Anthocyanin Composition and MybA-related Genotype in Kyoho Grape and Its Derivatives

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Abstract. In this study, we measured the anthocyanin composition and content in the ‘Kyoho’ grape cultivar and its derivatives via ultra-performance liquid chromatography–mass spectrometry and characterized the MybA-related genes at the color locus via capillary electrophoresis and quantitative real-time polymerase chain reaction. A total of 30 anthocyanins (15 monoglucoside and 15 diglucoside) were detected. Peonidin-3-O-[(6''-O-coumaroyl)-glucoside-5-O-glucoside was the most abundant component, and the content of malvidin-3-O-(c-6''-O-coumaroyl)-glucoside-5-O-glucoside was low in all cultivars. All 49 cultivars contained VvmybA1, VvmybA2, and Vvmyb43, whereas only the black-skinned cultivars contained VvmybA2. The anthocyanin content in the cultivars that contained VvmybA2 was significantly higher than other cultivars. These results could provide information for future color breeding programs in grapes.

Grape (Vitis vinifera L.) is one of the most important fruit crops, and it is cultivated widely around the world. Because V. vinifera presents a lack of resistance to native diseases, soil pests, and low winter temperatures, many breeders have attempted to improve the resistance of the V. vinifera species via hybridization between V. vinifera and American native species (Inesit and Pratt, 1975; Snyder, 1937). Therefore, numerous V. vinifera × Vitis labruscana accessions have been found or developed. In particular, many tetraploid V. vinifera × V. labruscana accessions, such as Kyoho and its derivative cultivars, were developed to produce large berries and are now popular grapes in China, Japan, South Korea, and other Asian areas. Thus, like V. vinifera, V. vinifera × V. labruscana grapes also play an important role in the worldwide grape market (Azuma et al., 2011).

With more than 767.2 km² under cultivation and more than 12.55 million tons of grapes produced in 2014, grape growing is one of the most remunerative farming enterprises in China. Nearly 80% of grapes produced in China are used for table consumption, whereas the remaining grapes are processed as raisins, juice, and wine. More than 1500 varieties are cultivated in China, and the current varieties represent the results of a selection process imposed by humans and ecogeographical conditions on diverse germplasm (Guo et al., 2012). At present, the cultivated area of the Kyoho and its derivatives accounts for ≈50% of the grape-growing area in China (Liu et al., 2014). Breeders have developed 163 new cultivars through many years of effort (Meng et al., 2017; Yang, 2005).

Berry color is an important appearance quality property and represents an important goal of grape variety breeding. Cultivated grapes today show substantially greater diversity in fruit color and include white (yellowgreen), pink, red, purple, and black. The anthocyanin content and composition determine the color profile of table grapes and influence their sensory qualities as well as their nutritional and commercial value.

Current research on the total content of anthocyanins in grape berry skin has been performed quantitatively via spectrophotometry, and the anthocyanin composition and content have been studied in only a few varieties. Moreover, the anthocyanin composition and content for large quantities of germplasm have rarely been studied. Eleven anthocyanins have been detected in Tempranillo (Esteban et al., 2001); 20 in Shiraz (Downey and Rochfort, 2008); 25 in Kyoho (Wang et al., 2012); 13 in Touriga Nacional and Tinta Roxor (Jordão and Correia, 2012); and 20 in Syrah, Cabernet Sauvignon, and Merlot (Fraige et al., 2014).

Grape skin color is determined by the quantity of anthocyanins, and the biosynthesis of these anthocyanins is controlled by structural and regulatory genes. Grape berry coloring represents a complex system, with different grape species and even different cultivars of the same grape cultivar group presenting different coloring mechanisms. In recent years, the discovery of MybA-related genes, an important regulatory gene family,

Table 1. Grape cultivars used in this study.

| Code | Cultivar               | Skin color     | Code | Cultivar               | Skin color     |
|------|-----------------------|----------------|------|-----------------------|----------------|
| 1    | Suihu                 | White          | 26   | Izu Nishiki           | Black          |
| 2    | Ougokyo               | White          | 27   | Hata No. 8            | Black          |
| 3    | Zuijinxiang           | White          | 28   | Huangguan             | Black          |
| 4    | Aki Queen             | Red            | 29   | Shexiu                | Black          |
| 5    | Olimpia               | Red            | 30   | Fujiiminori           | Black          |
| 6    | Benni Fuji            | Red            | 31   | Guixiangyi            | Black          |
| 7    | Red Queen             | Red            | 32   | Takasumi              | Black          |
| 8    | Benzuiluo             | Red            | 33   | Takatsuma             | Black          |
| 9    | Benyamabiko           | Red            | 34   | Kokuho                | Black          |
| 10   | Beni Izu              | Red            | 35   | Black Olimpia × Kokuho| Black          |
| 11   | Beniyoshi             | Red            | 36   | Black Olimpia         | Black          |
| 12   | Ikawa 1060            | Red            | 37   | Kuroshiro             | Black          |
| 13   | Ryuho                 | Red            | 38   | Heifeng               | Black          |
| 14   | Honey Red             | Red            | 39   | Dark Ridge            | Black          |
| 15   | Mizhi                 | Red            | 40   | Heiguixiang           | Black          |
| 16   | Ikawa 1015            | Red            | 41   | Honey Black           | Black          |
| 17   | Shandong Dazi         | Purple         | 42   | Black Marshal         | Black          |
| 18   | Jinfeng               | Purple         | 43   | Jinfeng               | Black          |
| 19   | Jvmeigui              | Purple         | 44   | Jingya                | Black          |
| 20   | Xiyanghong            | Purple         | 45   | Jingyou               | Black          |
| 21   | Shinnam Smile         | Purple         | 46   | Kyoho                 | Black          |
| 22   | Yongyou No. 1         | Purple         | 47   | Tianfeng              | Black          |
| 23   | Stao                  | Purple         | 48   | Pione                 | Black          |
| 24   | Fenghou               | Purple         | 49   | Wase Takasumi         | Black          |
| 25   | Hojyu                 | Purple         |      |                       |                |

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has increased our understanding of anthocyanin biosynthesis. Kobayashi et al. (2002) reported that MybA genes are involved in the regulation of anthocyanin biosynthesis in ‘Kyoho’ grape via the expression of the UFGT gene. Moreover, the transcription factors VvmybA1, VvmybA2, VvmybA3, VvmybA1-1, VvmybA1-2, and VvmybA2 have been shown to regulate anthocyanin biosynthesis via the expression of the UFGT gene in grape (Cutanda-Perez et al., 2009; Kobayashi et al., 2002, 2004; This et al., 2007; Walker et al., 2007). Fournier-Level et al. (2009) reported that continuous variations in anthocyanin content in V. vinifera could primarily be explained by a single gene cluster of VvmybA1, VvmybA2, and VvmybA3 genes. The number of functional alleles affects the ability of the plant to accumulate anthocyanin in grape berry skin (Azuma et al., 2011; Carrasco et al., 2015; Fournier-Level et al., 2010).

The objective of the present report was to explore the characteristics of anthocyanin composition and content via ultra-performance liquid chromatography (UPLC)-mass spectrometry (MS), characterize the MybA-related genes at the color locus via capillary electrophoresis and quantitative real-time polymerase chain reaction (qRT-PCR) in Kyoho and its derivatives to identify the germplasm resources with higher total anthocyanin content and higher monoterpene content, and acquire information for future color breeding programs in grapes.

**Materials and Methods**

*Plant materials.* Forty-nine varieties were analyzed from the Kyoho grapevine series (Table 1). Young leaves and ripe berries were collected from the vineyards at the National Grape Germplasm Repository (113°70'E and 34°72'N) of the Zhengzhou Fruit Research Institute of the Chinese Academy of Agricultural Sciences. Young leaves were collected from the vineyard, immediately frozen in liquid nitrogen, and then kept at −80°C until use. For anthocyanin extraction, a total of 100 berries from each cultivar were randomly sampled at harvest time, and the peeled skins were immediately frozen in liquid nitrogen and stored at −80°C until use.

**Extraction and detection of anthocyanins.** The extraction and detection of anthocyanins was performed as described by Sun et al. (2016). The anthocyanin content was determined by an ACQUITY Ultra-Performance Liquid Chromatography system (Waters, Milford, MA) linked to both a PDA detector (Waters) and a Micromass Quattro microTM API benchtop quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK), with an electrospray ionization source operating in multiple reaction monitoring mode. DNA extraction and PCR analysis. Genomic DNA (gDNA) was extracted from the leaves by the cetyl trimethylammonium bromide method (Muhammad et al., 1994). The primer sequences for VvMYBA1a, VIMYBA1-2, VIMYBA1-3, and VIMYBA2 are shown in Table 2. One primer in each pair was tagged with a fluorophore (FAM, HEX, or NED). PCR reactions were performed in a total volume of 15 µL that consisted of 50 ng DNA, 200 µM deoxyribonucleoside triphosphate, 0.2 µM of each primer, and 0.5 units of Ex Taq polymerase (TaKaRa, Kyoto, Japan). The PCR cycling conditions were as follows: an initial denaturation at 95°C for 5 min; a reaction cycle of 94°C for 30 s, 56°C for 30 s, and 72°C for 90 s, which was repeated 35 times; and a final extension at 72°C for 10 min. PCR fragments of VvMYBA1a were separated by electrophoresis on an 0.8% agarose gel and photographed under ultraviolet light. Other fragments were detected via capillary electrophoresis using an ABI 3730XL DNA analyzer (Applied Biosystems, Carlsbad, CA).

**Quantitative real-time PCR analysis of MybA-related genes.** The distribution of MybA-related genes at the color locus in Kyoho and its derivatives was analyzed by quantitative real-time PCR with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) and a Quanti Tect SYBR Green PCR kit (Qiagen, Hilden, Germany), as described in the manufacturers’ manuals. The primer sequences for VvMYBA1a, VIMYBA1-2, VIMYBA1-3, and VIMYBA2 are shown in Table 2. The relative amounts of these MybA-related genes were determined by the standard curve-based method. The standard DNA set for a calibration curve was prepared as a 5-fold dilution series of the gDNA of ‘Suho’ (for VvMYBA1a analysis) and ‘Kyoho’ (for VIMYBA1-2, VIMYBA1-3, and VIMYBA2 analysis) to cover the expected range of template concentrations. The PCR cycling conditions for VvMYBA1a and VIMYBA1-2 were 95°C for 15 min; 45 cycles of 95°C for 15 s, 54°C for 20 s, and 72°C for 31 s; and a final cycle for melting curve analysis. The cycling conditions for VIMYBA1-3 and VIMYBA2 were 95°C for 15 min; 45 cycles of 95°C for 15 s, 54°C for 20 s, and 72°C for 31 s; and a final cycle for melting curve analysis.
Types of anthocyanins from grape skins.

Five types of anthocyanins were detected in the Kyoho grapevine series, including 15 monoglucosides and 15 diglucosides. Specific information for each anthocyanin is shown in Table 3, including the peak number, retention time, molecular ions, and fragment ions. Peonidin was the most abundant component, and accounted for 32.9% of the total anthocyanins. The second-most abundant component was malvidin, which accounted for 23.6% of the total anthocyanins. The third-most abundant component was cyanidin, which accounted for 15.1% of the total anthocyanins. The fourth-most abundant component was cyanidin, which accounted for 14.6% of the total anthocyanins. The petunidin content was low for all the Kyoho grapevine series and accounted for 9.1% of the total anthocyanins. ‘Jingyou’ (45), ‘Shandong Dazi’ (17), and ‘Black Olympia’ (29), ‘Kuroshiro’ (37), and ‘Jingya’ (44). The remaining varieties contained 18 to 29 types of anthocyanins.

Table 4 shows the ranges and average contents of anthocyanins in the different grapevine series cultivars. In the Kyoho grapevine series, monoglucoside derivatives were more abundant than diglucoside derivatives, with these derivatives accounting for 62.0% and 38.0% of the total anthocyanins, respectively. These findings are consistent with the results of Liang et al. (2008). Peonidin-3-O-((t-6''-O-coumaroyl))-glucoside-5-O-glucoside was the most abundant component and accounted for 7.79% of the total anthocyanins. The third-most abundant component was cyanidin, which accounted for 3.47% of the total anthocyanins. The second-most abundant component was petunidin, which accounted for 1.58% of the total anthocyanins. The petunidin content was low for all the Kyoho grapevine series and accounted for 12.8% respectively. The malvidin-3-O-(c-6''-diglucoside levels at 23.1%, 29.3%, and 12.2%, respectively. The malvidin-3,5-O-diglucoside, which accounted for 10.8% of the total anthocyanins. ‘Jingyou’ (45), ‘Shandong Dazi’ (17), and ‘Honey Black’ (35) presented the highest malvidin-3,5-O-diglucoside, which accounted for 32.9% of the total anthocyanins. The third-most abundant component was cyanidin, which accounted for 15.1% of the total anthocyanins. The fourth-most abundant component was cyanidin, which accounted for 14.6% of the total anthocyanins. The petunidin content was low for all the Kyoho grapevine series and accounted for 9.1% of the total anthocyanins.
et al., 2015; Fournier-Level et al., 2010). We investigated the presence or absence of these genes and their relative DNA amounts in Kyoho and its derivatives by Quantitative real-time PCR (Fig. 2). In the VvMYBA1a analysis, the relative DNA amount of VvMYBA1a in ‘Suiho’ was assumed to be 1, and the relative amounts of VvMYBA1a in the other accessions were calculated compared with ‘Suiho’). In the VlMYBA2, VlMYBA1-2, and VlMYBA1-3 analyses, the relative amounts of VlMYBA2, VlMYBA1-2, and VlMYBA1-3 in ‘Kyoho’ were each assumed to be 1, and the relative DNA amounts of these genes in the other accessions were calculated compared with ‘Kyoho’.

In white-skinned cultivars ‘Suiho’ (1), ‘Ougyoku’ (2), and ‘Zuijinxiang’ (3), only the nonfunctional gene VvMYBA1a was detected, the functional MybA-related genes VlMYBA1-2, VlMYBA1-3, and VlMYBA2 were not detected. From these results, the relative DNA amount of VvMYBA1a in three cultivars was 1.00, 0.995, and 0.962, respectively.

In red-skinned cultivars, the relative DNA amount of VvMYBA1a, VIMYBA2-1, and VIMYBA2-3 were 0.497–0.826, 0.463–1.226, and 0.023–1.00, respectively. VvMYBA2 was not detected in any of these accessions. In purple-skinned cultivars, the relative DNA amount of VvMYBA1a, VlMYBA1-2, and VlMYBA1-3 were 0.535–0.786, 0.597–1.546, and 0.284–1.151, respectively. VlMYBA2 was detected in ‘Shandong Dazi’ (17), ‘Jinfeng’ (18), and ‘Yongyou No. 1’ (22), the relative DNA amount was 1.120, 0.839, and 1.130, respectively. In black-skinned cultivars, the relative DNA amount of VvMYBA1a, VIMYBA1-2, VIMYBA1-3, and VIMYBA2 were 0.282–0.663, 0.219–1.537, 0.337–1.135, and 0.015–1.102, respectively. Except for ‘Jinfeng’ (45), all black-skinned cultivars contained VIMyBA2.

### Discussion

**Type and composition of anthocyanins in different accessions.** The composition and content of anthocyanins in the grape cultivars varied greatly. Five types of anthocyanins were detected via UPLC-MS in the Kyoho grapevine series. Monoglucoside derivatives were more abundant than diglucoside derivatives. Peonidin-3-O-(6′-O-coumaroyl)-glucoside-5-O-glucoside was the most abundant component, and malvidin-3-O-(6′-O-coumaroyl)-glucoside, 5-O-glucoside was low in all Kyoho grapevine varieties, which is consistent with the results of Liang et al. (2008). Previous results showed that colored cultivars accumulate anthocyanins in their skins, whereas white-skinned cultivars do not (Azuma et al., 2008; Boss et al., 1996).

However, trace amounts of anthocyanins were detected in the white varieties analyzed. For example, in ‘Suiho’ (1), ‘Ougyoku’ (2), and ‘Zuijinxiang’ (3), 1.328 µg·g⁻¹ fresh weight (FW), 1.576 µg·g⁻¹ FW, and 2.015 µg·g⁻¹ FW anthocyanins were detected, respectively. These results may have been related to the stronger and more rapid separation capability.
and the immediate and higher-sensitivity detection of the UPLC-MS method. In the analysis of the Kyoho grapevine series, the highest anthocyanin content was found in ‘Shen Xiu’ (29), ‘Jingyou’ (45), and ‘Black Olimpia × Kokuho’ (35) at 3681 μg·g⁻¹ FW, 3272 μg·g⁻¹ FW, and 2091 μg·g⁻¹ FW, respectively. These excellent resources can be used as parents for grape color breeding.

Fig. 2. Relative DNA amount of VvMYB1a, VvMYB1-2, and VvMYB1-3. Note: Error bars show standard error.
Genotype distribution of MybA-related genes. The MybA genotypes of 49 accessions of the Kyoho grapevine series were determined by real-time quantitative PCR and capillary electrophoresis. The results showed that all 49 cultivars contained VvmybA1, VvmYbA2, and VvmYbA3. Moreover, significant values for the VvmYbA2 gene were detected in most black-skinned grapevine samples and few purple-skinned cultivars, and this gene was not present in the white-skinned and red-skinned cultivars. In future grape breeding, the color of progeny seedlings can be determined by detecting the MybA-related genes in early selection.

Anthocyanin contents and MybA-related genotype. Researchers have previously shown that MybA-related genes in grape contain functional and nonfunctional alleles. The number of functional alleles affects the ability of the plant to accumulate anthocyanin in grape berry skin (Azuma et al., 2011; Carrasco et al., 2015; Fournier-Level et al., 2010). Our results showed the lowest anthocyanin content was the white-skinned cultivars, which contained only one nonfunctional VvMYBA1 2, with an average 1.64 mg g\(^{-1}\) FW. The second was red-skinned cultivars, which contained nonfunctional VvMYBA1 and functional VMYBA1-2 and VMYBA1-3, with an average 195.33 mg g\(^{-1}\) FW. The highest anthocyanin content was black-skinned cultivars, which contained nonfunctional VvMYBA1a and functional VvMYBA1-2, VvMYBA1-3, and VvmYbA2, with an average 1259.73 mg g\(^{-1}\) FW. The higher the relative DNA amount of functional alleles, the higher anthocyanin content in the Kyoho grapevine series. These results could provide information for future color breeding programs in grapes.

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