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ORIGINAL ARTICLES

ABA Initiates Anthocyanin Production in Grape Cell Cultures

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Abstract Abscisic acid (ABA) has a well-known positive impact on grape ripening, especially color development, but its role in the initiation of anthocyanin synthesis remains unclear. To elucidate this point, ABA treatment was applied to a simple *Vitis vinifera* model, consisting of Cabernet Sauvignon cell suspensions that do not spontaneously produce anthocyanins under laboratory conditions. Endogenous ABA levels, the expression of some genes in the upstream part of the anthocyanin pathway, and anthocyanin content were determined. Exogenous ABA treatment sharply increased cell ABA content and induced both structural and regulatory genes involved in anthocyanin production. These changes were promptly detected, as early as 6 h after ABA treatment, whereas anthocyanin production was observed only after 4 days in culture. These results demonstrate that ABA promotes anthocyanin synthesis in grape cell culture.

Keywords Abscisic acid · Flavonoid · *Vitis vinifera* · Promoter · Phytohormone

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Introduction

Abscisic acid (ABA) is a phytohormone that plays an important role in various physiological processes in plants. It has been well documented that ABA is involved in plant tolerance and adaptation to a variety of stresses (Zhang and others 2006), as well as stomatal closure, seed dormancy, salt tolerance, flowering, and photosynthesis (Zeevaart and Creelman 1988). Moreover, in nonclimacteric fruits, ABA is considered to be involved in ripening (Coombe and Hale 1973; Hale and Coombe 1974), and in grapes, endogenous ABA concentration increases sharply during the *véraison* period (Broquedis 1987; Gagné and others 2006; Deytieux-Belleau and others 2007), suggesting a possible role in the onset of ripening. Transcripts and proteins linked to the ABA biosynthesis pathway have been found in ripening berries (Deluc and others 2007; Giribaldi and others 2007), though some evidence exists that ABA is synthesized in the leaves and may be transported to the clusters via the phloem vessels (Shiozaki and others 1999; Antolin and others 2003). The hypothesis that ABA triggers ripening in grapes has been circumstantiated by the demonstration that exogenous ABA treatment enhanced several processes involved in berry ripening, such as sugar accumulation, decreases in organic acid and tannin levels, as well as anthocyanin accumulation (Kataoka and others 1982; Matsushima and others 1989; Kim and others 1998; Esteban and others 2001; Hiratsuka and others 2001a; Ban and others 2003; Jeong and others 2004; De La Hera Orts and others 2005; Gagné and others 2006; Peppi and others 2006; Lacampagne and others 2010). Application of exogenous ABA on clusters not only increases the anthocyanin content of the skins (Hiratsuka and others 2001a; Peppi and others 2006), it also leads to earlier color development compared to untreated grapes (Gagné and

others 2006). This change is due to an increase in the expression of *UFGT* (UDP-glucose:flavonoid-3-*O*-glycosyltranferase encoding an enzyme specific to the anthocyanin pathway) and *VvMYBA1* (encoding a transcriptional regulator controlling anthocyanin biosynthesis) genes, as well as other genes encoding upstream-located enzymes [for example, *PAL*, phenylalanine ammonia-lyase; *CHI*, chalcone isomerase; *CHS*, chalcone synthase (Ban and others 2003; Jeong and others 2004)]. Considering anthocyanin production, ABA treatment of field-grown berries activates some, but not all, the components in the ripening process: chlorophyll loss (Deytieux-Belleau and others 2007) and berry softening (Peppi and others 2006) are activated in treated grapes, whereas titratable acidity and berry weight at harvest are not significantly affected (Gagné and others 2006; Peppi and others 2006; Koyama and others 2009). All these studies concerned grapes, a complex organ (several different tissues and hormonal factors), so it is still unclear whether ABA alone is the main factor in color initiation or whether other elements such as sugars (Zheng and others 2009) and ethylene (El Kereamy and others 2003; Chervin and others 2005) are required. As far as we know, ours is the first experiment to use a simple grape model to clarify our understanding of the role of ABA in grape color initiation. This model is likely to be valuable in further studies, focusing on the impact of a single environmental parameter on the anthocyanin pathway. Moreover, this model is relevant because experiments can continue year-round under controlled conditions.

Cabernet Sauvignon cell suspension cultures that do not spontaneously produce anthocyanins under laboratory conditions are a convenient system to use for our study. Non-anthocyanin-producing Cabernet Sauvignon cell suspensions were treated with ABA to assess the role of ABA in promoting anthocyanin synthesis and validate our model. Changes in endogenous ABA were measured throughout cell growth. The impact of ABA treatment on the anthocyanin pathway was evaluated by determining the anthocyanin content and the expression patterns of several genes encoding enzymes involved in anthocyanin biosynthesis (*VvPAL*, *VvC4H*, *VvCHI1*, *and VvCHI2*) and encoding a transcription regulator known to be mostly responsible for controlling this process (*VvMYB A1*).

Materials and Methods

Cell Culture and ABA Treatment

Cell suspension cultures of *Vitis vinifera* L. cv Cabernet Sauvignon (CS6) (Krisa and others 1999) were maintained under continuous fluorescent light (5000 lux), on an orbital shaker (110 rpm), at 25 ± 1°C. Suspension cultures were subcultured weekly in 250-ml Erlenmeyer flasks containing 50 ml cell suspension in B5 medium, supplemented with 20 g l^{-1} sucrose, 250 mg l^{-1} casein hydrolyzate, 0.5 mg l^{-1} 1-naphthalene-acetic acid, and 0.12 mg l^{-1} benzylaminopurine, by inoculating the cells at a 1/5 (v/v) ratio into fresh medium. For experimental purposes (ABA treatment as well as controls), 7-day-old cell suspensions were inoculated [1/7 (v/v) ratio] into an induction medium containing twice the sucrose concentration of the maintenance medium (Krisa and others 1999). ±-*cis*,*trans*-ABA (Sigma–Aldrich, Saint Quentin Fallavier, France) (Hiratsuka and others 2001a; Jeong and others 2004; Gagné and others 2006; Koyama and others 2009; Lacampagne and others 2010) was dissolved in methanol, sterilized by filtration (0.22 µm), and added to 7-day-old cell cultures grown in the induction medium. The final concentration in the culture medium, 10^{-4} M, was found to modulate polyphenol biosynthesis in previous studies on grapes (Hiratsuka and others 2001a; Gagné and others 2006; Zhang and others 2009a; Lacampagne and others 2010). Control cell cultures were prepared following a similar procedure to that used for ABA-treated cells except that these cells received only methanol, the carrier solvent of ABA, instead of ABA. Methanol solvent was sterilized and added at 0.1% final concentration (w/v) to 7-day-old cell cultures cultivated in the induction medium. At 0, 6, 12, 18, 24, 48, 96, 192, and 288 h after the addition of methanol, suspensions were filtered under a vacuum to harvest the cells and collect the culture medium. Filtered cells were rapidly washed with cold distilled water, weighed, frozen in liquid nitrogen, and stored at −80°C until analysis. Viability of control and treated cells was measured (Trypan blue). An aliquot of harvested cells was used to determine the corresponding dry weight. The experiment was carried out in triplicate and all flasks were prepared in triplicate for each experiment.

Abscisic Acid Extraction and Quantification

Free ABA content was determined using the method described by Antolin and others (2003). One gram of cells was ground to powder in liquid nitrogen and extracted in 60 ml 80% methanol (v/v), containing BHT (2,6-di-*tert*-butylphenol) as an antioxidant, with continuous stirring at 4°C overnight. After filtration, the extract was concentrated under reduced pressure at 35°C to remove methanol and adjusted to pH 3.0 (±0.05). The residue was dissolved in ultrapure water and mixed with polyvinyl polypyrrolidone (5% w/v) at 4°C for 20 min. After filtration and adjustment of pH to 2.5 (±0.05), the extract was subjected to three consecutive liquid-liquid extractions with diethyl ether (v/v). The organic phases containing free ABA were

pooled and dried under reduced pressure. The residue was dissolved in 1 ml diethyl ether and stored at −80°C until analysis. Prior to high-pressure liquid chromatography (HPLC) analysis, the extract was dried under nitrogen, dissolved in 300 µl methanol, and filtered through a 0.45-µm membrane. The extract was automatically injected and processed by a high-pressure liquid chromatograph (Thermo Fisher Scientific, Illkirch, France) equipped with a reverse-phase column (4.6 × 250 mm Hypersyl® BDS C18, 5 µm). A gradient solvent system was used with methanol as solvent A and 0.1 M H_3PO_4 in ultrapure water as solvent B. The elution program had the following proportions of solvent A: 0-7 min, 60%; 7-7.5 min, 60-65%; 7.5-11 min, 65%; 11-11.5 min, 65-60%; 11.5-20 min, 60%. The flow rate was 1 ml min^{-1} and the column was kept at room temperature. The detection was made at 254 nm. Data acquisition and processing were done using Chromquest 4.2 software (Thermo Fisher Scientific, Illkirch, France).

The ABA calibration curve was established with commercial ± -*cis*,*trans*-ABA and used to quantify endogenous free ABA in control or treated cells. A sample of ± -*cis*,*trans*-ABA external standard was included every five samples in each HPLC sequence to check both the retention time and the concentration of ABA. The purity of the ABA peak was first controlled by mass spectrometry coupled to HPLC to validate the procedure and then independently checked on randomly chosen samples. The extraction and the quantification were repeated three times per sample.

Anthocyanin Analysis

The anthocyanin extraction procedure was adapted from Gény and others (2003). Anthocyanins from the inner cells were extracted from 1 g frozen cells incubated in 20 ml MeOH/12 N HCl (99.9:0.1 v/v) at room temperature for 14 h with agitation. Anthocyanins from the culture medium were extracted by mixing the filtered medium with an equivalent volume of MeOH/12 N HCl (99.9:0.1 v/v) and stirring at room temperature for 14 h under agitation. The extraction medium was then filtered on a 100-µm Flacon® filter and stored at −80°C until analysis. Each sample was extracted in triplicate. Anthocyanin concentrations were determined by spectrophotometry according to the Ribéreau-Gayon and Stonestreet method (1965). Three replicates per extraction were done.

Expression Analysis

Total RNAs were isolated from cells using the RNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France), according to the protocol described by Tattersall and others (2005), with slight modifications. One gram of cells was mixed with RLT lysis buffer containing 1% β-mercaptoethanol (v/v) and the supplier's instructions were then followed.

RNA extracts were quantified by spectrophotometry and RNA quality was controlled by agarose gel electrophoresis. Samples were then treated with RNase-free DNase I (Promega, Charbonnières, France) according to the manufacturer's protocol, extracted using phenol/chloroform, and precipitated with ethanol. DNA contamination was checked by PCR amplification (40 cycles) using the *VveEF1γ* primers (Table 1) and RNA quality was controlled.

One microgram RNA was reverse transcribed with oligo$(dT)_{15}$ using MMLV reverse transcriptase (Promega) following the supplier's instructions. cDNA synthesis was controlled by PCR using 1 µl cDNA in a 50-µl reaction with *VveEF1γ* primers.

Transcript levels in cells were evaluated by semiquantitative analysis. All primers (Table 1) were designed from cDNA sequences and their specificity was previously checked by PCR amplification of a grape cDNA library, followed by gel electrophoresis and sequence analysis. The PCR reaction mixture contained the primers (2.5 mM each), four dNTPs (200 µM each), 5 mM Tris–HCl buffer (pH 8.3), 0.5 mg ml^{-1} BSA, 3 mM KCl, 0.3 mM $MgCl_2$, 0.1 mM tartrazine, 0.1% sucrose (w/v), 0.05% Ficoll, 0.5 unit *Taq* DNA polymerase, and 2.5 µl template DNA in a total volume of 25 µl. PCR was conducted in a thermal cycler (MiniCycler, MJ Research, Marnes-La-Coquette, France) for 30 cycles with the following parameters: denaturation at 95°C, 30 s; annealing at 54°C, 30 s; and elongation at 72°C, 45 s, with an initial denaturation step of 4 min and a final elongation step of 7 min. Amplification products were then analyzed by electrophoresis on a 1% agarose (w/v) gel prepared in 0.5× TAE buffer [Tris Base 2.42 g l^{-1}, 0.057% (v/v) acetic acid, 0.5 mM pH 8.0 EDTA], containing BET (1 ppm), with 1 µg λ phage DNA digestion products loaded as control. The gel was run at 100 mV in 0.5× TAE, detected under UV light, and quantified using Bio1D software (Vilber Lourmat, Marne-La-Vallée, France) by measuring the fluorescence intensity of PCR bands (in arbitrary units) and comparing them with the intensity of DNA markers made from λ phage DNA digestion products that were previously quantified by spectrophotometry. The ratio between the fluorescence of PCR bands measured for treated cells and that measured for control cells was then calculated for each gene at each date to evaluate the changes in expression level. Three replicates were carried out per analysis and per sample.

Statistical Analysis

Results were compared by one-way ANOVA and Student's *t* tests using Excel (Microsoft Corp., Redmond, WA, USA).

Table 1 PCR primers

Gene name	GenBank or TIGR accession no.	Orientation	Sequence 5′ → 3′
VveEF1γ	AF176496	Forward	GAA GGT TGA CCT CTC GGA
		Reverse	CAG AAG AGC CTC TCC CTC
VvPAL	X75967	Forward	CAT ATC CAC TGA TGC AGA AG
		Reverse	TCC CCT CAC ACA TTG CAG TA
VvC4H	AM468511	Forward	CTG CAA GGA AGT TAA GGA GA
		Reverse	ACC ATG CGT TCA CCA GGA TT
VvCHI1	X75963	Forward	TCA GGT CGA GAA CGT CCT AT
		Reverse	TTC TTC GCT TCA GGG GAA AC
VvCHI2	Tc38410	Forward	CCT GAA ATT GTG GGC CAT CT
		Reverse	ATC TCA GCA GTG CAT GAA GT
VvMYBA1	AB097923	Forward	TAG TCA CCA CTT CAA AAA GG
		Reverse	GAA TGT GTT TGG GGT TTA TC

TIGR = The Institute for Genomic Research

Results

Changes in Free ABA Cell Content Following ABA Treatment

We initially measured changes in free ABA content, in both control and ABA-treated cells, from the time of ABA addition (0 h) to 48 h (Fig. 1). A concentration of 10^{-4} M ABA was chosen to study its impact on cell metabolism on the basis of preliminary experiments and previous studies (Hiratsuka and others 2001a; Gagné and others 2006; Zhang and others 2009a, b; Lacampagne and others 2010). These studies demonstrated its effectiveness in modulating the hormonal status of grapes (that is, ABA concentrations), as well as affecting tannin and anthocyanin biosynthesis in terms of gene expression, enzyme activity, and polyphenol accumulation. This treatment did not modify the time-course of cell growth and no more than 5% of cells died (Trypan blue coloration) (data not shown). ABA stability was also checked by measuring ABA concentrations in flasks containing only the culture medium; no ABA degradation was detected during the 288 h of treatment under our laboratory conditions (data not shown).

Untreated (control) cells exhibited low and constant free ABA concentrations, below 0.6 nmol g^{-1} FW, throughout the 48-h measurement period. Adding ABA to the culture medium greatly affected free ABA levels in cells in terms of both concentrations and time-course of accumulation. In treated cells, the intracellular free ABA content rose sharply, around 40-fold higher than the control concentrations, as early as 6 h after the addition of ABA. Concentrations increased constantly, up to 400-fold greater than the control concentrations, by 18 h, then remained almost constant until 48 h. Free ABA in the culture medium, detected only in ABA-treated flasks, decreased slightly but constantly throughout the experiment (data not shown).

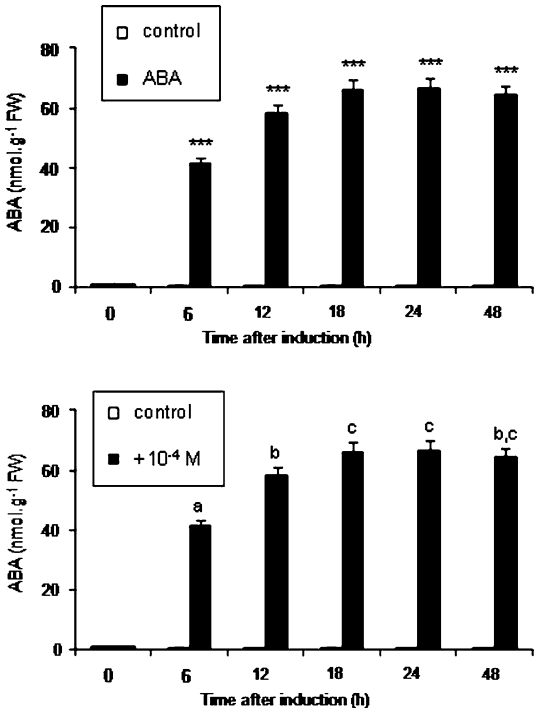

Fig. 1 ABA accumulation in cells, expressed in nanomoles per gram fresh weight. Cabernet Sauvignon cell cultures were supplemented with methanol (control) or ABA at 10^{-4} M (ABA) at 0 h and ABA accumulation was monitored in the cells for 48 h. Data represent the mean of three determinations ± standard error (bars). *Asterisks* indicate significant statistical differences between treatments on the same date using Student's *t* test; ** $P < 0.01$, *** $P < 0.001$

Changes in the Expression of Genes Encoding Upstream Anthocyanin Pathway Enzymes and VvMYBA1 Following ABA Treatment

Transcripts of some upstream anthocyanin pathway genes (*VvPAL*, *VvC4H*, *VvCHI1*, and *VvCHI2*) and the *VvMYBA1*

transcription regulator gene were assessed by semiquantitative analysis. Expression of the *VvγeEF1* gene was used as a constant internal standard. Results are shown in Fig. 2. Only the differences between ABA and untreated cells are described next.

All the genes we focused on were shown to be upregulated by ABA and their mRNA accumulation profiles were quite similar. These genes were rapidly and significantly induced, as early as 6 h after ABA addition, and then their expression patterns were modulated differently. The *VvPAL* gene was the most affected by ABA treatment: from 6 to 48 h, transcript levels remained high and almost constant in the presence of ABA. Expression of the *VvC4H* gene increased during the first 12 h after the ABA challenge and then decreased to reach a level similar to that measured in control cells. Both *VvCHI1* and *VvCHI2* transcripts accumulated until 18 h in culture, then decreased to lower levels than in the control. The expression profile of *VvMYBA1* was quite similar, with mRNA accumulation detected mainly during the first 18 h after ABA addition.

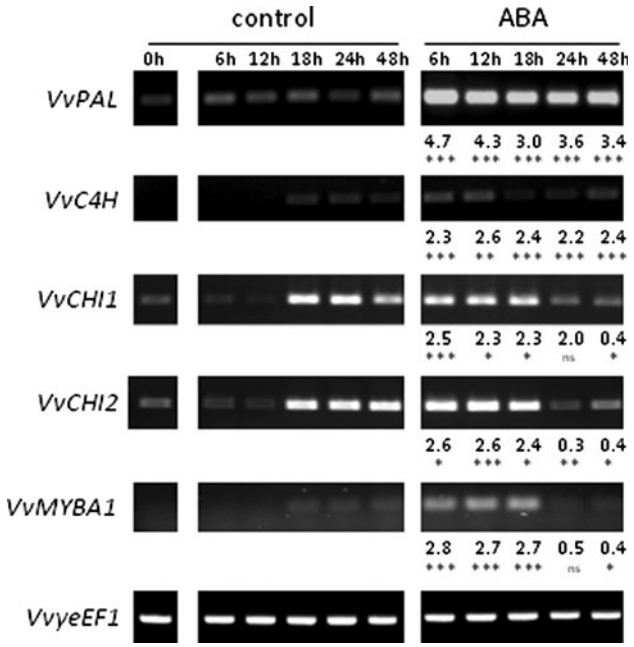

Fig. 2 Expression of the VvPAL, VvC4H, VvCHI1, VvCHI2, VvMYBA1, and VvγeEF1 genes in cell cultures treated with methanol (control) or ABA at 10^{-4} M (ABA). Changes in gene expression levels in ABA-treated cell cultures compared to control cells, corresponding to the ratio of fluorescence of the PCR bands measured for treated cells divided by the fluorescence of the PCR bands measured for the control cells, are shown below the electrophoresis gels. Analyses were carried out in triplicate and quantified as described in Materials and Methods. Results are the mean ± standard error of three determinations and *asterisks* indicate significant statistical differences between treatments on the same date using Student's *t* test; * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$

Changes in Anthocyanin Concentrations in Cells Following ABA Treatment

During the 2 days after ABA treatment, significant changes were detected in free ABA concentrations and in the expression of genes involved in the first part of the anthocyanin pathway in treated cells compared to controls (Figs. 1, 2). However, during this period, no coloring or anthocyanin compounds were detected and the cells remained green. Culture then continued for an extended period of time (288 h to 12 days) under the same conditions. Four days after ABA addition (96 h), treated cells visually changed and started to turn red. This process lasted at least until 12 days in culture, whereas control cells remained green, as shown in Fig. 3A. To determine whether this red color was due to anthocyanins, cells were subjected to anthocyanin extraction (Fig. 3B) and quantification (Fig. 3C). Our results confirmed that this red color was due to the presence of anthocyanins. Anthocyanin biosynthesis was clearly induced by ABA: anthocyanins were detected only in cells supplemented with ABA from 96 h in culture up to 288 h. The culture medium was also

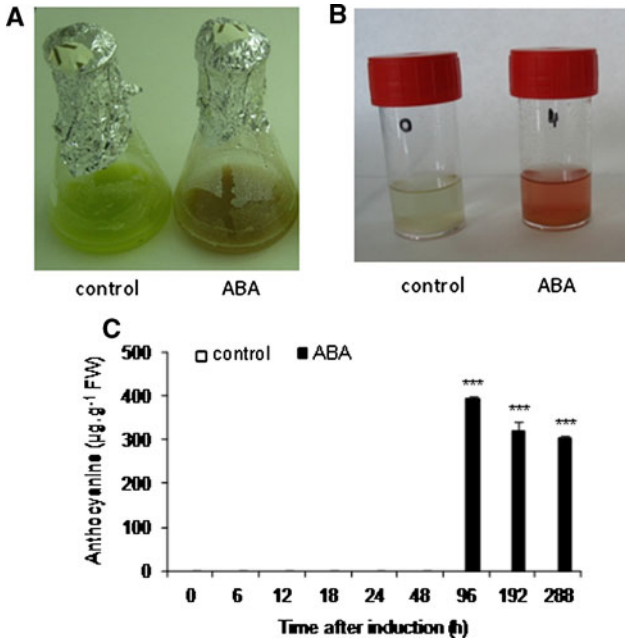

Fig. 3 Anthocyanin accumulation in Cabernet Sauvignon cells. **A** Cabernet Sauvignon cell cultures 12 days (288 h) after addition of methanol (control) or ABA at 10^{-4} M (ABA). **B** Anthocyanin extracts from cell cultures 12 days after addition of methanol (control) or ABA at 10^{-4} M (ABA). **C** Anthocyanin concentrations of Cabernet Sauvignon cells supplemented with methanol (control) or ABA at 10^{-4} M (ABA). Data are expressed as micrograms per gram fresh weight and represent the mean ± standard error of three determinations (bars). *Asterisks* indicate significant statistical differences between treatments on the same date using Student's *t* test; *** $P < 0.001$

analyzed for anthocyanins (data not shown) but none were detected, in either control or treated samples.

Discussion

In recent years, the mechanisms that control grape ripening have attracted a great deal of attention. Since Coombe and Hale (1973) demonstrated that ABA concentrations increased gradually in grapes before *véraison* and rapidly decreased as ripening began, this phytohormone has been studied extensively (Castellarin and others 2007; Deluc and others 2007; Koyama and others 2009; Owen and others 2009; Wheeler and others 2009; Lacampagne and others 2010). It has been confirmed that ABA synthesis is activated at *véraison* (Deluc and others 2007; Giribaldi and others 2007; Lund and others 2008; Koyama and others 2009) and that exogenous ABA hastens ripening (Matsushima and others 1989; Gagné and others 2006). One of the best-known roles of ABA is its ability to upregulate anthocyanin production. This role was demonstrated by exogenous ABA treatment of grapes, either in field tests (Gagné and others 2006; Peppi and others 2006; Deytieux-Belleau and others 2007) or in vitro (that is, sliced berries) (Hiratsuka and others 2001a; Koyama and others 2009), applied at early (Gagné and others 2006) or late (Peppi and others 2006) phenological stages and at different concentrations (Jeong and others 2004; Peppi and others 2006). The involvement of other phytohormones had been suggested. For instance, ethylene may take part in the control of ripening (El Kereamy and others 2003; Chervin and others 2005, 2008, 2009). Studies of ripening grapes revealed that complex hormonal signaling was activated at the time of *véraison*: the expression of genes related to ABA, auxin, ethylene, brassinosteroid, and, to a lesser extent, gibberellin and cytokinin metabolism was shown to be significantly modified at the onset of *véraison* (Symons and others 2006; Pilati and others 2007; Koyama and others 2009).

However, the exact role of ABA—as either the unique promoter of anthocyanin synthesis or simply a participant in a network controlling the triggering of pigmentation—remains unclear. In our study we isolated its impact by working on a simple model, that is, in vitro grape cell cultures. We selected Cabernet Sauvignon cells that did not spontaneously produce anthocyanins. Unlike inactivated mutants such as white cultivars, this model makes it possible to study the triggering of anthocyanin production. To the best of our knowledge, previous work assessing the impact of ABA on grape coloring used anthocyanin-producing grapes grown in field or in vitro (Hiratsuka and others 2001a; Ban and others 2003; Jeong and others 2004; Gagné and others 2006; Peppi and others 2006; Koyama and others 2009; Zheng and others 2009). The successful use of non-anthocyanin-producing tissues as a tool for studying the influence of ABA has been reported for several plants (Paek and others 1997; Kim and others 2006; Hung and others 2008) but not grapes. For instance, Nagira and others (2006) showed that changes in endogenous ABA concentrations played a decisive role in the induction of anthocyanin synthesis in regenerated *Torenia fournieri* shoots that do not produce anthocyanin under normal growing conditions.

In our work, the supplemented ABA was not degraded under culture conditions (data not shown) and was efficiently and rapidly incorporated into treated cells: endogenous free ABA content in the treated cells was significantly increased compared to the negligible content in controls, at any date (Fig. 1). The time-course of ABA accumulation in cells showed a fast, massive, lasting increase. This pattern was concordant with previous data that reported a rapid increase in endogenous ABA concentrations in ABA-treated cell cultures of other plants (Vágner and others 1998; Aroca and others 2008; Guo and others 2009; Zhang and others 2009b; Hao and others 2010). Considering the lasting impact of treatment, although some authors observed a slight, constant decrease following supplementation (Guo and others 2009), Vágner and others (1998) noticed that ABA levels remained high for 2 weeks in *Picea abies* embryos following ABA removal from the culture medium. Our observations indicated that ABA was imported into cells, but no conclusion was drawn concerning the mode of ABA intake: facilitated diffusion or transporter-mediated import may occur, whereas simple diffusion may not be possible due to the chemical structure of ABA (presence of carboxylic, ketone, and alcohol groups). Intake occurred rapidly following treatment and continued until a plateau was reached, possibly reflecting the osmotic equilibrium between inner cells and the culture medium.

The increase in endogenous ABA measured in our ABA-treated grape cells reflected observations in ABA-treated field-grown grapes, where an earlier, higher accumulation of ABA occurred in ABA-treated grapes compared to controls (Gagné and others 2006; Deytieux-Belleau and others 2007). The exogenous supply of ABA in our grape cell suspensions may mimic processes occurring in grapes during color change, corresponding to a massive import from the leaves (Koussa and others 2004; Antolin and others 2003) or to the triggering of ABA biosynthesis (Giribaldi and others 2007; Deluc and others 2007), because an ABA peak characterizes the onset of *véraison* in grape skins (Coombe and Hale 1974; Gagné and others 2006; Wheeler and others 2009).

ABA is known to have a positive impact on the expression of genes involved in anthocyanin production. It was confirmed by the upregulation of all genes we studied

as early as 6 h post-treatment, clearly correlating with the increase in cell ABA. This pattern was in accordance with results obtained in ABA-treated grape skins (Ban and others 2003; Jeong and others 2004), leading us to conclude that our cell model accurately reflected the processes occurring in grapes.

ABA-treated cells exhibited an earlier and transient increase in *VvCHI1*, *VvCHI2*, *VvC4H*, and *VvMYBA1* transcripts compared to control cells, suggesting earlier activation of their metabolism, as reported in ABA-treated compared to untreated grape skins (Ban and others 2003; Jeong and others 2004). A similar coordinated pattern was previously observed for downstream anthocyanin pathway genes (from *VvCHS* to *VvUFGT*, and *VvMYBA1*) in field-grown berries (Jeong and others 2004). *VvPAL* expression remained high and almost constant. PAL is the first enzyme in the phenylpropanoid pathway that leads to anthocyanins, as well as to many other polyphenol compounds such as stilbenes and tannins. One hypothesis is that a constant, high level of *VvPAL* induction may be required to produce sufficient precursors for the various branches of the polyphenol pathway, including anthocyanins. In grapes, PAL activation was shown to be promoted by ABA application (Hiratsuka and others 2001a, b; Gagné and others 2006; Koyama and others 2009), which also induced an increase in flavonoid compounds, mainly flavonols and anthocyanins (Koyama and others 2009). Furthermore, the ABA-induced *VvPAL* activation directly correlated with the increase in anthocyanin concentrations in both in vitro and field-grown grapes (Hiratsuka and others 2001a, b; Gagné and others 2006; Koyama and others 2009), as well as in other plants, for example, strawberries (Jiang and Joyce 2003) and *Gingko biloba* (Hao and others 2010).

The most striking result obtained in these Cabernet Sauvignon cell cultures, which do not spontaneously produce anthocyanins, was that anthocyanin production was initiated following ABA treatment. Numerous publications have reported a positive impact of this plant hormone on grape color development, that is, an increase in anthocyanin concentrations and earlier color expression due to the induction of specific structural and regulatory gene expression (Kataoka and others 1982; Matsushima and others 1989; Kim and others 1998; Esteban and others 2001; Hiratsuka and others 2001a; Ban and others 2003; Jeong and others 2004; De La Hera Orts and others 2005; Gagné and others 2006; Peppi and others 2006).

In our experiment, red color and anthocyanin accumulation were detected only in treated cells, suggesting that ABA may be the single promoter of anthocyanin production. A similar conclusion was reached by Nagira and others (2006) following exogenous ABA application to non-anthocyanin-producing *Torenia fournieri* shoots.

However, anthocyanin production was delayed (4 days) compared to the rapid changes observed in ABA levels and gene expression (after a few hours). The impact of ABA treatment on Cabernet Sauvignon cells was also delayed compared to cultures of anthocyanin-producing Gamay teinturier cells, under the same conditions, which exhibited increased anthocyanin levels compared to controls as early as 6 h after ABA addition (data not shown). Similarly, these changes occurred 1 day after ABA exposure for grapes cultured in vitro (Hiratsuka and others 2001a). This 4-day delay in color development in Cabernet Sauvignon cells may reflect the extensive changes required to launch the cell mechanisms and structures responsible for initiating anthocyanin production. The fact that no delay was observed in gene expression, whereas secondary metabolite accumulation started later, was previously observed by Fisher and others (2006), who investigated the expression of genes related to polyphenol biosynthesis and the accumulation of the corresponding metabolites (flavonols, flavanols, flavanones) in apple. The 4 days required for anthocyanin production suggest that this late synthesis may be due to post-translational modifications and/or protein biosynthesis.

In previous studies (Matsushima and others 1989; Hiratsuka and others 2001a; Ban and others 2003; Jeong and others 2004; Gagné and others 2006; Peppi and others 2006; Zheng and others 2009), anthocyanin synthesis was activated by ABA in spontaneous anthocyanin-producing tissues. In our experiment, anthocyanin production was triggered in non-anthocyanin-producing Cabernet Sauvignon cells, leading us to conclude that ABA initiated anthocyanin biosynthesis.

These data also correlated with the fact that maintaining high levels of ABA in contact with cells induced stable, constant anthocyanin production. Indeed, Nagira and others (2006) reported constant anthocyanin production in *Torenia fournieri* shoots maintained in contact with ABA for several days, whereas they became green again after transient ABA exposure. Koyama and others (2009) also reported a rapid, lasting increase in anthocyanin concentrations in grape skins grown in vitro following ABA supplementation of the culture medium.

These results raised the issue of whether ABA was the unique initiator molecule of anthocyanin biosynthesis in grapes. Indeed, other molecules have been hypothesized to control the triggering of grape ripening. Two main compounds have been considered as possible promoters of this process: sugars and, to a lesser extent, ethylene. Sugars have been shown to promote in vitro anthocyanin production in several plants, for example, *Torenia fournieri* shoots (Nagira and Ozeki 2004) and *Arabidopsis* (Solfanelli and others 2006), as well as grapes (Zheng and others 2009). Sugar sensing is closely associated with the ABA signaling

pathway (Cakir and others 2003; Carrari and others 2004). Synergistic effects of ABA and sugars on grape skin coloring and anthocyanin accumulation have also been reported (Kataoka and others 1982; Hiratsuka and others 2001a). Some authors suggest that ABA modulates sugar metabolism, import, and accumulation in grape berries (Palejwala and others 1985; Hiratsuka and others 2001a; Cakir and others 2003; Pan and others 2005; Yu and others 2006; Deluc and others 2009). A recent study also demonstrated that a sugar-induced protein kinase involved in sugar transport in grapes was also directly and positively controlled by ABA (Lecourieux and others 2010). However, previous experiments concluded that sugars did not play an initiating role in anthocyanin production in our grape model. In those experiments, Cabernet Sauvignon cells from the same clone, cultured under the same laboratory conditions as those used in this study, were grown in a maintenance medium containing 20 g l^{-1} sucrose, which was then supplemented with 20 g l^{-1} sucrose and the elicitor (Faurie and others 2009). These treated cells never turned red or produced anthocyanins, whereas the anthocyanin content of Gamay teinturier cells (Larronde and others 1998) increased significantly and rapidly after the same sucrose supplementation.

A transient increase in endogenous ethylene concentrations has been detected in grape skins at *véraison* (Hale and Coombe 1974; Alleweldt and Koch 1977), and cross-talk between ABA and ethylene is assumed to trigger color change (Symons and others 2006; Deytieux-Belleau and others 2007; Pilati and others 2007; Lund and others 2008; Koyama and others 2009). Exogenous and endogenous ethylene has been reported to have a positive impact on grape enlargement and composition as well as on anthocyanin production (Szyjewicz and Kliewer 1983; El Kereamy and others 2003; Chervin and others 2005, 2009). However, recent data suggest that ABA accumulation in nonclimacteric fruits, including grapes, precedes the ethylene peak and that ABA upregulates the expression of genes encoding the enzymes that catalyze ethylene synthesis (Koyama and others 2009; Zhang and others 2009b). Furthermore, ethylene accumulation at the beginning of ripening was not confirmed by Zhang and others (2009a), who also suggested that ethylene production may be controlled by ABA. In Cabernet Sauvignon cells from the same clone, cultured under similar conditions, ethephon, applied as a single inducer, failed to induce *VvPAL* expression or trigger red coloring, that is, anthocyanin accumulation, suggesting that ethylene was unable to trigger anthocyanin production under those conditions (Faurie and others 2009).

Taken together, our results indicate that ABA is the single initiator of anthocyanin biosynthesis in these grape cell cultures that do not spontaneously produce anthocyanins. Sugars and ethylene may not be responsible for initiating anthocyanin accumulation but rather for promoting higher concentrations. Further investigations are required to clarify their role in this process.

This work brings new insight into the initiation of anthocyanin biosynthesis in grapes: ABA has been shown to play a decisive role in triggering anthocyanin accumulation in cells that do not produce it spontaneously. Exogenous ABA treatment rapidly affected cell ABA content and promoted anthocyanin production. It also induced the upregulation of several structural genes in the upstream anthocyanin pathway (*VvPAL*, *VvC4H*, *VvCHI1*, *VvCHI2*), as well as the gene encoding a transcription factor involved in controlling anthocyanin production (*VvMYBA1*). In addition, these results are in accordance with numerous previous studies reporting a positive effect of ABA on anthocyanin production, thus validating our model (non-anthocyanin-producing Cabernet Sauvignon cells) as a convenient tool for studying the role of key molecules, cross-talk between molecules, and/or factors such as environmental parameters involved in controlling grape color development. This tool may help to elucidate the physiological events that occur during *véraison*. These cell cultures also constitute a simple model, grown under controlled conditions, easily available year-round, unlike field-grown grapes that can be collected only during a short season.

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References

Alleweldt G, Koch R (1977) Der äthylengehalt reifender Weinbeeren. Vitis 16:263–271

Antolin MC, Baigorri H, De Luis I, Aguirrezabal F, Gény L, Broquedis M, Sanchez-Diaz M (2003) ABA during reproductive development in non-irrigated grapes (*Vitis vinifera* L. cv. Tempranillo). Austr J Grape Wine Res 9:169–176

Aroca R, Vernieri P, Ruiz-Lozano JM (2008) Mycorrhizal and non-mycorrhizal *Lactuca sativa* plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. J Exp Bot 59:2029–2041

Ban T, Ishimura M, Kobayashi S, Shiozaki S, Goto-Yamamoto N, Horiuchi S (2003) Abscisic acid and 2,4-dichlorophenoxyacetic acid affect the expression of anthocyanin biosynthetic pathway genes in "Kyoho" grape berries. J Hortic Sci Biotechnol 78:586–589

Broquedis M (1987) Utilisation de la dialyse et de la chromatographie ultra-rapide pour le dosage de l'acide abscissique : Application à la vigne. Vitis 26:19–26

Cakir B, Agasse A, Gaillard C, Saumonneau A, Delrot S, Atanassova R (2003) A grape ASR protein involved in sugar and abscisic acid signaling. Plant Cell 15:2165–2180

Carrari F, Fernie AR, Iusem ND (2004) Heard it through the grape? ABA and sugar cross-talk: the ASR-story. Trends Plant Sci 9:57–59

Castellarin SD, Pfeiffer A, Sivilotti P, Degan M, Peterlunger E, Di Gaspero G (2007) Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grape under seasonal water deficit. Plant Cell Environ 30:1381–1399

Chervin C, El-Kereamy A, Roustan JP, Latche A, Lamon J, Bouzayen M (2005) Ethylene seems required for the berry development and ripening in grape, a non-climacteric fruit. Plant Sci 167:1301–1305

Chervin C, Tira-Umphon A, Terrier N, Zouine M, Severac D, Roustan JP (2008) Stimulation of the grape berry expansion by ethylene and effects on related gene transcripts, over the ripening phase. Physiol Plant 134:534–546

Chervin C, Tira-Umphon A, Chatelet P, Jauneau A, Boss PK, Tesniere C (2009) Ethylene and other stimuli affect expression of the UDP glucose-flavonoid 3-*O*-glucosyltransferase in a non-climacteric fruit. Vitis 48:11–16

Coombe BG, Hale CR (1973) The hormone content of ripening grape berries and the effects of growth substance treatments. Plant Physiol 51:629–634

De La Hera Orts ML, Martinez-Cutillas A, Lopez-Lorca JM, Perez-Prieto LJ, Gomez-Plaza E (2005) Effect of deficit irrigation on anthocyanin content of Monastrell grapes and wines. J Int Sci Vigne Vin 39:47–55

Deluc LG, Grimplet J, Wheatley MD, Tillett RL, Quilici DR, Osborne C, Schooley DA, Schlauch KA, Cushman JC, Cramer GR (2007) Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. BMC Genomics 8:429

Deluc LG, Quilici DR, Decendit A, Grimplet J, Wheatley MD, Schlauch KA, Mérillon JM, Cushman JC, Cramer GR (2009) Water deficit alters differentially metabolic pathways affecting important flavor and quality traits in grape berries of Cabernet Sauvignon and Chardonnay. BMC Genomics 10:212

Deytieux-Belleau C, Gagné S, L'hyvernay A, Donèche B, Gény L (2007) Possible roles of both abscisic acid and indol-acetic acid in controlling grape berry ripening process. J Int Sci Vigne Vin 41:141–148

El Kereamy A, Chervin C, Roustan JP, Cheynier V, Souquet JM, Moutounet M, Raynal J, Ford C, Latché A, Pech JC, Bouzayen M (2003) Exogenous ethylene stimulates the long-term expression of genes related to anthocyanin biosynthesis in grape berries. Physiol Plant 119:175–182

Esteban MA, Villanueva MJ, Lissarrague JR (2001) Effect of irrigation on changes in the anthocyanin composition of the skin of cv. Tempranillo (*Vitis vinifera* L.) grape berries during ripening. J Sci Food Agric 81:409–420

Faurie B, Cluzet S, Mérillon J-M (2009) Implication of signaling pathways involving calcium, phosphorylation and active oxygen species in methyl jasmonate-induced defense responses in grape cell cultures. J Plant Physiol 166:1863–1877

Fisher TC, Halbwirth H, Roemmelt S, Sabatini E, Schlangen K, Andreotti C, Spinelli F, Costa G, Forkmann G, Treutter D, Stich K (2006) Induction of polyphenol gene expression in apple (*Malus* × *domestica*) after the application of a dioxygenase inhibitor. Physiol Plant 128:604–617

Gagné S, Estève K, Deytieux C, Saucier C, Gény L (2006) Influence of abscisic acid in triggering "véraison" in grape berry skins of *Vitis vinifera* L. cv. Cabernet Sauvignon. J Int Sci Vigne Vin 40:7–14

Gény L, Darrieumerlou A, Doneche B (2003) Conjugated polyamines and hydroxycinnamic acids in grape berries during *Botrytis cinerea* disease development: differences between "Noble rot" and "Grey mould". Austr J Grape Wine Res 9:1–5

Giribaldi M, Perugini I, Sauvage FX, Schubert A (2007) Analysis of protein changes during grape berry ripening by 2-DE and MALDI-TOF. Proteomics 7:3154–3170

Guo D, Liang J, Li L (2009) Abscisic acid (ABA) inhibition of lateral root formation involves endogenous ABA biosynthesis in *Arachis hypogaea* L. Plant Growth Regul 58:173–179

Hale CR, Coombe BG (1974) Abscisic acid—an effect on the ripening of grapes. Bull R Soc NZ 12:831–836

Hao G, Du X, Zhao F, Ji H (2010) Fungal endophytes-induced abscisic acid is required for flavonoid accumulation in suspension cells of *Ginkgo biloba*. Biotechnol Lett 32:305–314

Hiratsuka S, Onodera H, Kawai Y, Kubo T, Itoh H, Wada R (2001a) ABA and sugar effects on anthocyanin formation in grape berry cultured in vitro. Sci Hortic 90:121–130

Hiratsuka S, Onodera H, Kawai Y, Kubo T, Itoh H, Wada R (2001b) Enzyme activity changes during anthocyanin synthesis in "Olympia" grape berries. Sci Hortic 90:255–264

Hung KT, Cheng DG, Hsu YT, Kao CH (2008) Abscisic acid-induced hydrogen peroxide is required for anthocyanin accumulation in leaves of rice seedlings. J Plant Physiol 165:1280–1287

Jeong ST, Goto-Yamamoto ON, Kobayashi S, Esaka M (2004) Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. Plant Sci 167:247–252

Jiang Y, Joyce DC (2003) ABA effects on ethylene production, PAL activity, anthocyanin and phenolic concentrations of strawberry fruit. Plant Growth Reg 39:171–174

Kataoka I, Sugiura A, Utsunomiya N, Tomana T (1982) Effect of abscisic acid and defoliation on anthocyanin accumulation in Kyoho grapes (*Vitis vinifera* L. × *V. labruscana* Bailey). Vitis 21:325–332

Kim SK, Kim JT, Jeong SH, Nam YS, Kim SH (1998) Effects of ethephon and ABA application on coloration, content, and composition of anthocyanin in grapes. J Korean Soc Hortic Sci 39:547–554

Kim JS, Lee BH, Kim SH, Oh KH, Yun Cho K (2006) Response to environmental and chemical signals for anthocyanin biosynthesis in nonchlorophyllous corn (*Zea mays* L.) leaf. J Plant Biol 49:16–25

Koussa T, Colin L, Broquedis M (2004) Endogenous levels of abscisic acid in various organs of *Vitis vinifera* L. (cv. Cabernet Sauvignon) between flower buds separated and grape closed stages. J Int Sci Vigne Vin 38:141–146

Koyama K, Sadamatsu K, Goto-Yamamoto N (2009) Abscisic acid stimulated ripening and gene expression in berry skins of the Cabernet Sauvignon grape. Funct Integr Genomics. doi: 10.1007/s10192-009-0145-8

Krisa S, Waffo-Téguo PW, Decendit A, Deffieux G, Vercauteren J, Mérillon JM (1999) Production of ^{13}C-labelled anthocyanins by *Vitis vinifera* cell suspension cultures. Phytochemistry 51:651–656

Lacampagne S, Gagné S, Gény L (2010) 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. J Plant Growth Regul 29:81–90

Larronde F, Krisa S, Decendit A, Chèze C, Deffieux G, Mérillon JM (1998) Regulation of polyphenol production in *Vitis vinifera* cell suspension cultures by sugars. Plant Cell 17:946–950

Lecourieux F, Lecourieux D, Vignault C, Delrot S (2010) A sugar-inducible protein kinase, VvSK1, regulates hexose transport and sugar accumulation in grape cells. Plant Physiol 152:1096–1106

Lund ST, Peng FY, Nayar T, Reid KE, Schlosser J (2008) Gene expression analyses in individual grape (*Vitis vinifera* L.) berries during ripening initiation reveal that pigmentation intensity is a valid indicator of developmental staging within the cluster. Plant Mol Biol 68:301–315

Matsushima J, Hiratsuka S, Taniguchi N, Wada R, Suzaki N (1989) Anthocyanin accumulation and sugar content in the skin of grape cultivar 'Olympia' treated with ABA. J Jpn Soc Hortic Sci 58:551–555

Nagira Y, Ozeki Y (2004) A system in which anthocyanin synthesis is induced in regenerated *Torenia* shoots. J Plant Res 117:377–383

Nagira Y, Ikegami K, Koshiba T, Ozeki Y (2006) Effect of ABA upon anthocyanin synthesis in regenerated *Torenia* shoots. J Plant Res 119:137–144

Owen SJ, Lafond MD, Bowen P, Bogdanoff C, Usher K, Abrams SR (2009) Profiles of abscisic acid and its catabolites in developing merlot grape (*Vitis vinifera*) berries. Am J Enol Viticult 60:277–284

Paek NC, Lee BM, Bai DG, Cobb BG, Magill CW, Smith JD (1997) Regulatory roles of abscisic acid for anthocyanin synthesis in maize kernels. Maydica 42:385–391

Palejwala VA, Parikh HR, Modi VV (1985) The role of abscisic acid in the ripening of grapes. Physiol Plant 65:498–502

Pan QH, Li MJ, Peng CC, Zhang N, Zou X, Zou KQ, Wang XL, Yu XC, Wang XF, Zhang DP (2005) Abscisic acid activates acid invertases in developing grape berry. Physiol Plant 125:157–170

Peppi MC, Fidelibus MW, Dokoozlian N (2006) Abscisic acid application timing and concentration affect firmness, pigmentation, and color of "Flame seedless" grapes. Hortic Sci 42:1440–1445

Pilati S, Perazzolli M, Malossini A, Cestaro A, Dematte L, Fontana P, Dal Ri A, Viola R, Velasco R, Moser C (2007) Genome-wide transcriptional analysis of grape berry ripening reveals a set of genes similarly modulated during three seasons and the occurrence of an oxidative burst at véraison. BMC Genomics 8:428

Riberéau-Gayon P, Stonestreet E (1965) Le dosage des anthocyanes dans le vin rouge. Bull Soc Chim 9:2649–2652

Shiozaki S, Kamata Y, Ogata T, Horiuchi S, Kawase K (1999) Localization of abscisic acid in grape berry by immunohistochemical techniques. J Jpn Soc Hortic Sci 68:1–9

Solfanelli C, Poggi A, Loreti E, Alpi A, Perata P (2006) Sucrose-specific induction of the anthocyanin biosynthetic pathway in *Arabidopsis*. Plant Physiol 140:637–646

Symons GM, Davies C, Shavrukov Y, Dry IB, Reid JB, Thomas MR (2006) Grapes on steroids. Brassinosteroids are involved in grape berry ripening. Plant Physiol 140:150–158

Szyjewicz E, Kliewer WM (1983) Influence of timing of ethephon application on yield and fruit composition of 'Chenin blanc' grapes. Am J Enol Viticult 34:53–56

Tattersall EA, Ergul A, Alkayal F, Deluc L, Cushman JC, Cramer GR (2005) Comparison of methods for isolating high-quality RNA from leaves of grape. Am J Enol Viticult 56:400–407

Vágner M, Vondráková Z, Strnadová Z, Eder J, Macháčková I (1998) Endogenous levels of plant growth hormones during early stages of somatic embryogenesis of *Picea abies*. Adv Hortic Sci 12: 11–18

Wheeler S, Loveys B, Ford C, Davies C (2009) The relationship between the expression of abscisic acid biosynthesis genes, accumulation of abscisic acid and the promotion of *Vitis vinifera* L. berry ripening by abscisic acid. Austr J Grape Wine Res 15:195–204

Yu XC, Li MJ, Gao GF, Feng HZ, Geng XQ, Peng CC, Zhu SY, Wang XJ, Shen YY, Zhang DP (2006) Abscisic acid stimulates a calcium-dependent protein kinase in grape berry. Plant Physiol 140:558–579

Zeevaart JA, Creelman RA (1988) Metabolism and physiology of abscisic acid. Ann Rev Plant Physiol Plant Mol Biol 39:439–473

Zhang J, Jia W, Yang J, Ismail AM (2006) Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Res 97:111–119

Zhang M, Leng P, Zhang G, Li X (2009a) Cloning and functional analysis of 9-*cis*-epoxycarotenoid dioxygenase (NCED) genes encoding a key enzyme during abscisic acid biosynthesis from peach and grape fruits. J Plant Physiol 166:1241–1252

Zhang M, Yuan B, Leng P (2009b) The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. J Exp Bot 60:1579–1588

Zheng Y, Tian L, Liu H, Pan Q, Zhan J, Huang W (2009) Sugars induce anthocyanin accumulation and flavanone 3-hydroxylase expression in grape berries. Plant Growth Regul 58:251–260