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-(t-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 *VvmybA3*, whereas only the black-skinned cultivars contained *VlmybA2*. The anthocyanin content in the cultivars that contained *VlmybA2* 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 (Einset 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 (yellow–green), 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 Roriz (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	Suiho	White	26	Izu Nishiki	Black
2	Ougyoku	White	27	Hutai No. 8	Black
3	Zuijinxiang	White	28	Huangguan	Black
4	Aki Queen	Red	29	Shenxiu	Black
5	Olimpia	Red	30	Fujiminori	Black
6	Benni Fuji	Red	31	Guixiangyi	Black
7	Red Queen	Red	32	Takasumi	Black
8	Benizuihō	Red	33	Takatsuma	Black
9	Beniyamabiko	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	Ryuhō	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	Jifeng	Black
19	Jvmeigui	Purple	44	Jingya	Black
20	Xiyanghong	Purple	45	Jingyou	Black
21	Shinam 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*, *VlmybA1-1*, *VlmybA1-2*, and *VlmybA2* 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 monomer 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 PDAe λ photo diode array detector (Waters) and a Micro-mass Quattro microTM API benchtop triple 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*, *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* 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 μ L that consisted of 50 ng DNA, 200 μ M deoxyribonucleoside triphosphate, 0.2 μ M of each primer, and 0.5 units of *ExTaq* 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 were 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*, *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* 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 'Suiho' (for *VvMYBA1a* analysis) and 'Kyoho' (for *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* analysis) to cover the expected range of template concentrations. The PCR cycling conditions for *VvMYBA1a* and *VlMYBA1-2* 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 *VlMYBA1-3* and *VlMYBA2* were 95 °C for

Table 2. Primers used for detection of *MYB*-related genes by means of polymerase chain reaction.

Gene name	Primer	Sequence (5'-3')	Fragment size/base pair	References
<i>VvMYBA1a</i>	F1	AAAAAGGGGGGCAATGTAGGGACCC	1560	Kobayashi et al., (2004)
	R1	GAACCTCCTTTTGAAGTGGTGACT		
<i>VlMYBA1-2</i>	F2	CACCACCTGAAAAAGAAGGTC	251	Azuma et al., (2008)
	R2	TCTTGATCCAGCTCAGCTAAC		
<i>VlMYBA1-3</i>	F3	GGACGTTAAAAATGGTTGCACGTG	999	Kobayashi et al., (2004)
	R3	GAACCTCCTTTTGAAGTGGTGACT		
<i>VlMYBA2</i>	F4	GCTGAGCATGCTCAAAATGGAT	161	Azuma et al., (2011)
	R4	TCCACCATATGATGACACCC		
<i>VvmybA1</i>	K	CTCGATGGAGAGCTTAGGAGTTAG	959	Sun et al., (2016)
<i>VvmybA2</i>	S	TAAATCAGATCAAGTGATTACTT	1241	
<i>VvmybA3</i>			750	

Table 3. The characteristics of the anthocyanins of mass spectrometry detected in berry skin.

Peak number	Anthocyanins ^z	t _R (min)	Molecular ion M ⁺ (m/z)	Fragment ions M (m/z)	Absorbance maxima (nm)
1	Dp-3,5-diglu	2.94	627	303,465	282,520
2	Cy-3,5-diglu	3.54	611	287,449	282,516
3	Pt-3,5-diglu	3.79	641	317,479	274,523
4	Dp-3-glu	4.41	465	303	280,523
5	Pn-3,5-diglu	4.63	625	301,463	278,513
6	Mv-3,5-diglu	4.74	655	331,493	275,524
7	Cy-3-glu	5.40	449	287	279,515
8	Pt-3-glu	5.93	479	317	277,526
9	Pn-3-glu	7.07	463	301	279,515
10	Mv-3-glu	7.44	493	331	278,530
11	c-Dp-3,5-coum-diglu	8.57	773	303,465,627	279,530
12	t-Dp-3,5-coum-diglu	9.61	773	303,465,627	279,530
13	c-Cy-3,5-coum-diglu	9.80	757	287,449,611	280,524
14	c-Pt-3,5-coum-diglu	10.07	787	317,479,625	280,530
15	t-Cy-3,5-coum-diglu	10.87	757	287,449,611	280,524
16	t-Pt-3,5-coum-diglu	10.93	787	317,479,625	280,530
17	c-Dp-3-coum-glu	11.21	773	303,465,627	282,530
18	c-Pn-3,5-coum-diglu	11.47	783	317,479,641	280,530
19	c-Mv-3,5-coum-diglu	11.53	801	639,493,331	280,530
20	t-Mv-3,5-coum-diglu	12.15	801	655,493,331	280,530
21	t-Dp-3-coum-glu	12.20	773	303,465,627	282,530
22	t-Pn-3,5-coum-diglu	12.34	783	317,479,641	280,530
23	c-Cy-3-coum-glu	12.45	595	287,449	283,522
24	c-Pt-3-coum-glu	12.84	625	317,479	280,531
25	t-Cy-3-coum-glu	13.56	595	287	283,522
26	t-Pt-3-coum-glu	13.98	625	317	280,531
27	c-Pn-3-coum-glu	14.45	609	301,463	279,523
28	c-Mv-3-coum-glu	14.65	639	331	280,531
29	t-Pn-3-coum-glu	15.56	609	301,463	279,523
30	t-Mv-3-coum-glu	15.79	639	331	280,531

^zc = cis isomers; coum = coumaroyl; Cy = cyanidin; diglu = diglucoside; Dp = delphinidin; glu = glucoside; Mv = malvidin; Pn = peonidin; Pt = petunidin; t = trans isomers.

15 min; 45 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 31 s; and a final cycle for melting curve analysis. Quantitative real-time PCR was carried out with three replicates per sample, and average data were calculated by using calibration curves and normalized against the amount of Chalcone synthase 3 (Azuma et al., 2011; Jeong et al., 2004; Yakushiji et al., 2006).

Results

Types of anthocyanins from grape skins.

Five types 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 13.7% of the total anthocyanins (Fig. 1).

Comparison of the anthocyanin composition in different grape varieties. Twelve to 30 anthocyanins were detected in the Kyoho grapevine series. Twelve or 13 anthocyanins were detected in the white cultivars ‘Suiho’ (1), ‘Ougyoku’ (2), and ‘Zuijinxiang’ (3). Thirty anthocyanins were detected in three black cultivars, ‘Shenxiu’ (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 10.8% of the total anthocyanins (Table 4). ‘Jingyou’ (45), ‘Shandong Dazi’ (17), and ‘Black Olympia × Kokuho’ (35) presented higher peonidin-3-O-(*t*-6''-O-coumaroyl)-glucoside-5-O-glucoside levels at 19.9%, 29.3%, and 12.2%, respectively. The second-most abundant component was malvidin-3,5-O-diglucoside, which accounted for 9.1% of the total anthocyanins. ‘Jingyou’ (45), ‘Shandongdazi’ (17), and ‘Honey Black’ (41) presented the highest malvidin-3,5-O-diglucoside levels at 23.1%, 25.2%, and 12.8%, respectively. The malvidin-3-O-(*c*-6''-O-coumaroyl)-glucoside, 5-O-glucoside content was low for all the Kyoho grapevine series and accounted for 0.009% of the total anthocyanins.

Identification of MybA-related genotype at the color locus. Many studies have shown that MybA-related genes regulate anthocyanin

Fig. 1. The content of different anthocyanins in Kyoho and its derivatives.

Table 4. Average content of different type anthocyanins kinds of grapes ($\mu\text{g}\cdot\text{g}^{-1}$ FW).

Anthocyanin ^z	Avg (range)	Percent (%)
(c)Mv- coum-diglu	0.062 (0–0.580)	0.009
(c)Cy-coum-diglu	0.428 (0–4.623)	0.058
(c)Pt-coum-diglu	0.565 (0–3.738)	0.08
(t)Mv- coum-diglu	0.610 (0–4.142)	0.08
(c)Pt-coum-glu	1.530 (0–8.738)	0.21
Dp-diglu	11.629 (0–240.006)	0.28
(c)Cy-coum-glu	2.067 (0–17.588)	0.30
(c)Dp-coum-glu	2.205 (0–26.394)	0.53
(t)Dp-coum-diglu	24.266 (0–378.887)	0.53
(t)Cy-coum-glu	25.455 (0–118.792)	0.55
Pt-diglu	3.857 (0–41.944)	0.57
(c)Dp-coum-diglu	3.866 (0–58.509)	0.68
Dp-glu	33.960 (0–500.448)	1.21
(t)Dp-coum-glu	34.962 (0–1078.254)	1.21
Mv-glu	36.417 (0.029–224.041)	1.23
(c)Pn-coum-glu	4.015 (0–25.427)	1.58
(t)Cy-coum-diglu	4.220 (0–58.240)	3.31
(c)Mv- coum-glu	4.969 (0–21.853)	3.47
Cy-diglu	8.886 (0–107.194)	4.63
(c)Pn-coum-diglu	8.913 (0–36.928)	4.76
(t)Pt-coum-diglu	9.018 (0–129.695)	4.96
(t)Pt-coum-glu	40.024 (0–470.348)	5.45
Pn-glu	40.762 (0.147–393.384)	5.55
Pt-glu	45.404 (0.010–596.219)	6.19
(t)Pn-coum-glu	51.771 (0–201.252)	7.05
Pn-diglu	57.215 (0.039–755.977)	7.79
(t)Mv- coum-glu	64.496 (0–283.436)	8.79
Cy-glu	66.280 (0.205–499.218)	9.03
Mv-diglu	66.813 (0.038–471.700)	9.10
(t)Pn-coum-diglu	78.928 (0–650.774)	10.75

^zc = cis isomers; coum = coumaroyl; Cy = cyanidin; diglu = diglucoside; Dp = delphinidin; glu = glucoside; Mv = malvidin; Pn = peonidin; Pt = petunidin; t = trans isomers.

biosynthesis in grape. MybA-related genes *VvmybA1*, *VvmybA2*, *VvmybA3*, *VlmybA1-2*, *VlmybA1-3*, and *VlmybA2* have been shown to regulate anthocyanin biosynthesis via the expression of the *UFGT* gene in grape. Although *VvmybA1*, *VvmybA2*, and *VvmybA3* are highly homologous, their CDS sequences have distinct different regions in their third exon. On their third exon, there exists some variation, such as fragment insertion and deletion. Compared with *VvMybA1*, the third exon of the *VvMybA2* gene has an insertion of a long segment, whereas the *VvMybA3* gene has a deletion of a long segment, and the last few hundred base pairs of these three genes show obvious specificity. We designed primers in the specific region of the third exon of each gene that could distinguish the three genes. The genotypes of

MybA-related genes in the 49 cultivars are provided in Table 5. The results showed that all 49 cultivars contained *VvmybA1*, *VvmybA2*, and *VvmybA3*, and none of the white-skinned and red-skinned cultivars contained *VlmybA2*. In purple-skinned cultivars, except ‘Shandong Dazi’ (17), ‘Jinfeng’ (18), and ‘Yongyou No. 1’ (22) contained *VlmybA2*, no other cultivars contained *VlmybA2*. Except for ‘Jifeng’ (43), all black-skinned cultivars contained *VlmybA2*.

Quantitative real-time PCR analysis of the MybA-related genes at the color locus. Researchers have previously shown that MybA-related genes in grapes 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

Table 5. Genotyping of MybA-related genes and anthocyanin content in accessions.

Code	Cultivars	Skin color	<i>VvmybA1</i>	<i>VvmybA2</i>	<i>VvmybA3</i>	<i>VlmybA2</i>	<i>VlmybA1-2</i>	<i>VlmybA1-3</i>	Anthocyanin content ($\mu\text{g}\cdot\text{g}^{-1}$ FW)
1	Suiho	White	+	+	+	—	—	—	1.328
2	Ougyoku	White	+	+	+	—	—	—	1.576
3	Zuijinxiang	White	+	+	+	—	—	—	2.015
4	Aki Queen	Red	+	+	+	—	+	+	94.914
5	Olimpia	Red	+	+	+	—	+	+	68.231
6	Benni Fuji	Red	+	+	+	—	+	+	214.628
7	Red Queen	Red	+	+	+	—	+	+	137.739
8	Benizuihō	Red	+	+	+	—	+	+	290.248
9	Beniyamabiko	Red	+	+	+	—	+	+	76.334
10	Beni Izu	Red	+	+	+	—	+	+	210.519
11	Beniyoshi	Red	+	+	+	—	+	+	388.863
12	Ikawa 1060	Red	+	+	+	—	+	+	81.087
13	Ryuhō	Red	+	+	+	—	+	+	267.655
14	Honey Red	Red	+	+	+	—	+	+	332.316
15	Mizhi	Red	+	+	+	—	+	+	64.572
16	Ikawa 1015	Purple	+	+	+	—	+	+	312.148
17	Shandong Dazi	Purple	+	+	+	+	+	+	1377.869
18	Jinfeng	Purple	+	+	+	+	+	+	725.289
19	Jvmeigui	Purple	+	+	+	—	+	+	929.512
20	Xiyanghong	Purple	+	+	+	—	+	+	99.882
21	Shinam Smile	Purple	+	+	+	—	+	+	126.438
22	Yongyou No. 1	Purple	+	+	+	+	+	+	639.645
23	Stao	Purple	+	+	+	—	+	+	302.911
24	Fenghou	Purple	+	+	+	—	+	+	1036.309
25	Hojyu	Purple	+	+	+	—	+	+	444.033
26	Izu Nishiki	Black	+	+	+	+	+	+	561.985
27	Hutai No. 8	Black	+	+	+	+	+	+	983.501
28	Huangguan	Black	+	+	+	+	+	+	658.631
29	Shenxiu	Black	+	+	+	+	+	+	3680.926
30	Fujiminori	Black	+	+	+	+	+	+	1260.415
31	Guixiangyi	Black	+	+	+	+	+	+	713.210
32	Takasumi	Black	+	+	+	+	+	+	860.591
33	Takatsuma	Black	+	+	+	+	+	+	762.287
34	Kokuho	Black	+	+	+	+	+	+	1114.749
35	Black Olimpia × Kokuho	Black	+	+	+	+	+	+	2091.495
36	Black Olimpia	Black	+	+	+	+	+	+	1285.452
37	Kuroshiro	Black	+	+	+	+	+	+	1213.995
38	Heifeng	Black	+	+	+	+	+	+	1237.417
39	Dark Ridge	Black	+	+	+	+	+	+	1310.254
40	Heiguixiang	Black	+	+	+	+	+	+	493.658
41	Honey Black	Black	+	+	+	+	+	+	1315.980
42	Black marshal	Black	+	+	+	+	+	+	1196.97
43	Jifeng	Black	+	+	+	—	+	+	364.909
44	Jingya	Black	+	+	+	+	+	+	1741.889
45	Jingyou	Black	+	+	+	+	+	+	3272.287
46	Kyoho	Black	+	+	+	+	+	+	861.202
47	Tianfeng	Black	+	+	+	+	+	+	1357.876
48	Pione	Black	+	+	+	+	+	+	862.634
49	Wase Takasumi	Black	+	+	+	+	+	+	1031.088

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 *VvMYB1a* analysis, the relative DNA amount of *VvMYB1a* in ‘Suiho’ was assumed to be 1, and the relative amounts of *VvMYB1a* in the other accessions were calculated compared with ‘Suiho’. In the *VIMYB2*, *VIMYB1-2*, and *VIMYB1-3* analyses, the relative amounts of *VIMYB2*, *VIMYB1-2*, and *VIMYB1-3* 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 *VvMYB1a* was detected, the functional MybA-related genes *VIMYB1-2*, *VIMYB1-3*, and *VIMYB2* were not detected. From these results, the relative DNA amount of *VvMYB1a* in three cultivars was 1.00, 0.995, and 0.962, respectively.

In red-skinned cultivars, the relative DNA amount of *VvMYB1a*, *VIMYB1-2*, and *VIMYB1-3* were 0.497–0.826, 0.463–1.226, and 0.023–1.00, respectively. *VIMYB2* was not detected in any of these accessions. In purple-skinned cultivars, the relative DNA amount of *VvMYB1a*, *VIMYB1-2*, and *VIMYB1-3* 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 *VvMYB1a*, *VIMYB1-2*, *VIMYB1-3*, and *VlmybA2* were 0.282–0.663, 0.219–1.537, 0.337–1.135, and 0.015–1.102, respectively. Except for ‘Jifeng’ (43), all black-skinned cultivars contained *VlmybA2*.

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-(t -6’’-O-coumaroyl)-glucoside-5-O-glucoside was the most abundant component, and malvidin-3-O-(c -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 $\mu\text{g}\cdot\text{g}^{-1}$ fresh weight (FW), 1.576 $\mu\text{g}\cdot\text{g}^{-1}$ FW, and 2.015 $\mu\text{g}\cdot\text{g}^{-1}$ FW anthocyanins were detected, respectively. These results may have been related to the stronger and more rapid separation capability

Fig. 2. Relative DNA amount of *VvMYB1a*, *VlMYBA2*, *VlMYBA1-2*, and *VlMYBA1-3*. Note: Error bars show standard error.

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 $\mu\text{g}\cdot\text{g}^{-1}$ FW, 3272 $\mu\text{g}\cdot\text{g}^{-1}$

FW, and 2091 $\mu\text{g}\cdot\text{g}^{-1}$ FW, respectively. These excellent resources can be used as parents for grape color breeding.

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 *VlmybA2* 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 *VvMYBA1a*, with an average $1.64 \mu\text{g}\cdot\text{g}^{-1}$ FW. The second was red-skinned cultivars, which contained nonfunctional *VvMYBA1a* and functional *VIMYBA1-2* and *VIMYBA1-3*, with an average $195.33 \mu\text{g}\cdot\text{g}^{-1}$ FW. The highest anthocyanin content was black-skinned cultivars, which contained nonfunctional *VvMYBA1a* and functional *VIMYBA1-2*, *VIMYBA1-3*, and *VlmybA2*, with an average $1259.73 \mu\text{g}\cdot\text{g}^{-1}$ 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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