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Effects of Abscisic Acid Treatment on Berry Coloration and Expression of Flavonoid Biosynthesis Genes in Grape

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Abstract

In order to enhance berry coloration of bright-red grape cultivars, the effects of abscisic acid (ABA) treatment on the quantity and composition of anthocyanins as well as the expression of genes related to flavonoid biosynthesis in the berry were examined. Exogenous ABA treatment increased anthocyanin content, especially petunidin- and malvidin-type anthocyanins. Quantitative real-time PCR analysis revealed that ABA treatment around véraison resulted in the upregulation of genes encoding enzymes responsible for both general flavonoid and anthocyanin biosynthesis. On the other hand, the gene expressions of enzymes involved in proanthocyanidin synthesis were drastically decreased at véraison and remained extremely low even with ABA treatment. Thus, increases in the total amount and composition ratios of petunidin- and malvidin-type anthocyanins were mainly caused by ABA-induced upregulation of *uridine diphosphate glucose flavonoid glucosyl transferase, glutathione S-transferase* 4, *O-methyl transferase* and *flavonoid* 3', 5' *hydroxylase* expression, resulting in the deep coloration of berry of skin.

Keywords

1. Introduction

The color of grape berry is determined by the quantity and composition of anthocyanins [1]-[3], which belong to

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the class of flavonoid molecules that are synthesized via the phenylpropanoid pathway (**Figure 1**). Two classes of genes are required for anthocyanin biosynthesis; one includes structural genes encoding enzymes that directly participate in the formation of anthocyanins and other flavonoids, and the other comprises regulatory genes controlling the transcription of structural genes. Uridine diphosphate-D-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) is a key enzyme determining carbon flow to anthocyanins, and its expression is regulated by myeloblastosis (Myb)-related transcription factors (VvMybA and VlMybAs) [4]-[7]. Additionally, VvMyb5a and VvMyb5b regulate the expression of enzymes involved in both anthocyanin and proanthocyanidin biosynthesis [8]-[10], while VvMybPA1 [11] [12], VvMybPA2 [13], VvMybPAR [14] and VvMybF [15] regulate the expression of enzymes responsible for proanthocyanidin and flavonol biosynthesis. Anthocyanidin reductase (ANR), which catalyzes the production of the building blocks of proanthocyanidins, competes with UFGT as one of the key enzymes that modulate carbon flow to proanthocyanidins [16]. Synthesized anthocyanins are sequestered in vacuoles by the non-enzymatic flavonoid carrier protein glutathione *S*-transferase (GSTs) and tonoplast-localized multidrug and toxic compound extrusion family transporters such as AnthoMATE [17]-[19].

Various environmental and physiological factors affect anthocyanin biosynthesis and grape coloration [20] [21]. Anthocyanins accumulate in grape skin after véraison, the onset of ripening in non-climacteric fruit,
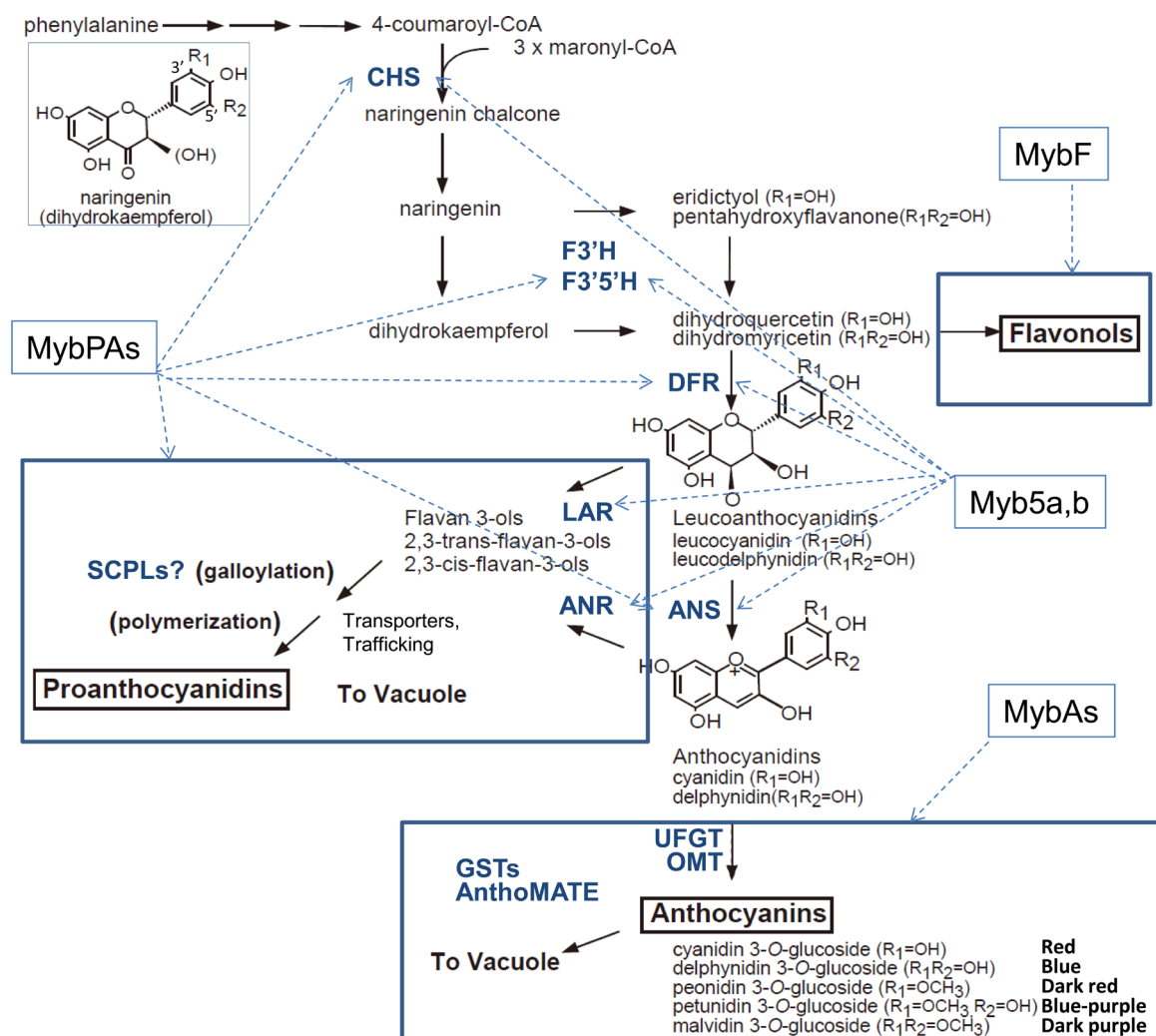
Figure 1. Principal pathway of flavonid biosynthesis and related Myb transcription factors in grape. ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; anthoMATE, anthocyanin multidrug and toxic compound extrusion family transporters; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3'H, flavonoid 3'-hydroxylase; F3',5'H, flavonoid 3',5'-hydroxylase; GST, glutathione *S*-transferase; OMT, flavonoid *O*-methyltransferase; SCPL, serine carboxipeptidase-like acyltransferase; UFGT, flavonoid 3-*O*-glucosyltransferase.

when grape berry maturation is accompanied by an increase in deformability and sugar content and decrease in acidity [22] [23]. These changes are regulated by physiological factors such as sugar accumulation and plant hormone levels [21]. This process is assumed to be triggered by endogenous abscisic acid (ABA), the level of which increases starting from the initial stage of fruit ripening and remains transiently elevated after véraison [21]. ABA application to grape clusters [21] [24] [25] and cell cultures [26] [27] is known to enhance anthocyanin accumulation via activation of the anthocyanin biosynthesis pathway. Therefore, ABA is a good candidate plant growth regulator for controlling the development of skin color in grapes. Here, we report the effects of ABA treatment on the quantity and composition of anthocyanins as well as the expression of genes related to anthocyanin biosynthesis, in order to develop the method for improving the coloration of a red grape berry variety. Our results suggest that the factor(s) other than ABA may also be involved in determining the carbon flow of flavonoid synthesis, especially proanthocyanidin biosynthesis, during grape berry development.

2. Materials and Methods

2.1. Plant Materials and ABA Treatment

One 25-year-old "Aki Queen" grape (*Vitis vinifera* × *Vitis labruscana*) grown at the Experimental Farm of Ishikawa Prefectural University was used for the treatment. Full-bloom clusters were dipped in 25 ppm gibberellic acid$_3$ (GA$_3$) solution including 5 ppm forchlorfenuron for inducing seedless, then dipped again in the same solution without forchlorfenuron 10 days after full-bloom (DAF), to stimulate berry growth. Clusters were sprayed with 200 or 400 ppm ABA solution (about 6 ml; BAL Planning Co., Japan) at 39, 48, or 55 days after full-bloom (DAF) based on our previous research (unpublished data). Véraison occurred around 42 DAF and clusters were harvested at 83 DAF. For all experiments, control clusters received no ABA treatment and there were eight replicates for each treatment.

2.2. Analysis of Total Soluble Solids (TSS) and Titratable Acidity

The pulp of peeled berries was squeezed and TSS and titratable acidity of the juice were determined. TSS content was measured with a digital refractometer (PAL-1; Atago Co., Japan). Titratable acidity was determined by titration and is expressed as the mass (in grams) of tartaric acid equivalent per 100 ml of juice. The significance of differences in TSS and titratable acidity between samples was determined using Tukey's test.

2.3. Analysis of Anthocyanin Content and Composition

Anthocyanins were extracted from 200 mg of freeze-dried berry skin in 15 ml of 50% (v/v) acetic acid at 4˚C for 24 h according to previously reported [28]. Total anthocyanin content was measured at 520 nm using a spectrophotometer (Biospec-1600; Shimadzu, Japan). For high-performance liquid chromatography (HPLC) analysis, the extract was passed through a 0.45-μm filter, and samples were subjected to reverse-phase HPLC using a Prominence LC20A instrument (Shimadzu, Japan) with a C18 Inertsil ODS-2 analytical column (5 μm, 250 × 6.0 mm inner diameter; GL Sciences, Japan) protected by a guard column containing the same material as previously reported [28]. Analysis was carried out at 35˚C, with mobile phase flow at 0.8 ml·min^{-1}. Elution was carried out with solvent A [1.5% (v/v) phosphoric acid] followed by solvent B [1.5% (v/v) phosphoric acid, 20% (v/v) acetic acid, and 25% (v/v) acetonitrile]. The concentration of solvent B was linearly increased from 25% to 85% over 40 min. Peaks detected by HPLC were identified by comparing the retention time and elution order of the standards cyanidin-3-monoglucoside (Cy3G), peonidin-3-monoglucoside (Pn3G), delphinidin-3-monoglucoside (Dp3G), petunidin-3-monoglucoside (Pt3G), and malvidin-3-monoglucoside (Mv3G). The other constituents, peonidin-3,5-diglucoside (Pn35G), cyanidin-3-(*p*-coumarylglucoside)-5-glucoside (Cy3*p*G5G), delphinidin-3-*p*-coumarylglucoside (Dp3*p*G), peonidin-3-(*p*-coumarylglucoside)-5-glucoside (Pn3*p*G5G), malvidin-3-(*p*-coumarylglucoside)-5-glucoside (Mv3*p*G5G), cyanidin-3-*p*-coumarylglucoside (Cy3*p*G), petunidin-3-*p*-coumarylglucoside (Pt3*p*G), peonidin-3-*p*-coumarylglucoside (Pn3*p*G), and malvidin-3-*p*-coumarylglucoside (Mv3*p*G) were estimated comparing the retention time and elution order as previously described [2] [28]. The concentration of each anthocyanin group, cyanidin (Cy3G, Cy3*p*G5G and Cy3*p*G), peonidin (Pn3G, Pn35G, Pn3*p*G5G and Pn3*p*G), delphinidin (Dp3G and Dp3*p*G), petunidin (Pt3G and Pt3*p*G), and malvidin (Mv3G, Mv3*p*G5G and Mv3*p*G), was calculated based on peak areas and calibration curves of Cy3G, Pn3G, Dp3G, Pt3G and Mv3G, respectively. The significance of differences in anthocyanin concentration and composition between

samples was determined using Student's *t*-test for concentration and Tukey's test for composition

2.4. Gene Expression Analysis

Total RNA was extracted from 400 mg of berry skin using the hot borate method [29]. After treatment with DNaseA (Thermo Scientific, USA), single-stranded cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, USA) in the presence of RNasin ribonuclease inhibitor (Promega, USA) and an oligo-dT primer. Quantitative real-time (qRT-)PCR was carried out with the StepOnePlus Real-Time PCR System (Applied Biosystems, USA) and the SYBR Green system with SYBR Pre-mix Ex-Taq II (Takara Bio, Japan). Primer sequences are listed in **Table 1**. The primer sets for *chalcone synthase* 3 (*CHS*3) and *open stomata* (*OST*)1 were prepared (synthesized) based on the previous reports [24] [30]. The amplification protocol was as follows: 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and 60°C for 30 s. The specific amplification of target genes was verified by polyacrylamide gel electrophoresis. Expression levels were normalized to that of *glyceraldehyde 3-phosphate dehydrogenase*, which was used as the internal reference. *OST*1 [30] and *Chitinase* (*CHT*)4 [31] were used as reporter genes for the ABA signal. Three independent RNA extractions were used for the analyses. All PCR reactions were replicated twice with the same cDNA samples and Student's *t*-test was used to determine significant differences in expression between the means.

Table 1. Sequences of the primers used in gene expression analysis.

| Gene	Forward (5' → 3')	Reverse (5' → 3')	Accession no.
Transcription factors			
*VlMybA*1-2	AGCCTGTTAGCTGAGCTGGA	CCTCCCTGGATTTGTTCGAT	AB073012
*VlMybA*1-3	CTGAGCATACTCAAATGGATCAAG	CACCTCCCTGGATTTGTTCTAC	AB427165
*VlMybA*3	CAAAGCTATAAAGTCTCGCAAGC	CACCTCCCTGGATTTGTTCTAC	FJ687580
*MybPA*1	TCGGAGATGATCAAGGTCAAGA	CGCTCAAGCAGTTGCAGATACT	NM_001281231
*MybPA*2	CCGTCTCCTAAAATGGCAGTTC	ATGAAAGAATTGGCGGTTTCG	NM_001281024
*Myb*5a	AGCAACGATGACGAAGACGAT	AGGGAACACATCCTCATTGATC	NM_001281179
*Myb*5b	ACCGATGATGTCTTCTCTTCATTTT	ACCATGGTGCTGTTGTTGGA	NM_001280925
Enzymes			
*CHS*3	TCGGCTGAGGAAGGGCTGAA	GGCAAGTAAAGTGGAAACAG	-
DFR	CAAGAACACCAACTGCCAGTGT	GCCCGGCAAAATTCCATAT	NM_001281215
ANS	GGCCTCAGGTTCCAACTATTGA	CAACTCCTCCCGGCATCTC	NM_001281218
F3'H	AGGCAAGCTCACCGACGTT	CTGTGCTGGATGAGGTGTCAGT	AB213603
F3'5'H	AACCCAGATTTTCTGGACGTAA	GCAGTAAATAAATTCAGGAGGAGTG	XM_010653221
UFGT	AAGCCGGGTACCGTTTATATG	CAACTGCCTCATGTGCTAGG	AB047091
FaOMT	TGAGAGAGCAGGCAGAGTCC	CGCGACGCCATCTCCAATAG	HM142924
ANR	TGCGACTTTGTCTTCCATGTTG	ATTGCTGGCTTGATCATGTCAT	NM_001280956
*SCPL*7	GCTTCCCATCTTACACAGAAAATGT	CAAAGCCCGGAGACCTGTT	XM_002269403
*SCPL*16	GTGCCGGAACTTCAATTATGC	CCACTCGGACATTTAATGCTTCT	XM_002272080
Carrier and transporters			
*GST*4	GGAGGAGAAAGCGGTAGTTGATC	GCATGACCAGTGTGTACACCAA	NM_001280940
*AM*1	ATCGGTTGGTGCAAAGAAGG	ATCTCTAGGCAAAGCATCACAGC	NM_001303078
ABA response			
*CHT*4	TCAAGTAGCGGCAGTGGTAGC	GGCACTGAGAAACGCTGCAC	NM_001281146
*OST*1	GCAACGCTGGTCGGTTTAGT	TAAGCCGTGGCATTGGACTT	-
Reference			
GADPH	CCAAGGCTGGAATAGCACTC	CCATGTGGACAATCAAGTCG	XM_002263109

3. Results

3.1. ABA Treatment Enhances Anthocyanin Accumulation and Berry Coloration

ABA treatment enhanced berry coloration (**Figure 2**). In the 39 DAF treatment group, 400 ppm ABA increased total anthocyanin content, whereas 200 and 400 ppm ABA decreased TSS (**Table 2**). Similar results were observed for the 48 DAF treatment group. In the 55 DAF group, ABA concentrations of 200 and 400 ppm increased total anthocyanin content while 400 ppm decreased TSS. In these experiments, anthocyanin accumulation was more effectively induced by treatment with 400 ppm than 200 ppm ABA; in the 55 DAF treatment, total anthocyanin content was increased 5 fold relative to untreated berry skin by 400 ppm ABA. Titratable acidity was affected by application of only 200 ppm ABA at 39 DAF (**Table 2**).

The amounts and composition ratios of various anthocyanin groups were examined by HPLC analysis (**Figure 3**). Exogenous ABA treatment stimulated accumulation of all type of anthocyanins in the berry skin. Interestingly, irrespective of the timing of treatment, the concentrations of petunidin- and malvidin-type anthocyanins, which are synthesized through the actions of flavonoid 3',5' hydroxylase (F3'5'H) and flavonoid 3',5' *O*-methyltransferase (FaOMT), were significantly increased. In contrast, the composition ratios of cyanidin and peonidin group were decreased while their amounts were increased by ABA treatment; consequently, the composition ratios of both groups became lower than the control value (<50% vs. 74%) ($P < 0.05$).

3.2. ABA Treatment Differently Modulates Myb Transcription Factors Involved in Anthocyanin Biosynthesis

qRT-PCR analysis was carried out using cDNA prepared from berry skin without treatment (control) or treated with 400 ppm ABA at 39 DAF. Consistent with previous reports that endogenous ABA content increased after véraison [22] [23], the expression level of the ABA-responsive gene *CHT*4 [30] was increased in control berry skin about 2 weeks after véraison (at 54 DAF, **Figure 4**). *CHT*4 expression level in ABA treated berry skin was
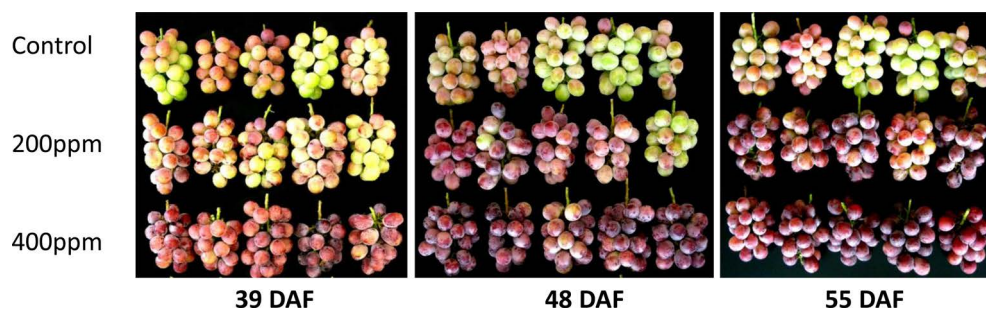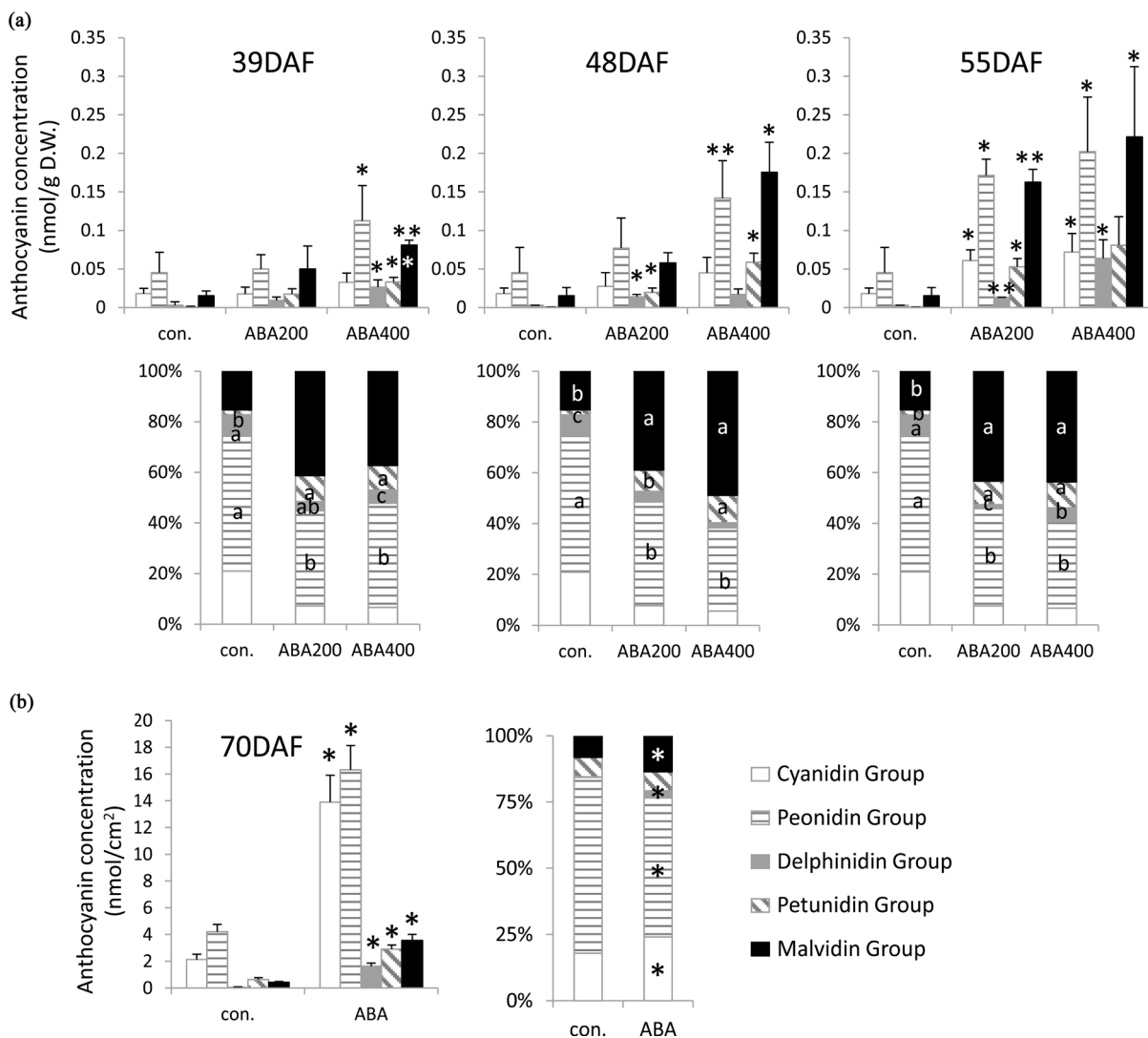

Figure 2. Effects of ABA treatment on color development of "Aki Queen" grape berries. Clusters were treated with 200 or 400 ppm ABA at 39, 48, or 55 DAF and harvested at 83 DAF.

Table 2. Effects of ABA treatments on total anthocyanin contents, TSS and titratable acidity.

Timing of treatment	ABA concentration	Anthocyanin (OD$_{520}$)	TSS (˚Brix)	Titratable acidity (g 100 ml^{-1})
	control	0.63 ± 0.27b[*]	18.38 ± 0.75a	0.37 ± 0.05b
39 DAF	200 ppm	0.80 ± 0.30b	16.52 ± 0.07b	0.44 ± 0.02a
	400 ppm	1.68 ± 0.44a	16.74 ± 0.28b	0.38 ± 0.02ab
	control	0.63 ± 0.27b	18.38 ± 0.75a	0.37 ± 0.05
48 DAF	200 ppm	1.06 ± 0.29b	17.18 ± 0.48b	0.38 ± 0.04
	400 ppm	2.44 ± 0.44a	16.68 ± 0.06b	0.36 ± 0.02
	control	0.63 ± 0.27b	18.38 ± 0.75a	0.37 ± 0.05
55 DAF	200 ppm	2.40 ± 0.06a	17.76 ± 0.95a	0.39 ± 0.03
	400 ppm	3.04 ± 0.99a	16.38 ± 0.32b	0.38 ± 0.02

[*]Values represent means ± SD of 3 replicates for anthocyanin and 5 replicates for TSS and titratable acidity. Data followed by different letters (a, b) in each column differ significantly by the Tukey's test ($P \leq 0.05$).

Figure 3. Effects of ABA treatment on the amounts and composition ratios of anthocyanin groups in "Aki Queen" grape berries. Clusters were treated with 200 or 400 ppm ABA at 39, 48, or 55 DAF and harvested at 83 DAF Stars indicate significance between treatment group ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, n = 3, Student's *t*-test).", as second sentence. Bars labelled with different lower-case letters indicate significance differences between treatment groups ($P \leq 0.05$, n = 3, Tukey's test).

increased by 6 fold relative to the level in untreated within 3 days (at 42 DAF), after which the magnitude of the difference decreased (to 3-fold higher than in the control at 54 DAF). The expression level of *OST*1, another ABA-responsive gene, was higher before véraison (at 27 and 33 DAF), and decreased thereafter (**Figure 4**) in control berry skin. *OST*1 expression was slightly increased by ABA treatment to 1.5 times the level in untreated berry skin within 3 days (at 42 DAF) and further increased to 3-fold higher at 54 DAF.

The qRT-PCR analysis revealed that all seven Myb-related transcription factor genes examined were expressed at different levels in control berry skin (**Figure 4**). *Myb5a*, *Myb5b*, *MybPA*1, and *MybPA*2 were expressed at higher levels before véraison and decreased gradually thereafter, with kinetics that was similar to those of *OST*1. In contrast, *VlMybA*1-2, *VlMybA*1-3, and *MybA*3 levels were slightly higher after véraison, with kinetics that were similar to those of *CHT*4.

ABA treatment at least transiently increased Myb transcription factor gene expression, albeit to different degrees for each gene. For instance, *Myb5a* and *Myb5b* levels increased within 3 days of ABA treatment (at 42 DAF) to 1.6 and 1.5 times those in untreated berry skin, respectively, but were comparable to control levels at 54 DAF. On the other hand, *MybPA*1 and *MybPA*2 expression was 2.1 and 3.3 times higher, respectively, at 42 DAF and 4.3 and 2.1 times higher, respectively, at 54 DAF than the levels in untreated berry skin. *VlMybA*1-2,
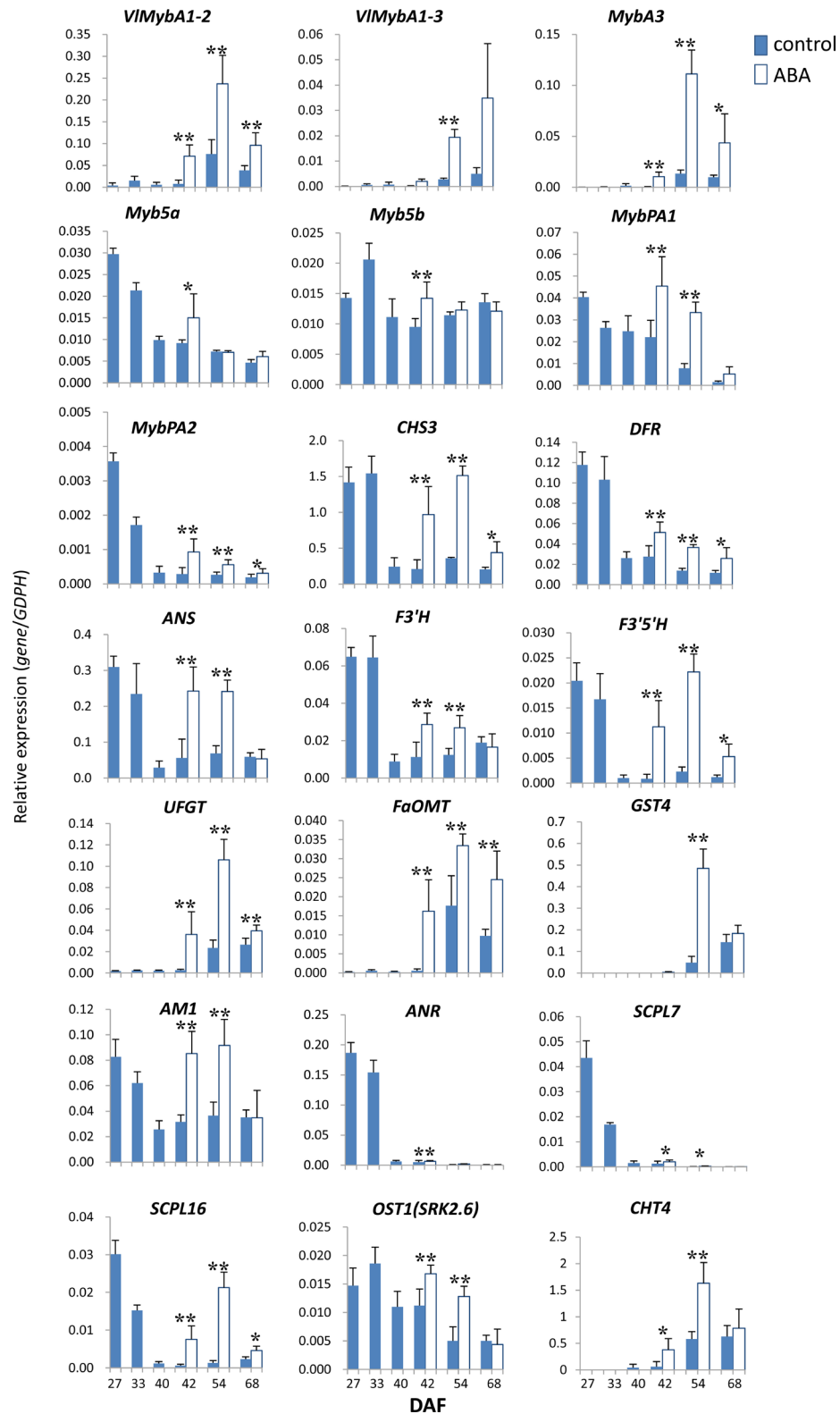
Figure 4. qRT-PCR analysis of genes related to anthocyanin biosynthesis in grape berry skin. In this experiment, samples were treated with 400 ppm ABA at 39 DAF. Expression levels were normalized to that of *glyceraldehyde 3-phosphate dehydrogenase* (*GADPH*). Each bar represents the mean value ± SD of three replicates. Bars labelled with asterisks indicate significant differences with respect to untreated berry skin samples. $^{**}P < 0.01$, $^{*}P < 0.05$ (Student's *t*-test).

*VlMybA*1-3, and *MybA*3 levels at 42 DAF were increased by 8.7, 13.9, and 32.1 fold, respectively, relative to those in untreated berry skin, but decreased to 3.1, 6.8, and 8.3 times the control values, respectively, at 54 DAF.

3.3. Anthocyanin Biosynthetic Enzyme Genes Show Variable Patterns of Expression during Berry Development That Are Altered by ABA Treatment

Among the 12 structural genes examined, four [*UFGT*, *FaOMT*, *GST*4, and *anthoMATE*1 (*AM*1)] are involved in anthocyanin biosynthesis and transport, three [*ANR*, *serine carboxypeptidase-like acyltransferase* (*SCPL*)7, and *SCPL*16] are implicated in the formation of proanthocyanidin, and the remaining five [*CHS*3, *dihydroflavonol reductase* (*DFR*), *anthocyanidin reductase* (*ANS*), *flavonoid 3'hydroxylase* (*F3'H*), and *F3'5'H*] function in a common flavonoid biosynthesis pathway (**Figure 1**). The expression kinetics in control and ABA-treated berry skin followed three distinct patterns. The levels of *UFGT* and *FaOMT* were upregulated in control berry skin about 2 weeks after véraison (at 54 DAF; **Figure 4**). ABA treatment increased their expression to 14.4 and 27.6 times those in untreated berry skin, respectively, within 3 days (at 42 DAF) and the magnitudes of increase gradually decreased thereafter (to 4.5 and 1.9 times higher than control, respectively, at 54 DAF). These expression kinetics were similar to those described above for *CHT*4.

*GST*4 expression was similar to that of *UFGT*, *FaOMT*, and *CHT*4, but was undetectable in control berry skin until 42 DAF. Therefore, the effect of ABA treatment was first detected at 54 DAF, at which time the level of GST4 was 10.1 times higher than in untreated berry skins, and then decreased to levels in untreated berry skin at 68 DAF.

The other nine genes—*i.e.*, *CHS*3, *DFR*, *ANS*, *F3'H*, *F3'5'H*, *AM*1, *ANR*, *SCPL*7, and *SCPL*16—showed similar expression patterns. The expression of these genes in control berry skin was higher before (at 27 and 33 DAF) and decreased after véraison (**Figure 4**). ABA treatment increased the expression levels to varying degrees; the kinetics were similar to those described above for *OST*1. It is worth noting that *F3'H* and *F3'5'H*—whose products act antagonistically in flavonoid biosynthesis—showed varied response to ABA treatment (**Figure 1**). The expression of F3'5'H increased by 13.0 and 9.6 folds upon ABA treatment at 42 DAF and 54 DAF, while the expression of F3'H increased by 2.5 and 2.2 folds, respectively.

4. Discussion

Application of a plant growth regulator is one strategy for controlling skin color development in grape berries, and viticulturists have sought to establish ABA-based method for enhancing the red color of table and wine grapes. In our results, ABA treatment at a higher concentration (400 ppm) increased total anthocyanin content while decreasing TSS. Treatment at a lower concentration (200 ppm) late in the growing season (55 DAF) increased the total anthocyanin content by 3.8 fold without decreasing TSS. These results indicate that ABA treatment around véraison negatively affects the sugar accumulation of "Aki Queen" berries, while the treatment at later maturation stage can enhance coloration without the decrease of TSS (including unpublished data).

Anthocyanin group composition in grape berry skin was altered by ABA treatment around véraison; the concentrations of petunidin and malvidin (dark blue/purple color) were increased, which resulted the decreased composition of cyanidin and peonidin (red color). The former two and the latter two anthocyanins are synthesized by *F3'5'H* and *F3'H*, respectively, which were enzymes that functioned antagonistically (**Figure 1**). Consistent with these changes *F3'5'H* expression was increased to a greater extent than that of *F3'H* by ABA treatment (13 vs. 2.5 times the level in untreated berry skin). Similar results were obtained in a previous study [2] that reported not only an increase in total anthocyanin content but also a change in the composition ratios of various anthocyanins in "Aki Queen" berries (*i.e.*, increases in petunidin and malvidin types and decreases in peonidin and cyanidin types). On the contrary, others had shown that ABA treatment did not influence anthocyanin composition in "Aki Queen" and "Pinot Noir" grape berry skin [32] [33]. These contradictory results may be due to the presence of seeds in the berries, since the latter studies do not employ treatment for seedless; it is well known that berry ripening is affected by the presence or absence of seeds [34] [35]. In this context, it is worth noting that exogenous ABA treatment modifies total anthocyanin concentration and anthocyanin composition in "Crimson Seedless" grape berries [36].

In this work, two genes were used as reporters for ABA response. *CHT*4 was identified as an ABA-inducible gene by a gene chip analysis using berry skin from the "Cabernet Sauvignon" [30], while *OST*1 was identified as a low temperature-inducible gene by oligo-DNA microarray analysis using detached "Pione" berries [30]. The

two genes showed distinct expression kinetics; *i.e.*, in control berry skin, *CHT*4 expression was upregulated after véraison within 3 days, and was significantly increased by ABA treatment, whereas *OST*1 expression was higher before véraison and then decreased, and was slightly increased by ABA treatment. Given that *OST*1 expression was upregulated by low temperature and ABA levels were higher in low temperature-treated grape berries, it was concluded that *OST*1 was involved in flavonoid biosynthesis in berry skin via low temperature-induced ABA signaling [30]. The *Arabidopsis OST*1 ortholog encoded an ABA-activated protein kinase, but ABA treatment had no detectable effect on *OST*1 mRNA abundance or on *OST*1 promoter—reporter activity, indicating that *Arabidopsis OST*1 expression was not directly regulated by ABA [37]. It is therefore likely that expression of the grape *OST*1 gene is associated with berry ripening and is not directly regulated by ABA; instead, some signal induced by its application might indirectly increase *OST*1 expression.

The expression kinetics of flavonoid biosynthesis-related genes examined in the present study was similar to those of either *CHT*4 or *OST*1. The former group included the Myb-related transcription factor genes *VlMybA*1-2, *VlMybA*1-3, and *MybA*3 and the structural genes *UFGT*, *FaOMT*, and *GST*4. The three Myb genes are contiguously clustered on chromosome 2, although *MybA*3 is considered as a pseudogene [4] [7]. In grapes, MybA1s regulate the anthocyanin synthesis pathway [4]-[7]. ABA has been reported to induce increases in *VvmybA*1 mRNA levels [25] [30], strongly supporting our conclusion that the expression of genes in this group is directly modulated by ABA. *UFGT* encodes a key enzyme that mediates carbon flow to anthocyanins in grapes [38] [39]; *FaOMT* encodes an OMT involved in anthocyanin methylation [40]; and *GST*4 encodes a non-enzymatic carrier involved in the transport and storage of anthocyanins in vacuoles [17]. UFGT, OMT, and GST have been implicated in grape berry coloration [40] [41], and their expression was increased in transgenic grapevines ectopically expressing the *VvmybA*1-2 gene [42]. These reports, together with our results, clearly indicate that ABA treatment induces the upregulation of genes encoding transcription factors as well as structural components involved in anthocyanin biosynthesis, leading to the accumulation of anthocyanin in berry skin.

Genes with *OST*1-type kinetics included four Myb-related transcription factor genes (*Myb*5a, *Myb*5b, *MybPA*1, and *MybPA*2) and nine structural genes (*CHS*3, *DFR*, *ANS*, *F3'H*, *F3'*, *5'H*, *AM*1, *ANR*, *SCPL*7, and *SCPL*16). Of these, *Myb*5a, *Myb*5b, *CHS*3, *F3'H*, *F3',5'H*, *DFR*, and *ANS* are involved in the general flavonoid biosynthesis pathway [9] [10] [14] [43] whereas *MybPA*1, *MybPA*2, and *ANR* are associated with the proanthocyanidin pathway [11]-[13] [16] [44]. *SCPL*7 and *SCPL*16 are orthologs of *DkSCPL*1 and *DkSCPL*2, which have been linked to proanthocyanidin accumulation in persimmon fruit [45] [46]. *AM*1 encodes a multidrug and toxic compound extrusion family transporter that is thought to be involved in anthocyanin transport to vacuoles [18]; however, we found that it was expressed at higher levels before véraison when there was no anthocyanin accumulation, suggesting that AM1 may also function in the transport of flavonoids other than anthocyanins. As discussed above, the expression of *OST*1-type genes in berries that were not treated with ABA was associated with berry ripening, with some signal stimulated by ABA treatment potentially enhancing the expression of these genes via an indirect mechanism. It was previously reported that ABA treatment of very young berries (at 14 or 17 DAF; 53 and 46 days before véraison) increased the expression of *VvMyb*5a, *VvMybPA*1, and *VvANR* genes [14] [47]. Since we applied ABA at a later stage (3 days before véraison), our results suggest that the effects of ABA on the expression of these genes depend on the timing of treatment. Interestingly, the levels of *ANR*—which encodes a key enzyme modulating carbon flow to proanthocyanidin—and *SCPL*7 were drastically reduced at véraison and remained extremely low even with ABA treatment, while the expression of other *OST*1-type genes were increased to a level comparable to those observed before véraison in response to ABA treatment. These results imply the influence of a factor(s) other than ABA on the regulation of flavonoid—especially proanthocyanidin—biosynthesis.

In conclusion, ABA treatment around véraison significantly increased the amount of petunidin- and malvidin-type anthocyanins, which resulted in deep coloration of berry skin. The changes were mainly caused by ABA-induced upregulation of *UFGT*, *GST*4, *FaOMT* and *F3'5'H*, and also by a hitherto unknown regulation system governing the gene expression of proanthocyanidin biosynthesis. ABA treatment to other red grape varieties on late maturation stage also increased anthocyanin content and improved berry coloration without decrease of TSS (manuscript in preparation). These results indicate that ABA treatment a few weeks before harvest can lead to the production of berries with the desired color.

Acknowledgements

References

[1] Falginella, L., Gaspero, G.D. and Castellarin, S.D. (2012) Expression of Flavonoid Genes in the Red Grape Berry of "Alicante Bouschet" Varies with the Histological Distribution of Anthocyanins and Their Chemical Composition. *Planta*, **236**, 1037-1051. http://dx.doi.org/10.1007/s00425-012-1658-2

[2] Kitamura, H., Nakayama, M., Nishikawa, Y., Kondou, H., Koshita, M. and Hiratsuka, S. (2005) Relationship between the Deep Coloration and Anthocyanins in the Skin of "Aki Queen" Berries. *Journal of the Japanese Society for Horticultural Science*, **75**, 401-406. http://dx.doi.org/10.2503/jjshs.74.401

[3] Shiraishi, S., Watanabe, Y., Okubo, H. and Uemoto, S. (1986) Anthocyanin Pigments of Black-Purple Grapes Related to Variety "Kyoho" (*Vitis vinifera* L. × *V. labrusca* L.). *Journal of the Japanese Society for Horticultural Science*, **55**, 123-129. http://dx.doi.org/10.2503/jjshs.55.123

[4] Azuma, A., Kobayashi, S., Mitani, N., Shiraishi, M., Yamada, M., Ueno, T., Kono, A., Yakushiji, H. and Koshita, Y. (2008) Genomic and Genetic Analysis of *Myb*-Related Genes That Regulate Anthocyanin Biosynthesis in Grape Berry Skin. *Theoretical and Applied Genetics*, **117**, 1009-1019. http://dx.doi.org/10.1007/s00122-008-0840-1

[5] Kobayashi, S., Goto-Yamamoto, N. and Hirochika H. (2004) Retrotransposon-Induced Mutations in Grape Skin Color. *Science*, **304**, 982. http://dx.doi.org/10.1126/science.1095011

[6] Kobayashi, S., Goto-Yamamoto, N. and Hirochika, H. (2005) Association of *VvMybA*1 Gene Expression with Anthocyanin Production in Grape (*Vitis vinifera*) Skin-Color Mutants. *Journal of the Japanese Society for Horticultural Science*, **74**, 196-203. http://dx.doi.org/10.2503/jjshs.74.196

[7] Walker, A.R., Lee, E., Bogs, J., McDavid, D.A.J., Thomas, M.R. and Robinson, S.P. (2007) White Grapes Arose through the Mutation of Two Similar and Adjacent Regulatory Genes. *Plant Journal*, **49**, 772-785. http://dx.doi.org/10.1111/j.1365-313X.2006.02997.x

[8] Cavallini, E., Zenoni, S., Finezzo, L., Guzzo, F., Zamboni, A., Avesani, L. and Tornielli G.B. (2014) Functional Diversification of Grapevine MYB5a and MYB5b in the Control of Flavonoid Biosynthesis in a Petunia Anthocyanin Regulatory Mutant. *Plant Cell Physiology*, **55**, 517-534. http://dx.doi.org/10.1093/pcp/pct190

[9] Deluc, L., Barrieu, F., Marchive, C., Lauvergeat, V., Decendit, A., Richard, T., Carde, J.P., Mérillon, J.M. and Hamdi, S. (2006) Characterization of a Grapevine R2R3-MYB Transcription Factor That Regulates the Phenylpropanoid Pathway. *Plant Physiology*, **140**, 499-511. http://dx.doi.org/10.1104/pp.105.067231

[10] Deluc, L., Bogs, J., Walker, A.R., Ferrier, T., Decendit, A., Mérillon, J.M., Robinson, S.P. and Barrieu, F. (2008) The Transcription Factor VvMYB5b Contributes to the Regulation of Anthocyanin and Proanthocyanin Biosynthesis in Developing Grape Berries. *Plant Physiology*, **147**, 2041-2053. http://dx.doi.org/10.1104/pp.108.118919

[11] Bogs, J., Ebadi, A., McDavid, D. and Robinson S.P. (2006) Identification of the Flavonid Hydroxylases from Grapevine and Their Regulation during Fruit Development. *Plant Physiology*, **140**, 279-291. http://dx.doi.org/10.1104/pp.105.073262

[12] Bogs, J., Jaffé, F.W., Takos, A.M., Walker, A.R. and Robinson S.P. (2007) The Grapevine Transcription Factor VvMYBPA1 Regulates Proanthocyanidin Synthesis during Fruit Development. *Plant Physiology*, **143**, 1347-1361. http://dx.doi.org/10.1104/pp.106.093203

[13] Terrier, N., Torregrosa, L., Ageorges, A., Vialet, S., Verriès, C., Cheynier, V. and Romieu, C. (2009) Ectopic Expression of VvMybPA2 Promotes Proanthocyanidin Biosynthesis in Grapevine and Suggests Additional Targets in the Pathway. *Plant Physiolgy*, **149**, 1028-1041. http://dx.doi.org/10.1104/pp.108.131862

[14] Koyama, K., Numata, M., Nakajima, I., Goto-Yamamoto, N., Matsumura, H. and Tanaka, N. (2014) Functional Characterization of a New Grapevine MYB Transcription Factor and Regulation of Proanthocyanidin Biosynthesis in Grapes. *Journal of Experimental Botany*, **65**, 4433-4449. http://dx.doi.org/10.1093/jxb/eru213

[15] Czemmel, S., Stracke, R., Weisshaar, B., Cordon, N., Harris, N.N., Walker, A.R., Robinson, S.P. and Bogs, J. (2009) The Grapevine R2R3-MYB Transcription Factor VvMYBF1 Regulates Flavonol Synthesis in Developing Grape Berries. *Plant Physiology*, **151**, 1513-1530. http://dx.doi.org/10.1104/pp.109.142059

[16] Xie, D.Y., Sharma, S.B., Paiva, N.L., Ferreira, D. and Dixon, R.A. (2003) Role of Anthocyanidin Reductase, Encoded by *BANYULS* in Plant Flavonoid Biosynthesis. *Science*, **229**, 396-399. http://dx.doi.org/10.1126/science.1078540

[17] Conn, S., Curtin, C., Bézier, A., Franco, C. and Zhang, W. (2008) Purification, Molecular Cloning, and Characterization of Glutathione *S*-Transferases (GSTs) from Pigmented *Vitis vinifera* L. Cell Suspension Cultures as Putative Anthocyanin Transport Proteins. *Journal of Experimental Botany*, **59**, 3621-3634. http://dx.doi.org/10.1093/jxb/ern217

[18] Gomez, C., Terrier, N., Torregrosa, L., Vialet, S., Fournier-Level, A., Verriès, C., Souquet, J.M., Mazauric, J.P., Klein, M., Cheynier, V. and Ageorges, A. (2009) Grapevine MATE-Type Proteins Act as Vacuolar H⁺-Dependent Acylated Anthocyanin Transporters. *Plant Physiology*, **150**, 402-415. http://dx.doi.org/10.1104/pp.109.135624

[19] Gomez, C., Conejero, G., Torregrosa, L., Cheynier, V., Terrier, N. and Ageorges, A. (2011) *In Vivo* Grapevine Anthocyanin Transport Involves Vesicle-Mediated Trafficking and the Contribution of anthoMATE Transporters and GST. *The Plant Journal*, **67**, 960-970. http://dx.doi.org/10.1111/j.1365-313X.2011.04648.x

[20] He, F., Mu, L., Yan, G.L., Liang, N.N., Pan, Q.H., Wang, J., Reeves, M.J. and Duan, C.Q. (2010) Biosynthesis of Anthocyanins and Their Regulation in Colored Grapes. *Molecules*, **15**, 9057-9091. http://dx.doi.org/10.3390/molecules15129057

[21] Kuhn, N., Guan, L., Dai, Z.W., Wu, B.H., Lauvergeat, V., Gomès, E., Li, S.H., Godoy, F., Arce-Johnson, P. and Delrot, S. (2014) Berry Ripening: Recently Heard through the Grapevine. *Journal of Experimental Botany*, **65**, 4543-4559. http://dx.doi.org/10.1093/jxb/ert395

[22] Coombe, B.G. and Hale, C.R. (1973) The Hormone Content of Ripening Grape Berries and the Effect of Growth Substance Treatments. *Plant Physiology*, **51**, 629-634. http://dx.doi.org/10.1104/pp.51.4.629

[23] Sun, L., Zhang, M., Ren, J., Qi, J., Zhang, G. and Leng, P. (2010) Reciprocity between Abscisic Acid and Ethylene at the Onset of Berry Ripening and after Harvest. *BMC Plant Biology*, **10**, 257. http://dx.doi.org/10.1186/1471-2229-10-257

[24] Ban, T., Ishimaru, M., Kobayashi, S., Goto-Yamamoto, N. and Horiuchi, S. (2003) Abscisic Acid and 2,4-Dichlorophenoxyacetic Acid Affect the Expression of Anthocyanin Biosynthetic Pathway Genes in 'Kyoho' Grape Berries. *The Journal of Horticultural Science & Biotechnology*, **78**, 586-589. http://dx.doi.org/10.1080/14620316.2003.11511668

[25] Jeong, S.T., Goto-Yamamoto, N., Kobayashi, S. and Esaka, M. (2004) Effects of Plant Hormones and Shading on the Accumulation of Anthocyanins and the Expression of Anthocyanins Biosynthetic Genes in Grape Berry Skins. *Plant Science*, **167**, 247-252. http://dx.doi.org/10.1016/j.plantsci.2004.03.021

[26] Gagné, S., Cluzet, S., Mérillon, J.-M. and Gény, L. (2011) ABA Initiates Anthocyanin Production in Grape Cell Cultures. *Journal of Plant Growth Regulation*, **30**, 1-10. http://dx.doi.org/10.1007/s00344-010-9165-9

[27] Hiratsuka, S., Onodera, H., Kawai, Y., Kubo, T., Itoh, H. and Wada, R. (2001) ABA and Sugar Effects on Anthocyanin Formation in Grape Berry Cultured *in Vitro*. *Scientia Horticulturae*, **90**, 121-130. http://dx.doi.org/10.1016/S0304-4238(00)00264-8

[28] Shiraishi, M., Yamada, M., Mitani, N. and Ueno, T. (2007) A Rapid Determination Method for Anthocyanin Profiling in Grape Genetic Resources. *Journal of the Japanese Society for Horticultural Science*, **76**, 28-35. http://dx.doi.org/10.2503/jjshs.76.28

[29] Wan, C.Y. and Wilkins, T.A. (1994) A Modified Hot Borate Method Significantly Enhances the Yield of High-Quality RNA from Cotton (*Gossypium hirsutum* L.). *Analytical Biochemistry*, **223**, 7-12. http://dx.doi.org/10.1006/abio.1994.1538

[30] Azuma, A., Fujii, H., Shimada, T. and Yakushiji, H. (2015) Microarray Analysis for the Screening of Genes Inducible by Light or Low Temperature in Post-Veraison Grape Berries. *The Horticulture Journal*, **84**, 214-226. http://dx.doi.org/10.2503/hortj.MI-041

[31] Koyama, K., Sadamatsu, K. and Goto-Yamamoto, N. (2010) Abscisic Acid Stimulated Ripening and Gene Expression in Berry Skins of the Cabernet Sauvignon Grape. *Functional & Integrative Genomics*, **10**, 367-381. http://dx.doi.org/10.1007/s10142-009-0145-8

[32] Mori, K., Saito, H., Goto-Yamamoto, N., Kitayama, M., Kobayashi, S., Sugaya, S., Gemma, H. and Hashizume, K. (2005) Effects of Abscisic Acid Treatment and Night Temperatures on Anthocyanin Composition in Pinot Noir Grapes. *Vitis*, **44**, 161-165.

[33] Kitamura, H., Nakayama, M., Kondou, H., Nishikawa, Y., Koshita, M. and Hiratsuka, S. (2007) Effect of Abscisic Acid Treatment at Variable Stages on the Expression of Proper and Deep Color in 'Aki Queen' Grape Berry Skins. *Horticultural Research* (*Japan*), **6**, 271-275. http://dx.doi.org/10.2503/hrj.6.271

[34] Niimi, Y., Ohkawa, M. and Torikata, H. (1977) Changes in Auxin and Abscisic Acid-Like Activities in Grape Berries. *Journal of the Japanese Society for Horticultural Science*, **46**, 139-144. http://dx.doi.org/10.2503/jjshs.46.139

[35] Kataoka, I., Sugiura, A., Utsunomiya, N. and 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.

[36] Ferrara, G., Mazzeo, A., Matarrese, A.M.S., Pacucci, C., Punzi, R., Faccia, M., Trani, A. and Gambacorta, G. (2015) Application of Abscisic Acid (S-ABA) and Sucrose to Improve Color, Anthocyanin Content and Antioxidant Activity of cv. Crimson Seedless Grape Berries. *Australian Journal of Grape and Wine Research*, **21**, 18-29. http://dx.doi.org/10.1111/ajgw.12112

[37] Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F. and Giraudat, J. (2002) Arabidopsis OST1 Protein Kinase Mediates the Regulation of Stomatal Aperture by Abscisic Acid and Acts Upstream of Reactive Oxygen Species Production. *The Plant Cell*, **14**, 3089-3099. http://dx.doi.org/10.1105/tpc.007906

[38] Boss, P.K., Davies, C. and Robinson, S.P. (1996) Expression of Anthocyanin Biosynthesis Pathway Genes in Red and White Grapes. *Plant Molecular Biology*, **32**, 565-569. http://dx.doi.org/10.1007/BF00019111

[39] Boss, P.K., Davies, C. and Robinson, S.P. (1996) Anthocyanin Composition and Anthocyanin Pathway Gene Expression in Grapevine Sports Differing in Berry Skin Color. *Australian Journal of Grape and Wine Research*, **2**, 163-170. http://dx.doi.org/10.1111/j.1755-0238.1996.tb00104.x

[40] Castellarin, S.D., Matthews, M.A., Di Gaspero, G. and Gambetta, G.A. (2007) Water Deficits Accelerate Ripening and Induce Changes in Gene Expression Regulating Flavonoid Biosynthesis in Grape Berries. *Planta*, **227**, 101-112. http://dx.doi.org/10.1007/s00425-007-0598-8

[41] Ageorges, A., Fernandez, L., Vialet, S., Merdinoglu, D., Terrier, N. and Romieu, C. (2006) Four Specific Isogenes of the Anthocyanin Metabolic Pathway Are Systematically Co-Expressed with the Red Color of Grape Berries. *Plant Science*, **170**, 372-383. http://dx.doi.org/10.1016/j.plantsci.2005.09.007

[42] Cutanda-Perez, M.C., Ageorges, A., Gomez, C., Vialet, S., Terrier, N., Romieu, C. and Torregrosa, L. (2009) Ectopic Expression of *VlmybA*1 in Grapevine Activates a Narrow Set of Genes Involved in Anthocyanin Synthesis and Transport. *Plant Molecular Biology*, **69**, 633-648. http://dx.doi.org/10.1007/s11103-008-9446-x

[43] Czemmel, S., Heppel, S. and Bogs, J. (2012) R2R3 MYB Transcription Factors: Key Regulators of the Flavonoid Biosynthetic Pathway in Grapevine. *Protoplasma*, **249**, S109-S118. http://dx.doi.org/10.1007/s00709-012-0380-z

[44] Bogs, J., Downey, M.O., Harvey, J.S., Ashton, A.R., Tanner, G.J. and Robinson, S.P. (2005) Proanthocyanidin Synthesis and Expression of Genes Encoding Leucoanthocyanidin Reductase and Anthocyanidin Reductase in Developing Grape Berries and Grapevine Leaves. *Plant Physiology*, **139**, 652-663. http://dx.doi.org/10.1104/pp.105.064238

[45] Akagi, T., Ikegami, A., Suzuki, Y., Yoshida, J., Yamada, M., Sato, A. and Yonemori, K. (2009) Expression Balances of Structural Genes in Shikimate and Flavonoid Biosynthesis Causes a Difference in Proanthocyanidin Accumulation in Persimmon (*Diospyros kaki* Thunb.) Fruit. *Planta*, **230**, 899-915. http://dx.doi.org/10.1007/s00425-009-0991-6

[46] Ikegami, A., Eguchi, S., Kitajima, A., Inoue, K. and Yonemori, K. (2007) Identification of Genes Involved in Proanthocyanidin Biosynthesis of Persimmon (*Diospyros kaki*) Fruit. *Plant Science*, **172**, 1037-1047. http://dx.doi.org/10.1016/j.plantsci.2007.02.010

[47] Lacampagne, S., Gagné, S. and 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. *Journal of Plant Growth Regulation*, **29**, 81-90. http://dx.doi.org/10.1007/s00344-009-9115-6