Involvement of Abscisic Acid in Controlling the Proanthocyanidin Biosynthesis Pathway in Grape Skin: New Elements Regarding the Regulation of Tannin Composition and Leucoanthocyanidin Reductase (LAR) and Anthocyanidin Reductase (ANR) Activities and Expression

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Abstract Proanthocyanidins, or condensed tannins, are crucial polyphenolic compounds for grape and wine quality. Recently, significant advances were achieved in understanding the biosynthesis of their main subunits, (+)-catechin and (-)-epicatechin, produced by leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), respectively. Expression and enzyme activity studies have been published but no data were previously available on their regulation by hormonal status. As abscisic acid (ABA) is involved in controlling anthocyanin biosynthesis, we examined the effects of the ABA signal on the proanthocyanidin pathway. The aim of this research was to determine whether ABA application on green grapes affected tannin content and composition, LAR and ANR activity, and the expression patterns of the genes encoding them (*VvANR*, *VvLAR1*, *VvLAR2*) and the associated transcription regulators (*VvMYB5a*, *VvMYBPA1*) in grape skin during ripening. Our results show that ABA affects tannin content and is involved in the tannin biosynthesis pathway in grape skin by decreasing LAR and ANR activity and repressing the expression of related genes a few days after application. This treatment reduced the tannin content of green grapes without modifying their composition but had a positive impact on tannin biosynthesis during veraison, as previously demonstrated for anthocyanins, suggesting that ANR and LAR were coregulated by ABA.

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Introduction

Abscisic acid (ABA) is a growth regulator involved in various physiological processes in plants. Its role in stomatal closure, seed dormancy, drought tolerance, flowering, and photosynthesis have been studied extensively (Zeevaart and Creelman 1988). In nonclimacteric fruits, ABA contributes to ripening (Coombe and Hale 1973; Coombe 1976), and in grapes, a nonclimacteric fruit, endogenous ABA levels increase dramatically during the veraison period (Broquedis 1987; Gagné and others 2006; Deytieux and others 2007), suggesting a possible role in the onset of maturation. Moreover, numerous publications have reported a positive impact of this plant hormone on grape color development (Kataoka and others 1982; Han and others 1996; Matsushima and others 1989; Kim and others 1998; Esteban and others 2001; Hiratsuka and others 2001; Ban and others 2003; Jiang and Joyce 2003; Jeong and others 2004; De La Hera Orts and others 2005; Gagné and others 2006; Peppi and others 2006). Exogenous applications of ABA on clusters not only increase the anthocyanin content of the skins (Hiratsuka and others 2001; Peppi and others 2006) but also lead to earlier color development compared to nontreated grapes (Gagné and others 2006). This is supported by an increase in expression of the *UFGT* (UDP-glucose:flavonoid-3-0-glycosyltranferase coding for an enzyme specific to the anthocyanin pathway) and *VvMYBA1* genes (coding for a transcriptional regulator controlling anthocyanin biosynthesis), as well as

other genes coding upstream-located enzymes (PAL-phenylalanine ammonia-lyase, CHI-chalcone isomerase, CHS-chalcone synthase, and so on) (Jeong and others 2004; Fujita and others 2007). These findings established that ABA was a promotive regulator of anthocyanin biosynthesis.

The anthocyanin pathway is part of the flavonoid pathway that also produces proanthocyanidins (Boss and others 1996). These phenolic compounds, known as condensed tannins, play a major role in grape and wine quality. In addition to their natural protective properties in fruit, they also have a beneficial impact on animal and human health (Aron and Kennedy 2008) and contribute to mouthfeel by conferring bitterness, astringency, and color stabilization (Brossaud and Cheynier 2001; Malien-Aubert and others 2001). Despite their biological and organoleptic properties, their biosynthetic pathway still has been only partially elucidated. Recent works have characterized two enzymes, anthocyanin reductase (ANR) and leucoanthocyanin reductase (LAR), which are responsible for the production of (−)-epicatechin and (+)-catechin, respectively (Punyasiri and others 2004; Xie and others 2003; Pfeiffer and others 2006; Gagné and others 2009). To date, these two enzymes are the last known steps in proanthocyanidin synthesis, whereas the condensation processes remain unclear. ANR and LAR have been studied at both the genetic and the biochemical level in various plants, including grapes (Bogs and others 2005; Fujita and others 2007; Bogs and others 2007; Gagné and others 2009), leading to significant progress in our understanding of the proanthocyanidin metabolism. However, no data are available on the regulation mechanisms controlling proanthocyanidin biosynthesis. ABA may be assumed to play a role similar to its action in controlling the anthocyanin metabolism.

In this work, changes in endogenous ABA following ABA application were examined to determine its effects on the phenolic compound pathway. We focused on tannin content and composition, LAR and ANR activity, as well as expression patterns for their structural genes (*VvANR*, *VvLAR1*, *VvLAR2*) and transcription regulator genes (*VvMYB5a*, *VvMYBPA1*).

Material and Methods

Plant Material and Sample Collection

Vitis vinifera L. cv. Cabernet-Sauvignon grapes were sampled from a commercial vineyard in the Pessac-Léognan appellation near Bordeaux (France). The north/south-oriented vineyard was planted in 1990 and grafted onto 101-14 rootstock. Planting density was 6,500 vines per ha,

and the pruning method was Guyot double. The experimental design consisted of completely randomized blocks of ten plants. Six distinct blocks of ten plants were randomly selected within one field, with these blocks distributed on three rows. On each row there were two blocks: one treated with water considered the "control" and one treated with ABA solution and named "ABA-treated." All plants of each block were treated. Each block was made of adjacent vines and randomly put on one of the three rows.

Grape clusters were collected in 2006 at several phenological stages, as defined by Eichhorn and Lorenz (1977). Random samples of five grape clusters per condition (ABA-treated or control) were selected from the vines of each block at ten phenological stages. Three green stages were collected: pea-sized grapes (stage 31), ten days after stage 31, and berry touch (stage 33), corresponding to 24, 34, and 45 days after anthesis (DAA), respectively. Four samples were collected during veraison: 10% red ripe (RR) grapes (stage 35), 50% RR (stage 36), 80% RR, and 100% RR (stage 37), corresponding to 59, 61, 63, and 67 DAA, respectively. Two samples were taken during ripening: 2 weeks after the end of veraison (83 DAA) and at maturity (harvest, 110 DAA, stage 38). Table 1 summarizes the samples taken for this study. Clusters were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Grape skins were carefully removed for analysis using razor blades.

Experimental Treatments

Within each block, all the clusters of all plants were treated at fruit set (14 DAA) by spraying either an aqueous solution of synthetic abscisic acid (± -cis,trans-ABA, Sigma, Saint Quentin Fallavier, France) 2×10^{-4} mol l^{-1} (53 mg l^{-1}) for the treated plants or water for the control plants. The ABA concentration was determined after preliminary experiments, according to results obtained by Gagné and others (2006). Each cluster was sprayed with a 10 ml solution (ABA or water) containing 0.05% Tween 20 as a wetting agent. All spraying was carried out at dusk (sunset) to minimize ABA photodestruction.

ABA Analysis

Free ABA was determined as described by Antolin and others (2003). The skins of ten clusters were extracted with 60 ml methanol 80% v/v, then purified using polyvinylpolypyrrolidone (PVPP), and finally extracted with diethylether. ABA was then quantified by high-pressure liquid chromatography (HPLC) analysis combined with UV spectrophotometry at 280 nm. An external standard was used and assays were validated independently by mass spectrometry using purified hormone extract.

Enzyme Extraction

LAR and ANR were extracted simultaneously using the method described by Gagné and others (2009). Twenty grape skins (approximately 2 g) were ground to powder in liquid nitrogen, homogenized in 3 ml lysis buffer [0.1 M HEPES pH 7.3, 1% sucrose (w/v), 1% PEG (w/v), 25 mM CaCl$_2$], and mixed with 200 mg PVPP. The homogenate was centrifuged at $20,000 \times g$ for 10 min and the supernatant incubated with Dowex 1×2 mesh 200 (Sigma), balanced with the lysis buffer. After centrifugation at $20,000 \times g$ for 5 min, the supernatant was percolated through a Sephadex G-25 column (GE Healthcare Amersham Biosciences, Orsay, France). The recovered suspension was used as crude extract to determine enzyme activity. Extractions were carried out at 4°C and performed in triplicate.

LAR Assay

LAR activity (expressed in pkat g^{-1} FW) was determined by monitoring the conversion of dihydroquercetin to (+)-catechin. The assay mixture contained 10 μl dihydroquercetin (1 g l^{-1} in methanol), 10 μl NADPH 20 mM, and 110 μl Tris-HCl buffer 0.1 M (pH 7.5). The reaction was initiated by adding 70 μl crude extract, incubated at 25°C for 30 min, and stopped by adding 200 μl ethyl acetate with vigorous vortexing. Extraction was repeated and the ethyl acetate phases were pooled and dried under nitrogen gas. Residues were dissolved in 100 μl HPLC-grade methanol for HPLC analysis. LAR products were separated on a Beckman Ultrasphere ODS (250 × 4.6 mm, 5 μm) (Beckman Coulter, Fullerton, CA, USA) reversed-phase column and eluted with acetic acid (5% v/v) in water (solvent A) and methanol (solvent B), according to the following program: 5% B from 0 to 5 min, 5–10% B from 5 to 10 min, 10% from 10 to 16 min, 10–90% B from 16 to 21 min, 90% B from 21 to 31 min, 90–5% B from 31 to 36 min, and 5% B up to 45 min. The flow rate was set at 1 ml min^{-1}, the detection wavelength was 280 nm, and the injection volume was 50 μl. Identification and quantification were performed using an external (+)-catechin standard (Sigma). Data represent the mean of three assays per extract ± standard deviation (SD).

ANR Assay

The ANR enzyme assay consisted of quantifying the (−)-epicatechin formed from cyanidin. The 200-μl reaction mixture contained 40 μl cyanidin chloride (5 g l^{-1} in methanol), 50 μl NADPH 20 mM, and 210 μl 0.1 M Tris-HCl buffer (pH 6.0). Two hundred microliters of crude extract were added and the mixture was incubated at 35°C

for 45 min. Reaction products were extracted twice with 500 μl ethyl acetate, dried under nitrogen gas, and dissolved in 100 μl HPLC-grade methanol for HPLC analysis. The HPLC conditions were the same as those for the LAR assay. The elution program was as follows: 5% B from 0 to 5 min, 5–10% B from 5 to 10 min, 10% from 10 to 15 min, 10–15% B from 15 to 25 min, 15% B from 25 to 40 min, 15–90% B from 40 to 65 min, 90% B from 65 to 75 min, 90–5% from 75 to 80 min, and 5% up to 80 min. The external standard was (−)-epicatechin (Sigma). Data are expressed as pkat g^{-1} FW and represent the mean of three assays per extract ± standard deviation (SD).

RNA Extraction and cDNA Synthesis

Total RNAs were isolated from grape skins, as described by Azif and others (2000). No DNA contamination was detected by PCR amplification (40 cycles) with VveEF1γ (AF176496) and VvUbiquitin1 (BN000705) primers (Table 1). DNase-treated total RNA (1 μg) was reverse transcribed with oligo(dT)$_{15}$, using MMLV reverse transcriptase (Promega, Charbonnières, France), following the supplier's instructions. cDNA synthesis was controlled by PCR using 1 μl cDNA in a 50-μl reaction with VveEF1γ and VvUbiquitin1 primers.

Expression Analysis by Real-Time Quantitative PCR

Transcript levels in grape skins were measured by real-time qPCR, using the IQ-SYBR® Green supermix on a MyIQ™ Single Colour Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) monitored via the Bio-Rad iQ5 2.0 standard edition Optical System software (Bio-Rad). The reaction mixture (20 μl) contained 5 μl cDNA template (0.4 g μl^{-1}) and 0.25 μM each of the forward and reverse primers specific to each gene (Table 2). Calibration curves

Table 1 Plant material collected for this study

Stage of development	Number of days after anthesis	Phenological stages according to Eichhorn and Lorenz (1977)
Pea-sized berry	24	31
Pea-sized berry + 10 days	34	–
Berry touch	45	33
10% red ripe berries per cluster	59	35
50% RR berries per cluster	61	36
80% RR berries per cluster	63	
100% RR berries per cluster	67	37
100% RR berries per cluster + 2 weeks	83	
Maturity or harvest	110	38

were prepared for each primer set as described by Jeong and others (2004) for a library of grape cDNA. Thermal cycling conditions were as follows: 95°C for 3 min, followed by 60 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s. Annealing temperature (60°C) was determined hypothetically when designing primers. Amplification specificity (specific melt temperature) was checked for each gene product at the end of each run by running a melting cycle from 50 to 96°C and by gel electrophoresis and sequence analysis. PCR efficiency and standard curve linearity were also evaluated in preliminary experiments to ensure % efficiency between 85 and 115 and an r^2 value of at least 0.95.

Expression analyses from control and treated grapes were carried out simultaneously for each gene. Transcript levels (μmol cDNA) for each flavonoid gene were normalized to *VvUbiquitin1* and *VveEF1γ* constitutively expressed transcripts (182- and 87-bp products, respectively). Three replicates of all real-time qPCR reactions were carried out per sample.

Tannin Extraction and Total Tannin Assays

Tannins were extracted from approximately 1 g FW plant material, ground to powder in liquid nitrogen, as described by Gagné and others (2006). Samples were macerated twice in 40 ml MeOH/12 N HCl (99.9:0.1 v/v), with stirring, at room temperature, for 3 h. After incubation, the extract was filtered on a 100-μm Flacon® filter (VWR, Fontenay-sous-Bois, France) and stored at −80°C until analysis. Each sample was extracted three times. Total tannin content was determined by spectrophotometry according to the Ribéreau-Gayon and Stonestreet method (1966). Data are expressed in milligrams of total tannins per gram of fresh weight (mg g^{-1} FW) and represent the mean ± SD of three replicates per extract.

Quantification of Catechin and Epicatechin Monomers and Polymers

Flavano-3-ols were quantified by analyzing 50 μl methanol extracts of phenolic compounds, as described by Saucier and others (2001), using the same HPLC conditions as above and the following elution program: 30–60% B from 0 to 15 min, 100% B from 16 to 25 min, 30% B from 26 to 30 min. Data represent the mean ± SD of three replicates per extract.

The catechin and epicatechin composition of the tannin polymers was determined after thioacidolysis of 100 μl phenolic extract (prepared as described above) in 100 μl thiolysis reagent (benzylmercaptan/2 N HCl/MeOH, 0.5/2/7.5 v/v/v) at 60°C for 5 h, as previously described by Atanasova and others (2002). Aliquots of 150 μl of 100 mg l^{-1} 4-methylcatechol solution were added to stop the reaction. HLPC analysis gradients were modified for the thiolysis products as follows: 30–40% B from 0 to 15 min, 40–70% B from 15 to 30 min, 70–100% B from 30 to 40 min, 100% B from 40 to 45 min, 100–30% B from 45 to 46 min, 30% B from 46 to 50 min. Flavan-3-ols and thioethers were identified by retention time and absorption spectrum compared with authentic commercial molecules and thiolysis products previously purified and characterized in the laboratory. Data represent the mean ± SD of three replicates per extract.

Statistical Analysis

Results were compared by one-way analysis of variance (ANOVA) and Student t test, with $P < 0.05$ statistical

Table 2 PCR primers used for real-time PCR

Gene name	GenBank accession no.	Orientation	Sequence 5′ → 3′
VvUbiquitin1	BN000705	F	CTT GAT GGG ACA GGT CTG
		R	TGT CTT GGA GGC AGG ATA
VveEF1γ	AF176496	F	GAA GGT TGA CCT CTC GGA
		R	CAG AAG AGC CTC TCC CTC
VvLAR1	DQ129685	F	CTC CAA CGG ATT TCT TCC
		R	CGT CCA CTG TTT TCA TCG T
VvLAR2	DQ129686	F	TAA ACG AGC TGG CAT CAC
		R	GCA GCG GCT AGT AGG TCA
VvANR	DQ129684	F	CTT GAT GGG ACA GGT CTG GT
		R	TGT CTT GGA GGC AGG ATA GC
VvMYB5a	AY555190	F	CCG CCT AAC CTG GAT CAG TA
		R	CCA TGG GTG CTT TGT AGT CC
VvMYBPA1	AM259485	F	AGA TCA ACT GGT TAT GCT TGCT
		R	AAC ACA AAT GTA CAT CGC ACAC

F forward, *R* reverse

significance between treatments, using Excel (Microsoft Corp., Redmond, WA, USA).

Results

Effects of ABA Treatment on Skin Hormonal Status

Variations in free ABA content in grape skins during fruit development are shown in Fig. 1. After flowering, the ABA content decreased to very low levels in young clusters. During veraison, the ABA level increased dramatically, reaching a maximum level at 80% red ripe (63 DAA) and subsequently decreased to harvest. ABA treatment modified the time course of ABA accumulation (Fig. 1), attenuating the decrease in ABA content during the first growth period and bringing forward and enhancing ABA accumulation, resulting in significantly higher ABA levels at color change. The maximum level was observed 2 days earlier in treated samples than in controls and ABA levels peaked 20% higher at 50% RR. At harvest (maturity, 110 DAA), ABA levels were similarly low in both treated and control skins.

Effects of ABA Treatment on Tannin and Flavan-3-ol Composition

Total tannin, catechin, and epicatechin (both monomer and polymerized forms) contents in the skins were determined (Figs. 2 and 3a–d).

Concomitant with the variation in ABA content, total skin tannins decreased from berry set to the beginning of veraison (59 DAA), increased transiently during the veraison period (from 61 to 83 DAA), then remained stable until harvest (Fig. 2). Following ABA treatment, total tannin content in the skins decreased before veraison and then increased during veraison (Fig. 2). During ripening

from 83 DAA to harvest, the tannin content of both sets of skins remained similar.

Catechin monomers (Fig. 3a) were present in the skins in very small amounts, 50–250-fold lower than the polymerized forms (Fig. 3b). After an initial drop at stage 31 (24 DAA), monomer contents remained nearly constant throughout development, whereas the catechin content of condensed tannins decreased constantly but rose transiently at 61 DAA, that is, the onset of veraison. Catechin was markedly affected by the ABA treatment: monomers were tenfold lower than in controls and the polymerized forms were reduced by 20–80% in treated skins (Fig. 3a, b).

Epicatechin in grape skins was also mainly polymerized (Fig. 3c, d) and contents decreased continuously during growth. Epicatechin monomers decreased during the first active growth period, then increased again in the middle of veraison, maintaining higher but fluctuating levels up to harvest. Epicatechin polymers initially decreased drastically after berry set (24 DAA), then again after veraison, remaining low until harvest (110 DAA). Epicatechin was affected by the treatment, but to a lesser extent than catechin. Epicatechin monomer contents were quite similar in control and treated skins, whereas polymer subunits in treated skins were two- to fivefold lower than in controls (Fig. 3c, d).

Effects of ABA Treatment on LAR and ANR Activity

LAR and ANR enzyme activity in the skins was determined by a procedure validated elsewhere (Gagné and others 2009). LAR activity showed two activation periods (Fig. 4a): an initial activation peak in pea-sized clusters (24 DAA) and a second at 67 DAA, that is, the end of veraison. ABA treatment reduced LAR activity at nearly every stage in development (Fig. 4a), especially the first growth period (24–45 DAA) where it was twofold lower than in controls, suggesting early activation of LAR.
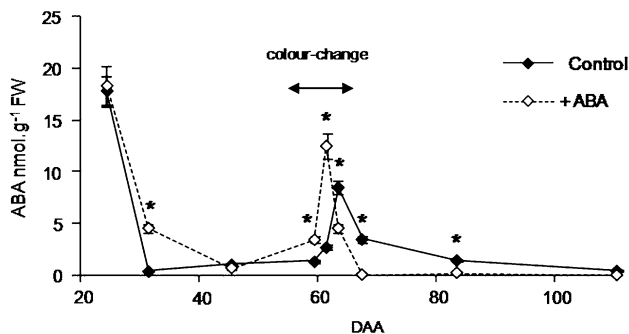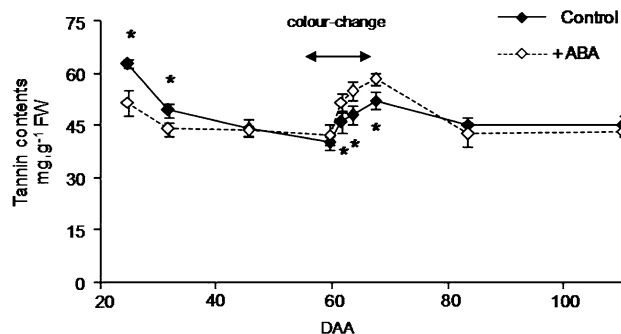

Fig. 1 ABA content, expressed in nanomoles per gram fresh weight, throughout growth of treated and control skins. Data represent the mean of three determinations ± standard deviation (error bars) and *asterisk* indicates for the same date a significant difference between treatments at $P < 0.05$ (Student t test)

Fig. 2 Total tannin content in treated and control skins throughout growth. Data represent the mean of three independent replicates ± standard deviation (error bars) and *asterisk* indicates for the same date a significant difference between treatments at $P < 0.05$ (Student t test)
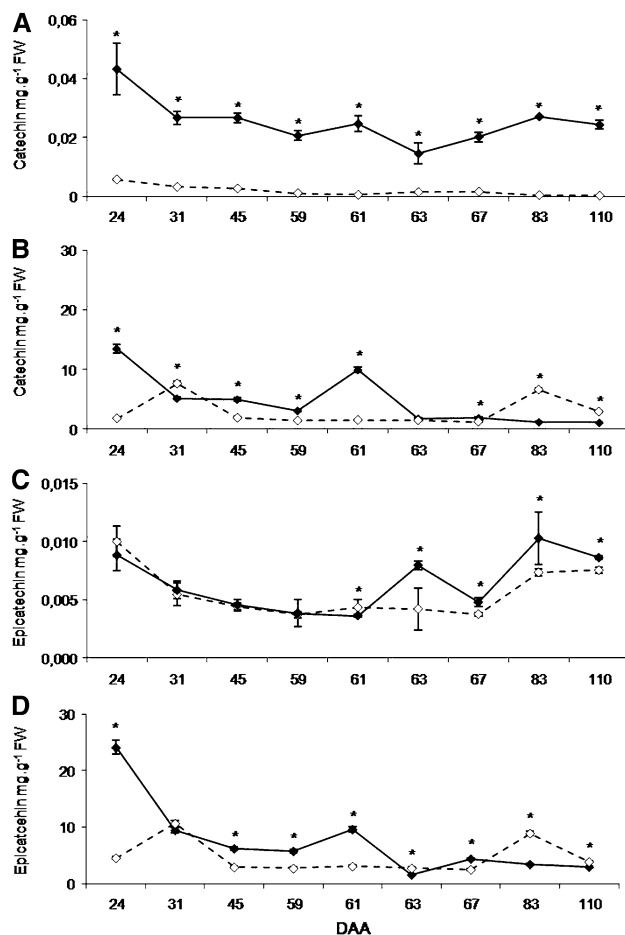
Fig. 3 Catechin (**a**, **b**) and epicatechin content (**c**, **d**) as free monomers (**a**, **c**) and polymerized tannin subunits (**b**, **d**) in the skins of treated and control grapes throughout growth. Data represent the mean of three independent replicates ± standard deviation (error bars) and *asterisk* indicates for the same date a significant difference between treatments at *P* < 0.05 (Student *t* test)

Fig. 4 LAR activity (**a**) and ANR activity (**b**), expressed in picokatals per gram fresh weight, in treated and control skins throughout growth. Data represent the mean of three determinations ± standard deviation (error bars) and *asterisk* indicates for the same date a significant difference between treatments at *P* < 0.05 (Student *t* test)

ANR was more active than LAR (35-fold higher enzyme activity than LAR at 24 DAA), with only one activation period during the initial growth stages, followed by a constant decrease (Fig. 4b). ABA affected ANR activation differently according to the stage (Fig. 4b): activity decreased drastically in green clusters but increased during veraison, and with a transient peak at 80% RR (63 DAA).

Effects of ABA Treatment on Gene Expression Patterns

The mRNA levels of *VvANR*, *VvLAR1*, *VvLAR2*, *VvMYB5a*, and *VvMYBPA1* were determined by real-time qPCR during skin development. Results for structural genes are shown in Fig. 5a–c and for encoding transcription factors in Fig. 6a, b.

VvLAR1, *VvLAR2*, and *VvANR* had different transcription patterns but all gene levels in the skins were highest during the first growth period. *VvLAR1* mRNA peaked at a very high level 10 days after berry set (31 DAA), then
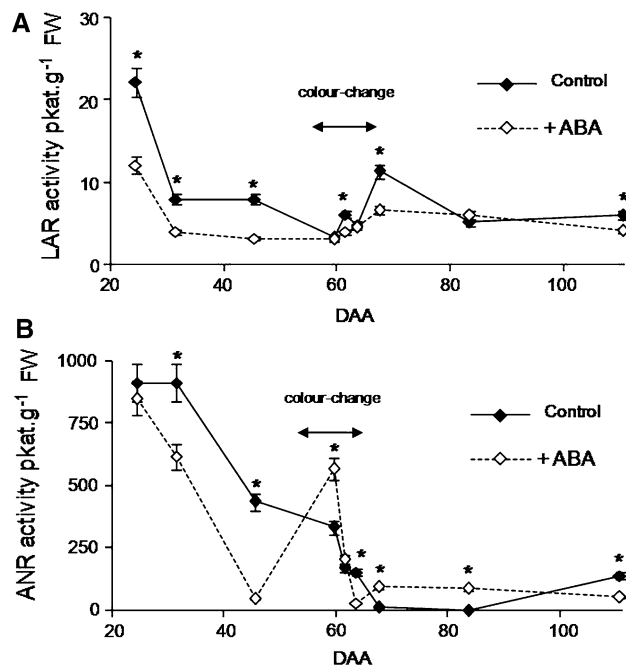
declined rapidly (Fig. 5a) and remained low. *VvLAR2* transcription exhibited two accumulation periods (Fig. 5b): the first at the same time as *VvLAR1* (31 DAA) and the second in mid-veraison (67 DAA). *VvANR* transcription decreased dramatically after berry set (24 DAA) and remained very low until harvest (Fig. 5c).

ABA treatment both delayed expression of all genes (all mRNA levels peaked at the end of berry touch and not at berry set, as in controls) and modified their levels. At the maximum accumulation stage, *VvLAR1* expression was 6.5-fold smaller in treated skins compared to controls (Fig. 5a), *VvLAR2* transcript levels were 5-fold higher (Fig. 5b), and *VvANR* expression was similar (Fig. 5c).

VvMYB5a and *VvMYBPA1* gene expression exhibited a similar pattern to that of *VvLAR2* during development, with two accumulation peaks: the first in green grape skins (31 DAA) and the second toward veraison (63 or 67 DAA) (Fig. 6). Both genes had similar expression levels but variations for *VvMYB5a* were smoother than for *VvMYBPA1*.

In treated grape skins, the profiles were very different from those in controls, with only one mRNA peak at berry touch (45 DAA) and no accumulation during veraison. ABA treatment also modified expression levels in green grape skins: *VvMYB5a* transcripts were 8.5-fold higher in treated skins than in controls (Fig. 6a), whereas *VvMYBPA1* levels decreased by half (Fig. 6b).
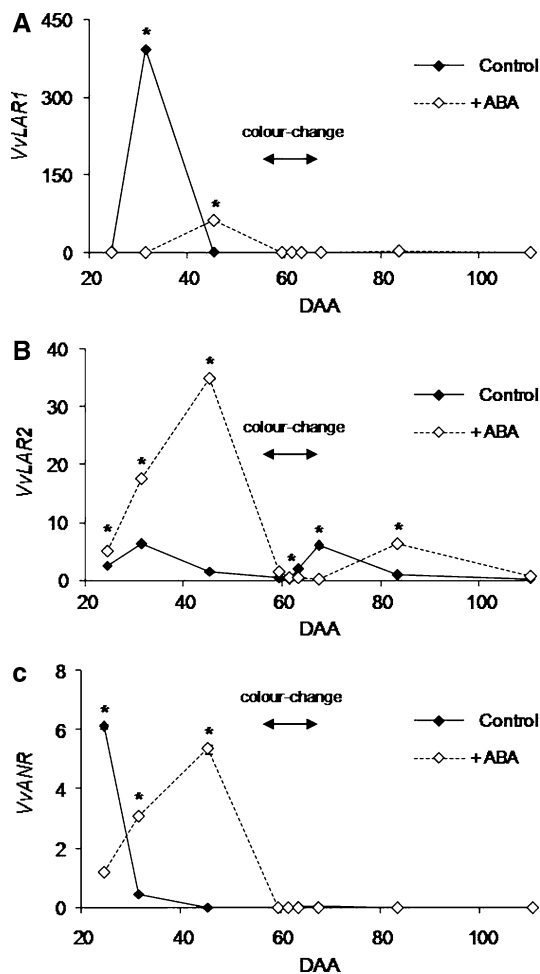
Fig. 5 Gene expression of **a** *VvLAR1*, **b** *VvLAR2*, and **c** *VvANR* in treated and control grape skins throughout growth. Expression, determined by real-time quantitative PCR, indicated the molar ratio of the mRNA level of each gene relative to that of the mean level of *VvUbiquitin1* and *VveEF1γ* in each sample. Data represent the mean of three determinations ± standard deviation (error bars) and *asterisk* indicates for the same date a significant difference between treatments at $P < 0.05$ (Student *t* test)

Fig. 6 Gene expression of **a** *VvMYB5a* and **b** *VvMYBPA1* in treated and control grape skins throughout growth. Expression, determined by real-time quantitative PCR, indicated the molar ratio of the mRNA level of each gene relative to that of the mean level of *VvUbiquitin1* and *VveEF1γ* in each sample. Data represent the mean of three determinations ± standard deviation (error bars) and *asterisk* indicates for the same date a significant difference between treatments at $P < 0.05$ (Student *t* test)

Discussion

Exogenous ABA application is a common strategy for modifying the hormonal balance of grapes. This technique has been widely and successfully used to study the impact of ABA on anthocyanin metabolism. Numerous data thus reported the positive impact of this growth regulator on berry coloring, the onset of ripening, and maturity parameters (Ban and others 2003; Jeong and others 2004; Gagné and others 2006; Deytieux and others 2007). In this study, clusters were treated early in development, at berry set (14 DAA), corresponding to stage 24 as defined by Eichhorn and Lorenz (1977). This was earlier than the usual treatment period, that is, around veraison (Hiratsuka and others 2001; Ban and others 2003; Peppi and others 2006).
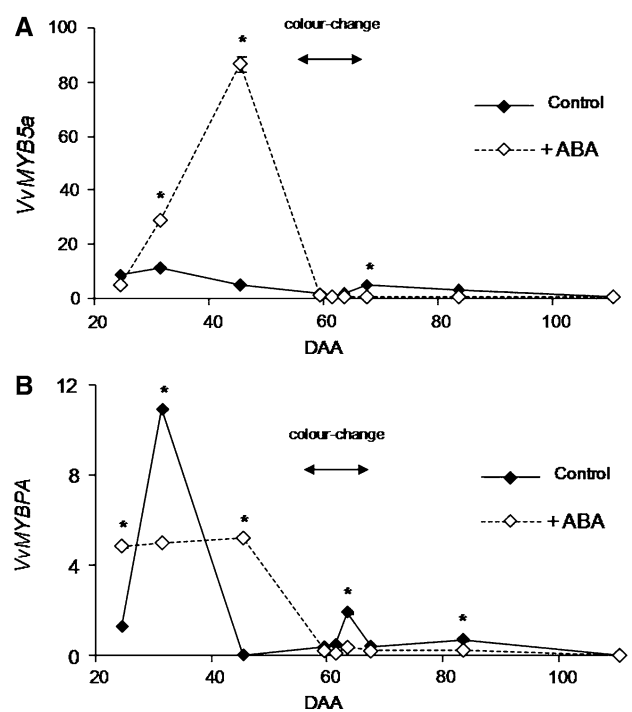
However, early treatment had already been proven effective (Gagné and others 2006) and its impact on skin physiology and metabolism confirmed. The high ABA content in control skins at berry set was attributable to either early influx or the initial ABA pool accumulated during flowering (Baigorri and others 2001; Antolin and others 2003). At veraison, ABA accumulation was observed in 80% RR clusters, in agreement with previous data (Broquedis 1987; Baigorri and others 2001; Antolin and others 2003), confirming the putative role of ABA in triggering maturation. Variations in the ABA content of treated skins were similar to those of control skins, but the time course was different. Contents decreased slower in treated than in control skins during the first growth period, possibly because of a greater initial accumulation following treatment. During veraison, ABA levels increased sooner in treated skins compared to controls and, consequently, the ABA peak characterizing this period occurred 2 days earlier. Thus, the physiological status of the treated skins was modified by applying ABA at berry set.

Although some data in the literature concerning variations in total skin tannins during development are still controversial (Fournand and others 2006), the profiles determined in

this study are concordant with previous results (Downey and others 2003; Roby and others 2004; Gagné and others 2009). Tannin content was high at berry set and then decreased throughout most of the ripening period, with a slight accumulation at the beginning of veraison. ABA treatment induced a shift in the pattern of tannin levels compared to controls. Profiles were parallel, but changes occurred earlier in treated skins than in controls. During the first active growth period, when tannin content tends to decrease, treated skins always had lower tannin content than controls. This suggests an early decrease in tannin after treatment, indicating that ABA applied directly to clusters modulates the tannin metabolism in the skins. On the other hand, tannin accumulation during veraison was greater and earlier in treated skins than in controls. The metabolism of treated skins was apparently activated by exogenous ABA treatment, in accordance with previous results showing that ABA triggered veraison and maturation (Jeong and others 2004; Gagné and others 2006; Deytieux and others 2007). However, Quirogua and others (2009) recently showed that spraying ABA on leaves had no effect on the anthocyanin content or total polyphenol index of grape juice, suggesting that different application methods may generate different signals and affect different pathways.

Our results revealed that total tannin content was affected by ABA treatment. The earlier changes observed in treated skins may be due to a general advance in the skin's physiological status or a specific effect of ABA on tannin biosynthesis. To clarify this point, we determined catechin and epicatechin contents, considering both free monomers and polymer subunits. In accordance with Downey and others (2003), catechin and epicatechin levels decreased throughout growth in control grape skins, with slight attenuation or even an increase in accumulation during ripening, as was the case in Syrah. After ABA treatment, both free monomer and polymer catechins decreased in all the development stages, except catechin polymerized subunits that reached higher levels in treated skins than in controls at two stages (31 and 83 DAA). This correlated with overexpression of the *VvLAR2* transcript, apparently affected by catechin polymerization but not LAR activity. In contrast to free monomer epicatechin content, which did not vary between treated and control skins, polymerized epicatechin levels decreased during the first growth period and at the end of veraison following ABA treatment, in correlation with ANR activity. Surprisingly, this was the only parameter in this study not modified by the treatment. The ineffectiveness of ABA application on monomers may be due to the reactivity of epicatechin. In many plants, this flavanol has been shown to be mainly polymerized (Xie and others 2004), remaining in free form only for a very short time.

Both LAR and ANR were active early in development, as previously described by Gagné and others (2009),

resulting in tannin accumulation in the skins of immature clusters. Although LAR activity decreased throughout growth, ANR was detected again at veraison, resulting in the increase in tannin content measured at that time. LAR and ANR profiles were concordant with early tannin biosynthesis, probably indicating that synthesis started earlier than the first stage studied here, that is, during flowering (Gagné and others 2009). Both LAR and ANR activities were modified by the ABA treatment: LAR activity was always lower than the enzyme activity detected in control skins. ANR activity dropped rapidly during the first growth period in treated skins and then increased sharply and transiently at the beginning of veraison, unlike the smooth decrease in control skins throughout growth. These results confirmed the impact of ABA on tannin biosynthesis, modulating the enzyme activity responsible for catechin and epicatechin production, as previously reported for other enzymes in the proanthocyanidin pathway, mainly PAL and CHI (Hiratsuka and others 2001; Gagné and others 2006). LAR exhibited a second activity peak during veraison, not observed in treated skins. Early, constant biosynthesis may occur in these skins, as little variation was observed throughout the growth period. A second ANR activity peak was detected again in treated skins during veraison, corresponding to the triggering of anthocyanin biosynthesis, whereas activity declined regularly in controls. Precursors, that is, anthocyanidins, accumulate and may be directed to the anthocyanin pathway, forming substrates for anthocyanidin synthase that result in anthocyanidin, or substrates for LAR and ANR that result in proanthocyanidin. It is well known that ABA activates anthocyanin synthesis and, consequently, anthocyanidin levels. Deluc and others (2006) hypothesized that a high accumulation of anthocyanidins provided precursors for both pathways, whereas at low contents precursors were preferentially directed toward anthocyanin synthesis. Consequently, the important accumulation of precursors induced by ABA treatment would activate ANR and explain the second activity peak detected for this enzyme (at the time of veraison).

Gene expression was also affected by ABA treatment, as indicated by the different pattern and abundance of *VvLAR1*, *VvLAR2*, and *VvANR* transcripts in treated and control skins. Treated skins displayed a delayed accumulation of these transcripts during the first growth period, reaching a maximum at berry set. This delay was not correlated with the LAR activity profile, as no increase in this activity was detected, either at that time or during veraison. The fact that enzyme activity does not reflect changes in mRNA abundance may indicate that there is another post-transcriptional or post-translational regulation level. This differential response may also suggest that two LAR isoforms are involved. These isoforms may be

specific to each development stage, as demonstrated for PAL (Hiratsuka and others 2001; Chen and others 2006), or regulated according to tissue specificity (Bogs and others 2005) or developmental stage. ANR patterns were more concordant, with a slight shift between expression and enzyme activation. The lower mRNA accumulation at berry set may explain the decrease in activity, whereas the dramatic accumulation at berry touch may trigger a sharp increase at the beginning of veraison.

Enzyme activation induced by ABA is supported mainly by an increase in anthocyanin pathway gene expression: ABA-treated skins exhibited a higher accumulation of PAL, CHS, CHI, dihydroflavonol reductase (DFR), flavanone 3-β-hydroxylase (F3H), and UFGT transcripts (Ban and others 2003; Jeong and others 2004) than untreated skins. These genes are controlled by a single transcriptional regulator, *VvMYBA1*, also regulated by ABA (Ban and others 2003; Jeong and others 2004). The two transcription factors related to tannin biosynthesis reported to date, *VvMYB5a* (Deluc and others 2006) and *VvMYBPA1* (Bogs and others 2007), may also be involved in controlling other genes in the phenolic compound biosynthesis pathway. Their expression patterns were also modified in ABA-treated skins compared to controls, and, remarkably, the profiles established for these genes closely paralleled those of structural genes in treated skins. Indeed, *VvMYB5a* expression increased at berry touch, confirming the involvement of this transcription factor in activating *VvLAR2* and *VvANR* (Deluc and others 2006). In contrast, the fact that *VvMYBPA1* transcripts remained at high levels until the end of the growth period following ABA treatment did not provide conclusive evidence about the role of this transcription factor in *VvLAR* and *VvANR* activation.

In conclusion, the modification of hormonal status by ABA spray treatment indicates that ABA is a berry tannin biosynthesis regulator. Each step (gene expression, enzyme activity, and tannin accumulation) in this pathway is affected by the treatment without modifying the procyanidin composition of grape skins. Although the role of ABA remains unclear, the results presented here confirm its molecular role, as observed in controlling anthocyanin biosynthesis. Full understanding of tannin biosynthesis requires further elucidation of the roles of *VvMYB5a* and *VvMYBPA1* or other transcription factors.

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