Leaf Removal Accelerated Accumulation of Delphinidin-based Anthocyanins in ‘Muscat Bailey A’ [Vitis × labruscana (Bailey) and Vitis vinifera (Muscat Hamburg)] Grape Skin

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Removing leaves around the grape cluster of Muscat Bailey A [Vitis × labruscana (Bailey) and Vitis vinifera (Muscat Hamburg)] cultivated in pergola style at the start of veraison increased the photosynthetically active radiation value by more than approximately 60- and 30-fold compared with those of the control and grape cluster from vines grown on ground covered with reflective film (reflective-film-treated grape cluster), respectively. The improved light exposure caused by leaf removal increased total anthocyanin concentration and changed the ratios of anthocyanin derivatives in the grape skins. Total anthocyanin concentration in the leaf-removal-treated grape skins 10 weeks after veraison increased by approximately 6.5-fold compared with that of the control. In addition, delphinidin-based anthocyanin concentrations in the leaf-removal-treated grape skins increased by approximately 7-fold compared with those of the control. Leaf removal up-regulated anthocyanin-synthesis-related genes in grape skins, such as CHS, F3′H, F3′,5′H, and UFGT. In particular, the overexpression of F3′,5′H in the leaf-removal-treated grape skins suggested that leaf removal contributed to the accumulation of delphinidin-based anthocyanins in grape skin. These findings are expected to improve viticultural practices with the aim of producing dark-colored red wine made from cultivars grown in pergola style.

Key Words: delphinidin-based anthocyanin, F3′,5′H, leaf removal, Muscat Bailey A, photosynthetically active radiation.

Introduction

Anthocyanins impart red/blue color to fruits, vegetables, and plants, and are classified into six types: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin, based on the number of hydroxyl groups on the B-ring. All of the above-mentioned anthocyanins except pelargonidin are present in grape berries and are divided into cyanidin-based anthocyanins and delphinidin-based anthocyanins (Nyman and Kumpulainen, 2001). The cyanidin-based anthocyanins consist of cyanidin and peonidin. On the other hand, the delphinidin-based anthocyanins are composed of delphinidin, petunidin, and malvidin. The color of anthocyanins is sensitive to pH. In wine that has a pH of 4 or lower, the cyanidin-based anthocyanins produce a red color, whereas the delphinidin-based anthocyanins produce a blue color. The anthocyanin composition ratio in grape skin is affected by the difference in expression levels of flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′,5′H) (Bogs et al., 2006; Castellarin and Gaspero, 2007; Castellarin et al., 2006; Jeong et al., 2006). Anthocyanin concentration is determined by the expression of UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) (Kobayashi et al., 2001). Therefore, the transcription levels of F3′H, F3′,5′H, and UFGT in grape skins during the growing season influence skin color and consequently affect the appearance of red wine.

Muscat Bailey A is a cross-hybridized grape cultivar [Vitis × labruscana (Bailey) and Vitis vinifera (Muscat Hamburg)] that was developed in Japan in 1927. Muscat Bailey A is cultivated for table and wine use and is one of the most largely produced Japanese red wines. Muscat Bailey A grapes have certain characteristics that
we focused on three grapevines and used approximately 20–30 grape clusters from 10–15 young shoots for each examination. Approximately 100 grape berries were randomly sampled from the grape clusters. Sampling was conducted five times from August 10 to October 8 (10 weeks after veraison) in the 2011 growing season. All plant materials were frozen immediately in liquid nitrogen and stored at −80°C until use.

Measurement of PAR and illuminance of grape cluster
Photosynthetically active radiation (PAR) and illuminance around the grape clusters subjected to each cultivation condition were measured. LI-190 SA and LI-210 SA sensors (LI-COR Inc., Lincoln, NE, USA) were used for the measurement of PAR and illuminance, respectively. In order to avoid the influence of climatic conditions on the measurements of PAR and illuminance, measurements were carried out around 11:00 am on the day following the start of veraison, which had fair weather.

Analysis of grape composition at harvest
Approximately 100 fresh grape berries were randomly sampled seven weeks after veraison (start of harvest phase) in the 2011 growing season. The grapes were divided into six groups, each composed of approximately 15 to 20 grape berries. After weighing the berries of each group, grape juice was obtained as described below. Briefly, the berries were pressed to 60% of total berry weight using a micropress (Quick juicer; Chibakogyo, Chiba, Japan). Total soluble solids (TSS) in juice were measured with a refractometer (PocketPAL-1; Atago, Tokyo, Japan) and expressed as Brix. pH of juice was measured with a pH meter (MH-60S; Toakogyo, Tokyo, Japan). Total acidity (TA) in juice was determined by neutralization titration with 0.1 N NaOH to pH 7.0 of 10 mL juice diluted with 10 mL distilled water, and expressed as g tartaric acid/L.

Quantification of anthocyanin derivatives and total anthocyanins
Anthocyanin derivatives (cyanidin, peonidin, delphinidin, petunidin, and malvidin) and total anthocyanins
in grape skins were analyzed by reversed-phase high-performance liquid chromatography (HPLC; LC-10Avp; Shimadzu, Kyoto, Japan) as described previously (Baranowski and Nagel, 1981; Liang et al., 2008; Wu and Prior, 2005) with slight modifications. Briefly, to collect the grape skin, approximately 100 fresh grape berries were randomly sampled five times during the grape-growing season. The skins of 10–15 berries were peeled off with tweezers, and each small piece of skin was pulverized in liquid nitrogen. One gram of pulverized skin was macerated in 10 mL of 0.1% HCl-methanol for 4 h at room temperature in the dark and the mixture was filtered through a 0.45 μm cellulose acetate filter (Advantec Toyo, Tokyo, Japan). Finally, these extract solutions were appropriately diluted using methanol before analysis. The conditions for HPLC (SCL-20Avp; Shimadzu) were as follows: CAPCELLPAC C18UG120, 5.0 μm, 4.6 × 250 nm column (Shiseido, Tokyo, Japan); column oven temperature, 40°C; mobile phase A [distilled water containing 0.4% (v/v) phosphoric acid]; mobile phase B [80% (v/v) acetonitrile + 20% mobile phase A]; and flow rate, 1.0 mL·min⁻¹. Linear gradient programs were set from 10% of mobile phase B to 60% of mobile phase B over a 65 min period and absorbance was detected at 525 nm.

The concentrations of cyanidin-based anthocyanins [cyanidin 3-glucoside, cyanidin 3-(6″-acetyl)glucoside, cyanidin 3-(6″-coumaroyl)glucoside,peonidin 3-glucoside,peonidin 3-(6″-acetyl)glucoside, and peonidin 3-(6″-coumaroyl)glucoside] and delphinidin-based anthocyanins [delphinidin 3-glucoside, delphinidin 3-(6″-acetyl)glucoside,delphinidin 3-(6″-coumaroyl)glucoside,petunidin 3-glucoside,petunidin 3-(6″-acetyl)glucoside,petunidin 3-(6″-coumaroyl)glucoside,malvidin 3-glucoside,malvidin 3,5-diglucoside,malvidin 3-(6″-acetyl)glucoside,malvidin 3-(6″-coumaroyl)glucoside,andmalvidin 3-(coumaroyl)-5-diglucoside]. Total anthocyanin concentrations in grape skins were determined by the amount of anthocyanin derivatives. Total anthocyanin and anthocyanin derivative concentrations in grape skin were expressed as mg malvidin-3-glucoside·g⁻¹. 

**RNA extraction**

One gram of skin was pulverized in liquid nitrogen. Total RNA was obtained from the pulverized samples as described previously (Kobayashi et al., 2009). Total RNA was purified with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as described previously (Tesniere and Vayda, 1991).

**Transcription analysis of anthocyanin-synthesis-related genes**

Transcription analysis and selection of anthocyanin-synthesis-related genes were performed as described previously (Kobayashi et al., 2009) with slight modifications. Briefly, first-strand cDNA was synthesized from 500 ng total RNA using a PrimeScript RT Reagent Kit (TaKaRa-Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. Nucleotide sequences of the PCR primers used in this study were designed by Primer Express software version 3.0 (Applied Biosystems Japan Ltd., Tokyo, Japan), and were as follows: chalcone synthase (CHS) primers (5′-TCTGAGCGAGATATGGGACACATG-3′ and 5′-CTGTCGTGCTTTCCCTTCT-3′, corresponding to bases 1469–1490 and 1563–1544 of VvCHS, GenBank accession no. AB015872, respectively), F3′H primers (5′-TATGGGCTGACCCCTACACGA-3′ and 5′-CCTG GGCAAACAACTCATT-3′, corresponding to bases 2566–2586 and 2664–2645 of VvF3′H, GenBank accession no. AB213605, respectively), F3′,5′H primers (5′-AGGGTGAGTGCAATAATGATTCT-3′ and 5′-CGCTGGATCCTCTTGATGT-3′, corresponding to bases 639–660 and 758–740 of VvF3′,5′H, GenBank accession no. AB213606, respectively), UFGT primers (5′-CTTCTTTCAGCACCAAGCACC-ATC-3′ and 5′-AGG CACACCGTTCGAGATA-3′, corresponding to bases 548–588 and 647–628 of VvUFGT, GenBank accession no. AB047099, respectively), and 18S rRNA primers (5′-CGAAAGCATTTGCCAAGGAT-3′ and 5′-AGGGTCGGAGTCAAATGAGTTC-3′, corresponding to bases 522–541 and 625–606 of Vv18S ribosomal RNA, GenBank accession no. AF207053, respectively). Real-time quantitative reverse transcription PCR (real-time quantitative RT-PCR) was performed using an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems) with SYBR Green detection modules according to the manufacturer’s instructions. Data were analyzed using the 7300 system software SDS 1.3.0 (Applied Biosystems). Expression levels were determined as the number of amplification cycles needed to reach a fixed threshold, as described previously (Pfaffl, 2001; Reid et al., 2006). Duplicate experiments of three samples (control, leaf-removal-treated, and reflective-film-treated) were performed in 96-well reaction plates.

**Statistical analysis**

The significant difference among the cultivation treatments was calculated by Tukey's statistical analysis.

**Results and Discussion**

**Light exposure conditions for each cultivation treatment**

The values of PAR and illuminance for each cultivation condition are listed in Table 1. The PAR value of the leaf-removal-treated grape cluster was more than approximately 60- and 30-fold higher than those of control and reflective-film-treated grape clusters, respectively. The PAR value of the reflective-film-treated grape cluster was also increased by more than 2-fold compared with that of the control. In addition, the illuminance value of the leaf-removal-treated grape cluster was 45- and 25-fold higher than those of control and reflective-film-treated grape clusters, respectively. The illuminance
value of the reflective-film-treated grape cluster was also increased by more than 1.5-fold compared with that of the control. These results suggested that our experiment was well designed.

Quantification of total anthocyanins and anthocyanin derivatives

The compositions of the treated grape berries at harvest are listed in Table 2. Although berry weight was not influenced by the treatments, TSS were higher in the leaf-removal-treated grape berries than in the control, whereas TA was lower. In addition, pH was higher in the leaf-removal-treated grape berries than in the control. The compositions of the reflective-film-treated grape

Table 1. Light exposure condition of grape clusters at start of veraison.

<table>
<thead>
<tr>
<th>Cultivation treatment</th>
<th>Light exposure condition</th>
<th>PAR (μmol·m⁻²·sec⁻¹)</th>
<th>Illuminance (klux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>26.1 ± 6.2 a</td>
<td>2.0 ± 0.7 a</td>
</tr>
<tr>
<td>Reflective film</td>
<td></td>
<td>57.4 ± 10.2 b</td>
<td>3.6 ± 0.3 b</td>
</tr>
<tr>
<td>Leaf removal</td>
<td></td>
<td>1634.8 ± 132.4 c</td>
<td>92.7 ± 11.9 c</td>
</tr>
</tbody>
</table>

Means followed by different letters in columns indicate significant differences at p ≤ 0.01 (n = 6).

Table 2. Berry compositions at harvest (n = 6).

<table>
<thead>
<tr>
<th>Berry composition</th>
<th>Berry weight (g)</th>
<th>TSS (Brix)</th>
<th>TA (g·L⁻¹)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.73 ± 0.34</td>
<td>16.3 ± 0.0</td>
<td>7.3 ± 0.1</td>
<td>3.63 ± 0.01</td>
</tr>
<tr>
<td>Reflective film</td>
<td>5.40 ± 0.47</td>
<td>16.8 ± 0.0</td>
<td>6.7 ± 0.1</td>
<td>3.68 ± 0.01</td>
</tr>
<tr>
<td>Leaf removal</td>
<td>5.29 ± 0.38</td>
<td>19.0 ± 0.0</td>
<td>5.5 ± 0.0</td>
<td>3.81 ± 0.01</td>
</tr>
</tbody>
</table>

Fig. 2. Total anthocyanins and anthocyanin derivatives in Muscat Bailey A grape skin. (A) Total anthocyanins. (B) Cyanidin-based anthocyanins. C3G: concentration of cyanidin 3-glucoside, cyanidin 3-(6″-acetyl)glucoside, and cyanidin 3-(6″-coumaroyl)glucoside, Pn3G: concentration of peonidin 3-glucoside, peonidin 3-(6″-acetyl)glucoside, and peonidin 3-(6″-coumaroyl)glucoside. (C) Delphinidin-based anthocyanins. D3G: concentration of delphinidin 3-glucoside, delphinidin 3-(6″-acetyl)glucoside, and delphinidin 3-(6″-coumaroyl)glucoside. Pt3G: concentration of petunidin 3-glucoside, petunidin 3-(6″-acetyl)glucoside, and petunidin 3-(6″-coumaroyl)glucoside. M3G: concentration malvidin 3-glucoside, malvidin 3,5-diglucoside, malvidin 3-(6″-acetyl)glucoside, malvidin 3-(6″-coumaroyl)glucoside, and malvidin 3-(coumaroyl)-5-diglucoside.
centrations in the leaf-removal-treated grape skins were increased by more than 3-fold relative to those in the control and reflective-film-treated grape skins (Fig. 2C). These results indicated that the improved light exposure caused by leaf removal could contribute to the accumulation of delphinidin-based anthocyanins in grape skins, as reported previously (Matus et al., 2009). Increasing light exposure by leaf removal might improve anthocyanin composition in grape skins regardless of cultivation style. As a result, these changes in anthocyanin composition in grape skin might contribute to wine quality, such as wine hue. In addition to Muscat Bailey A, leaf removal should be applied to other table-use cultivars, such as Kyoho, Kaiji, and Pione, which are similarly grown by pergola-style cultivation for table use in Japan, with the aim of improving their skin color. In contrast, Downey et al. (2004) reported that bunch shading increased cyanidin-based anthocyanins. Controlling grape skin color by shading and/or leaf removal is expected to contribute to the enhancement of commodity value.

Berries were between those of the leaf-removal-treated grape berries and the control. These results suggested that the increase in PAR value with pergola-style cultivation affected the maturity of grape berries. Palliotti et al. (2011) reported that early leaf removal conducted before flowering affected gas exchange and chlorophyll fluorescence, resulting in increases in grape berry compositions, such as must soluble solids, total anthocyanins, and total phenolics. Taken together, these results indicated that grape maturity might be promoted by the enhanced photosynthetic efficiency caused by leaf removal. Total anthocyanin concentration in the leaf-removal-treated grape skins showed a marked increase 10 weeks after veraison compared with those in the control and reflective-film-treated grape skins (Fig. 2A). Cyanidin-based anthocyanin concentrations in the leaf-removal-treated grape skins showed a minimal increase or hardly changed compared with those in the control and reflective-film-treated grape skins (Fig. 2B).

On the other hand, delphinidin-based anthocyanin concentrations in the leaf-removal-treated grape skins were increased by more than 3-fold relative to those in the control and reflective-film-treated grape skins (Fig. 2C). These results indicated that the improved light exposure caused by leaf removal could contribute to the accumulation of delphinidin-based anthocyanins in grape skins, as reported previously (Matus et al., 2009). Increasing light exposure by leaf removal might improve anthocyanin composition in grape skins regardless of cultivation style. As a result, these changes in anthocyanin composition in grape skin might contribute to wine quality, such as wine hue. In addition to Muscat Bailey A, leaf removal should be applied to other table-use cultivars, such as Kyoho, Kaiji, and Pione, which are similarly grown by pergola-style cultivation for table use in Japan, with the aim of improving their skin color. In contrast, Downey et al. (2004) reported that bunch shading increased cyanidin-based anthocyanins. Controlling grape skin color by shading and/or leaf removal is expected to contribute to the enhancement of commodity value.

Fig. 3. Transcription profiles of anthocyanin-synthesis-related genes in Muscat Bailey A grape skin. (A) Scheme of the flavonoid pathway leading to the synthesis of anthocyanins. PAL: phenylalanine ammonia lyase, C4H: cinnamate 4-hydroxylase, 4CL: 4-coumarate ligase, CHS: chalcone synthase, CHI: chalcone isomerase, F3′H: flavonoid 3′-hydroxylase, F3′,5′H: flavonoid 3′,5′-hydroxylase, F3H: flavonoid 3-hydroxylase, DFR: dihydroflavonol 4-reductase, LDOX: leucoanthocyanidin dioxygenase, UFGT: UDP-glucose: flavonoid 3-O-glucosyltransferase, OMT: O-methyltransferase. Genes tested in this study are in bold. (B) Five weeks after veraison. (C) Seven weeks after veraison. Real-time RT-PCR was performed as described in Materials and Methods. 18S rRNA was used as an internal control. Data were calculated as gene expression relative to each control gene expression.
Transcription analysis of anthocyanin-synthesis-related genes

The transcription profiles of anthocyanin-synthesis-related genes in grape skins during the growing season are shown in Figure 3. The expression of four anthocyanin-synthesis-related genes, \( CHS \), \( F3'H \), \( F3',5'H \), and \( UFGT \), in the leaf-removal-treated grape skins was the highest among the grapes tested throughout the grape-growing season (Fig. 3). Five weeks after veraison, leaf removal had up-regulated \( CHS \) expression by more than 4- and 1.5-fold relative to those of control and reflective-film-treated grape skins, respectively (Fig. 3B, \( CHS \)). The expression of \( UFGT \), the key enzyme for anthocyanin synthesis, in the leaf-removal-treated grape skins was up-regulated by more than 7-fold compared with those of control and reflective-film-treated grape skins seven weeks after veraison (Fig. 3C, \( UFGT \)). In addition, \( F3'H \) and \( F3',5'H \) expression was up-regulated by more than 2.5- and 4-fold compared with that of the control, respectively (Fig. 3C, \( F3'H \) and \( F3',5'H \)). Therefore, in addition to the up-regulation of \( F3',5'H \), the change in anthocyanin composition in grape skin might be affected by the expression ratios of \( F3'H \) and \( F3',5'H \). These results supported previous reports (Castellarin et al., 2006; Matus et al., 2009) and well explained anthocyanin concentration and composition ratios in grape skins (Fig. 2). Consequently, increasing the exposure of grape clusters to sunlight improved anthocyanin composition in grape skins regardless of cultivation style and cultivar. Controlling sunlight exposure during the growing season might improve anthocyanin concentration and composition ratios in Muscat Bailey A grape skins. These findings could contribute to improving viticultural practices with the aim of producing dark-colored Muscat Bailey A wines. Further investigation to determine the optimum canopy management for pergola-style cultivation, such as leaf removal position and timing, is expected to improve anthocyanin composition.

Literature Cited


