Plant Physiology and Biochemistry 105 (2016) 213–223

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Long-term effects of abscisic acid (ABA) on the grape berry phenylpropanoid pathway: Gene expression and metabolite content

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ARTICLE INFO

Article history:
Received 30 January 2016
Received in revised form
5 April 2016
Accepted 7 April 2016
Available online 19 April 2016

Keywords:
Grape berry
Abscisic acid
Phenylpropanoids

ABSTRACT

ABA has been proposed as the main signal triggering the onset of the ripening process in grapes, and modulating the secondary metabolism in grape berry skins. To determine the effect of ABA on secondary metabolism in berries, clusters of Carménère were sprayed with 0 μLL^{-1} ABA; 50 μLL^{-1} ABA and 100 μLL^{-1} ABA during *pre-véraison*, and the gene expression of the transcription factors and enzymes of the phenylpropanoid pathway were assessed from *véraison* to 70 days after *véraison* (DAV). Additionally, flavonols, tannins and anthocyanins were assessed from *véraison* until harvest (110 DAV). ABA accelerated sugar and anthocyanin accumulation at *véraison*. The grape transcript abundance of *VvDFR*, *VvANS*, *VvUFGT* and *VvMybA1*, all peaking around *véraison* mimicked the concentration of ABA throughout the season. The highest anthocyanin concentration occurred 35 DAV for all treatments, but higher pigment concentrations were observed in ABA-treated berries at *véraison* and from 60 to 70 DAV to harvest. *VvPAL* was also increased by treatment at the higher concentration of ABA from *véraison* to 40 DAV. Regarding flavanol synthesis, *VvLAR2* and *VvMyb4A* decreased from *véraison* until 40 DAV and then increased again until 70 DAV. Compared to the control, both ABA treatments resulted in a less-than-proportional reduction of the expression of both genes compared to the control and, after 40 DAV, in a more-than-proportional increase compared to the control, suggesting a long-term effect of the *pre-véraison* ABA spray on the berries. A concomitant increase in flavanols was observed in berries after 40 DAV, and this occurred at a higher extent in berries treated with the highest ABA concentration.

1. Introduction

The plant growth regulator abscisic acid (ABA) is involved in adaptive responses to stresses, including drought, inducing stomata closure (Sirichandra et al., 2009; Wasilewska et al., 2008). ABA also modulates plant growth and development, seed desiccation tolerance and dormancy (Barrero et al., 2005; Fujii and Zhu, 2009; Nakashima et al., 2009), as well as ripening in non-climacteric fruits, such as orange, cherry, strawberry, and grape (Coombe and Hale, 1973; Giribaldi et al., 2010; Kondo and Kawai, 1998; Rodrigo, 2003). Grape berry growth follows a double sigmoidal function, which is divided into three phases. The transition from the second to the third phase is termed *véraison* and is considered to be the onset of ripening. At this time, changes in primary and secondary metabolism occur; of these, sugar accumulation in the berry flesh and colouring in the skin of red varieties are the most prominent and are known to be preceded by (S)-cis-ABA accumulation (Wheeler et al., 2009).

For oenological purposes, regulated deficit irrigation is a common agronomical practice for red wine production because anthocyanin biosynthesis is up-regulated in water-stressed vines; this is probably mediated by drought-induced increases in the ABA content of grapes (Deluc et al., 2009; Roby et al., 2004). Exogenous applications of ABA to red grape berries increase the flavonoid content and the berry colouring of table and wine grapes (Giribaldi et al., 2010; Jeong et al., 2004; Koyama et al., 2010; Wheeler et al., 2009). Grape berry skin is the main site of flavonoid biosynthesis, and flavonoids are responsible for the main organoleptic properties of wine: a) bitterness and astringency are provided by flavanols, also known as condensed tannins; b) colour is provided by anthocyanins and c) the potential for wine aging is provided by both flavanols and flavonols due to their co-pigment properties (Brossaud et al., 2001).

The synthesis of flavonols, anthocyanins and tannins (end-products of the phenylpropanoid pathway), and the expression of

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http://dx.doi.org/10.1016/j.plaphy.2016.04.012
0981-9428/

the corresponding flavonoid pathway genes are conveniently separated in time, thus avoiding competition for common substrates (Feller et al., 2011). Tannins are synthesized at early berry developmental stages; due to their strong astringency and bitterness, these compounds are useful in preventing herbivory. Later, near *véraison*, flavonols and anthocyanins accumulate, acting as UV-B filters, and protect DNA from oxidative damage; anthocyanins also act as attractive pigments for fruit seed dispersal (Albert et al., 2009; Sarma and Sharma, 1999; Schemske and Bradshaw, 1999).

In recent years, many studies have reported the effects of ABA on flavonoid-related gene expression and metabolite accumulation in grape berries (Giribaldi et al., 2010; Koyama et al., 2010; Wheeler et al., 2009), thereby contributing to a better understanding of the mechanisms underlying the complex signalling network mediated by this plant growth regulator and how it affects the development and ripening of grape berries. ABA and its effect on grapes have been previously studied in terms of gene expression and phenolics for 28 days after *véraison* (DAV) and at 37 DAV (Koyama et al., 2010) and in terms of flavonols for 38 DAV (Fujita et al., 2006), gene expression and tannins for 40 DAV (Lacampagne et al., 2009), proteomics for 5 DAV (Giribaldi et al., 2010), metabolite contents for 53 DAV (Berli et al., 2009), and metabolite content and properties that affect quality in table grapes at harvest (Ferrara et al., 2015), among others. However, vine clusters used in wine production remain in the field for a much longer time than that assessed by many related reports describing the effect of ABA on grape berry metabolism.

Depending on variety and climate conditions, the period between *véraison* and harvest lasts from 30 to more than 70 days. Here, we report the effect of spraying ABA prior to *véraison* on the expression of genes involved in the phenylpropanoid pathway and, simultaneously (and along the ripening process) on the metabolites derived from this secondary metabolic route (including anthocyanins, flavanols and flavonols, all of which are of paramount importance for red wine quality). Here, we studied Carménère, a red grape variety that is useful as a model to study long-term metabolic processes because it is harvested not earlier than 90 DAV to allow for the spontaneous decrease of methoxypyrazines.

2. Material and methods

2.1. Field experiments

The experiments were carried out using 9-year-old, own-rooted *Vitis vinifera* plants cv Carménère in a commercial vineyard in the Maipo Valley in central Chile (33° 42′S, 70° 35′W) during the 2008–2009 growing season. Grapevines were trained to a vertical system using the Guyot double-pruning method with north-south-oriented rows and a planting density of 5000 plants ha^{-1}. The form of ABA used was S-(cis)-ABA 5% p/v, which was kindly supplied by Valent Biosciences (VBC-30062, Libertyville, Illinois, USA).

The experimental design consisted of completely randomized blocks of seven plants each including five replicates; the blocks were distributed in one row. On each block, 3 plants were chosen for the ABA treatments at distances of one plant apart, with two plants as borders. The treatments were as follows: i) control treatment (T0), 0.05% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, USA) in water; ii) 50 mg L^{-1} S-(cis)-ABA (T1) and iii) 100 mg L^{-1} S-(cis)-ABA (T2). All grape clusters of the selected plants were sprayed manually on the 19th and 22nd of January, approximately corresponding to 10 and 6 days before *véraison*. Berries were sampled for analysis on the following dates: -6, 0, 35, 65 and 105 days after *véraison* (DAV). Samples of 50 berries were randomly collected from all clusters on each plant and stored at −80 °C until analysis. Weight, pH, acidity and soluble solids were recorded for each sample.

2.2. S (+)-ABA extraction and determination

For ABA assays, a sample of 10 berries was randomly collected from different clusters per plant, immediately frozen with liquid nitrogen and stored at −80 °C until analysis. S-(cis)-ABA was analysed using an indirect competitive ELISA analysis based on a monoclonal antibody (DBPA1) that was raised against S-(cis)-ABA (Vernieri et al., 1989). Briefly, the berries were deseeded and finely pulverised in liquid nitrogen; 1.5 g of the powder was immediately extracted in distilled water (1:10 w/v) overnight at 4 °C in the dark. Plates were coated with 0.2 ml per well of ABA–4′-bovine serum albumin (BSA) conjugate, incubated overnight at 4 °C, and then washed three times with 75 mM phosphate-buffered saline (PBS) buffer, pH 7.0, containing 1 g L^{-1} BSA and 1% Tween-20. The third washing step was applied for 30 min at 37 °C. Next, 0.1 ml of ABA standard solution or sample and, subsequently, 0.1 ml of DBPA1 solution (lyophilized cell culture medium diluted in PBS buffer containing 10 g L^{-1} BSA and 0.5% Tween-20 at a final concentration of 50 μg mL^{-1}) was added to each well, and competition was allowed to occur at 37 °C for 30 min. The plates were washed again as described above, and 0.2 mL per well of anti-mouse IgG (whole molecule)-alkaline phosphatase (A4312, Sigma, USA) in PBS buffer containing 10 g L^{-1} BSA and 0.5% Tween-20 (final dilution, 1:2000) was added; the plates were then incubated for 30 min at 37 °C, washed again, and 0.2 mL of p-nitrophenyl phosphate (N2770, SIGMAFAST™, USA) was added per well. The plates were then incubated for 30 min at 37 °C before measuring the absorbance at 405 nm using an Epoch Microplate Spectrophotometer reader (BioTek, Winooski, USA).

2.3. Preparation of grape RNA and cDNA synthesis

For RNA extraction, 10 grape berries were randomly collected from each plant, frozen in liquid nitrogen, and the stored at −80° C until processed. Deseeded berries were finely pulverised in liquid nitrogen. Total RNA was extracted from 3 g of the fine powder using the perchlorate method described by (Davies (1996) with some modifications. RNA was precipitated by adding 0.1 vol. of 3 M sodium acetate (pH 5.2) and 2 vol. of ethanol 96% (v/v), and then sedimented, rinsed and dried. The pellet was resuspended in 0.5 mL of sterile water, mixed with 0.25 vol. of 10 M $LiCl_2$, and incubated overnight at 4 °C. The resulting homogenate was centrifuged at 15,000 *g* for 20 min at 4 °C, washed with 1 mL of ethanol 70% (v/v), and then centrifuged at 15,000 *g* for 10 min at 4 °C. The resulting pellet was dried, resuspended in 50 μL of sterile water and treated with DNAse I following the manufacturer's protocol (Promega, Madison, WI). The quantity and quality of total RNA were assessed using spectrophotometry (OD 260/280 and 260/240) and electrophoresis on 1.2% (w/v) formaldehyde-agarose gels.

For cDNA synthesis, 1.5 μg of total RNA was reverse transcribed using 0.5 μg oligo(dT)15 (Promega, Madison, WI) and M-MLV reverse transcriptase (Promega, Madison, WI) following the manufacturer's protocol. This cDNA was used as a template for both standard and real-time polymerase chain reactions.

2.4. Quantitative real-time (qReal-Time) PCR analysis

Relative gene expression was determined by *q*Real-Time PCR using a LightCycler 1.5 instrument (Roche, Basel, Switzerland). The specific primers used are shown in Table 1 and were designed using Primer Premier 5.0 software (Biosoft International, Palo Alto, CA). The PCR mixture (20 μL) contained 100 ng of the cDNA template, 2 μL of LightCycler® FastStart DNA Master SYBR Green I (Roche

Table 1
Oligonucleotide primers used for the Real-time PCR analysis of *Vitis vinifera* cv Carménère.

Primer name	Genbank accession no.	Sequence	Comment
VvActin2-S1	AF369525	5′-ACTGCTGAACGGGAAATTGT-3′	Actin 2 forward primer
VvActin2-A1		5′-AGATGGCTGGAAGAGGACT-3′	Actin 2 reverse primer
VvMYB1A-S1	AB097923	5′-AGATGCCGAAAAAGCTGCAG-3′	MYB1A forward primer
VvMYB1A-A1		5′-CTCCTTTTTGAAGTGGTGACT-3′	MYB1A reverse primer
VvMYB4a-S	XM_002278186	5′-TCAGAATCAGCCCACCATAC-3′	MYB4a forward primer
VvMYB4a-A		5′-CAACCCTAAGAAGTCATACCC-3′	MYB4a reverse primer
VvUFGT-S1	AB047098	5′-CGGAGGTCCTAGCACTG-3′	UFGT forward primer
VvUFGT-A1		5′-CTCATTAGCCCACTCTTTGT-3′	UFGT reverse primer
VvPAL 1S	X75967	5′-CCGAGCATCAACTAAATCCA-3′	PAL forward primer
VvPAL 1A		5′-GCAGAGTGCCACTAGGTAT-3′	PAL reverse primer
VvDFR S1	X75964	5′-ATTGAAGGGATGTTGGGCA-3′	DFR forward primer
VvDFR A1		5′-GAGTCGGTATGATAGTGATG-3′	DFR reverse primer
VvANS S1	EU156063	5′-CTATGAGGGCAAGTGGGTG-3′	ANS forward primer
VvANS A1		5′-GGTGGGAAGAGTGGTGGC -3′	ANS reverse primer
VvLAR2 S1	DQ129686	5′-ATTGGACCAGCTCACCCTAG-3′	LAR2 forward primer
VvLAR2 A1		5′-GCCTCCTCAATGAACCGTCT-3′	LAR2 reverse primer

Basel, Switzerland), 3 mM of Mg^{+2}, 0.5 μM of each primer, and 20 μL of water. The thermal cycle conditions were as follows: pre-incubation at 95 °C for 10 min, 40 cycles of 95 °C for 5 s, 58–62 °C for 10 s, and 72 °C for 8–20 s. Control reactions included a subset of PCR components lacking the cDNA template. The purity of the amplified products was confirmed by melting curve analysis and agarose gel electrophoresis. All reactions were performed twice.

For each gene, a standard curve was constructed using each specific primer set. Amplified DNA was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions and quantified using a spectrophotometer (UV-1601, Shimadzu, Japan). Purified standard DNA was diluted to appropriate concentrations (100, 10, 1, 0.1 and 0.01 pg/μL) and used to construct a real-time PCR standard curve. Finally, to determine relative expression levels, the initial amount of each gene was calculated based on the standard curve using the default (fit point/arithmetic) method of the LightCycler Software Version 3.5 (Roche Basel, Switzerland) and normalized to the values of *VvActin 2*.

2.5. Flavonoid content analysis

Berry phenolics were extracted as described by (Venencie et al. (1997) with modifications. Berry skin samples (n = 50) were weighted and ground in 15 mL of distilled water, 20 mL of hydro-alcoholic solution (H_2O: ethanol, 90: 10 v/v) and 2.5 g of tartaric acid; the final solution weight was adjusted to 100 g. The extracts were macerated for 2 h at room temperature using an orbital shaker, centrifuged, filtered through a 0.45-μm filter (Millipore, Bedford, MA) under vacuum and stored in amber bottles at 4 °C in total darkness until HPLC analysis.

To analyse anthocyanin compounds, 1.5 mL of the phenolic extract was filtered through a 20-μm membrane (Millipore, Bedford, MA), and 150 μL of each sample was injected into a HPLC-DAD system (Merck-Hitachi, Darmstadt, Germany) as described by (Peña-Neira et al. (2007). The HPLC-DAD system was equipped with an L-6200 pump, a L-7455 diode array detector and an L-7200 auto sampler, using a LiChrospher RP-18 (250 × 4.6 mm, 5 μm) column. The elution gradient was as follows: solvent A: water/formic acid (10%, v/v), solvent B: acetonitrile; from 0 to 8 min, 96% A, 4% B; from 8 to 27 min, 85% A, 15% B; from 27 to 40 min, 80% A, 20% B; the flow rate was 1.1 mL min^{-1} from 0 to 27 min and 1.5 mL min^{-1} from 27 to 40 min. Detection was carried out by scanning from 210 to 600 nm.

To analyse flavonols, 25 mL of the phenolic solution described above was extracted by mixing three times with 10 mL diethyl ether and 10 mL ethyl acetate. The organic fractions were combined, and the extracts were evaporated to dryness using an R210 rotary evaporator (Buchi, Flawil, Switzerland) equipped with a B491 bath (Buchi, Flawil, Switzerland) at <35 °C. The residue was dissolved in 2 mL of methanol/water (1:1, v/v) and filtered through a 0.20-μm membrane (Millipore, Bedford, MA); 35 μL of each sample was then injected into the HPLC-DAD for flavonol analysis as described by Peña-Neira et al., 2007. The liquid chromatography was performed on a HPLC-DAD (Agilent 1100) system equipped with a G1311A pump, a G1315B diode array detector and a G1313A auto sampler. Separation was performed on a reverse-phase Waters Nova-Pack C18 (300 × 3.9 mm, 4 μm) column. 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 as follows: 0–55 min, 100–20% A; 55–70 min, 20–10% A; and 70–90 min, 10–0% A. Detection was performed by scanning from 210 to 600 nm, and samples were analysed in duplicate.

Flavonol and anthocyanin compounds were identified by comparison of the spectra and retention times with those obtained by Peña-Neira et al. (2007). The following flavanol standards were purchased from Apin Chemicals (Abingdon, Oxford, UK), Sigma Chemicals (Poole, Dorset, UK), and Merck (Darmstadt, Germany): myricetin 3-O-galactoside, myricetin 3-O-glucoside, quercetin 3-O-galactoside, quercetin 3-O-glucoside, kaempferol 3-O-galactoside, and kaempferol 3-O-glucoside. Standard malvidin-3-glucoside was purchased from Extrasynthese (Lyon, France). Quantitative determinations were performed using the external standard method and commercial standards. The flavonol and anthocyanin calibration curves were obtained at 280 nm and 520 nm, respectively, by injecting different volumes of standard solutions under the same conditions as those used for the samples. Flavonol glycosides were quantified based on a standard quercetin-3-O-glucoside curve, and anthocyanins were quantified based on a standard malvidin-3-O-glucoside curve.

The total tannin content was determined by the method developed by (Bate-smith, 1981) with modifications. Briefly, 4 mL of a grape berry skin extract in hydro-alcoholic solution was divided between 2 tubes, to which 2 mL of distilled water and 6 mL of 37% HCL were added. One tube was heated for 30 min at 90 °C and then cooled in darkness; the absorbance of both tubes was recorded at 550 nm using a spectrophotometer (UV-1601, Shimadzu, Japan). Total tannin concentration was expressed in proanthocyanidin equivalents; five biological replicates were measured.

2.6. Statistical analysis

Flavonoid composition and expression profile data were statistically analysed by one-way, randomized-block ANOVA with $p < 0.05$. Significant differences between treatments were obtained by Tukey median analysis. Statistical analysis was performed using Minitab13 statistical software (*Minitab Inc.*, State College, PA, USA). For all assays, at least 5 biological replicates were used.

3. Results

ABA-sprayed berries exhibited maximal S-(cis)-ABA concentrations at *véraison* of nearly 3.5 μg g^{-1} FW in berries sprayed with 50 mg L^{-1} ABA and 8 μg g^{-1} FW in berries sprayed with 100 mg L^{-1} ABA; both values were significantly higher than that obtained for control berries (2 μg g^{-1} FW) (Fig. 1). All treatments exhibited decreased ABA content after *véraison*; however, at 35 DAV, the treatment with the highest ABA concentration maintained a significantly higher S-(cis)-ABA concentration compared to the control (2 and 1 μg g^{-1} FW, respectively). Finally, no differences were observed at 65 DAV (Fig. 1).

ABA application transiently accelerated berry ripening, as seen by the higher concentration of soluble solids *véraison*; an average of 11.6°Brix was measured for berries treated with the highest ABA concentration, compared to 9.3°Brix in control berries (Fig. 2A). This effect was also evident from the concomitant lower acidity observed in berries that were sprayed with both ABA concentrations compared to that of the control (Fig. 2B). Later in the ripening season, neither soluble solid nor acidity differences were observed between treatments (Fig. 2A and B); nevertheless, the physical properties of the berries were affected by ABA, resulting in a significantly higher skin-to-berry weight ratio in the berries treated with the 100 mgL^{-1} concentration of ABA compared to the control, from 65 DAV to 105 DAV (Fig. 2C).

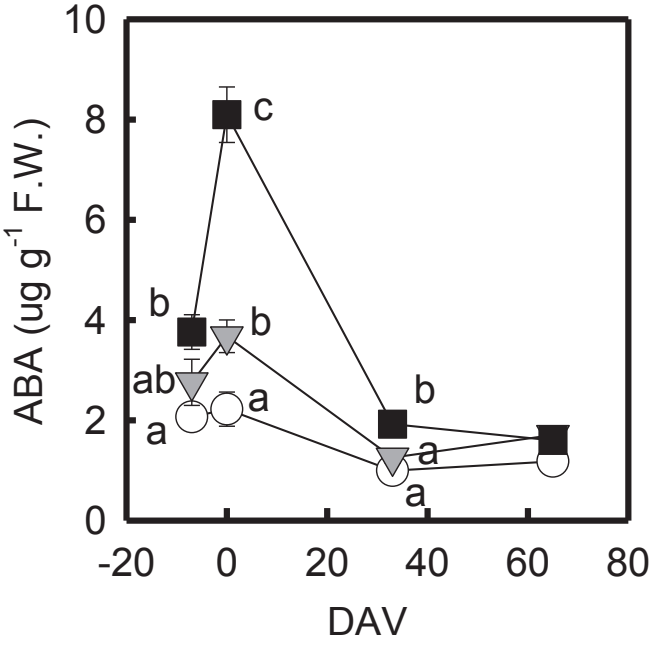

Fig. 1. Concentration of (S)-cis-ABA in the skin and flesh tissues of ABA-sprayed berries of Carménère berries throughout their ripening during the 2008–2009 season. (○) Control; (▼) 50 mg L^{-1} ABA; (■) 100 mg L^{-1} ABA. Vertical bars indicate the standard error of five biological replicates. Different letters indicate significant differences between treatments according to the Tukey test ($P < 0.05$).

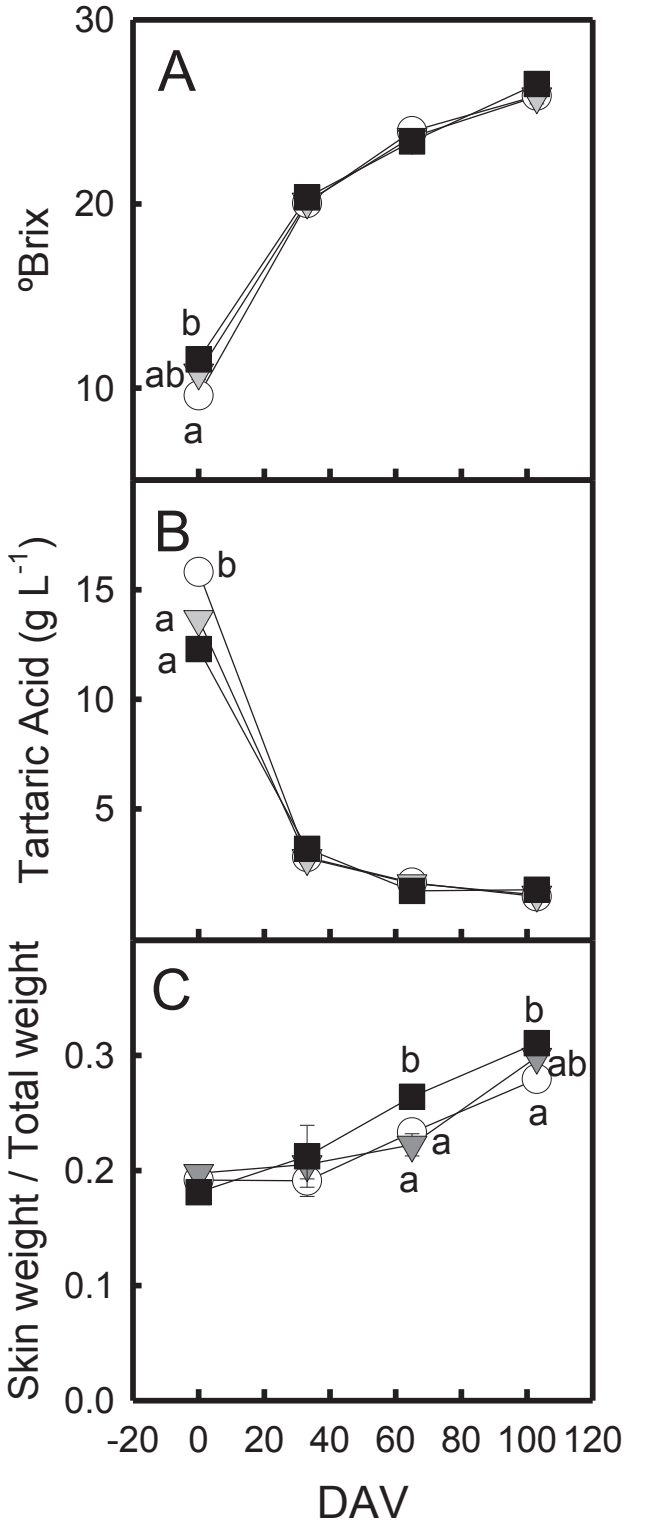

Fig. 2. Progress of berry development; (A) Soluble solids, (B) Acidity and (C) Skin weight: Total weight ratio for Carménère grapes sprayed with (○) 0 mg L^{-1} ABA, (▼) 50 mg L^{-1} ABA and (■) 100 mg L^{-1} ABA. Vertical bars indicate the standard error of five biological replicates. Different letters indicate significant differences between treatments according to the Tukey test ($P < 0.05$).

To assess the effect of ABA on the phenylpropanoid pathway, the expression levels of the structural genes *VvPAL* (encoding phenylalanine ammonia-lyase), *VvDFR* (encoding dihydroflavonol 4-

reductase), *VvANS* (encoding anthocyanidin synthase), *VvLAR2* (encoding leucoanthocyanidin reductase) and *VvUFGT* (encoding UDP glucose:flavonoid 3-O-glycosyltransferase) and the transcription factor genes *VvMybA1* and *VvMyb4a* were measured. *VvPAL* was immediately increased after the first application of 100 mg L^{-1} of ABA and reached the highest expression level at 35 DAV with a value four times that of the value observed in control berries (Fig. 3). All treatments reduced the expression level of *VvPAL* from 35 DAV to the end of the season, and no significant differences were found between the treatments (Fig. 3).

Downstream of the phenylpropanoid pathway, *VvDFR* and *VvANS* encode for enzymes that are involved in tannin and anthocyanin synthesis, and expression levels of these genes mimic the ABA content in berries (Figs. 1 and 3). The gene expression of *VvDFR* significantly differed between treatments at *véraison*; values increased proportionally to the hormone concentration (Fig. 3). Such differences lasted until 35 DAV, at which time the various ABA treatments showed similar expression values (both higher than the control); thereafter, no differences were observed between the treatments (Fig. 3). As for *VvANS*, even though the average expression values increased proportionally with ABA concentration at *véraison*, the differences were significant only at 35 DAV; both ABA treatments reached similar values (higher than that of the control) and further decreased to the end of the season without differences between the treatments (Fig 3).

As shown in Fig. 4, ABA induced an increase in the transcript levels of *VvUFGT* at *véraison*, reaching 2- and 3- fold higher values in grape berries treated with 50 mg L^{-1} ABA and 100 mg L^{-1} ABA, respectively, compared to the control. No differences in the expression level between treatments were observed at 35 DAV and 65 DAV (Fig. 4). *VvLAR2* (Fig. 4) encodes an enzyme that is involved in tannin synthesis; the expression of this gene was reduced right after *véraison* until 35 DAV; however, this decrease occurred more rapidly in the control than in the ABA-treated berries (Fig. 4). From 35 DAV, at which all treatments exhibited minimal and similar value, the expression levels increased until 65 DAV; significantly lower values were exhibited by the control berries.

Some MYB transcription factors are known as late-step regulators for the phenylpropanoid pathway; among these, *VvMybA1* induces anthocyanin synthesis in grape berries (Kobayashi et al., 2004). Throughout ripening, *VvMybA1* expression levels (Fig. 4) followed the same pattern as that observed for ABA concentration (Fig. 1) and *VvUFGT* expression (Fig. 4); levels increased sharply from pre-*véraison* to *véraison* before decreasing again at 35 DAV and remaining at low values until the end of the season (Fig. 4). At −6 DAV, *VvMybA1* transcripts levels were significantly higher only in berries treated with 100 mg L^{-1} of ABA compared to the control; however, at *véraison*, expression levels under the 50 mg L^{-1} and 100 mg L^{-1} ABA treatments were 2.5- and 4-fold higher than that of the control, respectively. *VvMyb4a*, a gene involved in repressing anthocyanin synthesis in *Vitis vinifera* (Matus et al., 2008) followed a similar pattern to that observed for *VvLAR2* (Fig. 4). The transcript abundance of *VvMyb4a* decreased from pre-*véraison* to 35 DAV before increasing again from 35 DAV to 65 DAV (Fig. 4). *VvMyb4a* expression was not only reduced to a lesser extent than in the ABA-treated berries from pre-*véraison* to 35 DAV (compared to control) but also reached higher values when the expression of this transcription factor increased again at the end of the season (particularly for the 100 mg L^{-1} ABA-treated berries) (Fig. 4).

Total anthocyanin concentration was higher in both ABA-treated berry skins at *véraison* and, including control berries, a dramatic increase was observed until 35 DAV, when an average concentration of 4 mg g^{-1} malvidin equivalents was reached, with no significant differences between treatments (Fig. 5A). Then, from 35 DAV to 105 DAV, the anthocyanin concentration declined; however, during that time, a slightly higher and significant pigment concentration was observed for the higher ABA concentration (Fig. 5A).

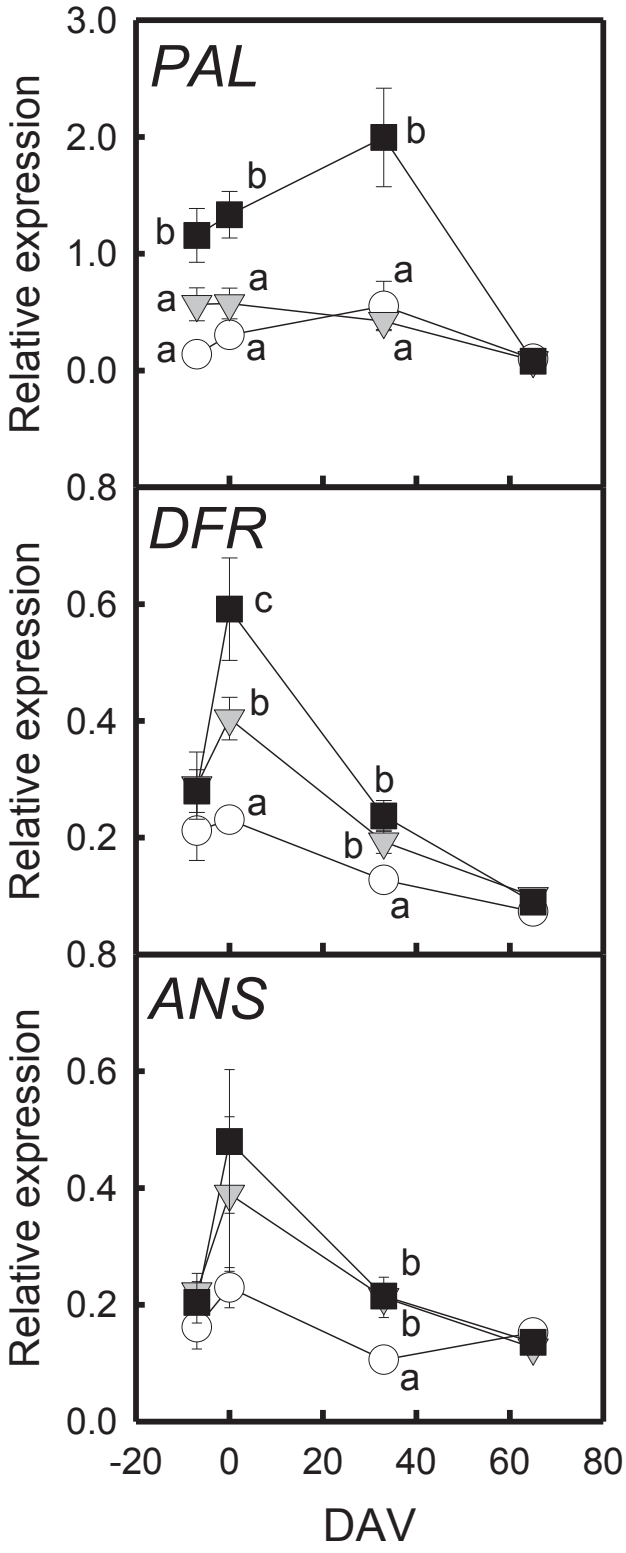

Fig. 3. Transcript profile of VvPAL, VvDFR and VvANS along ripening in deseeded grapes sprayed with (○) 0 mg L^{-1} ABA, (▼) 50 mg L^{-1} ABA and (■) 100 mg L^{-1} ABA. Vertical bars indicate the standard error of five biological replicates. Different letters indicate significant differences between treatments according to the Tukey test ($P < 0.05$).

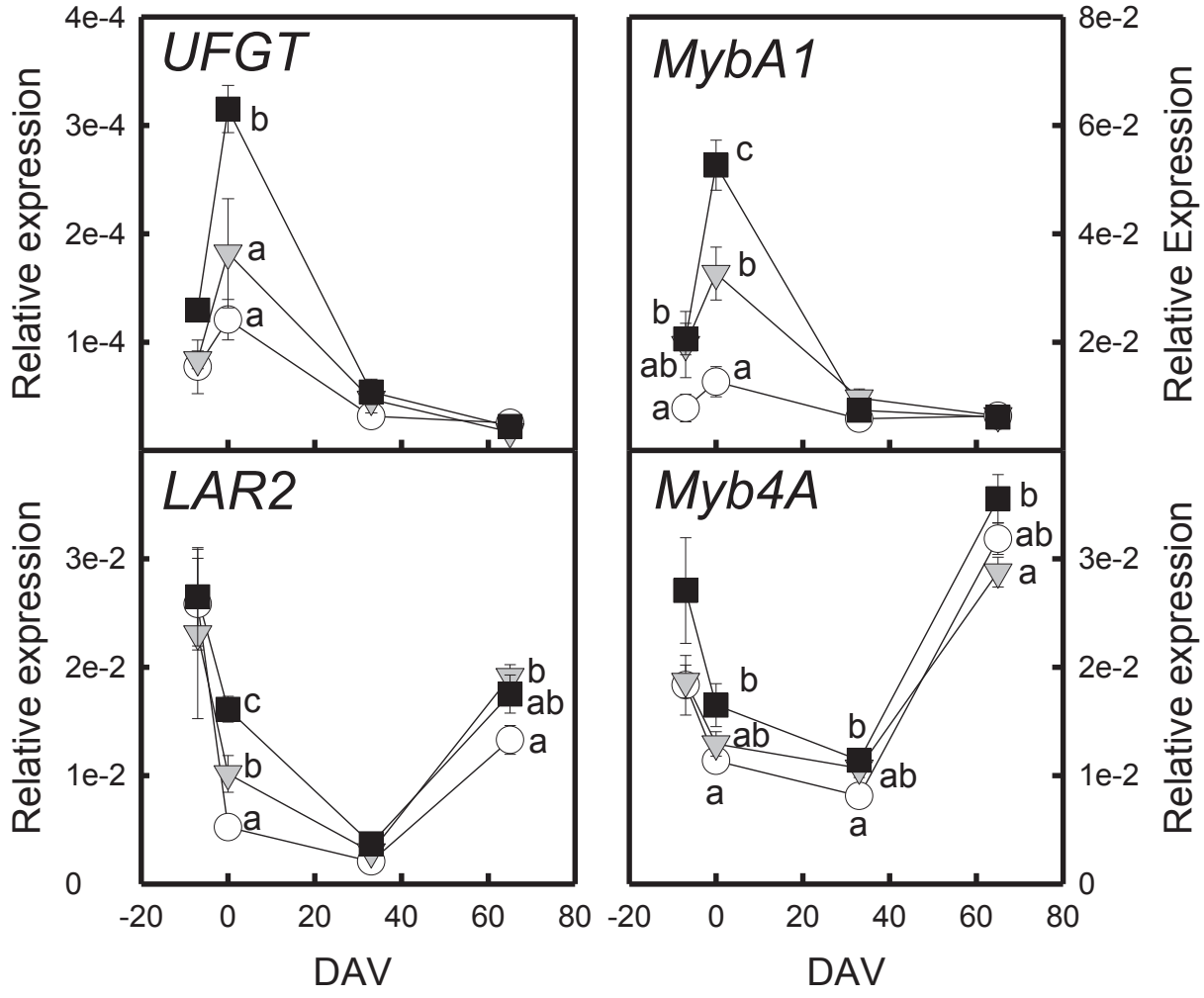

Fig. 4. The effects of ABA on the expression profile of key genes involved in anthocyanin and tannin synthesis (left panel) and MYB regulators that are related to flavonoid synthesis (right panel) in deseeded grapes from −6 to 65 DAV. (○) Control; (▼) 50 mg L^{-1} ABA; (■) 100 mg L^{-1} ABA. Vertical bars indicate the standard error of five biological replicates. Different letters indicate significant differences between treatments according to the Tukey test ($P < 0.05$).

Anthocyanins derived from cyanidin and delphinidin differ from each other in regard to the number of hydroxyl-substituted groups bound to the B-ring of the flavonoid skeleton; two and three groups are present in cyanidin- and delphinidin-based anthocyanins, respectively. As shown in Fig. 5B and C, both types of anthocyanins were found at nearly 1.8-fold higher concentrations in the skins of ABA-treated berries at *véraison* compared to the control; of these, tri-hydroxylated forms (Fig. 5B) reached higher concentrations than di-hydroxylated forms (Fig. 5C). The highest concentrations of the di- and tri-hydroxylated anthocyanins were reached at 35 DAV, and no significant differences were found between the treatments; the concentration of both forms then declined until the end of the season (Fig. 5B and C). The cyanidin-derived anthocyanins reached the highest concentrations in the 100 mg L^{-1} ABA-treated berries at 105 DAV (Fig. 5C). Fig. 5D shows that while the total anthocyanins decreased, ABA treatment resulted a lower proportion of tri-to dihydroxylated anthocyanins compared to the control.

The anthocyanin profile at harvest (Fig. 6) differed between the ABA-treated and control berries. ABA-treated berries exhibited lower concentrations of the non-methylated anthocyanins, delphinidin and petunidin. Conversely, the highest concentrations of the methylated anthocyanins, malvidin and peonidin, were found in the ABA-treated berries (Fig. 6A). The highest concentration of the acylated form of the most abundant anthocyanin in Carmenere, the acetyl glucoside- and p-coumaroyl glucoside-malvidin, was found in ABA-treated berries (Fig. 6B).

Tannins and flavonols are other important flavonoids derived from the phenylpropanoid pathway and compete for intermediates with anthocyanin synthesis. Although tannins are not induced immediately in berries after ABA spraying, higher concentrations (in terms of procyanidin equivalents) were observed at 35 DAV, 65 DAV and 105 DAV in the 100 mg L^{-1} ABA-treated berries (Fig. 7A). Additionally, a marked increase in proanthocyanidin concentration was observed from 35 DAV to 65 DAV, irrespective of the treatment. Flavonols, on the other hand, were dramatically increased from *véraison* to 35 DAV (Fig. 7B), similar to the findings for anthocyanins (Fig. 5); significantly higher concentrations were found in the ABA-treated berry skins at *véraison* and 35 DAV (Fig. 7b). However, from 35 to 65 DAV, the concentration of quercetin equivalents continued to increase in control berries, whereas the flavonol concentration decreased in the ABA-treated berries until the end of the season (Fig. 7B). The

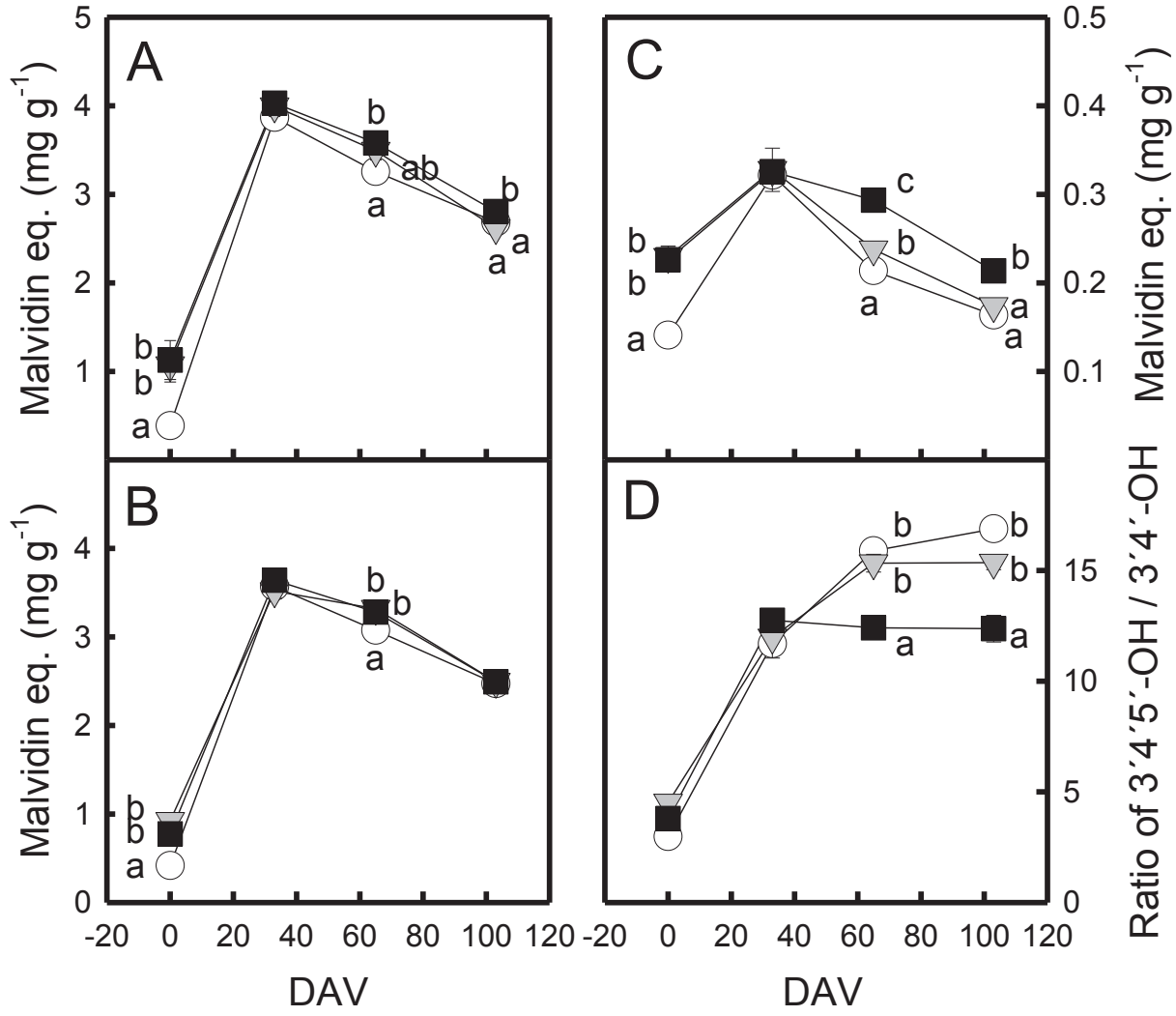

Fig. 5. (A) Total anthocyanins, (B) 3′4′5′-hydroxylated anthocyanins, (C) 3′4′-hydroxylated anthocyanins, and (D) the 3′4′5′-OH/3′4′-OH anthocyanins ratio during ripening in berry skins after spraying with (○) 0 mg L^{-1} ABA, (▼) 50 mg L^{-1} ABA and (■) 100 mg L^{-1} ABA at preveraison. Vertical bars indicate the standard error of five biological replicates. Different letters indicate significant differences between treatments according to the Tukey test ($P < 0.05$).

flavonol concentration of the control berries was higher than that of the ABA-treated berries at 105 DAV (Fig. 7B).

4. Discussion

ABA, a plant hormone that is commonly involved in drought responses (as reviewed by Chaves et al., 2010), naturally occurs in grape berries, and the free active form reaches a maximum near *véraison* (Coombe and Hale, 1973; Gagné and Esteve, 2006; Kondo and Kawai, 1998), as observed for the control berries in our study (Fig. 2). Interestingly, the ABA content of the treated berries was higher than that of the control, not only immediately after its application near *véraison* but also nearly 40 days later (although the concentration was lower at the latter time) (Fig. 1). ABA is known to have a rather short half-life in leaf and root tissues, depending on the water status of the plant; the values range approximately from 1 h to 15.5 h (Creelman et al., 1987; Ren et al., 2007). Therefore, the increased concentration of ABA in the treated berries after such a long time might result from the capacity of cell vacuoles to store ABA after conjugation with glucose (Bray and Zeevaart, 1985; Cutler and Krochko, 1999; Lehmann and Glund, 1986; Owen et al., 2009) or from a long-term autocatalytic effect of the sprayed ABA in the berry skins, even though other mechanisms could be involved. In fact, ABA can promote its own synthesis by inducing the expression of genes that code for NCED (Wheeler et al., 2009), a protein that catalyses the rate-limiting step in ABA synthesis; this effect lasts at least 15 days from the application of ABA to grape berries (Ren et al., 2007). Importantly, after 35 DAV, the ABA content in the grape berries that were treated with 100 mgL^{-1} of the hormone was nearly the same as that in the control berries at *véraison*; i.e., when the hormone was naturally at its highest concentration.

The effect of exogenous ABA at *véraison*, which hastens the ripening process, has previously and extensively been observed as transient increases in sugar accumulation concomitant to a loss of acidity and as transient increases in berry volume and colour intensity (Giribaldi et al., 2010; Koyama et al., 2010; Wheeler et al., 2009); these changes are similar to the changes in soluble solids and acidity (Fig. 2A and B), as well as anthocyanin concentration (Fig. 5), that were observed in the present study. In addition to chemical composition, physical properties such as the skin-to-pulp ratio are of interest for winemaking purposes because most of the metabolites that are involved in colour, flavour and smell are

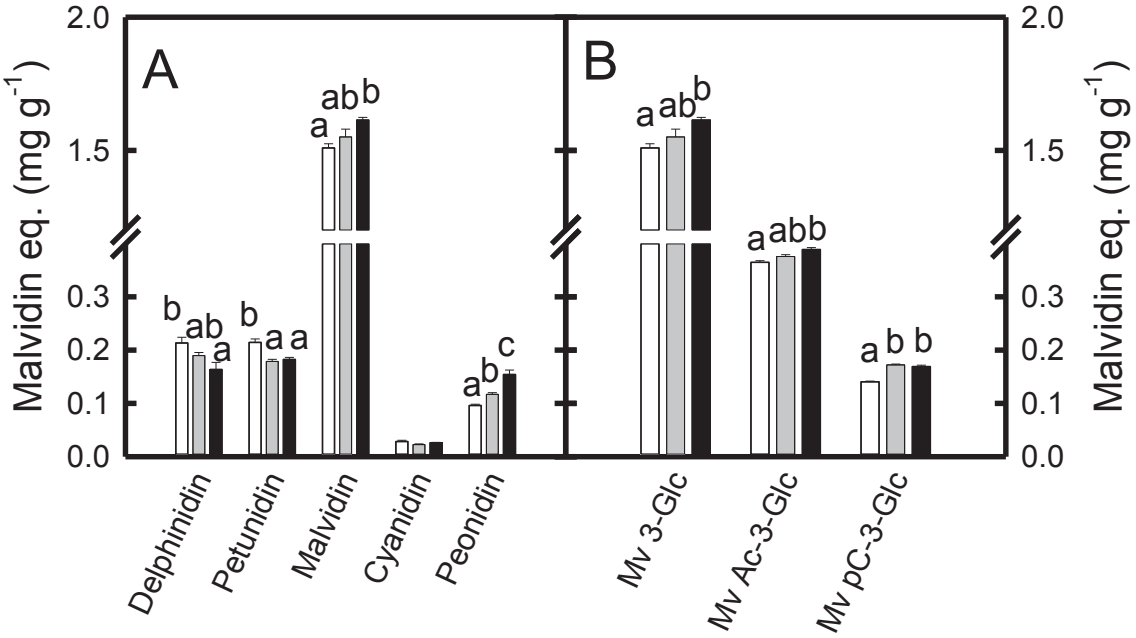

Fig. 6. Anthocyanins concentrations (grouped with respect to their modification: methylation (A) or acylation (B)) at harvest, in berry skins sprayed with ABA at veraison. White, grey and black vertical bars represent the Control, 50 mg L^{-1} ABA, and 100 mg L^{-1} ABA treatments, respectively, for five biological replicates. Different letters indicate significant differences between treatments according to the Tukey test ($P < 0.05$).

located in the berry skin (Ribéreau-Gayon, 1982; Souquet et al., 1996). We found that ABA resulted in a slightly higher skin weight-to-berry weight ratio, suggesting a potential for higher metabolite concentration, irrespective of berry size (Fig. 2C).

In addition to its role in the primary metabolism leading to transient sugar synthesis and grape berry colouring, ABA is also involved in the modulation of the phenylpropanoid pathway in berry skins (Jeong et al., 2004; Wheeler et al., 2009). The pathway is of paramount importance for feeding intermediates to the synthesis of various flavonoid compounds, such as flavonols, flavanols and anthocyanins. Similar to previous observations in red grapes (Jeong et al., 2004; Koyama et al., 2010), the structural genes coding for VvPAL, VvDFR and VvANS were upregulated by ABA (Fig. 3); this effect has previously been studied using proteomic analysis (Giribaldi et al., 2010) and causes increased anthocyanin content in grape berries (Jeong et al., 2004; Koyama et al., 2010; Peppi and Fidelibus, 2008; Wheeler et al., 2009). Clearly, the expression of genes coding for proteins that are involved in the phenylpropanoid pathway remains active, at least until approximately 35 DAV, in a pattern that resembles that of grape berry ABA content (Figs. 1 and 3).

Before anthocyanins are transported and compartmentalized, they must be stabilized through the addition of a glucose residue at the 3 position of the C ring. This reaction is catalysed by UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) (Larson and Coe, 1977). In addition, anthocyanins are synthesized from *véraison* onwards, resulting from the accumulation of *VvUFGT* transcripts; these transcripts are triggered by the expression of the *VvMybA1* transcription factor at *véraison* (Robinson and Davies, 2000). All of these events are closely related to the ABA content of grape berries (Jeong et al., 2004; Koyama et al., 2010; Wheeler et al., 2009). As seen in our study, (s)-cis-ABA concentration (Fig. 1) and the expression levels of *VvMybA1* and *VvUFGT* are closely related (Fig. 4). However, the expression of *VvMyb4A*, a repressor of anthocyanin synthesis (Matus et al., 2009), is increased post-*véraison* in the skins of *Vitis vinifera* L. cv. Cabernet-Sauvignon berries (Matus et al., 2008). Additionally, a recent study has found that this gene is upregulated by high temperatures, independently of light conditions, with a concomitant reduction of the anthocyanin content in grape skins of *Vitis X labruscana* cv. Pione (Azuma et al., 2012). In our study, we observed an increase in *VvMyb4a* transcripts from 36 to 65 DAV, and higher values were found in grapes that had been treated with 100 mg L^{-1} ABA (Fig. 4); simultaneously, *VvUFGT* transcript levels (Fig. 4) and anthocyanin content were decreased (Fig. 5A). A study conducted in strawberries suggested that this type of repressing MYB transcription factor prevents the accumulation of excess flavonoids, such as anthocyanins, which could have a cytotoxic effect if they were over-accumulated in the cytoplasm before transport into vacuoles (Aharoni et al., 2001). Interestingly, based on our results, an increase in the transcript abundance of *VvMyb4a* (Fig. 4) late in the season suggests that the repression of anthocyanin synthesis in fully coloured grape skins might divert substrates of the phenylpropanoid pathways to other end products, such as proanthocyanidins, as occurs towards the end of the season (Fig. 7). In addition, these results indicate that proanthocyanidin synthesis is not restricted only to pre-*véraison* as currently thought (Bogs et al., 2007, 2005; Terrier et al., 2009) but also occurs during the late stages of berry ripening.

Anthocyanins can be classified based on the hydroxylation of the *B* ring at two possible positions; this is controlled by cytochrome P450-type enzymes. Such modifications are dependent on parallel pathways downstream of F3′H and F3′5′H (Bogs et al., 2006; Castellarin et al., 2006). F3′H leads to cyanidin-derived anthocyanins in which the B-ring is di-hydroxylated at the 3′ and 4′ positions, yielding a mainly reddish colour; in contrast, F3′5′H leads to delphinidin-derived compounds containing a tri-hydroxylated B-ring with an additional hydroxyl group at the 5′ position, yielding a deep purple colour (Winkel-Shirley, 2001). The impact of water stress or ABA application on anthocyanin synthesis on grape berries, in terms of possible di- and tri-hydroxylation, has been controversial. Compared to ABA spraying, water stress results in

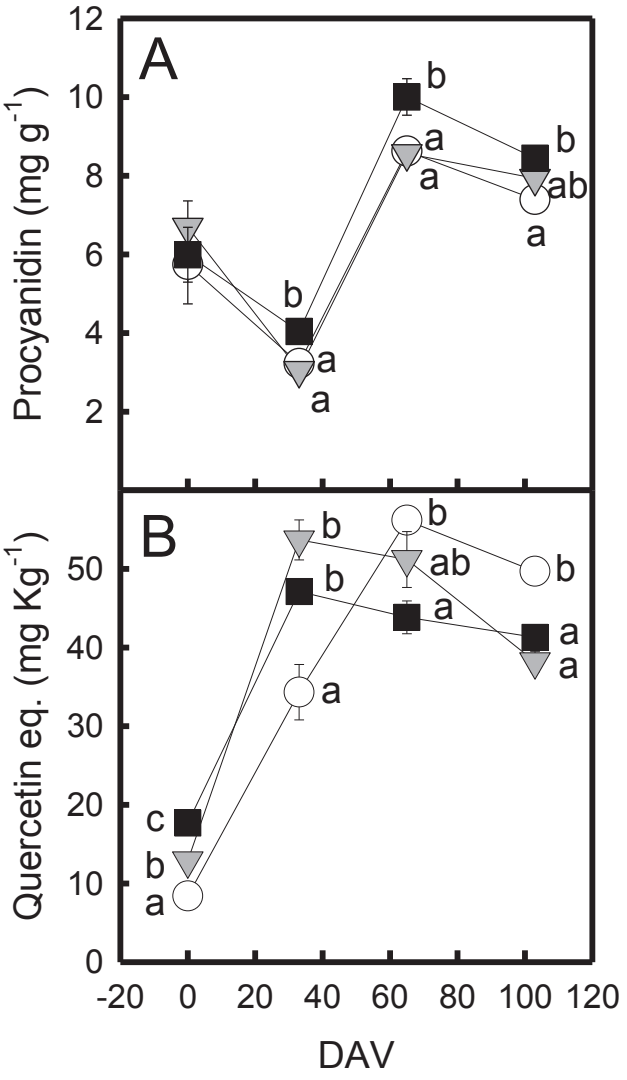

Fig. 7. Changes in (A) Tannins and (B) Flavonols in berry skins sprayed with ABA throughout ripening. (○) Control; (▼) 50 mg L^{-1} ABA; (■) 100 mg L^{-1} ABA. Vertical bars indicate the standard error of five biological replicates. Different letters indicate significant differences between treatments according to the Tukey test ($P < 0.05$).

complex signal cascades in the entire plant. Based on data published by Koyama et al. (2010), it appears that ABA reduces the ratio of tri-to di-hydroxylated anthocyanins in grape berries, at least until 37 DAV; in the same variety, Castellarin et al. (2007) observed an increase in this ratio under water stress accompanied by increases in the expression level of *VvF3′5′H*. Our results clearly show that in the Carménère variety, as in many other red varieties, the predominant anthocyanin corresponds to malvidin, a tri-substituted anthocyanin, and that the concentration of this compound decreases from 36 DAV until the end of the season (Fig. 5A). Pigment content results from the balance between synthesis and degradation. Because differences in the stability of di- and tri-hydroxylated anthocyanins are relevant only in extraction solvents (not in berry skin tissues), our results suggest that the synthesis of anthocyanins from 36 DAV onwards is slightly diverted towards cyanidin-derived pigments as a long-term effect of ABA (Fig. 5C).

Anthocyanins are classified according to their last modifications, such as methoxylation or 3–*O* glucose acylation. Such changes affect the polarity and solubility of the anthocyanins in aqueous and hydro-alcoholic solutions, such as must and wine. Non-methoxylated anthocyanins, such as delphinidin and cyanidin, contain ortho-di-hydroxyl groups that enhance their susceptibility to oxidation compared to methoxylated derivatives such as malvidin and peonidin, which are more stable (Sarni et al., 1995; Sroka, 2005). In addition, anthocyanins contain sugar residues that are acylated with aliphatic or aromatic acids, such as acetic or *p*-coumaric acids, respectively, at the $C_{6''}$ position in the glucose moiety. Like methoxylation, acylation of the sugar in anthocyanins can promote chemical stability (He et al., 2010). Therefore, ABA affects the final modifications of the anthocyanins and clearly favours the more stable forms, such as methoxylated and acylated anthocyanins (Fig. 6); this finding has potentially convenient implications for the winemaking industry.

Flavonol diversity also depends on flavonoid B-ring hydroxylation. Carménère and Cabernet Sauvignon have similar flavonol profiles; for example, quercetin 3-O-glucoside, which is synthesized downstream of F3′H, is the most abundant flavonol (Obreque-Slier et al., 2010). In addition, flavonol biosynthesis is dependent on the expression of *VvFLS1* expression, which is highest from 35 to 60 DAV in Shiraz berry skins and is accompanied by an increase of the flavonol content (Downey et al., 2003). Moreover, ABA treatments at *véraison* in Merlot and Cabernet Sauvignon increase *VvFLS4* and *VvFLS5* expression levels and flavonol content in berry skins at 35 DAV (Fujita et al., 2006). Similarly, from *véraison* until 35 DAV, our results show that berry skin flavonol concentrations are higher after ABA application (Fig. 7B). After that time, however, the ABA-treated berries exhibited lower flavonol concentrations until harvest. Such decreases might result from the higher expression levels of *VvDFR* that are observed until 35 DAV (Fig. 3); the product of this gene competes directly with FLS for the same substrates, dihydroflavonols (Crosby et al., 2011).

5. Conclusions

Controlled water deficit is an important form of field management in vitiviniculture and is generally recognized as useful for improving for grape berry quality; this effect is, at least in part, mediated by ABA. Many studies have investigated the involvement of ABA in the grape berry metabolism, thereby providing an important advancement of our understanding of the mechanisms underlying the ripening process in the non-climacteric grape berry model. Most studies have assessed either the effect of ABA on gene expression or on final metabolites by focusing on specific parts of the phenylpropanoid pathway, and other studies have investigated transcript abundance and grape berry composition; however, the latter were restricted to short-term studies.

It is interesting to note that the ABA was sprayed at the time when this hormone is naturally at its highest concentration in grapes; i.e., close to véraison, resulting in long-term effects. Tight regulation of the biosynthesis of flavanols, flavonols and anthocyanins might occur because these molecules share common intermediates. Indeed, ABA induces a selective increase in the activity of the synthetic pathway, as seen by increases in the transcript abundance of transcription factors and genes encoding catalytic enzymes of the phenylpropanoid pathway. Together with the effect of ABA on increasing the rate of sugar accumulation, anthocyanin synthesis is strongly increased; from the time at which the pigments start to decrease, it is likely that further synthesis favours the dihydroxylated forms. In contrast to previous findings, we found that flavanols resumed their synthesis 40 DAV and that the concentration of ABA at *véraison* was associated with the extent of this event, as seen from the flavanol concentration and the transcript abundance of the *VvMyb4A* transcription factor and *VvLAR2*. Finally,

the effect of ABA on increasing flavanol concentrations late in the season was accompanied by reductions in grape berry flavonols. It remains unclear whether this effect represents a direct repression of flavonols by ABA or if it results from the increased condensed tannin synthesis, which competes for intermediates with the flavonols. From 40 DAV onwards, it is likely that the availability of intermediates in the phenylpropanoid biosynthetic route is strongly reduced, based on the transcript abundance of *VvPAL*.

Further studies are warranted to improve our knowledge of this complex network that regulates flavonoid synthesis in grape berries and the involvement of ABA in its function. Our results suggest that the concentration of ABA at véraison has long-term effects, beyond 40 DAV, and that these are of importance to the grape berry quality for oenological purposes. These findings might be important for improving decision making regarding irrigation practices in grape berry production; in the wine-making industry, the use of controlled water deficits implies important reductions in yield.

Contribution

Alvaro Peña-Neira was involved in assistance and discussion of metabolite contents.

Freddy Ibañez was involved in gene expression work and discussion.

Luis Villalobos was responsible for ABA application, sampling, gene expression, analytical procedures, statistical analysis, and writing.

Claudio Pastenes was involved in planning, organizing, discussion and guidance of work and final writing.

Acknowledgements

This work was supported by funding obtained from the Innova-CORFO consortium Vinnova-Tecnovid (05CTE02-07). The authors thank Haras de Pirque Vineyards for providing plant material and field support, especially to Andrés Aparicio and Cecilia Guzmán.

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