Effects of Abscisic Acid/Ethephon Treatments on Berry Development and Maturation in the Yellow-green Skinned ‘Shine Muscat’ Grape

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Abscisic acid (ABA) and ethylene are well-known phytohormones that are involved in the maturation of grape berries and other fruits. However, the process of yellow-green skinned grape berry maturation is not well understood due to difficulties in determining grape maturity from changes in skin color. Skin browning during maturation is a major commercial problem in some yellow-green skinned grape cultivars including ‘Shine Muscat’. To resolve this issue, a better understanding of the mechanisms involved in grape maturation and skin-browning is needed. We treated ‘Shine Muscat’ grape clusters at the veraison stage (45–50 DAFB) with spray applications of ABA or ethephon. These treatments produced darker colors and increased the trans-resveratrol and flavonol contents of berry skins. The ABA and ethephon treatments significantly increased the severity of skin browning. Changes in the expression of genes involved in polyphenol biosynthesis and oxidation were consistent with increases in polyphenols and the severity of browning in berry skins. The expression of VvACO2 and VvYUC1 genes, which are involved in ethylene and auxin biosynthesis, respectively, were upregulated in berries with brown skins. Although ABA treatment also increased the size of the berries, the effect of ethephon treatment on berry maturation was similar to, or greater than, that of ABA treatment. In berry skins, the expression of VvACO3, which is involved in phytohormone biosynthesis, increased significantly in response to ABA treatment. Overall, the changes in gene expression produced by ABA and ethephon treatments differed. Therefore, different mechanisms may regulate the physiological responses to ABA and ethephon, although both treatments accelerate berry maturation.

Key Words: physiological disorder, phytohormone treatment, polyphenols, table grapes.
Fidelibus, 2008; Peppi et al., 2006; Roberto et al., 2012; Sun et al., 2010; Wheeler et al., 2009). The abscisic acid (ABA) content in colored grapes rapidly increases around the veraison stage (Coombes and Hale, 1973). Sugar and anthocyanin accumulation are stimulated by the external application of ABA (Koyama et al., 2010; Mori et al., 2005); therefore, ABA is an effective promoter of grape berry maturation. ABA treatment also leads to an increased accumulation of phenolic compounds in berries, including flavonols and proanthocyanidins (Berli et al., 2011; Koyama et al., 2010; Lacampagne et al., 2010). Ethylene is another well-studied phytohormone that is involved in fruit maturation in a range of plant species. Although grape berries are classified as non-climacteric fruits, ethylene may be produced during grape maturation (Chervin et al., 2004; Coomeb and Hale, 1973; El-Kereamy et al., 2003; Mehta and Chundawat, 1979). Exogenous ethylene treatment also affects the long-term expression of genes involved in anthocyanin biosynthesis and accumulation (Böttcher et al., 2013b; El-Kereamy et al., 2003). Ethephon (2-chloroethylphosphonic acid; 2-CEPA) treatment at the veraison stage accelerates maturation and reduces the temperature to the optimum date for grape harvest (Mehta and Chundawat, 1979). In addition, ABA and ethylene function interactively at the onset of berry ripening (Sun et al., 2010). Both ABA and ethephon treatments have diverse effects on fruits which vary according to the growth stage, cultivation year, and cultivar (Kitamura et al., 2007; Mehta and Chundawat, 1979; Peppi et al., 2006; Roberto et al., 2012). However, it is difficult to identify the specific effects of phytohormone interactions, and the optimal times for phytohormone treatments (i.e., the periods when tissues or organs are particularly sensitive) may be limited (Böttcher et al., 2013b; Giribaldi et al., 2009; Kitamura et al., 2007; Peppi et al., 2006; Weyers and Paterson, 2001).

‘Shine Muscat’ is a yellow-green skinned table grape cultivar grown in Japan. It has become more popular recently because the grapes have edible thin skins and the vines are easily cultivated to produce seedless berries (Yamada et al., 2008). The cultivation area has also increased dramatically. Unfortunately, this crop is susceptible to skin browning, a physiological disorder known as “Kasuri-sho” in Japanese. “Kasuri-sho” usually occurs during berry maturation, approximately 70–80 days after full bloom (DAFB) (Mochida et al., 2013; Suehiro et al., 2014). Skin browning markedly decreases the market value of the product. Therefore, there is a significant economic incentive to develop procedures that will reduce or prevent skin browning. Other yellow-green skinned table grape cultivars, e.g., ‘Suihou’ and ‘Seto Giants’, are also susceptible to the same disorder (Ogoro et al., 2007). To make progress in resolving the problem of skin browning, a better understanding of the berry maturation process in yellow-green skinned grape cultivars is needed. It is also difficult to determine the optimal time for harvesting yellow-green skinned cultivars because the external appearance of the grapes changes little during maturation. Therefore, the results of this research should also make it easier to select appropriate times for grape harvest.

In this study, we investigated the effects of ABA and ethephon treatments on the maturation of ‘Shine Muscat’ grapes at the veraison stage. Physiological changes in the grapes were detected by analyzing related gene expression levels.

Materials and Methods

Plant materials

In 2012, we selected two 7-year-old ‘Shine Muscat’ (Vitis labruscana Bailey × V. vinifera L.) plants for our experiments (vines Nos. 24 and 25). These vines had been grown under forcing culture at the Shimane Agricultural Technology Center. Forcing cultivation advances the harvest time by about one month compared with a rain-protected (normal) cultivation system. Flower clusters were treated with 200 ppm streptomycin (Meiji Seika Pharma, Tokyo, Japan), which was sprayed onto the plants 10 days before full bloom. The flower clusters were then immersed in 25 ppm gibberellic acid (GA3; Kyowa Hakko Bio Co., Ltd., Tokyo, Japan) solution containing 5 ppm 1-(2-chloro-4-pyridyl)-3-phenylurea (CPPU, Fulmet; Kyowa Hakko Bio Co., Ltd.) at full bloom to promote seedless berry production. The developing grape berry clusters were treated again by immersion in 25 ppm GA3 solution at 10–15 DAFB. The final number of berries was adjusted to 40–45 per cluster. The yield of grapes per plant was on average 2,293 and 2,298 kg/10a in vines 24 and 25, respectively. We hand-sprayed berry clusters at the veraison stage (45 DAFB) with the commercial fertilizer Miyobi (BAL Planning Co., Ltd, Aichi, Japan), which contains 10% natural ABA (S-ABA) diluted with water (a final equivalent concentration of 400 ppm S-ABA). Spraying continued until the berry clusters were completely wet. For the ethephon treatments, we sprayed the commercial plant growth regulator Ethrel 10 (Ishihara Sangyo Kaisha, Ltd., Osaka, Japan), which contains 10% ethephon (which produces ethylene during its degradation) diluted with water to a final equivalent concentration of 20 ppm. We sprayed three clusters per plant (i.e., six clusters in total) for each treatment. After the treatments, we periodically observed the appearance of whole berry clusters and harvested two berries from each cluster (i.e., six berries per plant) at 75, 90, and 120 DAFB. Berry weights and degree of browning were measured on each harvest date.

In 2013, we selected two 8-year-old plants as experimental material (vines Nos. 14 and 15). These vines had been grown under semi-forced and rain-protected culture at the Shimane Agricultural Technology Center. The streptomycin, GA3, and Fulmet treatments for
seedless berry production were identical to those used in 2012. The final number of berries was adjusted to 40–45 per cluster. The yield of grapes per plant was on average 1,870 and 2,274 kg/10a in vines 14 and 15, respectively. The berry clusters were treated with ABA and ethephon at the veraison stage (45 DAFB) using Miyobi (S-ABA) and Ethrel 10 (ethephon) at final equivalent concentrations of 800 and 40 ppm, respectively. Nine berry clusters per plant were subjected to each treatment. After the treatments, the appearance of whole berry clusters was observed periodically, and three berries were randomly collected from the upper, middle, and lower portions of each berry cluster: i.e., nine berries in total at 70, 90, and 110 DAFB (for vine 15)/120 DAFB (for vine 14) for measurement and analyses. The berry skins were collected and immediately frozen in liquid nitrogen, and then stored at −80°C until later use.

In 2014, we used three 9-year-old plants (vines Nos. 14, 15, and 16) grown under rain-protected culture at the Shimane Agricultural Technology Center. The procedures for the streptomycin, GA3, and Fulmet treatments for seedless berry production were identical to those used in previous years. The final number of berries was adjusted to 40 per cluster. The yield of grapes per plant was on average 1,420, 1,640, and 1,400 kg/10a for vines 14, 15, and 16, respectively. ABA and ethephon treatments were applied at the veraison stage (50 DAFB) as in previous years using the reagents (S)-(+)−ABA (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and 2-chloroethylphosphonic acid (2-CEPA, Tokyo Chemical Industry Co., Ltd.) at concentrations of 500 and 40 ppm, respectively. The ABA solution was prepared by dissolving (S)-(+)−ABA in a small amount of ethanol, followed by dilution in an aqueous solution (0.1% v/v, final concentration) of Tween 20 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and adjusted to pH 7.0 using HCl. The ethephon solution was prepared in the same way as the ABA solution. The control spray contained 0.1% (v/v) Tween 20 (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and 2-chloroethylphosphonic acid (2-CEPA, Tokyo Chemical Industry Co., Ltd.) at concentrations of 500 and 40 ppm, respectively. The ABA solution was prepared by dissolving (S)-(+)−ABA in a small amount of ethanol, followed by dilution in an aqueous solution (0.1% v/v, final concentration) of Tween 20 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and adjusted to pH 7.0 using HCl. The ethephon solution was prepared in the same way as the ABA solution. The control spray contained 0.1% (v/v) Tween 20. Ten clusters per plant were used for each treatment. Skin browning was first observed 90 DAFB in all plants. Four berries from each cluster were periodically harvested at 50 (pre-treatment) and 70 DAFB. After skin-browning, 4–8 berries with or without brown skin were harvested separately at 90 and 110 DAFB (120 DAFB for vine 16) for measurements and analyses. The berry skins were collected, immediately frozen in liquid nitrogen, and then stored at −80°C until later use.

Measurement of berry characteristics

Berries were weighed, after which the skin colors at their apices were determined using a color reader (CR-10; Konica Minolta Sensing Inc., Tokyo, Japan); this device gives Commission Internationale de l’Elcairage (CIE) L*, a*, and b* values. The total soluble solids content (TSSC; °Brix) of the juice extracted from the berries was also measured by refractometry (N-1E; Atago Co., Ltd. Tokyo, Japan). The severity of skin browning in each whole berry cluster was determined using criteria developed by Mochida et al. (2013; Fig. 1A). Individual berries were assigned to categories according to the severity of browning as shown in Figure 1B.

Measurement of trans-resveratrol and flavonol contents in berry skins

We extracted polyphenols (including resveratrol and flavonol), in accordance with the methods described by Romero-Pérez et al. (2001). The frozen berry skin samples (1.0 g each) were homogenized in a 25 mL ethanol/water mixture (80:20, v/v) and then incubated at 60°C for 30 min. After centrifugation (8,000 rpm, 15 min), each supernatant was transferred to a flask and the solvent was completely evaporated in a rotary evaporator (Tokyo Rikakikai Co., Ltd. Tokyo, Japan). The solid residue was dissolved in 2 mL of 50% ethanol and transferred to a micro-centrifuge tube. After centrifugation (12,000 rpm, 15 min), each supernatant was transferred to a new micro-centrifuge tube and stored at −80°C for later analyses.

The resveratrol content was determined using an Ultra-Fast Liquid Chromatography (UFLC) system (Prominence UFLC LC-20AD; Shimadzu Co., Kyoto, Japan) equipped with a degasser (DGC-20 A3), an auto sampler (SIL-20AC HT), a column oven (CTO-20A), a diode array detector (SPD-M20A), and a 50 × 3.0-mm Inertsil ODS-3 column (GL Science, Tokyo, Japan). The extracts were analyzed using a previously described UFLC procedure (Suehiro et al., 2014).

Total flavonol content was determined using the simple spectrophotometric method described by Mammen and Daniel (2012). A total of 0.25 mL of 10% AlCl3 (w/v), 0.1 mL of 1 M potassium acetate, and 0.25 mL of distilled water were added to each 0.5 mL sample, followed by incubation at room temperature for 1 h. The absorbance of each solution was measured at 415 nm using a Shimadzu UV-VIS scanning spectrophotometer (UV-1800; Shimadzu Co.). Quercetin (Sigma Chemical Co., St. Louis, MO, USA) was used as the calibration standard, and the total flavonol content in each sample was calculated as the equivalent amount of quercetin (mg·g−1 FW). Trans-resveratrol was not detected using this method.

Because the original resveratrol and flavonol contents data for 90 and 110–120 DAFB in 2014 were derived from separately harvested samples with or without brown skin, the data for each harvest date were merged after taking into account the proportion of brown skinned grapes in each grape berry cluster.

RNA extraction and gene expression analysis

Total RNA samples were prepared from berry skins...
using the hot borate extraction method described by Wan and Wilkins (1994). Each total RNA sample was quantified using a spectrophotometer and adjusted to 200 ng L$^{-1}$. cDNA samples were synthesized from 1.0 μg of total RNA after treatment with DNase I (TaKaRa Bio Inc., Shiga, Japan). Reverse transcription was performed in 20 μL reaction volumes using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) with mixed oligo (dT) 20 and random primers (9 mer) following the manufacturer’s protocol. After the reactions were completed, 20 μL of water was added to each sample. Thus, a 1.0 μL cDNA sample was equivalent to 25 ng of total RNA. Extractions were performed on three samples from each berry cluster sampling point.

The expression levels of target genes were quantified by real-time quantitative reverse transcription PCR (qRT-PCR) using a TaKaRa Thermal Cycler Dice device (TP8000; TaKaRa Bio Inc.) as described in our previous study (Suehiro et al., 2014). Primer sets were prepared for the following polyphenol biosynthesis-related genes: phenylalanine ammonia-lyase (PAL) genes (Sparvoli et al., 1994); chalcone synthase (CHS) genes (Suehiro et al., 2014); stilbene synthase (STS) genes (Henanff et al., 2009); polyphenol oxidase (PPO) genes (Suehiro et al., 2014); VvMYB14 and VvMyb15 genes that encode Myb-like transcription factors involved in stilbene biosynthesis (Höll et al., 2013) and phytohormone-related genes: the 9-cis-epoxycarotenoid dioxygenase (NCED), and NCED2 genes, which encode enzymes that are involved in ABA biosynthesis (Speirs et al., 2013); type 2C protein phosphatase (PP2C) genes, which encode negative regulators of ABA signaling (Boneh et al., 2012; Gambetta et al., 2010); 1-aminocyclopropane-1-carboxylate oxidase (ACO) genes from the ethylene biosynthesis pathway (Muñoz-Robredo et al., 2013); the ethylene insensitive 3 family protein (EIN3) gene, which encodes a transcription factor in the ethylene signaling pathway (Chervin and Deluc, 2010); the GA 2-oxidase (GA2ox), GA3ox, and GA20ox from the GA biosynthesis pathway (Giacomelli et al., 2013); the gibberellin acid insensitive (GAI) gene, which encodes a transcription factor with a DELLA domain that is involved in GA signaling (Boss and Thomas, 2002); the gibberellin-insensitive dwarf 1 (GID1) gene, which encodes one of the GA receptors (Acheampong et al., 2015); the cytokinin histidine kinase receptors (CHKs) genes, which encode membrane-bound homodimeric proteins that are involved in cytokinin signaling (Böttcher et al., 2015); the VvYUC1 gene, which encodes a key enzyme that is involved in auxin biosynthesis (Böttcher et al., 2013a); and the auxin response factor (ARF) gene, which encodes a transcription factor that is involved in auxin signaling (Wan et al., 2014). Primer sets for the VvPALS, VvPP2Cs, VvGA2ox, VvGA20ox, VvGA3ox, VvGA11, VvGID1, and VvCHKs genes were generated for this study using DNA sequence information obtained from previous studies (Acheampong et al., 2015; Boneh et al., 2012; Boss and Thomas, 2002; Böttcher et al., 2015; Giacomelli et al., 2013; Sparvoli et al., 1994). The primer sequences for each of the target genes are listed in Supplementary Table 1. The Elongation Factor 1 (EF1) (Hanana et al., 2007) and Ubiquitin (Ubq) (Czemmel et al., 2009) genes were used as internal control (reference) genes. The reactions were performed at least twice for each sample. Threshold cycle (Ct) values were measured using software provided by the manufacturer (ver. 5.11; TaKaRa Bio Inc.), and expression levels were calculated after normalization to the two internal control genes.

Because the gene expression data for PALs, CHSs, STSs, Myb14, Myb15, PPO1, and PPO2 for 90 and 110–120 DAFB in 2014 were derived from separately harvested samples with or without brown skin, the data used were merged after taking into account the proportion of brown skinned grapes in each grape berry cluster.

Results

Effects of ABA/ethephon treatments on berry development

Neither ABA nor ethephon treatment had significant effects on TSSC (°Brix); however, both ABA and ethephon treatment significantly increased or decreased sugar contents, depending on the individual plant (Fig. 2A; Table 1). Berry weights at 70, 90, and 110–120 DAFB increased significantly after ABA, but not ethephon, treatment (Fig. 2B; Table 1). The berry skin colors also changed in response to the treatments. The L* values of ABA- and ethephon-treated berries were lower than those of control berries, indicating that skin color had darkened. The treatments also affected a* and b* values, which tended to increase and decrease, respectively (Table 1). Converting the CIE L*a*b* values to CIE xyY color space models showed that the skin colors changed from green-yellow at 70 DAFB, becoming redder at 110–120 DAFB. ABA treatment also reduced the color intensity. Skin color was markedly darker after ABA treatments compared with controls (Fig. 2C). Once the berry skins began to brown, the darkened area spread across the apex of each berry as maturation proceeded. The severity of browning in whole berry clusters and individual berries at 90 DAFB was higher after ABA and ethephon treatments than in controls (Fig. 2D). Finally, vine 14 showed the most severe browning in both years. Skin browning was more severe after ABA and ethephon treatments at 90 DAFB, whereas there were no clear differences at 110–120 DAFB (Table 1).

Trans-resveratrol and flavonol contents

The trans-resveratrol and flavonol contents of berry skins differed among vines and experimental years. Overall, ABA and ethephon treatments enhanced the
accumulation of these compounds (Table 2). In 2013, ABA treatments affected the trans-resveratrol content of vine 15, but no other obvious treatment effects were evident at 70 DAFB. ABA treatments increased the trans-resveratrol and flavonol contents of most of the vines at 90 DAFB. In 2014, ethephon treatments increased the trans-resveratrol and flavonol contents to values greater than those measured for the ABA-treated and control groups at 110–120 DAFB.

Expression of genes involved in polyphenol biosynthesis and oxidation

The expression levels of genes involved in polyphenol biosynthesis and oxidation are shown as mean values from 2013 (vines 14 and 15) and 2014 (vines 14, 15, and 16) in Figure 3. After ABA treatment at veraison, VvPALs, VvCHSs, VvSTSs, and VvMyb14 expression levels were elevated. In particular, VvCHSs and VvMyb14 gene expression levels were significantly elevated at 90 DAFB (Fig. 3A–D). In contrast, VvMyb15 expression levels did not differ significantly in response to either treatment (Fig. 3E). VvPPO1 expression increased significantly at 90 DAFB after ABA treatment, whereas the expression of VvPPO2 increased significantly after ethephon treatment (Fig. 3F, G). The response of VvPPO1 was similar to that of VvCHSs and VvMyb14 (Fig. 3B, D, F).

Expression of phytohormone-related genes

In this gene expression analysis, we have presented data from two experimental years separately because samples with and without brown skin were analyzed separately in 2014. There were no significant differences in the levels of VvNCED1, VvEIN3, VvGA11, VvGID1, and VvARF1 gene expression among treatments over the two years (Figs. 4A, G–I and 5A, G–I, L).

VvNCED1 expression decreased as the berries ma-

![Fig. 1. Skin-browning of ‘Shine Muscat’ berries. (A) Clusters of grape berries with brown skin. The degree of browning was determined in accordance with the methods described by Mochida et al. (2013); left, degree of browning was 0.1 at 90 days after full bloom (DAFB); center: severity of browning was 1.0 at 120 DAFB; right: degree of browning was 2.8 at 120 DAFB. Scale bar indicates 10 cm. (B) The berry-skin browning index. The degree of skin browning was categorized (0–5) according to the proportions of berry apices with brown skin: 0, no brown skin; 1, 1–20% brown skin; 2, 21–40%; 3, 41–60%; 4, 61–80%; 5, 81–100%. Scale bar indicate 1.0 cm.

![Fig. 2. Effects of ABA/ethephon treatments on TSSC, berry size, color, and the degree of browning. Mean values of TSSC (n = 15–35), berry size (n = 132–360), color (n = 132–360), and browning degree (clusters; n = 50–66; berries: n = 8–149) from two years (i.e., 2013 and 2014). TSSC (A). Berry size (B). Skin color changes depicted in a CIE xyY color space chromaticity diagram (C). Degree of browning in clusters and individual berries (D). The result of 45–50 DAFB indicates pre-treatment data (A and B). Different letters indicate significant differences between treatment groups within the same period according to Tukey’s HSD test at P < 0.05. Values are mean ± SE.](https://example.com/figure2)
Table 1. Effects of ABA/ethephon treatments on TSSC, berry size, color, and the severity of browning in individual vines.

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<td>14.4</td>
<td>16.8*</td>
<td>14.4</td>
<td>15.5</td>
<td>16.8*</td>
<td>14.4</td>
<td>15.5</td>
<td>16.8*</td>
<td>14.4</td>
<td>15.5</td>
<td>16.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2014</td>
<td>14.4</td>
<td>15.5</td>
<td>16.8*</td>
<td>14.4</td>
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<td>14.4</td>
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</tr>
</tbody>
</table>

The results from the 2012 sample show 75 DAFB data.

* Indicates no data. Asterisks indicate significant differences between treatment and controls in t-tests at P < 0.05.
tured (Figs. 4A and 5A); however, in 2013 $VvNCED1$ expression levels were higher in the ABA- and ethephon-treated groups than in the control groups at 70 and 90 DAFB (Fig. 4A). $VvNCED2$ expression also decreased as the berries matured (Figs. 4B and 5B); however, in 2014 $VvNCED2$ expression levels in the control

Table 2. Effects of ABA/ethephon treatments on trans-resveratrol and flavonol contents of berry skins from each individual vine.

<table>
<thead>
<tr>
<th>DAFB</th>
<th>Year</th>
<th>Vine No.</th>
<th>Biological replicates for analysis (n)</th>
<th>trans-Resveratrol contents (µg·g⁻¹ FW)</th>
<th>Flavonol contents (µg·g⁻¹ FW)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>ABA</td>
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<tr>
<td>70</td>
<td>2013</td>
<td>14</td>
<td>3</td>
<td>0</td>
<td>2.3</td>
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<td></td>
<td></td>
<td>15</td>
<td>3</td>
<td>1.2</td>
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</tr>
<tr>
<td></td>
<td>2014</td>
<td>14</td>
<td>(2)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
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<td></td>
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<td>(2)</td>
<td>0.2</td>
<td>0.1</td>
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<td>16</td>
<td>(2)</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>14–16</td>
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<td>0.2</td>
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<tr>
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<tr>
<td></td>
<td>2014</td>
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<td>(2)</td>
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<td>5.9</td>
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<td>3.6</td>
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</table>

* The data from 2014 for vines 14, 15, and 16 included only two biological replicates. Therefore, no statistical analyses were performed for the individual vines.

Values represent mean contents. The data for 90 and 110–120 DAFB in 2014, which were originally derived from separately harvested samples with or without brown skin, were merged after taking into account the proportion of brown skin in each grape berry cluster. Asterisks indicate values that differed significantly from controls according to $t$-tests ($P < 0.01$).

Fig. 3. Expression of genes related to polyphenol biosynthesis and oxidation. During 2013 and 2014, gene expression levels were analyzed using qRT-PCR after ABA and ethephon treatments. The genes were: $VvPALs$ (A), $VvCHSs$ (B), and $VvSTSs$ (C), which are key genes in the flavonoid and stilbene biosynthesis pathways; $VvMyb14$ (D) and $VvMyb15$ (E), which are transcription factor genes that regulate the polyphenol biosynthesis pathways; and $VvPPO1$ (F) and $VvPPO2$ (G), which are involved in the oxidation of polyphenols. The data were normalized to $VvEF1$ and $VvUbq$ gene expression levels and shown relative to expression levels at 45–50 DAFB (pre-treatment), which were set at a value of 1.0. ABA treatment was Miyobi in 2013 and (S)-(−)-ABA in 2014. Ethephon treatment was Ethrel 10 in 2013 and 2-CEPA in 2014. The control was water. All data are merged mean values from expression analyses over two years (i.e., 2013 and 2014). The original data for 90 and 110–120 DAFB in 2014, which were derived from separately harvested samples with or without brown skin, were merged after taking into account the proportion of brown skin in each grape berry cluster and used for the calculation. Different letters indicate values that differed significantly among the treatments at each sampling date according to Tukey’s HSD test ($P < 0.05$). Values are mean ± SE ($n = 15–42$).
groups significantly increased at 110–120 DAFB (Fig. 5B). VvPP2Cs expression increased as the berries matured (Figs. 4C and 5C); however, in 2014 VvPP2Cs expression levels in skin that was not brown after ABA and ethephon treatments were significantly lower at 110–120 DAFB (Fig. 5C).

The VvACO1, VvACO2, and VvACO3 genes showed different expression patterns in 2013 and 2014 (Figs. 4D–F and 5D–F). VvACO1 expression decreased as the berries matured only in 2014, and some significant differences in VvACO1 expression were observed among the different treatments (Fig. 5D). In 2014, VvACO2 expression increased significantly in samples with brown skin at 90 and 110–120 DAFB (Fig. 5E). The vertical axis scale on the 2013 VvACO3 figure panel is one hundred-fold greater than that of the 2014 figure panel because the level of VvACO3 expression at 45 DAFB (pre-treatment) was very low in 2013 (Fig. 4F). ABA treatment significantly increased the expression of VvACO3 at 90 DAFB in both years (Figs. 4F and 5F). In 2013, VvACO3 expression peaked at 70 DAFB in the ethephon treatment and control groups, whereas the expression peak for the ABA treatment group was at 90 DAFB. In 2014, VvACO3 expression peaked at 70 DAFB only in the control group, whereas expression levels gradually decreased as the berries matured in the ABA and ethephon treatment groups. Among the three VvACO homologs, the expression pattern of VvEIN3 was the most similar to that of VvACO2 (Figs. 4E, G and 5E, G).

In 2014, VvYUC1 expression levels at 110–120 DAFB decreased significantly after ABA treatment and increased significantly in berries with brown skins (Fig. 5K). The same expression pattern was observed for VvARF1 (Fig. 5L). The expression patterns of VvGID1 and VvCHKs were similar (Figs. 4I, J and 5I, J). In 2013, VvGID1 expression levels decreased after ABA treatment at 70 and 90 DAFB (Fig. 4I); however, this was not observed in 2014 (Fig. 5I). In 2013, the expression of VvCHKs also decreased after ABA treatment at 70 and 90 DAFB (Fig. 4I), and decreased significantly after ABA and ethephon treatments at 110–120 DAFB in 2014. In addition, the expression of VvCHKs increased in berries with brown skins (Fig. 5J).

We also investigated GA oxidase genes (VvGA2ox,
VvGA3ox, and VvGA20ox), but were unable to detect their expression (data not shown).

**Discussion**

**Effects of ABA/ethephon treatments on berry properties**

The effects of phytohormone treatments depend on the timing of hormone application, the cultivar, and growth conditions (Wheeler et al., 2009). Across the two years of our experiment, ABA treatments affected berry skin colors, which became darker (Fig. 2C; Table 1). Therefore, ABA treatments altered berry maturation in yellow-green skinned grapes, as previously reported for grapes with other skin colors (Berli et al., 2011; Coombe and Hale, 1973; El-Kereamy et al., 2003; González et al., 2012; Jeong et al., 2004; Kataoka et al., 1982; Kitamura et al., 2007; Koyama et al., 2010; Lacampagne et al., 2010; Mehta and Chundawat, 1979; Mori et al., 2005; Peppi and Fidelibus, 2008; Peppi et al., 2006; Roberto et al., 2012; Sun et al., 2010; Wheeler et al., 2009). Berry size also increased after ABA treatments (Fig. 2A; Table 1). The effects of exogenous ABA application on berry size have also been reported in previous studies (Peppi and Fidelibus, 2008; Wheeler et al., 2009). Wheeler et al. (2009) suggested that ABA enhanced the flow of carbon into berries and indirectly changed their osmotic potential. In addition, ABA can affect the activity of the cell wall-modifying enzyme polygalacturonase (Deytieux et al., 2005), and reducing the strength of the cell wall may lead to cell expansion and an increase in berry volume (Gambetta et al., 2010). ABA treatment also enhanced trans-resveratrol and flavonol accumulation in the berry skins, which may be commercially beneficial (Table 2). The enhanced accumulation of polyphenols in ‘Shine Muscat’ resembles responses by other grapes with colored skins, most of which accumulate anthocyanins (Berli et al., 2011; Koyama et al., 2010). The accumulation of polyphenols in response to ABA treatment was driven by the upregulation of gene expression in the corresponding biosynthetic pathways (Fig. 3). Secondary metabolite accumulation is one of the indicators of berry maturation (Conde et al., 2007; Kuhn et al., 2014). Therefore, our results show that exogenous ABA application at veraison accelerates berry maturation in...
yellow-green skinned grapes.

Ethephon treatments also affected berry skin color and size, but to a lesser extent than ABA treatments (Fig. 1B, C; Table 1). The accumulation of trans-resveratrol and flavonol in berry skins was also enhanced by ethephon treatments (Table 2). Although there were annual variations, the final sizes of the effects observed at 110–120 DAFB were larger after ABA than the ethephon treatments (Table 2). Ethylene has a general effect on berry ripening (Chervin and Deluc, 2010; Hale et al., 1970), but not on cell or berry sizes (Liu et al., 2016). Surprisingly, the changes in gene expression levels after ethephon treatments were small compared to those that followed ABA treatments and they did not correlate with polyphenol accumulation (Fig. 3). El-Kereamy et al. (2003) observed that ethephon treatment (3.6 ppm) of the colored-skin ‘Cabernet Sauvignon’ grape at veraison produced long-term increases in the expression of anthocyanin biosynthesis genes. In our experiments, some gene expression changes may have been obscured by annual variations or differences in cultivars and experimental conditions.

Effects of ABA/ethephon treatments on skin browning

The severity of browning increased after ABA and ethephon treatments (Fig. 1D; Table 1). At 110–120 DAFB, final browning was more severe after ethephon than ABA treatments. The expression of the VvPPO1 gene, which encodes an enzyme with an important role in plant tissue browning, increased significantly in response to ABA treatments at 90 DAFB (Fig. 3F). In contrast, ethephon treatments significantly increased the level of VvPPO2 gene expression at 90 DAFB (Fig. 3G). Because skin browning was also severe after ethephon treatments, the increase in VvPPO2 expression level is probably more closely associated with browning. Previous studies have shown that VvPPO2 plays a role in skin browning (Katayama-Ikegami et al., 2017; Suehiro et al., 2014), and the expression of PPO genes can be induced in response to biotic and abiotic stresses (Taranto et al., 2017; Yan et al., 2017). ABA can also mediate plant defense responses (Vishwakarma et al., 2017). The upregulation of VvPPO1 gene expression in grape skins in response to ABA treatments was considered a defense reaction; however, no significant differences in VvPPO1 gene expression were observed between the ethephon treatment and control groups (Fig. 3F).

Skin browning induced the upregulation of VvACO2 and VvYUC1 gene expression (Fig. 5E, K). Correlations have been observed in grapes between ACO gene expression levels and ethylene production (Chervin et al., 2004), and between VvYUC1 gene expression levels and auxin content (Böttcher et al., 2013a); therefore, the increased expression of these genes suggests that the ethylene and auxin biosynthetic pathways are upregulated in berries with brown skin. Böttcher et al. (2013a) also suggested that grape berry maturation is regulated by the interaction between ethylene and auxin production. In Arabidopsis roots, ethylene and auxin can stimulate flavonol accumulation (Lewis et al., 2011). Skin browning and flavonol accumulation may be positively regulated by the activation of auxin and ethylene biosynthesis in grape berries.

In addition, the expression of the VvCHKs genes increased in berries with brown skin at 110–120 DAFB (Fig. 5J). In Arabidopsis, the expression of Arabidopsis histidine kinase (AHK) genes, which are cytokinin receptor genes that are orthologous to VvCHKs, were induced by dehydration. Among these, AHK1 is a positive regulator, whereas other AHKs function as negative regulators (Tran et al., 2007). AHKs can mediate stress responses by both ABA-dependent and ABA-independent signaling pathways. A dehydration stress response probably occurs in the brown skin of grape berries. However, the physiological changes caused by the upregulation of VvCHKs genes remain unclear.

Effects of ABA/ethephon treatments on phytohormone metabolism

The expression of VvNCED1 and 2 decreased during berry maturation (Figs. 4A, B and 5A, B). VvNCED1 is probably the predominantly expressed VvNCED homolog (Sun et al., 2010; Wheeler et al., 2009). The expression of the VvNCED genes in ‘Cabernet Sauvignon’ decreased as the berries matured after veraison (Wheeler et al., 2009). Although VvNCEDs gene expression was not clearly affected by the ABA/ethephon treatments in our study, the expression patterns of VvNCED1 and 2 in ‘Shine Muscat’ appeared to be similar to those in grapes with colored skins.

The expression of PP2C is induced by abiotic stresses (e.g., temperature and dehydration) (Azuma, 2018; Boneh et al., 2012). There are several PP2C homologs in grapes, and the VvPP2Cs genes analyzed in this study belonged to the same phylogenetic group as the Arabidopsis genes AtAB11, AtAB12, AtHAB1, and AtHAB2. The expression of VvPP2Cs genes increases during berry maturation, and ABA signaling may be linked to the regulation of VvPP2C gene expression (Gambetta et al., 2010). In Arabidopsis, AtHAB1 gene expression may be regulated by a negative feedback loop mediated by ABA signaling (Schweighofer et al., 2004). In addition, ABA-dependent stress signaling may increase the activity of PPOs, stimulating browning (Bower and Cutting, 1988). Based on these observations, we suggest that ABA signaling is suppressed in the latter stages of berry maturation, when skin browning has not been initiated.

During ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate is converted to ethylene by aminocyclopropane-carboxylate oxidase (ACO). There are several ACO genes, and our results showed that VvACO3 ex-
pression changed significantly after ABA treatment (Figs. 4F and 5F). The results of our two-year expression analysis show high VvACO3 expression levels at 90 DAFB, suggesting that ethylene production was initiated at that time. Although it is present at low levels, ethylene is produced in maturing grape berries and affects the progress of maturation (Chervin et al., 2004). Exogenous ethylene promotes endogenous ethylene production in berries (El-Kereamy et al., 2003). Sun et al. (2010) also observed that ABA or ethylene treatment increased ACO gene expression and ethylene production in ‘Muscat Hambrug’. In our ‘Shine Muscat’ study, ethylene production may have been increased by the upregulation of VvACO3 expression gene at 90 DAFB after ABA treatment. In contrast, very small amounts of ethylene were generated at 60–70 DAFB when VvACO1 and VvACO2 gene expression levels were high in ‘Thompson Seedless’ grapes; the expression of VvACO3 remained unchanged (Muñoz-Robredo et al., 2013). We did not identify a specific effect that correlated with VvACO3 expression in this study. Future studies will be needed to understand how the production of ethylene and other plant hormones affect berry development.

In Arabidopsis seeds, ABA treatment suppresses the expression of GID1 (Voegele et al., 2011). In addition, the expression of cytokinin signaling genes is suppressed in Arabidopsis when the cytokinin content is decreased by environmental stress or in response to ABA (Nishiyama et al., 2011). In this study, ABA treatments also affected VvGID1 and VvCHKs gene expression levels (Figs. 4I, J and 5I, J). In contrast, ethephon treatment had no significant effects on the expression of these genes. A regulatory network involving ABA, GA, and cytokinin may be important in grape berry skins. This suggestion is supported by the observation that significant decreases in VvPP2Cs and VvCHKS gene expression occur at 110–120 DAFB in berries without brown skin after ABA treatment (Fig. 5C, J).

In this study, the changes we observed in the expression of phytohormone signal-related genes were small compared with changes in the expression of genes involved in biosynthetic pathways. The expression of genes involved in the ethylene and auxin signaling pathways was not altered significantly by our treatments. Liu et al. (2016) suggested that there is a close relationship between the expression of VvEIN3 and VvACO2. In this study, we observed similar expression patterns for VvEIN3 and VvACO2. However, the significant increase in VvACO3 expression after ABA treatment probably affected endogenous ethylene levels (Figs. 4F and 5F). In addition, decreases in VvACO2 and VvYUC1 expression after ABA treatment at 110–120 DAFB in 2014 may have affected both ethylene and auxin levels (Fig. 5E, K). ABA and auxin have opposing effects on grape berry maturation (Coomb and Hale, 1973; Davies et al., 1997). However, the gene expression analysis results from this study suggest that ABA treatment promotes an increase in auxin levels during skin browning. Auxin may play an important role in berry browning. In addition, the crosstalk between ABA, ethylene, and other phytohormones may play an important regulatory role in grape berry maturation (Fortes et al., 2015). Further studies on the effects of phytohormone treatments, including the quantification of endogenous phytohormones, will be required to enhance our understanding of the factors that regulate berry maturation and skin browning in yellow-green skinned grape cultivars.

**Conclusions**

In yellow-green skinned grapes, ABA/ethephon treatments at the veraison stage significantly changed the appearance of berries at harvest by increasing the severity of browning and the trans-resveratrol and flavonol contents of berry skins. In berries with brown skin, interactions between ethylene and auxin may have been induced because the expression levels of the VvACO2 and VvYUC1 genes had increased. In addition, ABA treatment affected the size of the berries. Further studies will be required to identify the genes that regulate the molecular mechanisms underlying berry enlargement and skin development. ABA treatment at the veraison stage altered the expression of several genes involved in phytohormone biosynthesis and signaling more significantly than ethephon treatment. ABA treatment at veraison may affect ethylene biosynthesis by upregulating VvACO3 gene expression during berry maturation at approximately 90 DAFB. Overall, the ABA and ethephon treatments produced different changes in gene expression. Although berry maturation and skin browning were accelerated by both treatments, different mechanisms may regulate the physiological responses to ABA and ethephon in berry skins.

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