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ORIGINAL PAPER

Haplotype composition at the color locus is a major genetic determinant of skin color variation in *Vitis* ×*labruscana* grapes

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Abstract Skin color is one of the most important fruit traits in grape, and has become greatly diversified due to hybridization and human selection. Many studies concerning the genetic control of grape color in European species (*Vitis vinifera* L.), especially the role of *MYB*-related genes, have been reported. On the other hand, there have been few studies of the *MYB*-related genes in grapes belonging to *V.* ×*labruscana* L.H. Bailey, a subgroup of grapes that originated from the hybridization of *V. labrusca* with *V. vinifera*. In the present study, we found a novel functional haplotype, HapE2 (consisting of the genes *VlMYBA2* and *VlMYBA1–3*), in diploid *V.* ×*labruscana*. Moreover, we developed a method to determine the haplotype compositions of tetraploid grapes by means of quantitative real-time PCR, and investigated the relationship between haplotype composition and skin color. The color locus in *V.* ×*labruscana* grapes usually consists of functional haplotypes (HapE1 and/or HapE2), and non-functional haplotype HapA. The number of functional haplotypes in the genome was found to be correlated with the level of anthocyanin in the skin. Anthocyanin contents of grapes that contained HapE2 were significantly higher than those containing HapE1. These results suggest that the number and kind of functional haplotypes at the color locus are the major genetic factors that determine skin color variation. These findings provide new knowledge about the unique genetic control of color in *V.* ×*labruscana* grapes, and should contribute to development of new cultivars that have the desired color and anthocyanin content.

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Introduction

In the worldwide grape industry, the European species *Vitis vinifera* L. is the dominant grape used to produce wine, raisins, and fresh table grapes. However, we know today that the resulting failures of early colonists to establish *V. vinifera* in the United States resulted from a lack of resistance to native diseases, soil pests, and low winter temperatures in the northernmost areas (Einset and Pratt 1975). To overcome these difficulties, seedlings or selections from wild American native grape species were selected during the first half of the nineteenth century. Thereafter, many breeders attempted to improve the species through hybridization between *V. vinifera* and American native species (Snyder 1937). The most commonly used native species was the fox grape (*V. labrusca* L.). *V.* ×*labruscana* L.H. Bailey is a subgroup of grapes that originated from the hybridization of *V. labrusca* with other species, most commonly *V. vinifera*. As a result of these efforts, more than 1,500 *V.* ×*labruscana* accessions were found or developed (Hedrick 1908, 1925; Snyder 1937). Furthermore, many *V.* ×*labruscana*-related tetraploid grapes such as ‘Kyoho’ and ‘Pione’ were developed to produce a large berry and are now popular grapes in Japan

and other Asian areas. Thus, *V.* ×*labruscana* grapes also play an important role in the worldwide grape market as well as *V. vinifera*.

Today, grape berry skin color has become greatly diversified due to hybridization and human selection. The color variation among black, red, and white (yellow–green) grape berries results from the quantity and composition of anthocyanins in the berry skin. Anthocyanin biosynthesis in many plants is controlled by regulatory genes that belong to three major groups of transcription factors, namely the MYB, basic helix-loop-helix (bHLH), and WD40 classes (Koes et al. 2005). It has been shown that the presence or absence of anthocyanins in grape berries segregates as a monogenic trait determined by a locus in linkage group 2 (Doligez et al. 2002; Fischer et al. 2004; Salmaso et al. 2008). Two *MYB*-related transcription-factor genes that regulate anthocyanin biosynthesis, *VvMYBA1* and *VvMYBA2*, have been isolated from *V. vinifera* grapes (Kobayashi et al. 2004; Walker et al. 2007). Walker et al. (2006) reported that the color locus of *V. vinifera* is composed of these two genes, both of which control berry color. Recently, it was suggested that the color locus is a cluster of *MYB* and *MYB*-like genes, including *VvMYBA1* and *VvMYBA2*, spanning a 200-kb region located on chromosome 2 (Azuma et al. 2009; Fournier-Level et al. 2009; Matus et al. 2008).

The origin of white-skinned genotypes was recently demonstrated to be the result of disruption of the two functional *MYB*-related genes. It was suggested that the insertion of *Gret1*, a Ty3-*gypsy*-type retrotransposon, in the promoter region of *VvMYBA1* led to its transcriptional inactivation; the resulting non-functional allele was named *VvMYBA1a* (Kobayashi et al. 2004, 2005). Several genetic studies have revealed that white-skinned individuals are homozygous for *VvMYBA1a*, whereas colored-skinned individuals contain at least one copy of the functional allele *VvMYBA1c* (Azuma et al. 2007; Kobayashi et al. 2004; Lijavetzky et al. 2006; This et al. 2007). Walker et al. (2007) reported that a single-nucleotide polymorphism (SNP) mutation in the coding sequence of *VvMYBA2* inactivated gene transcription, and named the resulting non-functional allele *VvMYBA2w*. Walker et al. (2007) also isolated functional alleles (*VvMYBA1c* and *VvMYBA2r*) and non-functional alleles (*VvMYBA1a* and *VvMYBA2w*) of *VvMYBA1* and *VvMYBA2* from a bacterial artificial chromosome (BAC) library of 'Cabernet Sauvignon' (*V. vinifera*), and revealed that no functional allele of either gene was present in any white-skinned accession of *V. vinifera* examined.

Because the genes present within each copy of the color locus are inherited together, it is helpful to consider them as part of a single large allele (haplotype). We originally named the functional allele Haplotype C (HapC) and the non-functional allele Haplotype A (HapA; Azuma et al. 2008). Very recently, Fournier-Level et al. (2010) reported that HapC can be divided into two subgroups ("haplogroups"): N (Fig. 1; designated HapC-N in the present study) and Rs (designated HapC-Rs). HapC-N contains the functional alleles *VvMYBA1c* and *VvMYBA2r*, whereas the HapC-Rs contains the functional allele *VvMYBA1c* and the non-functional allele *VvMYBA2w*. Based on this understanding of haplotype structure, Fournier-Level et al. (2010) also suggested that the *Gret1* insertion in the *VvMYBA1* promoter occurred after the emergence of *VvMYBA2w*.

From *V.* ×*labruscana* grapes, four functional *MYB*-related genes, *VlMYBA1-1*, *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2*, have been isolated so far (Azuma et al. 2008; Kobayashi et al. 2002). In a previous study, we showed that *VlMYBA1-2* and *VlMYBA1-3* exist at nearby positions within the color locus (Azuma et al. 2008). This combination was originally named Haplotype E (HapE), and confirmed that the color locus in most *V.* ×*labruscana* grape accessions consisted of HapE and HapA haplotypes. Furthermore, we investigated the relationship between haplotype combination and anthocyanin content by making crosses between parents with different combinations, and found that berries from progeny seedlings with two functional haplotypes had a higher anthocyanin content than those from seedlings with only a single functional haplotype (Azuma et al. 2008).

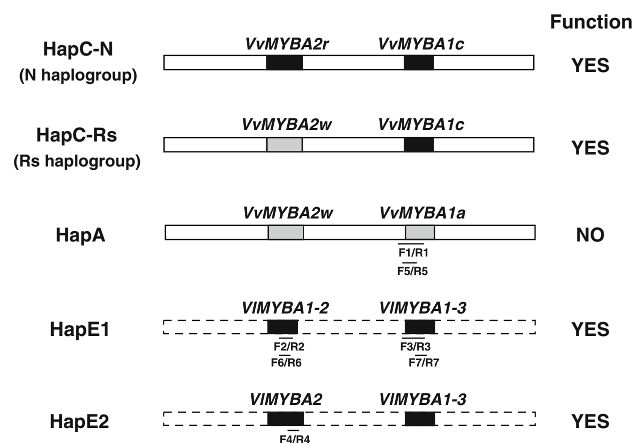

Fig. 1 Haplotypes at the color locus. HapC-N and HapC-Rs are equivalent to the N haplogroup and Rs haplogroup, respectively, described by Fournier-Level et al. (2010). Haplotype E1 (HapE1) is equivalent to "HapE" described by Azuma et al. (2008). *Gray colored boxes* indicate non-functional *MYB*-related genes. *Black colored boxes* indicate functional *MYB*-related genes. Primer positions used in PCR and qRT-PCR analyses are indicated below the maps. *F1–F7* forward primers, *R1–R7* reverse primers. *Dotted lines* depicting HapE1 and HapE2 indicate that the exact positions of *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* within the color locus are unknown

Although many studies concerning the mechanisms controlling the color of grape berry skin have been reported, the underlying genetic mechanisms responsible for color variation are not completely understood. In particular, there have been few reports of genetic analysis of the *MYB*-related genes in *V.* ×*labruscana*. For example, although *VlMYBA1-1* and *VlMYBA2* were isolated from a tetraploid 'Kyoho' (*V.* ×*labruscana*) cDNA library by Kobayashi et al. (2002), it had not been shown whether these *MYB*-related genes also exist in HapE. Furthermore, there have been no reports about the relationship between the haplotype composition and skin color of tetraploid grape cultivars. Previously, we analyzed the distribution of haplotypes in 35 grape accessions by means of a PCR-based method and successfully detected the haplotype combinations of diploid grapes (Azuma et al. 2008). However, we were unable to determine the haplotype composition of tetraploid grapes by this method because we could detect only the presence or absence of a particular haplotype, not how many copies were present in the tetraploid genome.

In the present study, we investigated the distribution of *MYB*-related genes (*VvMYBA1a*, *VlMYBA1-1*, *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2*) using progeny seedlings and found a novel haplotype at the color locus in *V.* ×*labruscana* grapes. Furthermore, we developed a method to determine the detailed haplotype compositions in tetraploid grapes by means of quantitative real-time PCR (qRT-PCR). Finally, we investigated the relationship between the haplotype compositions of the grapes (including both diploid and tetraploid accessions) and anthocyanin content, and found novel characteristics of haplotype compositions that were related to color variation.

Materials and methods

Plant materials

The molecular mechanism of color variation in *V.* ×*labruscana* grapes was analyzed in 26 progeny seedlings and 25 accessions (Tables 1, 3). Young leaves and ripe berries were collected from the vineyards at the Grape and Persimmon Research Station, National Institute of Fruit Tree Science (NIFTS), Hiroshima, Japan. Leaves from which genomic DNA (gDNA) was to be extracted were frozen in liquid nitrogen and stored at −80°C until use. For anthocyanin extraction, a total of ten berries from each progeny seedling and accession were randomly sampled at harvest time, and the peeled skins were immediately frozen in liquid nitrogen and stored at −80°C until use.

PCR analysis of the *MYB*-related genes at the color locus in progeny seedlings

We used plants raised by grafting seedlings from a cross between 'Muscat of Alexandria' (*V. vinifera*; white-skinned) and 'Campbell Early' (*V.* ×*labruscana*; black-skinned) onto 'Teleki 5BB' rootstocks (2- to 3-year after the grafting, $n = 26$) for the investigation of the segregation of *MYB*-related genes at the color locus. gDNA from young leaves of the parents and progeny was extracted as described previously (Kobayashi et al. 2002) and used as the template for PCR. The primer sequences for *VvMYBA1a*, *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* are shown in Table S1. PCR reactions were performed in a total volume of 10 μL, comprising 5 ng DNA, 200 μM dNTPs, 0.2 μM of each primer, and 0.5 units of ExTaq polymerase (Takara, Shiga, Japan). The PCR cycling conditions for *VvMYBA1a* and *VlMYBA1-3* detection were an initial phase at 95°C for 3 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s; and a final phase at 72°C for 5 min. The cycling conditions for *VlMYBA1-2* and *VlMYBA2* were an initial phase at 95°C for 3 min; 36 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final phase at 72°C for 5 min. PCR fragments were separated by electrophoresis in 1.2% agarose gel in TAE buffer and photographed under UV light. For the segregation analysis of the progeny seedlings, we used Chi-square tests to assess the agreement between the observed and expected haplotype ratios.

qRT-PCR analysis of *VlMYBA1-1* in progeny seedlings

The distribution of *VlMYBA1-1* at the color locus in progeny seedlings were analyzed by qRT-PCR with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and a QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) as described in the manufacturers' manuals. The primers for this analysis were F (5′-CTATTGGCATGGTCACCAT-3′) and R (5′-GAATGTGTTTGGGGTTTATC-3′). This primer set amplified the common region of the *VlMYBA1-1* and *VlMYBA2* coding sequences because it was unable to develop primers specific to *VlMYBA1-1*. The amounts of DNA corresponding to these *MYB*-related genes were determined by the standard curve-based method. Plasmid DNA of *VlMYBA1-3* for the reference gene and *VlMYBA2* for the target genes was used as the template for the standard. A standard DNA set for a calibration curve was prepared as a fivefold dilution series of plasmid DNA samples of *VlMYBA1-3* and *VlMYBA2* to cover the expected range of template concentrations. The primer sequences for *VlMYBA1-3* were shown in Table S2. The cycling conditions were 95°C for 15 min; 45 cycles of

Table 1 PCR analysis of *MYB*-related genes in the seedlings of the 'Muscat of Alexandria' × 'Campbell Early' cross

		VvMYBA1a[a]	*VlMYBA1-3*[b]	*VlMYBA1-2*	*VlMYBA2*	Haplotype composition[c]	Anthocyanin content (mg/g fw)[d]
Parents	Muscat of Alexandria	+	−	−	−	A/A	
	Campbell Early	−	+	+	+	E1/E2	
Progeny seedlings	1	+	+	−	+	A/E2	5.07 ± 0.60
	2	+	+	−	+	A/E2	6.16 ± 0.43
	3	+	+	−	+	A/E2	8.89 ± 0.33
	4	+	+	+	−	A/E1	1.44 ± 0.09
	5	+	+	−	+	A/E2	3.67 ± 0.39
	6	+	+	−	+	A/E2	6.86 ± 0.85
	7	+	+	+	−	A/E1	3.07 ± 0.30
	9	+	+	+	−	A/E1	1.40 ± 0.04
	10	+	+	−	+	A/E2	7.17 ± 1.50
	11	+	+	+	−	A/E1	1.25 ± 0.13
	13	+	+	−	+	A/E2	5.40 ± 0.69
	14	+	+	−	+	A/E2	
	15	+	+	+	−	A/E1	
	17	+	+	+	−	A/E1	
	18	+	+	+	−	A/E1	
	19	+	+	+	−	A/E1	
	20	+	+	−	+	A/E2	
	21	+	+	−	+	A/E2	
	22	+	+	+	−	A/E1	
	23	+	+	+	−	A/E1	
	24	+	+	+	−	A/E1	
	26	+	+	+	−	A/E1	
	27	+	+	+	−	A/E1	
	28	+	+	−	+	A/E2	
	30	+	+	−	+	A/E2	
	32	+	+	+	−	A/E1	

[a] *VvMYBA1a*, non-functional *MYB*-related gene

[b] *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2*, functional *MYB*-related genes

[c] *A*, Haplotype A; *E1*, Haplotype E1; *E2*, Haplotype E2

[d] Average ± SE ($n = 3$), cyanidin-3-glucoside equivalent

95°C for 15 s, 56°C for 20 s, and 72°C for 31 s; and a final cycle for melting curve analysis. qRT-PCR was carried out with three replicates per sample. Data were calculated using calibration curves and normalized against the amount of *VlMYBA1-3*.

Determination of the haplotype composition in tetraploid grapes by means of the qRT-PCR method

Haplotype compositions at the color locus in tetraploid grapes were analyzed by qRT-PCR using the above-described PCR system and reaction kit. gDNA was extracted from young leaves of tetraploid grape accessions and used as the template. The primer sequences for *VvMYBA1a*, *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* are shown in Table S2. The relative amounts of these *MYB*-related genes were determined by the standard curve-based method. The standard DNA set for a calibration curve was prepared as a fivefold dilution series of the gDNA of 'Hakuho' (for *VvMYBA1a* analysis) and 'Black Beat' (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 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. qRT-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* (*CHS3*) (Jeong et al. 2004; Yakushiji et al. 2006).

Investigation of the relationship between haplotype composition and skin color

The skin colors of the progeny seedlings and grape accessions were visually assessed at harvest time. The registered skin color for each accession was also confirmed by using the database of the *Vitis* International Variety Catalogue (http://www.vivc.de/index.php). The total anthocyanin content in the berry skin in 2010 was analyzed according to Shiraishi et al. (2007). In summary, 1 g of berry skin from each of the progeny seedlings and accessions was macerated in 10 mL of 50% aqueous acetic acid (v/v) for 12 h at 4°C in the dark. We then diluted 100 μL of extracted material to a final volume of 1 mL in 50% aqueous acetic acid, and measured the total anthocyanin content by reading the absorbance at 520 nm with a spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). Total anthocyanin content was expressed as mg of cyanidin-3-glucoside (Extrasynthèse, Genay, France) equivalent per gram of fresh berry skin weight. The statistical significance of the relationship between the haplotype composition at the color locus and the total anthocyanin content was evaluated using Student's *t* test and the Tukey–Kramer test.

Results

PCR analysis of the *MYB*-related genes at the color locus in progeny seedlings

We investigated the segregation of *MYB*-related genes at the color locus in progeny seedlings from the cross 'Muscat of Alexandria' × 'Campbell Early'. The non-functional gene *VvMYBA1a* was detected in the white-skinned 'Muscat of Alexandria' parent, but the functional *MYB*-related genes *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* were not detected (Table 1). On the other hand, *VvMYBA1a* was not detected in 'Campbell Early', but *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* were all detected. *VvMYBA1a* and *VlMYBA1-3* were detected in all of the progeny seedlings (Table 1). These results suggest that 'Muscat of Alexandria' is homozygous for *VvMYBA1a* and that the haplotype composition at the color locus is HapA/HapA. Although both *VlMYBA1-2* and *VlMYBA2* were detected in the parent 'Campbell Early', *VlMYBA1-2* was not detected in progeny seedlings that contained *VlMYBA2*, and vice versa (Table 1). In a previous study, we showed that 'Campbell Early' was homozygous for the haplotype composition HapE (containing *VlMYBA1-2* and *VlMYBA1-3*; Azuma et al. 2008). In the present study, however, *VlMYBA1-2* and *VlMYBA2* were proven to be allelic and 'Campbell Early' was heterozygous for *VlMYBA1-2*/*VlMYBA2*. 'Campbell Early' was also revealed to be homozygous for *VlMYBA1-3* because this gene was detected in each of the progeny seedlings. These results suggest that in addition to HapE, a novel haplotype that contains *VlMYBA1-3* and *VlMYBA2* exists at the color locus in the 'Campbell Early' genome; we named this Haplotype E2 (HapE2) and renamed HapE as Haplotype E1 (HapE1; Fig. 1). From these findings, we predicted that 'Campbell Early' would be heterozygous for HapE1 and HapE2 (HapE1/HapE2), and therefore, that the progeny seedlings from the 'Muscat of Alexandria' (HapA/HapA) × 'Campbell Early' (HapE1/HapE2) cross would segregate as 1 HapA/HapE1:1 HapA/HapE2. The ratio observed was not significantly different from the 1:1 expected ratio, based on a Chi-square test (Table 2), providing evidence that our model is correct.

qRT-PCR analysis of *VlMYBA1-1* in progeny seedlings

To investigate whether *VlMYBA1-1* is contained in HapE1 or HapE2, a qRT-PCR that can detect the amount of *MYB*-related gene DNA was performed on the progeny seedlings. In this analysis, a primer set that detected both *VlMYBA1-1* and *VlMYBA2* was used because we were unable to develop primers specific to *VlMYBA1-1*. These primers did not amplify any DNA in the HapA/HapE1 progeny seedlings (Fig. 2), indicating that neither *VlMYBA1-1* nor *VlMYBA2* was contained in HapE1. In

Table 2 Segregation of the haplotypes in the seedlings of the 'Muscat of Alexandria' × 'Campbell Early' cross

Parents[a]	Haplotype composition	Total number of progeny	Number of progeny		Expected ratio	χ^2 value	*P* value[b]
			A/E1	A/E2			
Muscat of Alexandria	A/A	26	14	12	1:1	0.154	0.695
Campbell Early	E1/E2						

A Haplotype A, *E1* Haplotype E1, *E2* Haplotype E2

[a] 'Muscat of Alexandria' (seed parent), white-skinned; 'Campbell Early' (pollen parent), black-skinned

[b] The *P* value represents the significance of the difference between actual segregation results and the expected ratio (χ^2 test)

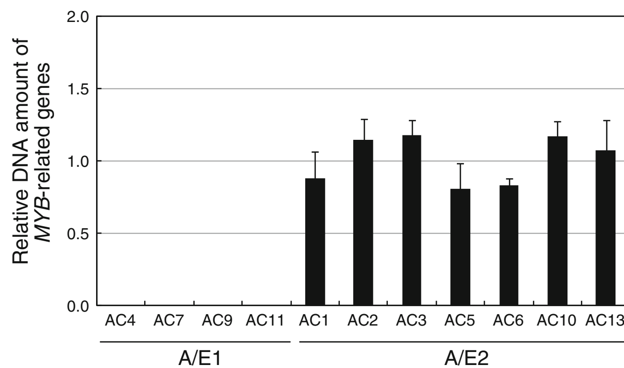

Fig. 2 Relative DNA amount of specific *MYB*-related genes in progeny of the cross 'Muscat of Alexandria' × 'Campbell Early'. The primer set used in this analysis detects both *VlMYBA1-1* and *VlMYBA2* but does not distinguish between them. Relative DNA amounts of *VlMYBA1-1* and *VlMYBA2*, compared to *VlMYBA1-3*, were calculated. AC1 to AC13 designate the individual progeny seedlings. *A* Haplotype A, *E1* Haplotype E1, *E2* Haplotype E2. *Error bars* show standard error (SE)

HapA/HapE2 seedlings, the relative DNA amounts of *VlMYBA1-1* and *VlMYBA2*, compared to *VlMYBA1-3*, were 0.81–1.17 (Fig. 2). This indicates that either *VlMYBA1-1* or *VlMYBA2* (but not both) is contained in HapE2. Furthermore, *VlMYBA2*-specific primers could detect *VlMYBA2* in HapA/HapE2 seedlings (Table 1). These results suggest that *VlMYBA2* is contained in HapE2 but that *VlMYBA1-1* is not. From these results, we concluded that *VlMYBA1-1* does not exist in either HapE1 or HapE2.

Determination of the haplotype compositions of tetraploid grapes by means of qRT-PCR

We determined the haplotype composition in several tetraploid grape varieties by means of the qRT-PCR method. As shown in Fig. 1, *VvMYBA1a*, *VlMYBA1-2*, and *VlMYBA2* are unique to HapA, HapE1, and HapE2, respectively. On the other hand, *VlMYBA1-3* is contained in both HapE1 and HapE2. Using these findings, we investigated the presence or absence of these genes and their relative DNA amounts in tetraploid grapes, and used this information to predict the haplotype compositions at the color locus. These predictions were based on the assumption that HapA, HapE1, and HapE2 were the only haplotypes present in this set of varieties.

In white-skinned 'Hakuho', only the non-functional gene *VvMYBA1a* was detected; the functional *MYB*-related genes *VlMYBA1-2*, *VlMYBA1-3*, and *VlMYBA2* were not detected (Fig. 3). From these results, the relative DNA amount of *VvMYBA1a* in 'Hakuho' was 4 (Fig. 3a), and the haplotype composition of 'Hakuho' was determined to be HapA/A/A/A (Table 3).

In black-skinned 'Black Beat', *VvMYBA1a* was not detected whereas *VlMYBA1-3*, *VlMYBA1-2*, and *VlMYBA2* were each detected (Fig. 3). Thus, the relative DNA amount of *VlMYBA1-3* in 'Black Beat' was assumed to be 4 (Fig. 3b). In black-skinned 'Kyoho', *VvMYBA1a*, *VlMYBA1-3*, *VlMYBA1-2*, and *VlMYBA2* were all detected. The relative DNA amount of *VvMYBA1a* in 'Kyoho', compared to 'Hakuho' (relative amount = 4), was 2.17, and the relative DNA amount of *VlMYBA1-3* in 'Kyoho', compared to 'Black Beat' (relative amount = 4), was 2.07 (Fig. 3a, b). These results suggest that the haplotype composition of 'Kyoho' is HapA/A/E1/E2, and so it was assumed that the relative DNA amount of *VlMYBA1-2* and *VlMYBA2* was 1 (Fig. 3c, d; Table 3). The relative DNA amounts of *VlMYBA1-2* and *VlMYBA2* in 'Black Beat', compared to 'Kyoho' (relative amount of 1 for each), were 2.38 and 1.90, respectively (Fig. 3c, d). From these results, we determined the haplotype composition of 'Black Beat' to be HapE1/E1/E2/E2 (Table 3).

In the colored-skinned selection 390-76 ('Fujiminori' × 'Aki Queen'), the relative DNA amounts of *VvMYBA1a*, *VlMYBA1-3*, *VlMYBA1-2*, and *VlMYBA2* were 1.10, 3.19, 2.00, and 0.93, respectively. Therefore, the haplotype composition of this accession was determined to be HapA/E1/E1/E2. In 'Pione', and 'Fujiminori', the relative DNA amounts of the four genes were similar to those in 'Kyoho', suggesting that the haplotype composition of these accessions was HapA/A/E1/E2. In 'Ruby Roman', 'Aki Queen', 'Ryuho', and 'Benizuiho', the relative DNA amounts of *VvMYBA1a*, *VlMYBA1-3*, and *VlMYBA1-2* were 2.72–3.34, 0.72–1.31, and 0.79–0.99, respectively. *VlMYBA2* was not detected in any of these accessions. From these results, the haplotype composition of these accessions was determined to be HapA/A/A/E1. Thus, the haplotype compositions of the color locus in tetraploid grapes were determined successfully using the qRT-PCR method.

Relationship between the haplotype composition at the color locus and berry color in progeny seedlings and grape accessions

We investigated the skin color of grapes set on 11 of the 26 progeny of 'Muscat of Alexandria' × 'Campbell Early'. Because the other 15 seedlings had not yet set fruits, we could not investigate their skin color in this study. All 11 of the progeny plants that tested accumulated anthocyanin in the berry skin (Table 1). This result demonstrates that both HapE1 and HapE2 from 'Campbell Early' were functional haplotypes. Interestingly, berries from most of the seedlings that contained HapA/HapE1 tended to be red-skinned, and those from HapA/HapE2 seedlings were black-skinned. The average anthocyanin content of berry skin was

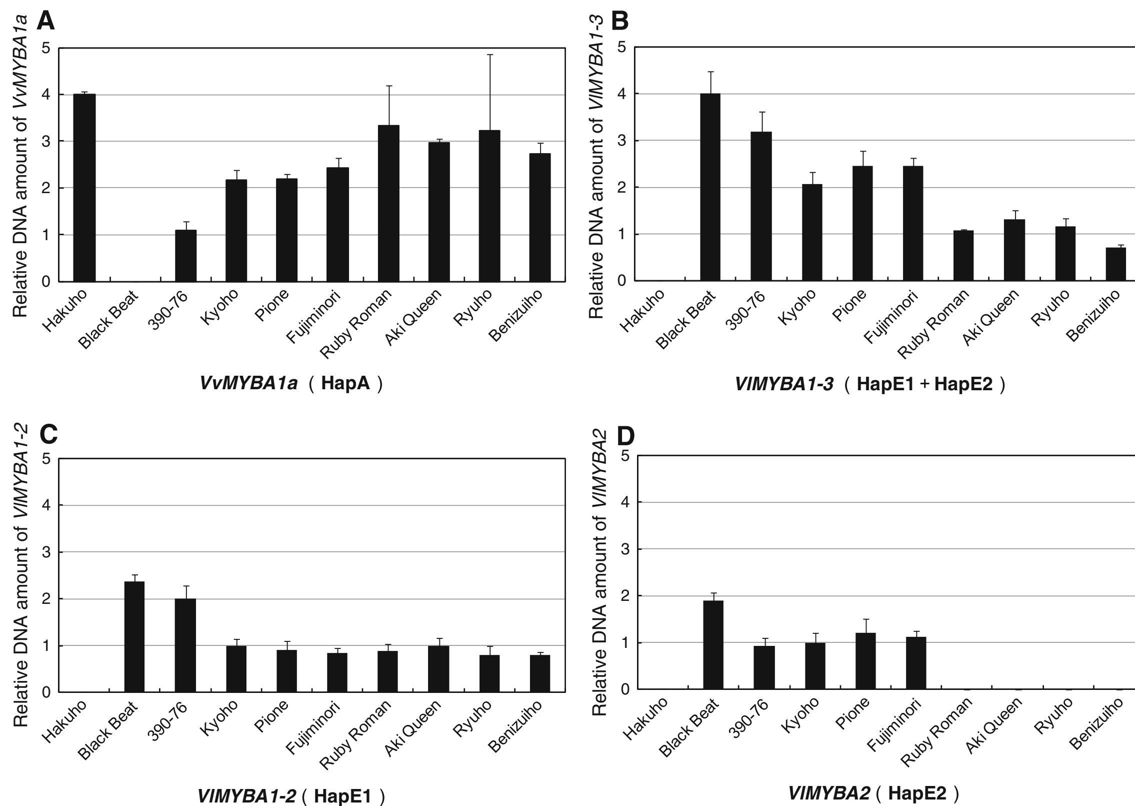

Fig. 3 Relative DNA amounts of *VvMYBA1a* (**a**), *VlMYBA1-3* (**b**), *VlMYBA1-2* (**c**), and *VlMYBA2* (**d**) in tetraploid grape cultivars. In the *VvMYBA1a* analysis (**a**), the relative DNA amount of *VvMYBA1a* in 'Hakuho' was assumed to be 4, and the relative amounts of *VvMYBA1a* in the other grape accessions were calculated compared to 'Hakuho'. In the *VlMYBA1-3* analysis (**b**), the relative amount of *VlMYBA1-3* in 'Black Beat' was assumed to be 4, and the relative amounts of *VlMYBA1-3* in the other grape accessions were calculated compared to 'Black Beat'. In the *VlMYBA1-2* (**c**) and *VlMYBA2* (**d**) analyses, the relative amounts of *VlMYBA1-2* and *VlMYBA2* in 'Kyoho' were each assumed to be 1, and the relative DNA amounts of these genes in the other grape accessions were calculated compared to 'Kyoho'. *Error bars* show standard error (SE)

1.79 mg/g fw in the berries from HapA/HapE1 seedlings and 6.17 mg/g fw in the berries from HapA/HapE2 seedlings (Fig. 4a). It was thus confirmed that, as suggested by the phenotypes, the anthocyanin contents of berries from HapA/HapE2 progeny seedlings were significantly higher than those from HapA/HapE1 seedlings.

The relationship between haplotype composition and skin color in the grape accessions is shown in Table 3. Among the tetraploid grapes, the haplotype composition of white-skinned accessions 'Hakuho' and 'Suiho' was HapA/A/A/A. The black-skinned accessions 'Kyoho', 'Takasumi', 'Shigyoku', 'Takatsuma', 'Pione', 'Dark Ridge', and 'Fujiminori' were HapA/A/E1/E2. The black-skinned accessions 'Black Beat', 'Ishiharawase', and 390-76 contained three or four functional haplotypes (HapE1/E1/E2/E2 or HapA/E1/E1/E2). On the other hand, red-skinned accessions 'Yoho', 'Aki Queen', 'Benizuiho', 'Gorby', 'Ryuho', 'Ruby Roman', and 'Queen Nina' contained only a single functional haplotype (HapA/A/A/E1). Interestingly, HapE2 was not detected in any of the red-skinned accessions examined. The average anthocyanin content of berry skin was 1.73 mg/g fw in the HapA/A/E1/E2 accessions and 0.20 mg/g fw in the HapA/A/A/E1 accessions (Fig. 4b). The average anthocyanin content in the HapA/A/E1/E2 accessions was significantly higher than that of the HapA/A/A/E1 accessions. It was confirmed that 'Black Beat', 'Ishiharawase', and 390-76, which contained three or four functional haplotypes, tended to have high anthocyanin content in the berry skin (Table 3).

Among the diploid grape accessions, black-skinned 'Campbell Early' contained HapE1/HapE2, and 'Houman' and 'North Black' contained HapA/HapE2 (Table 3). On the other hand, red-skinned 626-84 [('Katta Kurgan' × 'Takasago') × ('Takasago' × 'Campbell Early')] and 'North Red' contained HapA/HapE1. The average anthocyanin content in HapA/HapE2 accessions was significantly higher than that of the HapA/HapE1 accessions (Fig. 4b). These results suggest that the number of functional haplotypes affects the capability for accumulation of anthocyanin in grape berry skin. Furthermore, the ability of anthocyanin biosynthesis induction of HapE2 appears to be higher than that of HapE1.

Table 3 Relationship between haplotype composition and skin color in *V.* ×*labruscana* grapes

Accession	Ploidy	Color	Haplotype composition				Anthocyanin content (mg/g fw)[a]
Hakuho	4×	White	A	A	A	A	Undet.
Suiho		White	A	A	A	A	Undet.
Kyoho		Black	A	A	E1	E2	1.48 ± 0.15
Takasumi		Black	A	A	E1	E2	1.55 ± 0.23
Shigyoku		Black	A	A	E1	E2	1.95 ± 0.12
Takatsuma		Black	A	A	E1	E2	0.82 ± 0.03
Pione		Black	A	A	E1	E2	1.14 ± 0.22
Dark Ridge		Black	A	A	E1	E2	3.46 ± 0.57
Fujiminori		Black	A	A	E1	E2	1.75 ± 0.12
390-76		Black	A	E1	E1	E2	2.44 ± 0.27
Ishiharawase		Black	E1	E1	E2	E2	1.71 ± 0.08
Black Beat		Black	E1	E1	E2	E2	7.44 ± 1.79
Yoho		Red	A	A	A	E1	0.29 ± 0.04
Aki Queen		Red	A	A	A	E1	0.16 ± 0.01
Benizuiho		Red	A	A	A	E1	0.17 ± 0.01
Gorby		Red	A	A	A	E1	0.20 ± 0.03
Ryuho		Red	A	A	A	E1	0.15 ± 0.02
Ruby Roman		Red	A	A	A	E1	0.14 ± 0.01
Queen Nina		Red	A	A	A	E1	0.26 ± 0.01
Campbell Early	2×	Black	E1			E2	4.35 ± 0.27
Houman		Black	A			E2	3.49 ± 0.65
North Black		Black	A			E2	3.55 ± 0.31
Concord		Black	A			E1	1.20 ± 0.07
626-84		Red	A			E1	0.46 ± 0.08
North Red		Red	A			E1	0.27 ± 0.04

A Haplotype A, *E1* Haplotype E1, *E2* Haplotype E2, *Undet.* undetectable

[a] Average ± SE ($n = 3$), cyanidin-3-glucoside equivalent

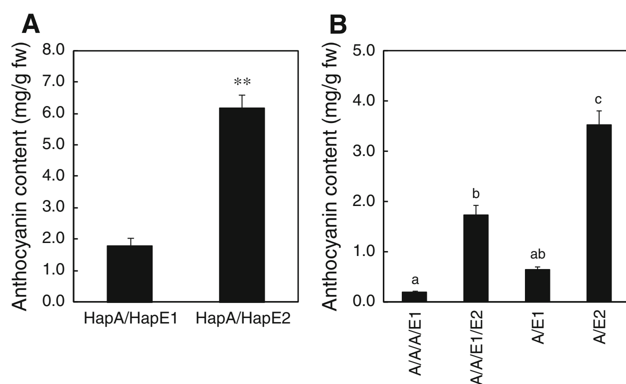

Fig. 4 Relationship between the haplotype composition at the color locus and the total anthocyanin content in berry skins of (**a**) 11 seedlings from the 'Muscat of Alexandria' × 'Campbell Early' cross, and (**b**) tetraploid and diploid grape accessions. *Double asterisks* in **a** indicate a significant difference from HapA/HapE2 ($P < 0.01$, Student's *t* test). *Bars* in **b** labeled with *different letters* are significantly different ($P < 0.05$, Tukey–Kramer test)

Discussion

Previously, we reported that many colored-skinned *V.* ×*labruscana* grapes contained HapE (consisting of *VlMYBA1-2* and *VlMYBA1-3*) at the color locus (Azuma et al. 2008). However, it had not yet been clarified whether *VlMYBA1-1* and *VlMYBA2*, which had been isolated from a cDNA library of *V.* ×*labruscana* 'Kyoho' (Kobayashi et al. 2002), were also present in HapE. In the present study, we found a novel functional haplotype, HapE2 (consisting of *VlMYBA2* and *VlMYBA1-3*), in some colored-skinned *V.* ×*labruscana* grapes. Surprisingly, *VlMYBA1-1* was not detected within gDNA samples of any of the *V.* ×*labruscana* grape cultivars, including 'Kyoho'. Kobayashi et al. (2002) also reported that the nucleotide sequence of *VlMYBA1-1* completely coincided with that of *VlMYBA2*, except for duplication of the X–Y region. Furthermore, an intron-like sequence (GT···AG) of *VlMYBA2* normally found at positions 692–862 was absent from *VlMYBA1-1*. From these findings, we suggest that the *VlMYBA1-1* might be derived from the same primary transcript as *VlMYBA2* through mis-splicing. The findings reported here provide further evidence that *VlMYBA1-1* does not represent an additional color locus gene.

The results of the present study demonstrate that the structure of the color locus in *V.* ×*labruscana* grapes differs from that in *V. vinifera*. The color locus in *V. vinifera*

grapes consists mainly of HapC and HapA (Azuma et al. 2008; Fournier-Level et al. 2010; Kobayashi et al. 2004; Lijavetzky et al. 2006; This et al. 2007; Walker et al. 2007). HapC-N is presumed to be an ancestral haplotype, consisting of functional genes *VvMYBA1c* and *VvMYBA2r* (Fig. 1). HapC-Rs contains both a functional component, *VvMYBA1c*, and a non-functional component, *VvMYBA2w* (Fournier-Level et al. 2010), and HapA contains two non-functional components, *VvMYBA1a* and *VvMYBA2w*. Here, we showed that the color locus in a set of *V.* ×*labruscana* grape accessions consisted of HapE1, HapE2, and HapA haplotypes (Table 3). In a previous study by our research group, HapE (including HapE1 and HapE2) was detected only in *V.* ×*labruscana* grapes (Azuma et al. 2008). HapE1 contained in 'Campbell Early' (HapE1/HapE2) was presumed to be inherited from 'Concord' (HapA/HapE1), which is a parent of 'Campbell Early'. Although the origin of 'Concord' is unclear, HapE1 might have originated from *V. labrusca* because 'Concord' is classified into the *V. labrusca* group. Another parent of 'Campbell Early' is an F1 cross, 'Belvidere' × 'Muscat Hamburg'. It was confirmed that the haplotype composition of 'Muscat Hamburg' is HapA/HapC (Azuma et al. 2008). Therefore, HapE2 in 'Campbell Early' might have originated from 'Belvidere' (*V. labrusca*); however, we could not investigate the haplotype composition of 'Belvidere' because our institutes do not have this accession. Nevertheless, these findings indicate that HapE1 and HapE2 originated from *V. labrusca*, which is one of the North American species. It was confirmed that HapA is contained in many *V.* ×*labruscana* grapes. Previously, it was reported that *VvMYBA1a* (which contains a *Gret1* insertion) was detected in many accessions of *V. vinifera* and *V.* ×*labruscana*, but it was not detected in any of the North American or East Asian *Vitis* species tested (Mitani et al. 2009). This suggests that the HapA contained in *V.* ×*labruscana* grapes originated from *V. vinifera*. North American grapes have been classified into many species including *labrusca*, *aestivalis*, *cinerea*, *doaniana*, *longii*, *riparia*, and *rupestris* (Winkler et al. 1974). It is unknown whether HapE1 and HapE2 are also contained in these North American species. Further analysis of the genomic structure of the color locus in North American species would be needed to clarify the genomic relationships and evolutionary differentiation of *Vitis* species.

Recently, qRT-PCR method has been used to determine the allelic compositions in the polyploidy plants such as Persimmon (*Diospyros kaki* Thunb.) and tetraploid *Arabidopsis thaliana* (Akagi et al. 2009; Henry et al. 2006). In the present study, we developed a method to determine the haplotype compositions in tetraploid grapes by means of qRT-PCR, and investigated the relationship between haplotype composition at the color locus and skin color in diploid and tetraploid grapes using the methods. As a result, the haplotype composition was revealed to be a major determinant of skin color variation. In our previous study, the relationship between the haplotypes and total anthocyanin content in diploid progeny seedlings was investigated, and seedlings with two functional haplotypes (HapC/HapE) were proven to have a higher anthocyanin content than seedlings with only a single functional haplotype (e.g., HapA/HapC or HapA/HapE; Azuma et al. 2008). Among the tetraploid grape accessions examined in this study, black-skinned accessions contained at least two functional haplotypes, whereas red-skinned accessions contained only a single functional haplotype (Table 3). Interestingly, several of the high-anthocyanin accessions, such as 'Black Beat' and 390-76, contained three or four functional haplotypes (Table 3). Therefore, it is thought that the number of functional haplotypes is one of the major genetic factors that determines the skin color of diploid and tetraploid grapes. Furthermore, the anthocyanin contents in the grape accessions containing HapE2 are higher than those in the accessions containing HapE1 (Fig. 4a, b). These findings suggest that the number and kind of functional haplotypes at the color locus are the major genetic determinant of skin color variation.

In *V. vinifera* grapes, Fournier-Level et al. (2009, 2010) found that black-skinned accessions tended to contain HapC-N whereas red-skinned accessions tended to contain HapC-Rs. The difference in color between HapC-N and HapC-Rs grapes can be explained by the number of functional *MYB*-related genes contained in each haplotype. In HapC-N, two functional *MYB*-related genes, *VvMYBA1c* and *VvMYBA2r*, are present, whereas only one functional *MYB*-related gene, *VvMYBA1c*, is present in HapC-Rs.

What is the reason for the difference in color between grapes containing HapE1 and those containing HapE2? Although we have no firm evidence that explains the difference between these two haplotypes, we can propose three hypotheses. One is that different expression levels of *VlMYBA1-2* and *VlMYBA2* lead to the difference in color between HapE1 and HapE2 berries. In a previous study, we were able to isolate 30 cDNA clones representing *VlMYBA2*, but only 4 clones of *VlMYBA1-2*, from the mature berry of 'Kyoho' (Kobayashi et al. 2002). This result implies that the expression level of *VlMYBA2* in berry skin is higher than that of *VlMYBA1-2*. Several reports have indicated that the expression level of *MYB*-related genes correlates with the anthocyanin content in berry skin (Azuma et al. 2009; Jeong et al. 2004; Matus et al. 2009; Yamane et al. 2006). From these findings, different expression levels of *VlMYBA1-2* and *VlMYBA2* might be a major factor explaining the difference in color between HapE1 and HapE2 berries.

The second hypothesis is that different *MYB*-related genes lead to differential regulation of the anthocyanin

biosynthesis pathway genes. Kobayashi et al. (2002) suggested that the sequence of the coding region differed between *VlMYBA1-2* and *VlMYBA2*. It is possible that DNA polymorphism, such as SNPs (causing amino acid substitutions), in the coding region of these *MYB*-related genes leads to differential regulation of the anthocyanin biosynthesis pathway genes. The amino acid substitution may affect the binding or promoting ability of these transcription factors with respect to the promoters of the pathway genes. Studies to clarify whether differences in anthocyanin accumulation are influenced by the differences in binding or promoting ability among these *MYB*-related genes are needed.

The third hypothesis is that some unidentified color-related genes might be contained in HapE1 and/or HapE2, and these genes might affect the color conferred by each haplotype. Since the publication of the genome sequence of *V. vinifera* (Jaillon et al. 2007; Velasco et al. 2007), it has become relatively easy to investigate the genomic structure of the color locus in *V. vinifera* grapes. Recently, it was found that the color locus in *V. vinifera* is a cluster of *MYB* and *MYB*-like genes, including *VvMYBA1* and *VvMYBA2*, spanning a 200-kb region located on chromosome 2 (Azuma et al. 2009; Fournier-Level et al. 2009; Matus et al. 2008). Fournier-Level et al. (2009) reported that the continuous variation in anthocyanin content in grape (*V. vinifera*) was explained mainly by a single gene cluster of three *VvMYBA* genes (*VvMYBA1*, *VvMYBA2*, and *VvMYBA3*) at the color locus, although *VvMYBA3* has not been functionally validated. The authors demonstrated that five polymorphisms in these *VvMYBA* genes accounted for 84% of the observed skin color variation. Unfortunately, it is not easy to investigate whether novel color-related genes or DNA polymorphisms are contained in HapE1 and HapE2 because the genomic sequence of the color locus in North American species, especially *V. labrusca*, is not yet known. More detailed analysis of the structures of HapE1 and HapE2 would be needed to clarify why the berry color produced by these haplotypes is different.

It is possible that the total anthocyanin content is affected by additive or synergistic effects of functional *MYB*-related genes at the color locus, as shown by the observed variation in the colors of grape skins within a haplotype. However, even if haplotype composition was same, anthocyanin contents displayed a continuous variation among grape accessions (Tables 1, 3). These results suggest that anthocyanin content is a quantitative trait that results from the sum of the expression of many enzymes related to anthocyanin biosynthesis as well as other enzymes. Recently, Matus et al. (2008) found nine anthocyanin-related *MYB*-related gene models distributed on chromosomes 2 and 14 of grape through the use of a *Vitis*–*Arabidopsis* phylogenetic tree. Furthermore, several reports indicated that VvMYB5a, VvMYB5b, VvMYBPA1, and VvMYBPA2 appear to regulate several genes in the common steps of the flavonoid pathway (Bogs et al. 2007; Deluc et al. 2006, 2008; Terrier et al. 2009). Moreover, the final anthocyanin content, the anthocyanin composition, and the expression levels of the genes related to the anthocyanin biosynthesis vary in response to internal and environmental conditions such as the levels of sugar, abscisic acid, temperature, water, and light (Castellarin et al. 2007; Hiratsuka et al. 2001; Jeong et al. 2004; Kataoka et al. 1984, 2003; Kliewer and Torres 1972; Matus et al. 2009; Mori et al. 2007; Yamane et al. 2006). These findings indicate that even if a plant has haplotypes at the color locus that could potentially cause it to accumulate a high anthocyanin content, factors at other loci might affect the final anthocyanin content and composition.

Interestingly, all of the tetraploid red-skinned accessions examined in this study contained HapA/A/A/E1, and many of black-skinned accessions contained HapA/A/E1/E2, although other patterns of haplotype composition would be possible in a tetraploid genome (Table 3). In the diploid grape accessions, the predominant haplotype composition was heterozygous for non-functional and functional haplotypes: very few grape accessions were homozygous for functional haplotypes (Azuma et al. 2008; Fournier-Level et al. 2010; Kobayashi et al. 2004; Lijavetzky et al. 2006; This et al. 2007; Walker et al. 2007). Improvement of commercially desirable fruit traits (e.g., skin color, berry size, and sugar contents) is one of the important objectives in a grape breeding program. In general, consumers of table grapes prefer well-pigmented grapes with high sugar content, and the high marketability of such fruits is thus important for farmers. On the other hand, excessive anthocyanin concentration in the grape berry might have a negative effect on other fruit traits. For example, it is possible that excessive anthocyanin accumulation in grape berries could cause a reduction in sugar content, because of the energy needed to synthesize a large amount of anthocyanin. Zheng et al. (2009) indicated that an optimum concentration of sugars was needed for accumulation of anthocyanin in grape berries. Therefore, seedlings that contain a large number of functional haplotypes might tend not to be selected in a breeding program because of the low sugar content of the berries. Thus, by selecting for high sugar content, the genetic variation in haplotype might be reduced. Further studies concerning the relationships among haplotype composition, anthocyanin content, and sugar content in grape berries are needed.

Our findings in the present study provide new knowledge about the unique genetic control of color development in *V.* ×*labruscana* grape berries. Furthermore, our findings provide a method for predicting berry skin color by testing seedlings at a very young stage for the haplotype

compositions at the color locus, enabling early selection of seedlings that will have the desired color and anthocyanin content in the grape berries.

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