studied. Treatments consisting of removing or moving aside the basal leaves which shade berry clusters were applied. Shading did not affect sugar accumulation or gene expression of *HEXOSE TRANSPORTER 1,* although in the leaf removal treatment, these events were retarded during the first weeks of ripening. Flavonols were the most drastically reduced flavonoids following shading and leaf removal treatments, related to the reduced expression of *FLAVONOL SYNTHASE 4* and its putative transcriptional regulator *MYB12*. Anthocyanin accumulation and the expression of *CHS2*, *LDOX*, *OMT*, *UFGT, MYBA1*, and *MYB5a* genes were also affected. Other regulatory genes were less affected or not affected at all by these treatments. Non-transcriptional control mechanisms for flavonoid synthesis are also suggested, especially during the initial stages of ripening. Although berries from the leaf removal treatment received more light than shaded fruits, malvidin-3-glucoside and total flavonol content was reduced compared with the treatment without leaf removal. This work reveals that flavonol-related gene expression responds rapidly to field changes in light levels, as shown by the treatment in which shaded fruits were exposed to light in the late stages of ripening. Taken together, this study establishes MYB-specific responsiveness for the effect of sun exposure and sugar transport on flavonoid synthesis.

Key words: bHLH, flavonoids, grape, leaf removal, MYB12, PAR, sugar, source, sink, WDR.

Introduction

Grapes (*Vitis vinifera* L.), both for fresh and wine consumption, are an important source of flavonoids, including anthocyanins, flavonols, and flavan-3-ols. These molecules are particularly relevant in this fruit species since they define colour (Somers and Evans, 1974) and affect taste (Baxter *et al.*, 1997; Vidal *et al.*, 2003; Hufnagel and Hofmann, 2008). In addition, they possess a high antioxidant capacity and contribute to protection against cardiovascular diseases and cancer (reviewed by Lin and Weng, 2006) when consumed as part of a Mediterranean diet. In order to increase berry flavonoid content in the vineyard, it is fundamental to understand the biosynthesis of these molecules and how this is affected by the environment and different viticultural practices.

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Flavonoid biosynthesis is derived from the phenylpropanoid pathway (Fig. 1), one of the most-characterized secondary metabolic routes in plant systems. Although different groups of proteins are responsible for producing, transporting, and storing flavonoids, the two most-studied classes correspond to the biosynthetic (structural) enzymes

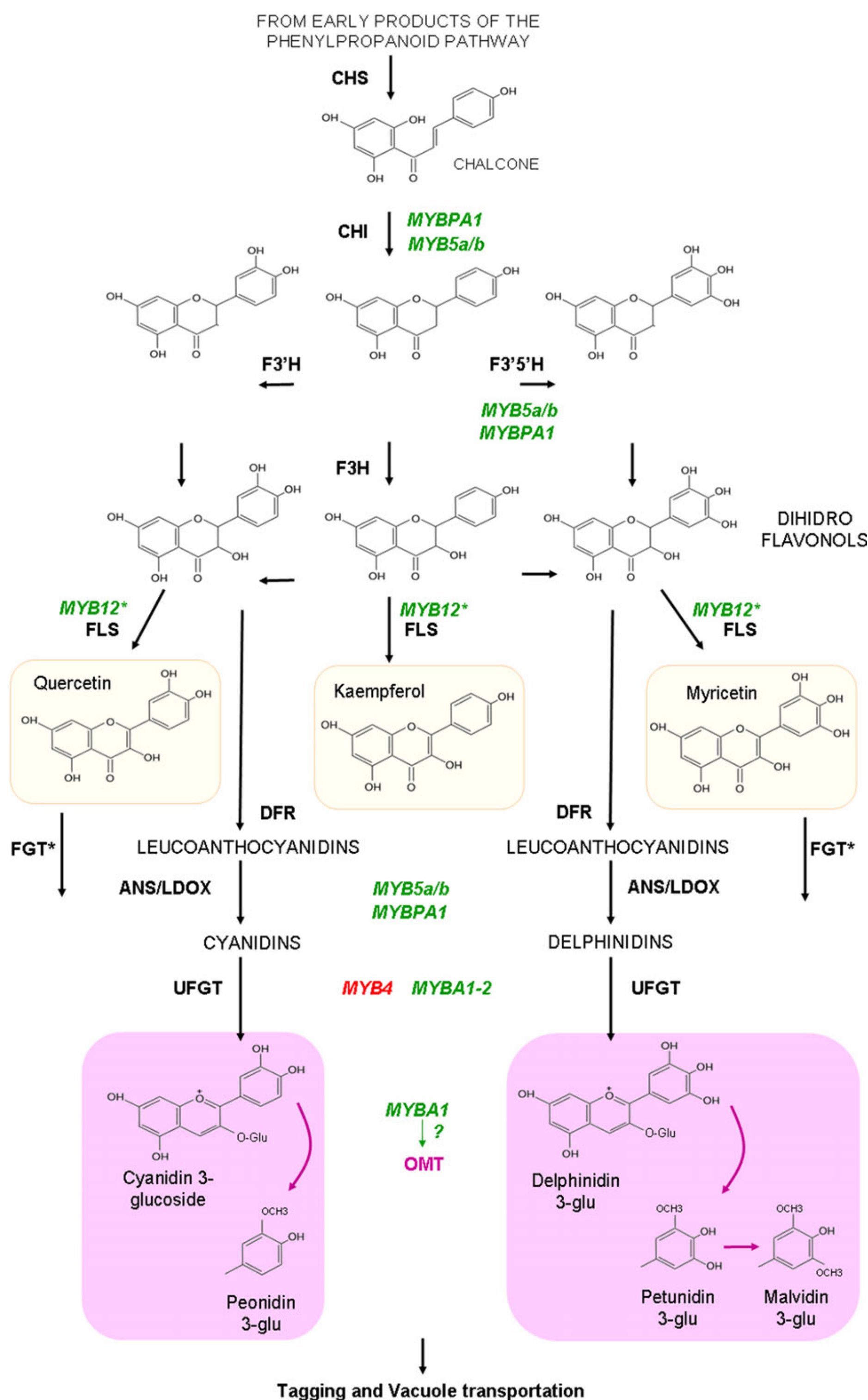

Fig. 1. Simplified overview of flavonol and anthocyanin biosynthesis within the phenylpropanoid pathway and its regulation in grape by characterized *MYB* genes (flavan-3-ols are not shown in this pathway). The repressor MYB4 is shown in red, while all MYB activators are shown in green. Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; F3H/F3′H/F3′5′H, flavonoid hydroxylases; DFR, dihydroflavonol-4-reductase; ANS/LDOX, anthocyanidin synthase/leucoanthocyanidin dioxygenase; UFGT, UDP glucose:flavonoid-3-*O*-glucosyltransferase; FLS, flavonol synthase; and OMT, *O*-methyltransferase.

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and the transcription factors controlling the pathway. Flavonoid transcriptional regulators have been extensively studied in plant species such as maize, petunia, *Arabidopsis* and, lately, in grapes and apples. From all the possible transcription factors responsible for controlling the pathway, R2R3 MYB, β helix-loop-helix (bHLH), and trypthophan-aspartic acid repeat (WDR) proteins have been the most-extensively analysed (Payne *et al.*, 2000; Baudry *et al.*, 2004).

In grapes, some MYB genes have been shown to be involved in flavonoid metabolism (Fig. 1). In particular, many of the white grape cultivars present in the world today arose from multiallelic mutations of the MYBA1 and MYBA2 genes (Kobayashi *et al.*, 2004; Lijavetzky *et al.*, 2006; Walker *et al.*, 2006, 2007; This *et al.*, 2007), which control the last biosynthetic step of anthocyanin synthesis, a glycosylation reaction mediated by the UDP-GLUCOSE FLAVONOID 3-*O*-GLUCOSYLTRANSFERASE (UFGT) enzyme (Kobayashi *et al.*, 2002). MYB5a (MYBCS-1; Deluc *et al.*, 2006), MYB5b (Deluc *et al.*, 2008), MYBPA1 (Bogs *et al.*, 2007) and MYBPA2 (Terrier et al., 2009) appear to regulate general branches of the pathway (Fig. 1), together with flavan-3-ol synthesis. In grapes, an AtMYB4 homologue (Genbank accession EF113078), found to be a repressor of *UFGT* (JT Matus *et al.*, unpublished results) was recently isolated and characterized. A putative flavonol-related MYB transcription factor (MYB12; Genbank accession FJ418175) was also found while performing a genome-wide analysis of grape MYB members (Matus *et al.*, 2008). Co-activators belonging to other transcription factor families (bHLH and WDR) have also been isolated recently (JT Matus *et al.*, unpublished results). From all these genes in grape, only *MYBA* genes have been studied in terms of their modulation by light and hormonal factors (Jeong *et al.*, 2004), as well as by temperature (Mori *et al.*, 2007).

The impact of environmental factors and viticultural practices on the flavonoid content and composition of grape berries has been widely studied in diverse wine-producing regions (reviewed by Downey *et al.*, 2006). Light and all those practices which promote its incidence on berries throughout grape ripening, significantly increase the accumulation of flavonoids (Cortell and Kennedy, 2006) and the expression of their biosynthetic genes (Downey *et al.*, 2004; Jeong *et al.*, 2004). Light-induced flavonoid synthesis requires changes in gene expression mediated by three major classes of photoreceptors: phytochromes, blue/UV-A light receptors, and UV-B light receptors (reviewed by Argüelo-Astorga and Herrera-Estrella, 1998). The best-characterized light receptors in plants are phytochromes (PHY; Quail, 1994). These receptors are able to promote gene expression by three different signal transduction pathways. Of these, the cyclic GMP (cGMP)-mediated pathway regulates genes such as those involved in anthocyanin biosynthesis and *CHALCONE SYNTHASE* (*CHS*) was the first gene shown to be dependent on PHY-cGMP signalling (Bowler *et al.*, 1994; Millar *et al.*, 1994; Christie and Jenkins, 1996).

In addition to light, temperature also influences flavonoid production, although in a negative manner. Mori *et al.* (2007) demonstrated that high temperature increases anthocyanin degradation in grape skin, together with a decrease in expression of flavonoid biosynthetic and *MYBA* genes. On the other hand, low temperature increases anthocyanin production, as has been observed in grape (Mori *et al.*, 2005; Yamane *et al.*, 2006) and other crop species (maize: Christie *et al.*, 1994; red orange: Lo Piero *et al.*, 2005; apple: Ubi *et al.*, 2006).

Field practices which control vegetative growth of grapevines directly affect the incidence of light on grape clusters. These include shoot, tip, and leaf removal. Leaf removal is generally recommended between the onset of ripening (veraison) and harvest, eliminating approximately one-third of the basal leaves. This practice is applied with the purpose of balancing foliage vigour relative to fruit production, increasing exposure of clusters to sunlight, facilitating ventilation, and diminishing the incidence of fungal diseases. Considering the fact that photosynthetic activity of basal leaves is lower than that of intermediate and apical leaves after berries begin to soften (Hunter and Visser, 1989), post-veraison leaf removal should increase light incidence on the clusters, without significantly affecting the photosynthetic activity of the whole plant. From this period onwards, sugars transported into grapes originate mainly from intermediate and apical leaves (Hunter and Visser, 1989). However, the effect of the time after veraison at which this practice is applied could have a substantial impact on grape physiology, since basal leaves lose their photosynthetic activity gradually during the ripening period.

In addition to this unresolved issue, there are no studies which correlate the changes observed in the content of grape flavonoids under different environmental conditions, with the expression of regulatory genes other than *MYBA* genes. In this work, the relationship between the expression of members of different transcription factor families and their target genes and flavonoid synthesis was studied under different post-veraison light exposure and leaf removal treatments.

Materials and methods

Experimental design and berry sampling throughout ripening

Different treatments were applied to a commercial Cabernet Sauvignon field at veraison (onset of ripening), located in the Maipo Valley, Chile (33°36' S, 70°39' W), during the 2006/2007 growing season. The vines were 10-year-old, drip-irrigated, and grown on their own roots using a bilateral cordon with a vertical shoot positioning trellis system in a north–south row orientation. Plants possessed a medium vigour, as classified by morpho-physiological measurements described in Peña-Neira *et al.* (2004). Normal commercial irrigation was homogenously applied at 66% potential evapo-transpiration. Nitrogen fertilization was applied during flowering at 66 kg ha^{-1} and plants were pruned before bud burst leaving two-node spurs per metre.

Veraison was determined as the time at which clusters were 30–50% coloured and sugar concentration reached *c.*

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5° Brix (5% w/w soluble solids). At this stage, treatments T1 (light exposed) and T4 (leaf removal) were imposed in order to increase the sunlight exposure of grape clusters (Fig. 2A). In T1, the basal leaves shading each cluster were moved into a different position by the use of nylon zip-ties (see Supplementary Fig. S1 at *JXB* online), while in T4, 80% of the basal leaves (those from the first third of each shoot with clusters) were removed. Leaf moving in T2 (delayed sunlight exposure) was applied during the sixth week after veraison (Fig. 2B), while clusters from T3 (shaded cluster) were untreated.

Grape clusters from the east side of each experimental row (exposed to sunlight during the morning until midday) were treated and sampled. In each biological replicate (row), ten grape clusters from six plants were used for treatments T1, T2, and T3. Treatments were imposed altogether in each row. A contiguous plant to each row was exclusively used for the T4 (leaf removal) treatment. A total of 60 berries were sampled weekly from ten grape clusters for 8 weeks after veraison. Weeks 2, 4, 6, and 8 were considered for HPLC analysis while weeks 1, 3, 5, and 7 were for RNA extraction and gene expression quantification by real-time PCR (Fig. 2A). Berries were immediately peeled and deseeded. Berry skins were frozen in liquid nitrogen, and stored at –80 °C until required for RNA extraction.

By the ninth week after veraison (technical maturity or commercial harvest), clusters which hadn't been sampled but were treated, were used for physical and chemical analyses. The weight of 200 berry skins, and the pH and soluble solid content of berry juice were recorded, the latter being determined by means of a temperature compensated digital refractometer (Atago, Japan).

Flavonoid content analysis

Berry phenolics were extracted as in Venencie *et al.* (1997), with modifications. Berry skin samples (n=60) were weighed and ground with 15 ml distilled water, 20 ml hydroalcoholic solution (EtOH:H_2O, 10:90 v/v) and 2.5 g tartaric acid, adjusting the final solution weight to 100 g. Extracts were macerated for 2 h at 30 °C by means of an orbital shaker, centrifuged, and filtered through glass microfibre. Samples were filtered through a 0.45 μm membrane under vacuum at <35 °C. A 2 ml aliquot was used to screen absorbance at 520 nm and 280 nm to quantify anthocyanins and phenolic compounds, respectively, as described by García-Barceló (1990). 150 μl of each sample were then injected into the HPLC-DAD for the analysis of anthocyanin compounds (Peña-Neira *et al.*, 2007).

Non-anthocyanin compounds were extracted from an aliquot (50 ml) of macerated and filtered grape skins, by

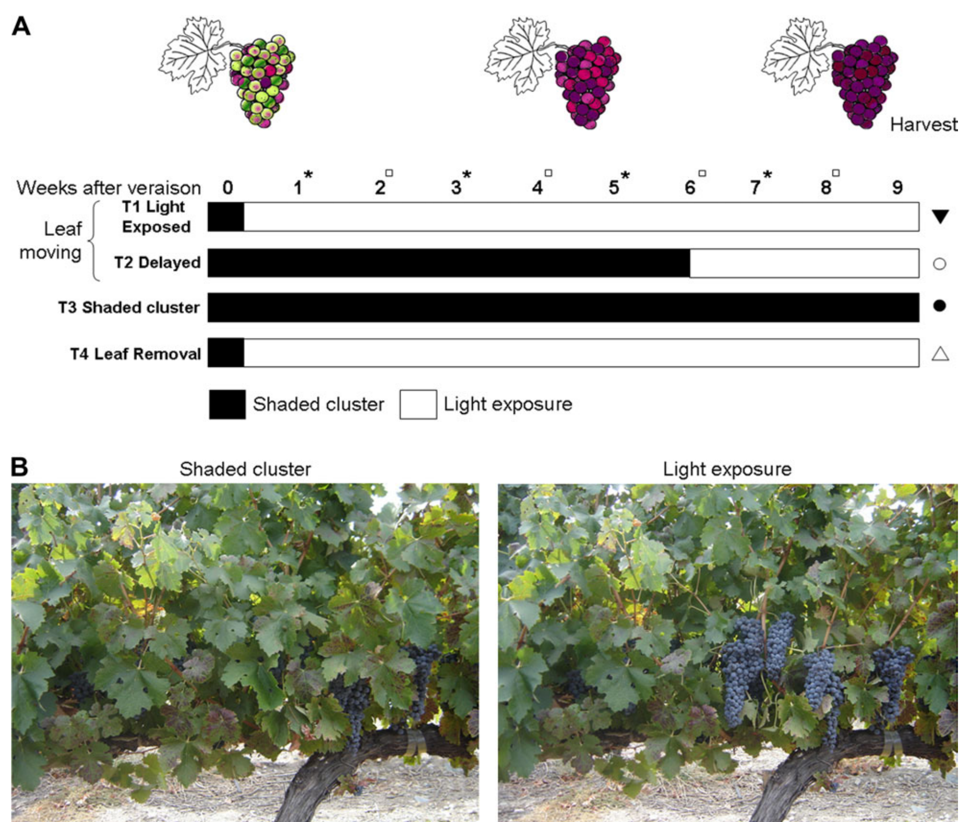

Fig. 2. (A) Experimental design and data sampling for different light exposure and leaf removal treatments. Coloured clusters represent the grape phenologies observed during the different periods of ripening. Even-numbered weeks (squares) were sampled for HPLC flavonoid analysis, while odd-numbered weeks (asterisks) were sampled for RNA extraction and gene expression quantification. Symbols on the right correspond to each treatment as used in Figs 4–7. (B) Field photograph of grapes before and after T3 treatment (leaves were moved aside but not removed).

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mixing the sample three times with 20 ml diethyl ether and 20 ml ethyl acetate. The organic fractions were then combined and extracts were evaporated to dryness under vacuum at <35 °C. The residue was dissolved in 1 ml methanol/water (1:1, v/v), and analysed by HPLC-DAD and HPLC-DAD-MS as described by Peña-Neira *et al.* (2000, 2004). 20 μl of each sample were injected.

The chromatographic system for HPLC-DAD analysis of anthocyanins consisted of an HPLC equipped with a 991 photodiode-array detector (Waters Corp. Milford, MA, USA) using a Chromolith Performance RP-18 (4.6×100 mm) column. The detection was carried out by scanning from 210 to 600 nm. The elution gradient consisted of the following solvents. Solvent A: water; solvent B: water/formic acid (5%, v/v), solvent C: acetonitrile, starting from 0 to 10 min, 77–50% B; 3–30% C; 10–12 min, 100% C at a constant flow of 3 ml min^{-1}. The same liquid chromatography system equipment was used for non-anthocyanin compound analysis. Separation was performed on a reverse-phase Waters Nova-Pack C18 (300×3.9 mm ID) with 4 packing. Two mobile phases were employed for elution. (A) Water/acetic acid (98:2 v/v) and (B) water/acetonitrile/acetic acid (78:20:2 by vol.). The gradient profile was 0–55 min, 100–20% A; 55–70 min, 20–10% A; 70–90 min, 10–0% A. Detection was performed by scanning from 210 to 360 nm with an acquisition speed of 1 s. Samples were analysed in duplicate.

The identification of derivate flavonol and anthocyanin compounds (see Supplementary Fig. S2 at *JXB* online) was carried out by comparison of their spectra and retention time with those obtained by Peña-Neira *et al.* (2004, 2007). The standards were purchased from Apin Chemicals (Abingdon, Oxford, UK), Sigma Chemicals (Poole, Dorset, UK), and Merck (Darmstadt, Germany): for flavonols, myricetin-3-*O*-galactoside, myricetin-3-*O*-glucoside, isorhamnetin-3-*O*-galactoside, quercetin-3-galactoside, quercetin-3-rutinoside, quercetin-3-glucoside, quercetin-3-rhamnoside, kaempferol-3-galactoside, and kaempferol-3-glucoside were used. For malvidin-3-glucoside the standard was purchased from Extrasynthése (Lyon, France). Quantitative determinations were performed using the external standard method with commercial standards. The flavonol and anthocyanin calibration curves were obtained at 280 nm and 520 nm, respectively, by injection of different volumes of standard solutions under the same conditions as for the samples analysed. Flavonol glycosides were quantified with the curve of quercetin-3-*O*-glucoside. Anthocyanins were quantified with the curve of malvidin-3-*O*-glucoside.

Nucleic acid extraction and cDNA synthesis

Total RNA was isolated from berry skins according to the procedure of Reid *et al.* (2006), using a CTAB-Spermidine extraction buffer. For cDNA synthesis, one μg of total RNA was reverse transcribed with random hexamer primers in an 18 μl reaction mixture using the StrataScript® reverse transcriptase (Statagene, USA) according to the manufacturer's instructions.

Quantitative comparison of gene expression throughout berry skin development

Relative transcript quantification of isolated genes was performed by real-time RT-PCR, using the Brilliant® SYBR® Green QPCR Master Reagent Kit (Stratagene) and the Mx3000P detection system (Stratagene) as described in the manufacturer's manual. Amplification of a fragment of the *UBIQUITIN1* gene (99 bp; TC53702, TIGR database, VvGi5) was used for normalization (Downey *et al.*, 2003). PCR conditions, standard quantification curves for each gene, primer efficiency values (see Supplementary Table SI at *JXB* online) and relative gene expression calculations were conducted according to Poupin *et al.* (2007). Briefly, standard quantification curves with serial dilutions of PCR products were constructed for each gene to calculate amplification efficiency according to:

$$\left(E = \left[10^{(-1/\text{slope})}\right] - 1\right) \quad (1)$$

This value was then used to obtain an accurate ratio between the expression of the gene of interest (*GOI*) and the housekeeping gene, using Equation (2):

$$\frac{(1+E_{GOI})^{-\Delta Ct}}{\left(1+E_{Ubiquitin}\right)^{-\Delta Ct}} = \frac{(1+E_{GOI})^{-(Ct\ GOI - Ct\ GOI\ calibrated)}}{\left(1+E_{Ubiquitin}\right)^{-(Ct\ Ubi - Ct\ Ubi\ calibrated)}} \quad (2)$$

Gene expression levels were normalized to the expression of the first sample for the full shaded treatment (T4), in order to obtain a calibrated ΔCt for each gene.

In all cases, R^2 values of standard curves were above 90%. *Ct* values for *UBIQUITIN* varied no more than one unit between all samples analysed for each real time experiment. All experiments were performed with three biological replicates and three technical replicates. Reaction specificities were tested with melt gradient dissociation curves, electrophoresis gels, and cloning and sequencing of each PCR product.

Statistical analysis

Flavonoid composition and expression profile data were statistically analysed by two-way ANOVA to test the significance of the effects of treatments at the different stages of berry ripening. Tukey media comparison analysis was performed to compare the treatments at the same berry ripening stage. Statistical differences between means were based on the least significant method when *F* values were significant with $P < 0.05$.

Results and discussion

Cluster light exposure levels have no effect on general chemical parameters at harvest

Vineyard row orientation has a pronounced effect on the photosynthetically active radiation (*PAR*) received by the

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two sides of the rows (Grifoni *et al.*, 2008). In addition, radiation and temperature are different on each side of a north–south oriented row (Pereira *et al.*, 2006). The vineyard used in this study had a north–south orientation and clusters on the eastern side were considered for analysis in this study.

A *PAR* measurement device was set up within the grape bunch, simulating the position of a single berry in the cluster (Fig. 3A). For 10 h, incident *PAR* was recorded every 5 min (Fig. 3B). Photographs of the experimental rows were taken until 14.00 h at 4 WAV (see Supplementary Video S1 at *JXB* online). *PAR* measurements revealed that exposed and shaded clusters received different intensities of radiation (Fig. 3). While exposed fruits were subjected to almost incident *PAR* levels between 11.00 h and 13.00 h (above 1500 μmol m^{-1} s^{-1}), shaded clusters received between 100–400 μmol m^{-1} s^{-1} during the same period of the day (Fig. 3B).

By harvest time (9 WAV), the remaining clusters did not show any differences in skin weight, pH or sugar concentrations in berry juice (Table 1), suggesting that, by the end of ripening, neither shade nor leaf removal affected final sugar or acid content. In addition, cluster morphology and size was not affected by any treatment, except for a possible premature lignification of peduncles in clusters from treatments T1 and T4 (see Supplementary Fig. S3 at *JXB* online).

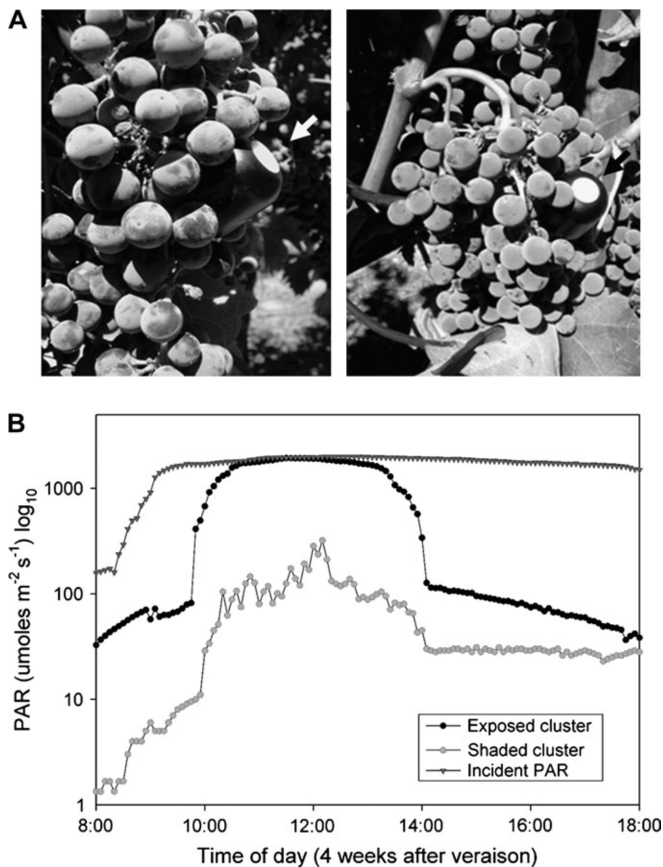

Fig. 3. PAR measurements taken at 4 weeks after veraison for shaded and exposed clusters from the east side of one of the experimental rows. Incident PAR is included. (A) Arrow indicates the position of the PAR meter in each cluster. (B) Daily measurements from 08.00 h to 18.00 h.

Anthocyanin content and genes regulating their synthesis are differentially affected by sunlight exposure and leaf removal

Several reports have shown that anthocyanins and flavonols are directly affected by exposure to sunlight or UV radiation (reviewed by Downey *et al.*, 2006). Light is a fundamental requirement for colour formation in grapes and other fruit crops such as apple. Despite this, differences in the experimental design, the analytical measurements used, the cultivar chosen, the geographical location of the experimental site and many other factors, have produced contradictory results regarding the relationship between anthocyanin content and light in grapes (Hunter *et al.*, 1995; Bergqvist *et al.*, 2001; Spayd *et al.*, 2002; Downey *et al.*, 2004). In addition, no studies have been carried out in which changes in flavonoid content have been correlated with expression of regulatory genes, other than *MYBA1* (Jeong *et al.*, 2004).

The sampling approach and timing conducted in this work was designed to detect sequential and/or temporal cause–effect relationships between gene transcript abundances and metabolite levels. Total anthocyanin accumulation was significantly higher in both light-exposed treatments (T1 and T4), including the glycosylated, acylated, and *p*-coumaroylated derivatives of all anthocyanins (Fig. 4). Anthocyanin glycosides in grapes are based on the di-hydroxylated derivates cyanidin and peonidin, and the tri-hydroxylated derivates delphinidin, petunidin, and malvidin, with the latter being the most abundant in wine grape cultivars (Roggero *et al.*, 1984; Hebrero *et al.*, 1988). When each one of these molecules was analysed, different responses under sunlight exposed, shaded, and leaf removal treatments were observed (Fig. 4; see Supplementary Table SII, at *JXB* online).

Sunlight exposure (T1) increased the levels of delphinidin-3-*O*-glucoside (Dp3G), cyanidin-3-*O*-glucoside (Cy3G), petunidin-3-*O*-glucoside (Pt3G), peonidin-3-*O*-glucoside

Table 1. General physical and chemical analyses of Cabernet Sauvignon grape berry samples from each light exposure treatment, taken at 9 weeks after *veraison*

Standard deviations are shown (±). Using a Tukey test, no significant differences were found in any of these measurements.

	T1	T2	T3	T4
Skin weight of 200 berries (g)	58.7±7.3	52.8±0.7	55.4±0.4	54.0±1.1
pH	3.68±0.02	3.59±0.01	3.63±0.01	3.66±0.03
Soluble solids (Brix degrees)	24.7±0.3	23.8±0.7	24.4±0.5	25.1±0.3

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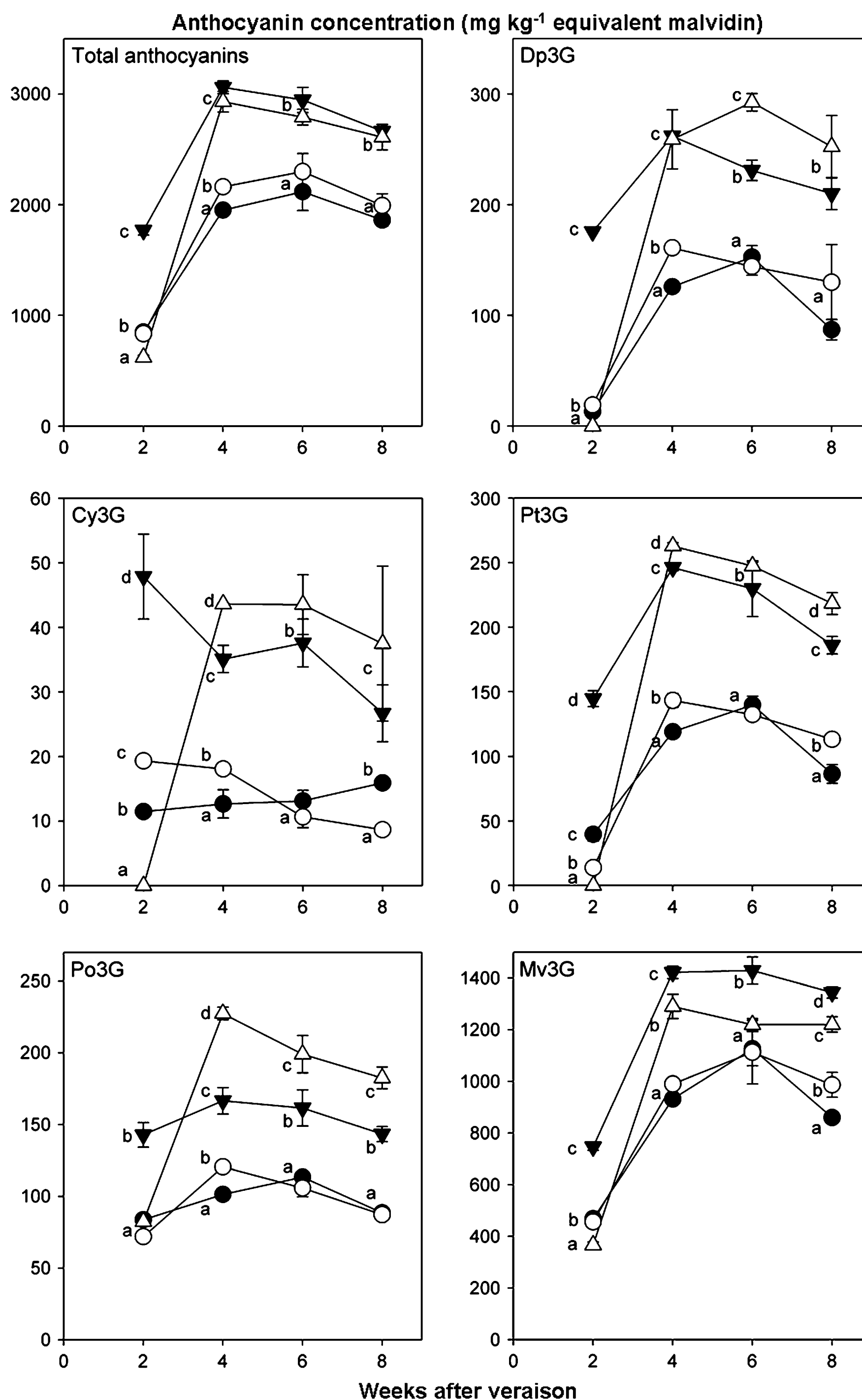

Fig. 4. Concentration of total and 3-*O*-glycosylated anthocyanin compounds from the different light exposure treated berry skins, taken from 2–8 weeks after veraison. (filled inverted triangles) T1 exposed; (open circles) T2 delayed; (filled circles) T3 shaded; (open triangles) T4 leaf removal. Anthocyanin concentrations are calculated in malvidin equivalents. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments as calculated by Tukey statistical analysis (P <0.05).

(Po3G), and malvidin3-*O*-glucoside (Mv3G) throughout all stages of berry ripening (Fig. 4). In the leaf removal treatment (T4), the levels of the most methylated di- and tri-hydroxylated derivates (Po3G and Mv3G, respectively) were significantly lower in the second week after veraison, even when they were compared with the delayed (T2) and shaded (T3) treatments. Dp3G, Pt3G, and Cy3G were not detected in the samples from the leaf removal treatment at this ripening stage. This observation suggests an effect of removing source organs (basal leaves) on flavonoid synthesis during the initial stages of berry ripening. Despite this, and with the exception of Mv3G, all anthocyanin

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levels in T4 increased to those observed in the fully exposed T1 treatment after the fourth week, suggesting the activation of a compensation process, at least regarding anthocyanin accumulation. Pt3G and Po3G abundances were even higher in the leaf-removal treatment compared to T1 at 8 WAV, prior to harvest. Mv3G levels were significantly higher in the exposed treatment, and although the leaf removal treatment increased Mv3G concentration by the fourth week, it never reached the same levels as in T1.

Although the least abundant anthocyanin observed was Cy3G, its concentration increased in the fully shaded treatment (T3) during ripening when compared to T1, T2, and T4. All other non-acylated glycosides began to decrease during the latter stages of ripening in all treatments. Similar results regarding possible shifts in anthocyanin composition have been suggested, in which low light and cool climates could increase the concentration of non-acylated cyanidin glycosides (Downey *et al.*, 2006).

Since significant differences were observed in the metabolic profiling of anthocyanins, it was reasonable to expect a differential expression in some of the flavonoid biosynthetic genes, under the treatments applied in this study (Fig. 5). Metabolites were analysed in even-numbered weeks, whereas gene expression was determined in odd-numbered weeks. This sampling approach enabled us to observe different possible regulatory mechanisms for flavonoid synthesis in a fruit development stage-specific manner. In general, biosynthetic genes were not affected by treatments during the first week of sampling, although metabolites already showed differences between treatments. This led us to propose a possible non-transcriptional regulation of flavonoid synthesis during the initial weeks of berry ripening. In fact, it has been shown that the activity of several enzymes of the pathway, such as PHENYLALANINE AMMONIA-LYASE (PAL), can be affected by light through a non-transcriptional mechanism (Sreelakshmi and Sharma, 2008). From the third week, biosynthetic genes already differ in expression, depending on each treatment. Regulation at the transcriptional level is thus suggested for the mid and latter stages of berry skin ripening.

CHS2 has been reported to be isoform of *CHALCONE SYNTHASE* which is most affected by light (Jeong *et al.*, 2004), and was used to test the effectiveness of the sunlight exposure treatments in changes of gene expression. In this study, *CHS2* expression was increased by light and it appears that this response is concomitant with the expression of *LEUCOANTHOCYANIDIN OXIDASE* (*LDOX*), *O-METHYLTRANSFERASE* (*OMT*), and *UFGT*, because of their remarkably similar expression profiles. The expression of these four biosynthetic genes declined throughout ripening until 7 WAV, at which point a small increase was observed. The decrease in gene expression correlates with the decay in the rate of accumulation of many of the anthocyanins studied between weeks 4 and 8, as seen in Fig. 4 with a decrease in the curve slope. At 3 WAV and 5 WAV, a significantly higher expression value for the T1 treatment was observed, indicating that these genes are being regulated by light. In addition, expression was affected negatively by the leaf removal treatment as shown in the T4 treatment (Fig. 5, upper and middle panels).

Expression of *MYBA1*, as a direct regulator of *UFGT* expression (Kobayashi *et al.*, 2002), is regulated by light (Jeong *et al.*, 2004; Fig. 5, bottom). In apple, a MYB factor responsible for anthocyanin synthesis has also been described as affected by light (Takos *et al.*, 2006), but in addition, it is shown here that MYBA1 was also affected in the leaf removal treatment at the third week after veraison (Fig. 5, bottom). As recently reported, this work supports a role for *MYBA* co-regulating, either directly or indirectly, the expression of genes other than *UFGT,* such as *OMT* (MC Cutanda-Perez *et al.*, 2009).

Changes in anthocyanin synthesis after leaf removal are related to the expression of a hexose transporter

Since both anthocyanin profiling and expression of anthocyanin-related genes were affected in the leaf removal treatments, it was possible that leaf organ removal itself repressed flavonoid synthesis. Since source organs were being removed in this treatment, a possible gene being affected could be a sugar transporter, which incorporates sugars exported from the leaves into the berries, contributing to the sink–source balance. The grape *HEXOSE TRANSPORTER1* (*HT1*) is expressed in all berry tissues and leaves and is involved in sugar import into the berry (Fillion *et al.*, 1999). As seen in Fig. 5 (bottom), *HT1* expression was affected only in the early stages of berry skin ripening (1 WAV), exclusively in the leaf removal treatment. This down-regulation could be due to a decrease in source organ potential in the period in which leaves are highly photosynthetically active (first weeks after veraison).

In addition to a possible role of HT1 in the first stages of mesocarp berry growth, before veraison (Vignault *et al.*, 2005; Conde *et al.*, 2006), removing source organs diminished *HT1* expression in berry skin, suggesting an important role of this transporter in this particular berry tissue. HT1 is also regulated post-transcriptionally by a hexokinase-independent mechanism (Conde *et al.*, 2006). Therefore, it is necessary to study the effect of this practice on the expression and regulation of invertases, sucrose transporters, and recently-isolated hexose transporters (Hayes *et al.*, 2007), to understand the global impact on sugar accumulation.

Even though mesocarp cells are specialized in incorporating and hydrolysing sucrose into glucose and fructose for storage during grape ripening, exocarp cell sugar transport could directly affect skin colour pigmentation in the same cells, as there is a direct relationship between sugar content and anthocyanin synthesis in grape (Vitrac *et al.*, 2000) and other species (Solfanelli *et al.*, 2006). This correlation has been proven to be mediated by MYB factors in *Arabidopsis* (Teng *et al.*, 2005). In grapes, biosynthetic genes such as *LDOX* and *DFR*, possess 'sucrose boxes' in their promoters (Gollop *et al.*, 2001, 2002). These are regulatory elements which determine sugar-specific gene expression responsiveness and are also found in the promoter of grape *HT1* (Atanassova *et al.*, 2003). By this means, at 1 WAV,

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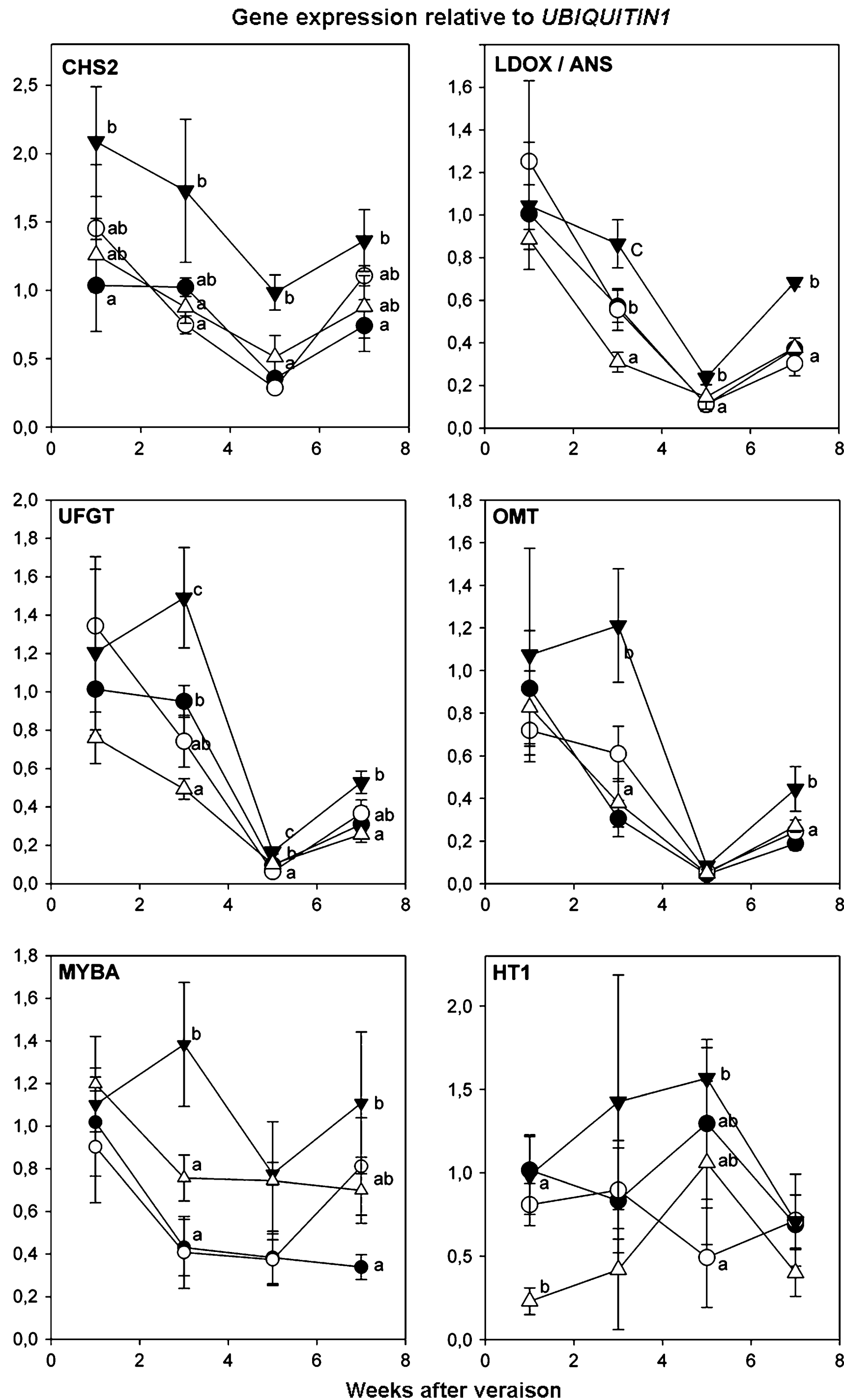

Fig. 5. Changes in the expression of anthocyanin biosynthetic genes, the MYBA regulator and *HEXOSE TRANSPORTER1*, under different light exposure or leaf removal treatments. (filled inverted triangles) T1 exposed; (open circles) T2 delayed; (closed circles) T3 shaded; (open triangles) T4 leaf removal. Transcript levels are expressed in relation to the *VvUBIQUITIN1* gene. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments as calculated by Tukey statistical analysis (P <0.05).

a decrease in *HT1* expression under the leaf removal conditions could diminish sugar import into exocarp cells, with the consequent repression of flavonoid-related expression at 3 WAV. Hormone signalling (e.g. ethylene or abscisic acid) may be playing an additional role in this response since organ removal constitutes a stress event to the plant. This issue should also be addressed in the future.

Genes affecting other branches of the flavonoid biosynthetic pathway are less affected by light exposure

In model species such as *Arabidopsis*, genes belonging to both the MYB and bHLH families, which modulate flavonoid content in different plant organs, have been reported to be differentially modulated by environmental

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conditions (e.g. by light in *Arabidopsis*; Cominelli *et al.*, 2008). In addition, a WDR factor regulating anthocyanin synthesis in *Perilla frutescens* was found to be up-regulated by light (Sompornpailin *et al.*, 2002). In this work, the expression of different members of the grape MYB, bHLH, and WDR families, which are able to control flavonoid synthesis when expressed in homologous or heterologous systems was also studied.

MYB5a (Deluc *et al.*, 2006), MYB5b (Deluc *et al.*, 2008), and MYBPA1 (Bogs *et al.*, 2007) are capable of activating the grapevine promoters of several biosynthetic genes of the flavonoid pathway, including *LDOX*. They also regulate flavan-3-ol synthesis by controlling *LEUCOANTHOCYANIDIN REDUCTASE* (*LAR*) and *ANTHOCYANIDIN REDUCTASE* (*ANR*) expression. The differences observed in *LDOX* expression under the light exposure treatments were only related to the differences observed in *MYB5a*, as seen in Fig. 6. In addition, *MYB5a* expression was affected at 1 WAV for the leaf removal treatment, suggesting a much faster response than *MYBA1* following changes in sugar transport. The differences observed in anthocyanin content could not be explained by alterations in *MYBPA1* expression, as the accumulation of transcripts of this gene were not affected in the different light exposure treatments. *MYB5b* expression was very similar to the expression previously reported by Deluc *et al.* (2008), and levels did not vary under the treatments applied in this study. It has been suggested that MYB5a and MYB5b, which regulate similar structural genes, exert their regulatory effect in different periods of berry ripening, with MYB5a predominating in the early stages and MYB5b towards the later stages (Deluc *et al.*, 2008). It is possible that MYB5a is being co-regulated with MYBA1 and that it could be affected by light and sugar transport into the berry.

Some MYB factors also possess repressor activities and inhibit phenolic compound synthesis (Jin *et al.*, 2000; Aharoni *et al.*, 2001). FaMYB1 is an anthocyanin repressor found in strawberry. It was suggested that its function was to regulate the excess levels of flavonoids which could have a cytotoxic effect if they over-accumulate in the cytoplasm and are not efficiently transported into vacuoles (Aharoni *et al.*, 2001). In *Arabidopsis*, AtMYB4 is also a repressor, regulating one of the first steps of the phenylpropanoid pathway, controlled by the CYNAMATE-4-HYDROXYLASE (C4H) enzyme, necessary to synthesize sinapate esters in response to UV light (Jin *et al.*, 2000). This gene is down-regulated by UV-B in order to increase the content of these protective UV-screening molecules only under stress conditions. Recently, a grape MYB4 homologue repressor was isolated and characterized (JT Matus *et al.*, unpublished results). Despite its similarity to AtMYB4, this gene negatively regulates *UFGT* expression, thus it is a direct repressor of anthocyanin synthesis. Despite its importance and the fact that is up-regulated during berry ripening (Matus *et al.*, 2008), it seems that light or changes in sugar import do not affect MYB4 expression throughout ripening (Fig. 6).

Other regulatory factors and co-activators have been found in grape. The *Arabidopsis* TRANSPARENT TESTA 8 (TT8) grape homologue MYCA1 was not affected by these light treatments. In *Arabidopsis*, *TT8* regulates flavonoid synthesis and is highly up-regulated by light (Cominelli *et al.*, 2008) and other environmental factors. In apple, at least two MYC (also known as bHLH) proteins are needed to induce anthocyanin synthesis (Espley *et al.*, 2007). *In silico* analysis of the grape genome suggests that grapes may also possess more than one bHLH affecting flavonoid synthesis (data not shown). It is possible that another bHLH gene could be regulated by light conditions. The WDR1 co-activator was only affected at 5 WAV between treatments T1 and T3. Therefore, MYB factors may participate in this regulation more directly than bHLH or WDR factors, in response to environmental conditions such as light. New members from these families should be isolated and tested to resolve this issue. In any case, differences observed between the responsiveness of transcriptional regulators and target gene expression levels may imply the effect of additional regulatory mechanisms.

Flavonols are the most drastically affected flavonoids under shadow treatments, an effect possibly mediated by the MYB12 transcription factor

Among flavonoids, the accumulation of flavonols was the most dramatically affected in berry skins under the treatments applied in this study (Fig. 7A). Flavonols have a high anti-oxidant capacity and have been associated with the velvet-type astringency of red wines (Hufnagel and Hofmann, 2008). Total flavonol content, including flavonol galactosides and glycosides, was significantly higher in T1 and very similar between T2 and T3 during the first 6 weeks. The leaf removal treatment had almost twice the flavonol content than in T2 at 8 WAV, although levels were not significantly different at 4 WAV. It has previously been reported that flavonols are more affected than anthocyanins under different light levels (Downey *et al.*, 2003; Pereira *et al.*, 2006), although this effect has not been studied before in relation to MYB expression.

FLS4 and FLS5 are the two most expressed flavonol synthase isoforms in berries (Fujita *et al.*, 2006), and the former is the most affected under low light conditions. From all the genes studied in this work, *MYB12* and *FLS4* were the most affected by light (Fig. 7B), even during the first week of berry skin ripening. *MYB12* was previously identified in the grape R2R3 MYB subfamily as a putative flavonol regulator (Matus *et al.*, 2008), given its close homology to AtMYB12 which controls *FLS* expression in *Arabidopsis* (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007). Although no functional analysis has yet been conducted for this gene, considering that *MYB12* and *FLS4* expressions and response patterns to light were very similar, it is suggested that *FLS4* could be a target of MYB12 in grape. Leaf removal also affected the expression levels of both genes, suggesting that sugar import again is responsible for activating this other branch of the phenylpropanoid pathway. In contrast to what was observed with the anthocyanin content, flavonol levels in T4 did not reach those found in T1

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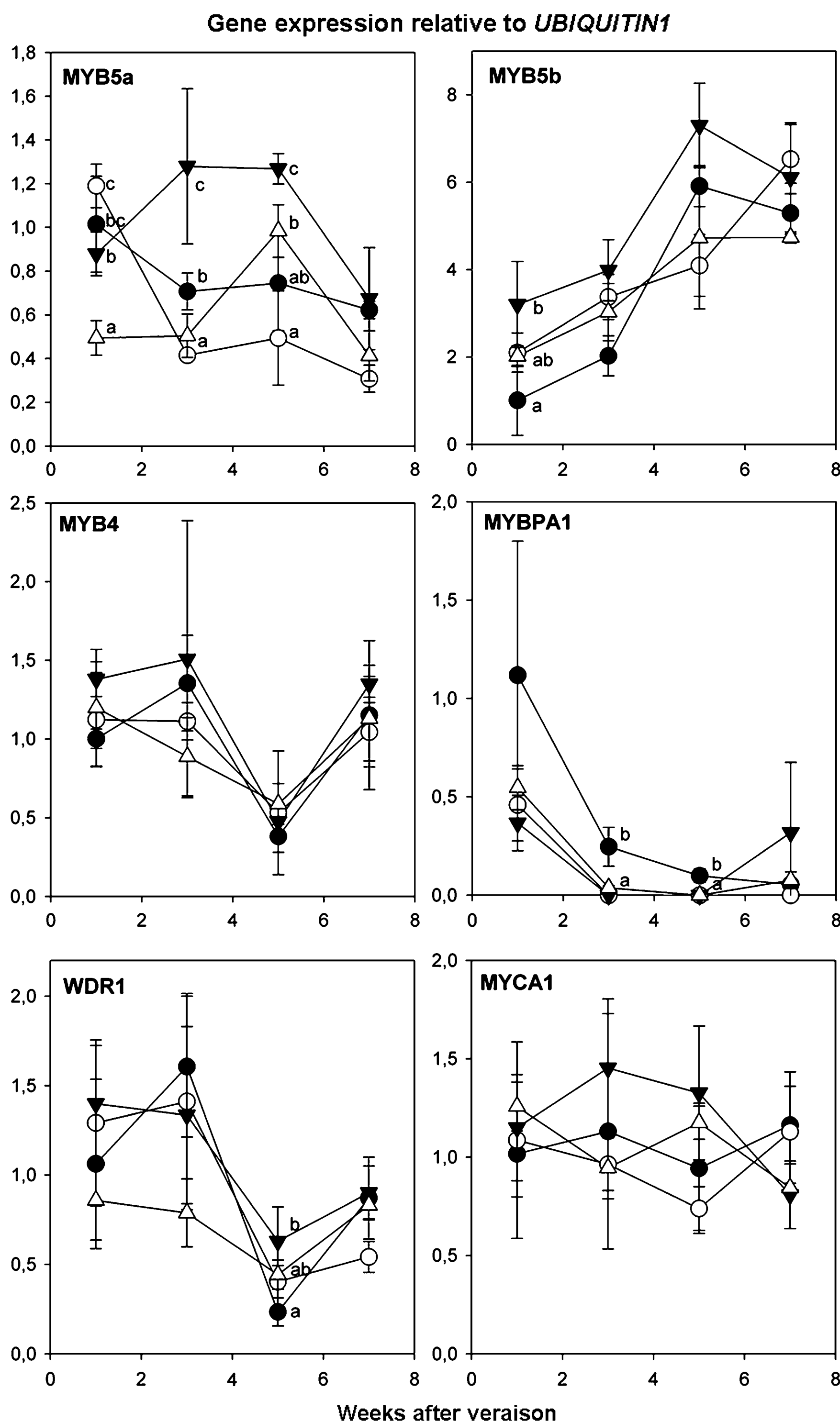

Fig. 6. Changes in transcript levels of MYB, MYC, and WDR regulators of different branches of flavonoid synthesis, under different light exposure or leaf removal treatments. (filled inverted triangles) T1 exposed; (open circles) T2 delayed; (filled circles) T3 shaded; (open triangles) T4 leaf removal. Transcript levels are expressed in relation to the *VvUBIQUITIN1* gene. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments as calculated by Tukey statistical analysis (P <0.05).

during the last stages of ripening, suggesting that the compensation mechanisms previously suggested for anthocyanins do not occur as efficiently for flavonol accumulation.

Flavonol synthesis has been reported to respond rapidly once shaded tissues are exposed to light (Downey *et al.*, 2004). This response was also observed in the T2 (delayed) treatment, in which light exposure applied in the sixth week after veraison quickly increased flavonol synthesis and expression of *MYB12* and *FLS4* to levels even greater than the T1 treatment. This change in expression was correlated

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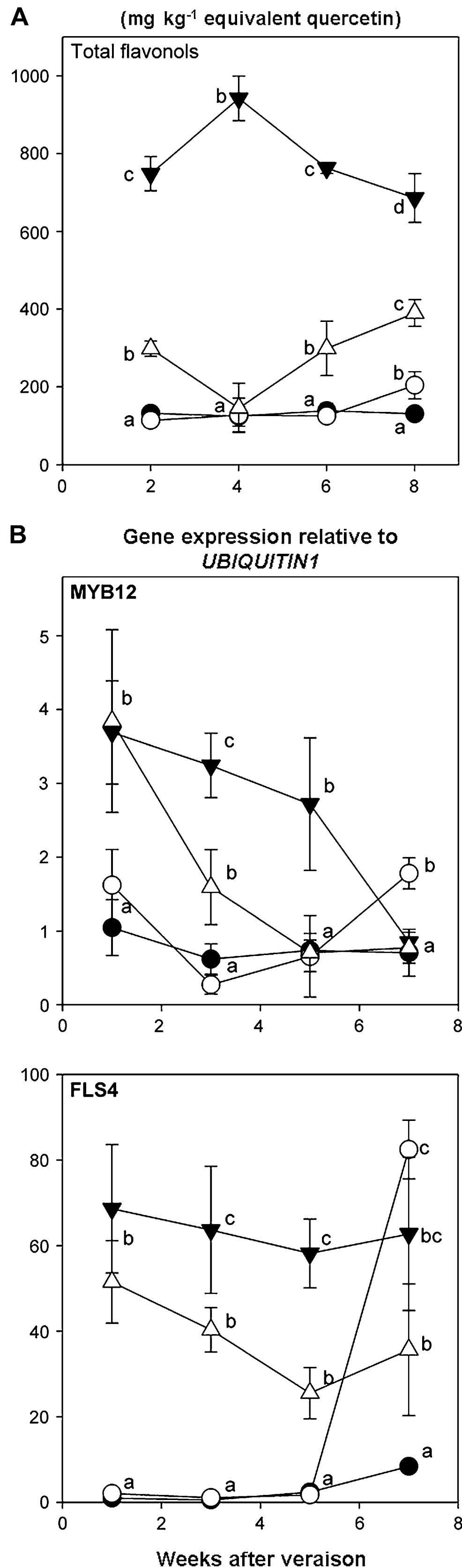

Fig. 7. Changes in total berry skin flavonol content (A) and transcription levels of the flavonol biosynthetic gene *FLS4* and its putative regulator *MYB12* (B) under different light exposure or leaf removal treatments. (filled inverted triangles) T1 exposed; (open circles) T2 delayed; (filled circles) T3 shaded; (open triangles) T4 leaf removal. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for each ripening stage as calculated by Tukey statistical analysis (P <0.05).

with a 2-fold increase in total flavonol content compared to T3 (Fig. 7A), although this level is still very low compared to T1 or T4 treatments.

Conclusion

Viticultural practices affect the plant directly if they constitute a stress event such as organ removal, but can also affect the plant indirectly as a consequence of the modified microenvironment. Gene regulation of a metabolic pathway under these conditions varies in terms of the intensity and timing of the practice imposed. In this study, it was possible to observe that leaf removal at veraison has an early diminishing effect on sugar transport and flavonoid synthesis, especially on flavonol accumulation, during the ripening of the berry skin. For most anthocyanins, nevertheless, these differences are compensated at harvest. Although leaf removal increases sun exposure of the cluster, it is necessary to evaluate the exact changes in sink–source relationships that may occur in this condition.

Light and sugar are capable of inducing significant changes in flavonoid-related gene expression. In this study, it was shown that *MYB* genes regulating flavonoid synthesis are differentially affected by light. MYBs regulating the final anthocyanin or flavonol biosynthetic steps are more affected than MYBs controlling several points of the pathway. Other regulatory genes isolated so far do not respond in the same manner as MYB factors. It is important to examine the presence and function of regulatory elements in the promoters of these light responsive genes in order to understand these differences. In addition, it is suggested that other regulatory mechanisms (not related to transcriptional control) could also be governing flavonoid synthesis at least during the initial stages of berry ripening.

This work exemplifies how the flavonoid content and the genes controlling their synthesis are affected and could be manipulated by viticultural practices such as canopy management. New research efforts will be needed fully to understand the interaction between the plant, the environment, and the field practices in order to modify the quality of grapes. As an interesting projection for continuing the study of the regulation of flavonoid synthesis, it is necessary to analyse whether other viticultural practices, such as irrigation regimes, modify the expression of any of the regulatory genes considered in this study. Screening the expression of biosynthetic genes and their transcription factors under different environmental conditions and field practices will increase our understanding of the complex regulatory network under which flavonoids are being synthesized and accumulated.

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Supplementary data

Supplementary material is available at *JXB* online.

Fig. S1. Basal leaf moving for treatments T1 and T2 using nylon zip-ties.

Fig. S2. HPLC chromatogram for (A) anthocyanidinic (Abs 520 nm) and (B) low molecular weight phenolic compounds, which include flavonol derivatives (Abs 280 nm).

Fig. S3. Cluster morphology at 9 weeks after veraison.

Table S1. Primers used for quantification of transcripts by means of real-time quantitative PCR.

Table S2. Concentration of all anthocyanin compounds from the different light exposure treated berry skins, taken from 2–8 weeks after veraison.

Video S1. Daily time-course of the experimental field in which sunlight treatments were imposed (north orientation).

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