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Anthocyanin contents and composition of *VlmybA1-2* and *VlmybA2* genes in *Vitis labrusca* hybrid grape cultivars and cross seedlings

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Abstract

The grape *Vitis labrusca* and hybrids from *V. vinifera* × *V. labrusca* have different haplotypes of *Myb* gene combinations from the *V.vinifera* grape. The *Myb* gene PCR results show that the white-skinned *V.labrusca* hybrid cultivar 'Cheongsoo' has a *Myb* allele contained in two pairs of haplotypes A (*VvmybA1a*) like the *V.vinifera* grape. However, other red-skinned *V.labrusca* hybrid grape cultivars have haplotype A2 (containing *VlmybA2*), haplotype A1-2 (containing *VlmybA1-2*), or both (Hap A1-2 and A2). The *V.labrusca* hybrid cultivars containing the haplotype A2 show higher anthocyanin contents. However, the anthocyanin content of the haplotype A2 was significantly higher than the haplotype A1-2 in the seedlings from the cross between the 'Campbell Early' grape (*V. labrusca*) × the 'Neo Muscat' grape (*V. vinifera*). These results show that the *VlmybA2* gene is closely related to anthocyanin contents and grape skin color in the *V.labrusca* hybrid grape cultivars and their cross seedlings.

Keywords: Allele, anthocyanin, breeding, *myb* gene, skin color.
Abbreviations: *MYB*_Myeloblastosis; Hap_Haplotype; *Gret*_Grape retrotransposon 1; TF_Transcription factor; UDP_Uridine diphosphate; UFGT_flavonoid 3-O-glucosyltransferase.

Introduction

The most of major grapevine cultivars derived from the species *Vitis vinifera* Linn. However, other species have unique characteristics, as a *Vitis labrusca* Linn. from eastern America, shows downy mildew resistance and a foxy flavor (Robinson 1986). Many grape cultivars have been generated by crossing *V. vinifera* and *V. labrusca*, which generates the *V. labruscana* Bailey hybrid cultivar that has several advantageous characteristics, including disease resistance to *V. vinifera* (Mullins and Willams, 1992). Grapevine cultivar shows various growth and fruit characteristics, but the most important factor is skin color. Skin color variation is caused by anthocyanin type and its contents (Jackson, 2008; Lancaster and Lister et al., 1997). Anthocyanins are a type of water-soluble flavonoid pigment (red, purple, or deep blue) found in flowers, fruits, and vegetables (Brugliera et al., 1999). In plants, anthocyanidin, as a precursor, is the primary location of pigment after glycoside hydrolysis, and is commonly found in the cytoplasm or vacuole. Anthocyanidin has three junction points with three major functional groups including the –H (hydrogen), -OH (hydroxyl), and -OCH_3 (methoxyl) groups. When -OCH_3 substituted for the hydroxyl group on the phenyl constituent, the derivatives can be divided into peonidin, petunidin, and malvidin. Regarding grape skin color, delphinidin, malvidin, and petunidin are deep blue, purple, and dark red. Also, cyanidin and peonidin showed red and purple color, but its concentration depending on the anthocyanin biosynthetic pathway (Brugliera et al., 1999; Figueiredo-Gonzalez et al., 2012; He et al., 2010; Jackson, 2008). Anthocyanin biosynthesis follows a sequential process from phenylalanine to p-coumaroyl-CoA, chalcones, flavanones, dihydroflavonols, leucoanthocyanins, and anthocyanidins using the enzymes. This pathway ultimately generates UDP-glucose using the enzyme flavonoid 3-O-glucosyltransferase (UFGT) (Koes et al., 2005; Sparvoli et al., 1994; Winkel-Shirley 2001). Because anthocyanidin is unstable in the biosynthesis, glycosylation is required to increase stability. Also, UFGT, the synthetase involved in the last step of anthocyanin biosynthesis, plays a role in glycosylating anthocyanidin during the over-maturing stage in fruit with red skin (Ford et al., 1998). Based on *ufgt* gene expression analysis, expression in the fruit skin increased after grape coloration (Boss et al., 1996a, b; Kobayashi et al., 2001; Ramazzotti et al., 2008), while for *V. vinifera*, *ufgt* gene sequences did not differ between the red and white cultivars (Kobayashi et al., 2001). For these reasons, many studies on transcription factors (TFs), which regulate transcription of the *ufgt* gene by associating with the transcription regulatory region, have been reported (Jeong et al., 2004; Kobayashi et al., 2002). Anthocyanin synthesis in plants is controlled by specific TF genes such as those in the *Myb* gene family, basic helix-loop-helix (bHLH) family, and WD40 family (Ramsay and Glover et al., 2005). Several plants possess *Myb* TF genes, including PAP1/PAP2 (*Arabidopsis*), AN2/AN4 (*Petunia*), and C1/PL (*Zea*; maize) which typically regulate anthocyanin biosynthesis (Albert et al., 2011; Brugliera et al., 1999; Dooner and Nelson, 1977; Gerats et al., 1983; Koes et al., 2005; Quattrocchio et al., 1998). In the grapevine cultivar 'Kyoho' (*Vitis* hybrid: *V. labrusca* × *V. vinifera*), the cDNA sequence of four different *Myb* genes (*Myb A, B, C,* and *D*) was determined. Only *Myb* A gene expression detected in the skin and flesh after veraison, but other genes were detected during all growth stages (Kobayashi et al., 2002). For *V. vinifera* (European species) and *V. labruscana* (European species × American species), each gene has a

different base sequence, and the organization of genes regulating skin color differs between species. Grapes grouped into white, red and black grapes depending on skin color, and the skin color of white grapes is unique since anthocyanin biosynthesis is absent (Slinkard and Singleton, 1984). The lack of anthocyanin biosynthesis in these grapes is because *Gret1* (a retrotransposon) inhibits the expression of the *VvmybA1* gene, a European species of the *MybA* gene, by associating with upstream regions of the coding sequence. This form of the gene known as *VvmybA1a* (Azuma et al., 2007; Kobayashi et al., 2004; Mitani et al., 2009). On the contrary, *VvmybA1c* (Azuma et al., 2007; Figueiredo-González et al., 2012; Shimazaki et al., 2011; Yakushiji et al., 2006) with the same sequence of the *VvmybA1* gene but lacking the *Gret1* insertion plays a significant role in the skin color of European grapes (Walker et al., 2006). On the other hand, among the cDNA sequences in 'Kyoho' grape (*Vitis labruscana*: *V. labrusca* × *V. vinifera*), genes that correlated with the R2R3 domain of the *Myb* gene have been identified. These genes include *VlmybA1–1*, *VlmybA1–2*, and *VlmybA2* (Geekiyanage et al., 2007; Kobayashi et al., 2002, 2004). The *VlmybA1–3* gene have been confirmed by comparing the base sequences of cDNAs in 'Concord' (*V. labrusca*) and 'Campbell Early' (*V. labruscana*). The differences in coding sequences between the *VlmybA1–3* and *VvmybA1c* genes include sequence insertions of 44 and 111 bp into the upstream region of the *VvmybA1c* gene (Azuma et al., 2008). For the two types of grapes (*V. vinifera* and *V. labrusca*), there are four haplotypes depending on the *myb* gene combination; haplotype A (Hap A) with the *VvmybA1a* gene, haplotype C (Hap C) with the *VvmybA1c* gene, haplotype A1–2 (Hap A1–2) with the *VlmybA1–2* and *VlmybA1–3* genes, and haplotype A2 (Hap A2) with the *VlmybA2* and *VlmybA1–3* genes (Azuma et al., 2011).

Although grape skin color is an important factor of characterizing each species, it is hard to confirm skin color of cross-seedling after crossing and collecting the seeds because seedlings have juvenility. In this study, we find a correlation between haplotypes and skin color of several grape varieties. Moreover, then we explored whether the haplotype detection can applied for early diagnosis skin color in the seedlings with 'Campbell Early' grape (*V. vinifera*) and 'Neo Muscat' grape (*V. labrusca*).

Results and Discussion

Haplotype analysis of grapevine species and cultivars

A total of 13 cultivar (6 cultivars of *V. vinifera* and 7 cultivars of *V. labruscana*) was analyzed with primer targeting haplotypes, haplotype A (*VvmybA1a*), haplotype C (*VvmybAlc*), haplotype A1-2 (*VlmybA1–2*), and haplotype A2 (*VlmybA2*). The haplotypes of red cultivars were predicted based on PCR analysis (Table 1). A single band representing *VvmybA1a* (Hap A; 1,559 bp) was detected all tested cultivars, but 'Campbell Early', 'Jinok' and 'Cabernet Sauvignon' not detected *VvmybAla* gene band, because it had *VvmybA1c* (Hap C) or *VlmybA1-3* (Hap C). These findings are in line with studies reported by Azuma, (2007) and Mitani, (2009). For *V. vinifera* and *V. labrusca*, the *VvmybA1* gene became the *VvmybA1a* gene after *Gret1* (Grape retrotransposon 1) insertion, and white cultivars created as skin color expression inhibited (Kobayashi et al., 2004, 2005, 2009). Haplotype A has nonfunctional *Myb* gene (*VvmybA1a*) so that the skin color of haplotype A/A cultivar becomes a white, but haplotype A/C had a skin color. For the red cultivars 'Ruby Okuyama' and 'Flame Seedless', as well as the skin color mutants of 'Italia' and 'Muscat of Alexandria', the skin colors were expressed normally (Kobayashi et al., 2004, 2005, 2009). On the other hand, *VlmybA1–3* was not identified two white cultivar 'Cheongsoo' (*V. labrusca* hybrid) and 'Neo Muscat' (*V. vinifera*) because it contained two pairs of the haplotype A (Hap A/A). *VlmybA1–3* is an allele of *VvmybA1*, and both genes are not present at the same locus. *V. labrusca* hybrid cultivars 'Hongisul', 'Campbell Early', 'Jinok', 'North Black', 'Schuyler', and 'Tamnara' observed a 1,005 bp of *VlmybA1–3* gene band. However, *VlmybA1–3* in *V. vinifera* cultivars 'Flame Seedless', 'Kaiji', 'Rizamat', 'Ruby Seedless', and 'Cabernet Sauvignon' migrated as an 850 bp band (Fig. 1). A previous report showed that *VlmybA1–3* was derived from 44 bp and 111 bp insertions from the coding sequence of the *VvmybA1c* gene in *V.vinifera* (Azuma et al., 2008). As we have seen, *VlmybA1-3* gene in *V.labrusca* species had a same function such as *VvmybA1c* in the *V.vinifera* but had some differences in the sequence.

We explored two allelic genes, *VlmybA1–2* and *VlmybA2*, a present downstream of *VlmybA1–3* and *VvmybA1a* in the *V. labrusca* hybrid cultivars. *VlmybA1–2* primer analysis did not clearly detect the gene in *V. vinifera* cultivars. However, *V. labrusca* hybrid cultivars such as 'Hongisul', 'Campbell Early', and 'Jinok' were detected 251 bp in size. Based on *VlmybA2* primer analysis, no bands were detected in the *V. vinifera* cultivars, but some *V. labrusca* hybrid cultivars such as 'Campbell Early', 'Jinok', 'North Black', 'Schuyler', and 'Tamnara' showed a 161 bp of *VlmybA2* gene band (Fig. 1). As a results, we identified the haplotype following as haplotype A/A in the white cultivar 'Cheongsoo', haplotype A1-2/A1–2 in the red cultivar 'Hongisul', haplotype A1–2/A2 in the black cultivars 'Campbell Early' and 'Jinok', and haplotype A2/A2 in 'North Black', 'Schuyler', and 'Tamnara' (Table 1). For the varieties of *V. labrusca* hybrid, the *VlmybA2* gene was commonly found in the black cultivars 'Campbell Early', 'Jinok', 'North Black', 'Schuyler', and 'Tamnara', as well as the red cultivar 'Hongisul', also in agreement with previous reports (Azuma et al., 2008, 2011). In the PCR results, we found differences of *Myb* gene composition and identified haplotypes in the *V. labrusca* hybrid cultivars.

Anthocyanin contents of the grapevine species and cultivars

For 'Campbell Early', 'Jinok', 'Schuyler', and 'Tamnara' with black skin color, as well as 'Hongisul' with red skin color, anthocyanin concentrations across haplotype variants were analyzed (Table 2). For 'Hongisul' (Hap A1-2/A1–2), the concentration of peonidin was highest while concentrations of delphinidin, malvidin, and petunidin were not measurable. For 'Campbell Early' and 'Jinok' (Hap A1–2/A2), the concentration of peonidin was highest while petunidin concentrations were not determined. However, for 'Campbell Early' and 'Jinok', the concentration of delphinidin, malvidin, and cyanidin varied depending on species, unlike 'Hongisul'. For 'Schuyler' and 'Tamnara' (Hap A2/A2), the concentration of peonidin was highest, similar to other species. Overall, the concentration of peonidin was high, and there were no significant differences in anthocyanin composition between gene groups (Table 2). However, the synthetases flavonoid 3' hydroxylase (F3'H) and flavonoid 3'5' hydroxylase (F3'5'H), involved in the anthocyanin biosynthetic pathway, play a role in synthesizing flavonols and flavan-3-ols. The synthesis of delphinidin-based anthocyanin and prodelphinidin (Jeong et al., 2006), and the concentration of delphindin-3-O-glucoside,

Table 1. Haplotype and skin color of grape cultivars between *V. vinifera* and *V. labrusca* hybrid.

Species	Color	Cultivar name	Allele (Haplotype)[y]	Genes
V. vinifera	White	Neo Muscat	AX/AX (Hap A/ Hap A)	*VvmybA1a* / *VvmybA1a*
	Red, Black	Flame Seedless, Kaiji, Rizamat, Ruby Seedless	AX/CX (Hap A/ Hap C)	*VvmybA1a* / *VvmybA1c*
	Red, Black	Cabernet Sauvignon	CX/CX (Hap C/ Hap C)	*VvmybA1c* / *VvmybA1c*
V. labruscan[z]	White	Cheongsoo	AX/AX (Hap A/ Hap A)	*VvmybA1a* / *VvmybA1a*
	Red	Hongisul	C'A1-2/C'A1-2 (Hap A1-2/ Hap A1-2)	*VlmybA1-3* / *VlmybA1-2*
	Black	North Black, Schuyler, Tamnara	C'A2/C'A2 (Hap A2/ Hap A2)	*VlmybA1-3* / *VlmybA2*
	Black	Campbell Early, Jinok,	C'A1-2/C'A2 (Hap A1-2/ Hap A2)	*VlmybA1-3* / *VlmybA2 and* *VlmybA1-3* / *VlmybA1-2*

[z]. *V.labruscana* is hybrid species by cross between *V.vinifera* and *V.labrusca*, [y]. Allele A has non-functional *Myb* gene because inserted Gret1; Allele X not affected grape skin color which located *Myb* genes. Allele C has normal *Myb* gene (*VvmybA1c*); Allele C' (*VlmybA1-3*) only contained in *V.labrusca.* This gene sequence insertions of 44 and 111 bp in the upstream region of the *VvmybA1c* gene; A1-2 and A2 locus gene are normal function *Myb* gene in *V. labrusca*.

Table 2. Anthocyanin contents of fruit skin in the *V.labrusca* hybrid grape cultivars according to the different haplotypes.

Haplotype[z]	Cultivar	Anthocyanin contents ($mg \cdot g^{-1}$ Fw)					Total Anthocyanin content[w] ($mg \cdot g^{-1}$ fw)	Skin color
		Del[y]	Mal	Pet	Cyn	Peo		
A1-2/A1-2	Hongisul	ND[x]	ND	ND	0.02±0.01 a	8.72±3.47 c	7.59± 2.23 c	Red
A1-2/A2	Campbell Early	0.43±0.02 a	1.64±0.14 a	ND	0.64±0.02 a	106.23±6.56 a	86.33± 7.20 a	Black
	Jinok	0.12±0.01 c	ND	ND	0.02±0.02 a	48.61±3.90 b	82.01±16.54 a	Black
A2/A2	Schuyler	0.27±0.04 b	0.88±0.06 a	ND	0.10±0.02 a	49.39±4.06 b	55.71±13.67 b	Black
	Tamnara	0.01±0.02 c	1.31±0.05 a	1.28±0.71	0.03±0.02 a	16.09±1.91 c	36.23± 4.31 b	Black

[z]Hap/Hap; Haplotype/Haplotype. A1-2 (*VlmybA1-2*), A2 (*VlmybA2*).
[y]Sum of contents in anthocyanin groups, Del; delphinidin, delphinidn-3-*O*-β-glucopyranoside, and delphinidin-3-*O*-β-rutinoside, Mal; malvidin, and malvidin-3,5,-*O*-di-β-glucopyranoside, Pet; petunidin, Cyn; cyanidin-3-*O*-β-glucopyranoside, cyanidin-3-*O*-β-rutinoside, and cyanidin-3-*O*-β-galac-topyranoside, Peo; peonidin, peonidin-3-*O*-β-glucopyranoside, peonidin-3,5,-*O*-di-β-glucopyranoside, and peonidin-3-*O*-β-gal-actopyranoside.
[x]ND; Not detected. Means with the same letter are not significantly different at the 5% by Tukey's HSD.
[w] Absorbance at 520nm convert to estimated total anthocyanin contents with internal standard malvidin-3-*O*-glucoside chloride.

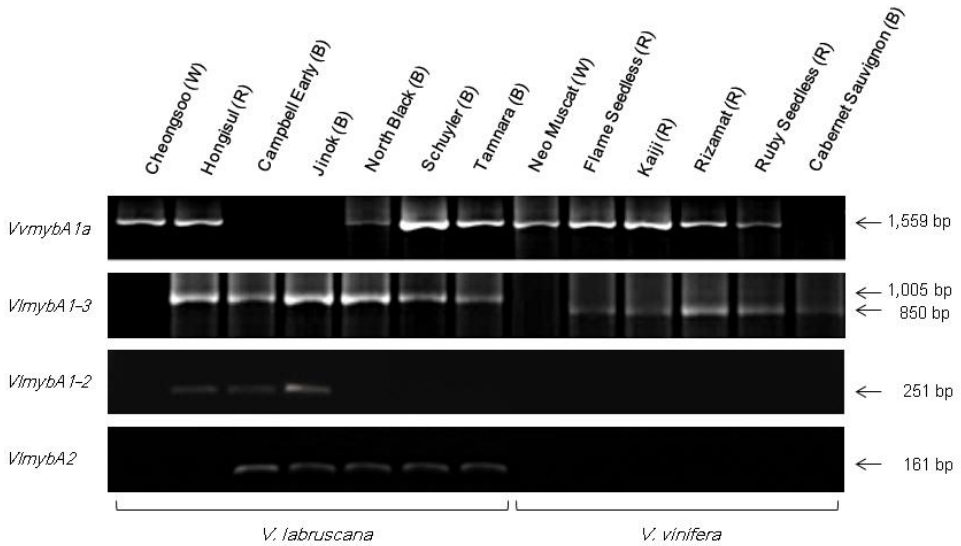

Fig 1. Genomic DNA PCR results of the *Myb* gene in the different grape species and cultivars by using leaf tissue. The characters in the bracket are skin color; W, White, R; Red, and B; Black. Haplotype A has *VvmybA1a* band, haplotype A1-2 has *VlmybA1-3* & *VlmybA1-2*, and haplotype A2 has *VlmybA1-3* and *VlmybA2*. *VlmybA1-3* has 155bp insertion in the *Vvmyba1c* of the *V.vinifera* grape.

Table 3. Anthocyanin content and total anthocyanin content of fruit skin in the seedlings of a cross between grape 'Campbell Early' (*V. labruscana;* A1-2/A2) × 'Neo Muscat' (*V. vinifera;* A/A).

Haplotype[z]	Anthocyanin contents (mg·g^{-1} Fw)					Total Anthocyanin content[x] (mg·g^{-1} Fw)
	Del[y]	Mal	Pet	Cyn	Peo	
A/A1-2	0	5.95	0.27	0.15	3.32	21.56
A/A2	0.05**[w]	17.70**	0.14[ns]	0.53**	3.75[ns]	60.46**

[z]Hap/Hap; Haplotype/Haplotype; A (*VvmybA1a*), A1-2 (*VlmybA1-2*), A2 (*VlmybA2*).
[y]Sum of contents in anthocyanin groups, Del; delphinidin, delphinidn-3-*O*-β-glucopyranoside, and delphinidin-3-*O*-β-rutinoside, Mal; malvidin, and malvidin-3,5,-*O*-di-β-glucopyranoside, Pet; petunidin, Cyn; cyanidin-3-*O*-β-glucopyranoside, cyanidin-3-*O*-β-rutinoside, and cyanidin-3-*O*-β-galac-topyranoside, Peo; peonidin, peonidin-3-*O*-β-glucopyranoside, peonidin-3,5,-*O*-di-β-glucopyranoside, and peonidin-3-*O*-β-gal-actopyranoside.
[x] Absorbance at 520nm convert to estimated total anthocyanin contents with internal standard malvidin-3-*O*-glucoside chloride.
[w] Student t-test **; Correlation is significant at the 0.01 level (2-tailed), *; Correlation is significant at the 0.05 level (2-tailed), ns; Not significant.

malvidin-3-O-glucoside, petunidin-3-O-glucoside, cyaniding-3-O-glucoside, and peonidin-3-O-glucoside (Figueiredo-González et al., 2012) vary between species. In the three red species 'Gran Negro', 'Mouratón', and 'Brancellao', the *Myb* gene plays a role in the last stage of the anthocyanin biosynthetic pathway, and anthocyanin composition is determined after UFGT enzyme is expressed (Bogs et al., 2006; Brugliera et al., 1999; Hoshino et al., 2003; Okinaka et al., 2003). Bogs (2006) and Lücker (2010) reported that anthocyanin was converted into delphinidin type or cyanidin type (through unique synthesis pathways), depending on F3'H or F3'5'H, respectively, after UFGT expressed. In our results shown that anthocyanin contents according to anthocyanin type were different to cultivar, but that not related to *Myb* gene because anthocyanin type already determined prior step of anthocyanin biosynthesis.

The total anthocyanin contents in *V. labrusca* hybrids was measured to explore a correlation between the haplotype (Hap A1–2, Hap A2), which has an effect on the skin color of *V. labrusca* hybrids, and the total anthocyanin content. As shown in Table 2, the total anthocyanin content in black skin cultivars 'Campbell Early' and 'Jinok' (Hap A1–2/A2), were higher than those in the black skin cultivars 'Schuyler' and 'Tamnara' (Hap A2/A2). On the other hand, the total anthocyanin contents in the red skin cultivar 'Hongisul' (Hap A/A1–2) was lowest. Compared to the haplotype analysis, total anthocyanin content in the black skin cultivar between the *VlmybA1–2* gene and the *VlmybA2* gene was higher than in the red skin cultivar with the *VlmybA1–2* gene. Based on statistical analysis, there was a significant difference between the total anthocyanin contents from the genotypes *VlmybA1–2* and *VlmybA2*. These experimental results correspond with the results of Azuma et al. (2011); *VlmybA2* commonly found in the black skin cultivars 'Campbell Early' and 'North Black', and the total anthocyanin contents in those cultivars were approximately 3–20 times higher than those in red species.

Correlation between haplotypes and anthocyanin contents of seedlings

Based on haplotype analysis of the crossed seedlings grape 'Campbell Early' (*V. labruscana*, A1–2/A2, red) × 'Neo Muscat' (*V. vinifera*, A/A, white), the haplotypes of the crossed seedlings were haplotype A1–2 or haplotype A2 depending on the presence of *VlmybA1–2* and *VlmybA2*. In the total 95 seedlings, haplotype A1–2 was present for 56, and haplotype A2 was present for 39 seedlings. On the other hand, fruited 37 seedlings, haplotype A1–2 identified 26, and haplotype A2 was 11 seedlings. It was a small number of populations for analysis genetic segregation, but total seedlings shown nearly 1:1 segregation each haplotype.

The contents of anthocyanin in the haplotype A2 seedlings were significantly higher than those in the haplotype A1–2 (Table 3). This is in line with the results discussed above, in that the total anthocyanin contents in 'Campbell Early' (Hap A1–2/A2), 'Jinok' (Hap A1–2/A2), 'Schuyler' (Hap A2/A2), and 'Tamnara' (Hap A2/A2) were higher than that in a red skin cultivar 'Hongisul', with a haplotype A1–2/A1–2. These results

indicate that total anthocyanin contents are affected by haplotype whether *VlmybA1–2* or *VlmybA2* genes, and total anthocyanin contents in the crossed seedlings containing the *VlmybA2* gene is high. In grape, anthocyanin synthesis was related at least three loci such as *VvmybA1*, *VvmybA2*, *VvmybA3* (Pelsy, 2010; Walker et al., 2007). The anthocyanin content is a quantitative trait and results from the sum of the expression of many enzymes related to anthocyanin biosynthesis. In case, haplotype A with *VvmybA2* from white cultivar was containing seedling, another *Myb* gene from *V. labrucana* has a strong influence on a skin color. Unless this study does not inform about the sequence of *VvmybA2* gene and *VlmybA2* gene, but *VvmybA2* gene in the white cultivar is not normally working (Walker et al., 2007). Previously results (Kobayashi et al., 2002), *VlmybA2* containing doubled X–Y domain region, DNA binding domain, which is a possible clue about more highly anthocyanin contents of haplotype A2/A2 type seedlings. In this fact, haplotype closely related to their anthocyanin contents and its identification can allow to predict skin color in the seedlings that breed with *V.labrusca*.

Materials and Methods

Plant materials

Six grapevine cultivars of *V. vinifera* and seven cultivars of *V. labruscana* (Table 1) used for identifying haplotype determination. Those cultivars were grown in the experimental vineyard of National Institute of Horticultural & Herbal Science (NIHHS), Suwon, Korea, and were cultured with conventional cultural practices on the vertical shoot position (VSP) trellis. Total 95 Seedlings from crossing black colored cultivar 'Campbell Early' (*V. labruscana*) and white colored cultivar 'Neo Muscat' (*V. vinifera*), were identified haplotype with PCR. For PCR analysis, young leaves collected from each grapevine cultivar and seedlings in the experimental vineyard in May 2013 and frozen in liquid nitrogen (LN_2), and stored at -80℃ for DNA extraction. Total 37 Bunches harvested same place in October 2013, skin separated from berries. Skins were blotted with paper towels to remove any residual pulp and measured skin weight. Collected skin with frozen in LN_2, and stored at -20℃ until anthocyanin contents analysis.

Haplotype analysis

Haplotype composition summarized in the table 1. Collected leaves were grinding with the pestle with LN_2. Genomic DNA was extracted using extraction kit (DNeasy Plant Mini Kit, Qiagen, USA) following manufacture's procedure. PCR reaction volume of 50 ul included 2.5 unit of Ex-Taq ^{TM} DNA polymerase (Takara, Japan), 5 ul of 10 x buffer, 4 ul of dNTP mixture, 10 pmol of each primer set, 100 ng of template DNA, and fill up to 50 ul with distilled water. PCR condition for *VvmybA1a* (Hap A; F*:* AAAAAGGGGGGCAATGTAGG-GACCC, R: GAACCTCCTTTTTGAAGTGGTGACT), and *VlmybA1-3* (Hap C'; F*:* GGACGTTAAAAAATGGTTGCA-CGTG, R: GAACCTCCTTTTTGAAGTGGTGACT) were programmed for one step of 3 min at 95℃, followed by 35 cycles of 30 sec of 94℃, 30 sec of 60℃, 90 sec of 72℃, and a final step of 5 min of 72℃. PCR condition for *VlmybA1-2* (Hap A1-2; F: CACCACTTGAAAAAGAAGGTC, R: TCTTGA-TCCAGCTCAGCTAAC) was programmed for one step of 3 min at 95℃, followed by 25 cycles of 30 sec of 94℃, 30 sec of 55.9℃, 30 sec of 72℃, and a final step of 5 min of 72℃. PCR condition for *VlmybA2* (Hap A2; F*:* GCTGAGCATGCT-CAAATGGAT, R: TCCCACCATATGATGTCACCC) was programmed for one step of 3 min at 95℃, followed by 25 cycles of 30 sec of 94℃, 30 sec of 57.9℃, 90 sec of 72℃, and a final step of 5 min of 72℃. All of amplified reaction was using thermal cycler (Takara, JP/TP600, Japan). 5 ul of PCR products were analyzed by electrophoresis on 1% agarose gel; bands stained with added EtBr, under the UV light.

Anthocyanin contents

Anthocyanin standards were used to 3-glucoside, 3, 5-di-glucoside of cyanidin delphinidin, malvidin, peonidin, and petunidin (Extrasynthese, Genay, France). These kinds of anthocyanins were principal anthocyanins in the grape species but not that of pelargonidin (He et al., 2010). The anthocyanin analysis was performed as described by Jeong et al. (2006). Anthocyanin extract solution prepared as following procedure: Each 0.5 g of berry skin placed into 50 ml test tube, added to 10 ml of 10% formic acid/methanol (v/v), and extracted from 24 h dark place. The mixture was centrifuged at 4,000 rpm for 10 min at 4℃. The supernatant filtered through a 0.45 ㎛ membrane filter (HAWP Millipore Co., Bedford). These solutions kept at 4℃ in the dark until anthocyanin analysis. The anthocyanin compositions were analyzed by high-performance liquid chromatography (HPLC; Agilent 1100 HPLC Chemstation, California, USA) and zorbax C18 column (4.6 × 250 nm, 5 μm, Agilent, California, USA). The column oven temperature is 30℃. In all cases, ten μl of extract were injected. The Solvent A was 10% formic acid/distilled water (D.W), the solvent B was 100% acetonitrile. Solvent A maintained at 95% throughout the analyzed, and the flow rate was 0.7 ml/min. The initial condition of solvent B was 5%, increased to 8% in 5 min, and risen to 9% in 15 min, and risen to 10% in 20 min, and increase to 15% for 30 min, and risen to 15% in 45 min, and risen to 20% in 50 min, and risen to 30% in 65 min, and risen to 40% in 66 min, and risen to 40% in 70 min. The column oven temperature is 30℃. 1 ml of extract solution transferred into a test tube, and 9 ml of 0.2 M sodium acetate/ distilled water solution (pH 1.0 with HCl) added for measurement of total anthocyanin contents. 1 ml of the reaction solution was thoroughly mixed and then putted on test cell immediately, and the absorbance at 520 nm read with a spectrophotometer (Agilent Technologies, US/8453, USA). The concentration of total anthocyanins expressed as $mg \cdot g^{-1}$ skin fresh weight using malvidin-3-*O*-glucoside chloride (Extrasynthese, Genay, France) as an external standard.

Statistical analysis

Anthocyanin content analysis was replicated three times for each sample. All of the data was analyzed to identify significant differences with R program (Ver. 2.13.0) for Windows. Tukey's HSD (Honest Significant Difference) test and the student t-test analysis ($p < 0.05$) of variance were performed to define anthocyanin content, according to haplotypes of cultivars and seedlings.

Conclusion

The grape cultivars *Vitis labrusca* and hybrid cultivars from *V. vinifera* x *V. labrusca* have different kinds of *Myb* genes, which regulate anthocyanin biosynthesis like the *V.vinifera* grape. An anthocyanin content of haplotype A2 (*VlmybA1-3* and *VlmybA2*) was significantly higher than haplotype A1-2

(*VlmybA1-3* and *VlmybA1-2*) in the *V.labrusca* hybrid cultivars and their cross seedlings from *V. labrusca* x *V. vinifera*. However, *VlmybA2* gene function is not clear in these results, but it is related to anthocyanin content as a *Myb* gene family. Based on this study, our findings show that grape skin color is controlled by haplotypes in *V.labrusca*, and we can find out skin color in juvenile years by genomic DNA PCR in the seedlings from the progenies of *V. vinifera* x *V. labrusca*.

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