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ORIGINAL PAPER

Genomic and genetic analysis of *Myb*-related genes that regulate anthocyanin biosynthesis in grape berry skin

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Abstract As a result of natural hybridization and human selection over millennia, the skin colors of grapes have become greatly diversified. The color is determined by the quantity and composition of anthocyanins. Color-skinned cultivars accumulate anthocyanins in their skins, whereas white-skinned cultivars do not. *Myb*-related transcription-factor genes such as *VvmybA1* regulate anthocyanin biosynthesis. *VvMYBA2r*, *VlmybA1-1*, *VlmybA1-2*, and *VlmybA2*, which are homologs of *VvmybA1*, also regulate anthocyanin biosynthesis. In this study, we isolated a novel *Myb*-related sequence, *VlmybA1-3*, from cultivars of *Vitis labruscana* (*Vitis vinifera* × *Vitis labrusca*) by means of inverse PCR, and confirmed by means of transient gene expression assay that the gene regulates anthocyanin biosynthesis in grape berry skin. Seedlings of *V. labruscana* with two functional haplotypes at a region of berry color loci accumulated more anthocyanins than seedlings with a single functional haplotype. In addition, we investigated the haplotypes at the region in 35 cultivars (both *V. vinifera* and *V. labruscana*), and found certain typical characteristics. These findings will contribute to the selection of seedlings with high anthocyanin quantities in breeding programs for wine and table grapes, and will help elucidate the origin and evolution of *Vitis* species.

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Introduction

Skin color is one of the most important qualities used as the basis for selection in breeding programs for wine and table grapes. Today, skin color in grapes has become greatly diversified, and includes black, red, pink, gray, and white (yellow–green) berries as a result of natural hybridization and human selection over millennia. The color of berry skins is determined by the quantity and composition of anthocyanins. Color-skinned cultivars accumulate anthocyanins in their skins, whereas white-skinned cultivars do not (Boss et al. 1996a). In many plants, such as maize (*Zea mays*), *Petunia* spp., snapdragon (*Antirrhinum* spp.), and *Arabidopsis thaliana*, the genetics and biochemistry of the anthocyanin biosynthesis pathway have been well characterized (Holton and Cornish 1995; Quattrocchio et al. 1993; Winkel-Shirley 2001). Anthocyanin biosynthesis in many plants is controlled by regulatory genes that belong to two major groups of transcription factors, namely the MYB and bHLH families (Borovsky et al. 2004; Holton and Cornish 1995; Ozeki et al. 2003; Ramsay et al. 2003; Robbins et al. 2003; Sainz et al. 1997; Schwinn et al. 2006; Spelt et al. 2000).

In grapes, *Myb*-related transcription-factor genes such as *VlmybA1-1*, *VlmybA1-2*, and *VlmybA2* have been isolated from the mature berries of tetraploid 'Kyoho' grape (*Vitis labruscana*: *Vitis labrusca* × *Vitis vinifera*), and it has been confirmed that *VlmybA1-1* regulates anthocyanin biosynthesis via expression of the gene for UDP-glucose:flavonoid 3-

O-glucosyltransferase (UFGT) (Kobayashi et al. 2002). Boss et al. (1996a, b) showed that the expression of the gene for UFGT is critical for anthocyanin biosynthesis in grapes. We also showed that *VvmybA1*, a homolog of *VlmybA1-1* that is found in many *V. vinifera* cultivars, plays the same function in anthocyanin biosynthesis (Kobayashi et al. 2004, 2005). Furthermore, *VvmybA1* transcripts were not present in any of the white-skinned cultivars we examined, but were present in all the color-skinned cultivars (Kobayashi et al. 2004). These results suggest that the expression of *VvmybA1* is essential for the development of coloration of grape skin in *V. vinifera* grapes. Doligez et al. (2002), Fischer et al. (2004) and Salmaso et al. (2008) showed that the presence or absence of anthocyanins segregated as a monogenic trait determined by a locus in linkage group 2. In previous research, we suggested that the expression of *VvmybA1* is blocked in the *VvmybA1a* allele, which contains *Gret1*, a Ty3-*gypsy*-type retrotransposon, in its promoter region (Kobayashi et al. 2004). In contrast, the alleles *VvmybA1b* and *VvmybA1c* are functional. *VvmybA1b* has a single long terminal repeat (a solo LTR, which may have occurred as a result of intra-recombination between 5′LTR and 3′LTR) of *Gret1* in the 5′-flanking region near the coding region of *VvmybA1*, and is expressed (Kobayashi et al. 2004, 2005). In contrast, *VvmybA1c* completely lacks *Gret1* and is most likely the original sequence of *VvmybA1* before the insertion of *Gret1* (Yakushiji et al. 2006). In most grapes that have been examined, white-skinned grape berries are homozygous for *VvmybA1a*, whereas color-skinned berries are heterozygous for *VvmybA1a*/*VvmybA1b* or *VvmybA1a*/*VvmybA1c* or are homozygous for *VvmybA1c* (Azuma et al. 2007; Kobayashi et al. 2004; Lijavetzky et al. 2006; This et al. 2007). Most recently, we investigated the relationship between the *VvmybA1* genotype and the phenotype of grape skin color (the presence or absence of anthocyanins) using seedlings and demonstrated that the *VvmybA1* genotype determines grape skin color and that *VvmybA1* is the major gene involved in anthocyanin biosynthesis (Azuma et al. 2007). Lijavetzky et al. (2006) and Salmaso et al. (2008) also indicated that the coloring of grape skin depends on the genotype of *VvmybA1* in an analysis of crossed seedlings of a color-skinned cultivar. Furthermore, Lijavetzky et al. (2006) showed that the sequence variation at the *VvmybA1* locus explains berry color in more than 95% of analyzed cultivars.

Several additional sequences of *VvmybA1* have been reported. Walker et al. (2006) and Yakushiji et al. (2006) showed that skin color mutations of 'Pinot Noir' and 'Cabernet Sauvignon', from black-skinned to white-skinned, are caused by deletion of the functional *VvmybA1* allele. Yakushiji et al. (2006) named the null allele in 'Pinot Blanc' (white-skinned bud sport of 'Pinot Noir') *VvmybA1d*. Lijavetzky et al. (2006) observed *VvmybA1* expression in a white cultivar, 'Roditis', and found that the last 135 coding nucleotides of *VvmybA1* had been deleted. They named the allele that encodes the truncated protein $VvmybA1^{ROD}$. This et al. (2007) reported an additional 44-bp insertion within the promoter region of *VvmybA1* in several cultivars, and that the insertion was frequently related to the red- and pink-skinned cultivars. Furthermore, 111 and 44-bp insertions were observed within the promoter region of *VvmybA1* in other cultivars (Lijavetzky et al. 2006; This et al. 2007). In a previous study, we detected an unknown fragment that differed from *VvmybA1a*, *VvmybA1b*, and *VvmybA1c* in color-skinned cultivars of *V. labruscana* such as 'Concord' by using allele-specific PCR primers for *VvmybA1*, and were unable to detect the fragment in any *V. vinifera* cultivars or in white-skinned cultivars of *V. labruscana* (Kobayashi et al. 2004). However, it is not clear whether the putative new allele represented by this fragment regulates anthocyanin biosynthesis, as do *VvmybA1b* and *VvmybA1c*.

In the present study, we isolated the putative *Myb*-related sequence that we detected in the color-skinned cultivars of *V. labruscana*, and examined whether it is functional. Moreover, we investigated the relationship between the total anthocyanin content in grape skin and the haplotypes at a region of berry color loci using crossed seedlings. In addition, we investigated the haplotypes in 35 cultivars (*V. vinifera* and *V. labruscana*), and discuss the typical characteristics of these cultivars and the relationships between them.

Materials and methods

Plant materials

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 required. In addition, gDNA samples of 'Koshu' and 'Ryugan' were supplied by Dr. Nami Goto-Yamamoto, National Research Institute of Brewing, Hiroshima, Japan. For anthocyanin extraction, a total of six berries from different bunches on each crossed seedling were randomly sampled at harvest time, and the peeled skins were immediately frozen in liquid nitrogen and stored at −80°C until use.

Isolation and sequencing of VlmybA genes

Inverse PCR was carried out according to the method of Triglia et al. (1988) to isolate the open reading frame (ORF) and upstream region of the unknown putative *Myb*-related gene in the *V. labruscana* grapes. The gDNA from young leaves of 'Concord' and 'Campbell Early' was

extracted as described previously (Kobayashi et al. 2002), and digested with *Sna*BI tailored to the sequences of a *VvmybA1c* allele (DDBJ accession number AB242302), and then were self-ligated to generate circular molecules. The first PCR was performed with the circularized gDNA fragments as a template. The primers for the first PCR were forward 5′-CACAAAACCGTACTTCCTATC-3′ and reverse 5′-GTTGGGGAACAGGCAAGTCTA-3′. The amplifications were performed in a total volume of 50 μL, comprising 10 ng of circularized gDNA, 400 μM dNTP mixture, 25 μL of 2 × GC buffer I, 0.2 μM each primer, and 0.5 units of Takara LA Taq polymerase (Takara, Kyoto, Japan). The PCR cycling conditions were an initial phase at 95°C for 3 min; 36 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 5 min; and a final phase at 72°C for 10 min. The second nested PCR was performed under the same conditions as the first PCR, except that the template and primers were changed in order to increase the specificity of the inverse PCR analysis. In this analysis, a sample of 1% of the first PCR products was used as the template, and the forward primer 5′-TCGAACCTGTTCTATCTCATG-3′ and the reverse primer 5′-TGATGTGCGTGTATAGA TGGT-3′ were used for the second nested PCR. The nested PCR products were then cloned into pCR2.1-TOPO vectors (Invitrogen, Carlsbad, CA, USA) and their sequences were determined.

To amplify the ORF and the upstream region of *VlmybA1-2*, we used the forward primer 5′-GGACGTTA AAAAATGGTTGCACGTG-3′ and reverse primer 5′-AA ATCAGATCAAGTGATTTACTTC-3′. PCR reactions were performed in a total volume of 30 μL, comprising 10 ng gDNA, 200 μM dNTPs, 0.2 μM each primer, and 0.5 units of ExTaq polymerase (Takara). The PCR cycling conditions were an initial phase at 95°C for 3 min; 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min; and a final phase at 72°C for 5 min. The PCR products were purified using SUPREC-PCR (Takara) and directly sequenced. Nucleotide and amino acid sequences were analysed using the GENETYX software system (Software Development Co. Ltd., Tokyo, Japan).

Vector construction and transient gene expression assays

In this part of the study, we used the DNA fragment containing the full-length *VlmybA1-3* structural gene isolated from gDNA of 'Concord' by inverse PCR as a template. From its sequence, we designed a forward primer (5′-CGC TCTAGA(*Xba*I)CTCCATGGAGAGCTTAGGAGTTAG-3′) and a reverse primer (5′-CGCGAGCTC(*Sac*I)TAAATCA GATCAAGTGATTTACTTC-3′) to amplify the structural gene. The amplified fragment was digested with *Xba*I and *Sac*I, then integrated into the binary vector pBI121 (Clontech, Mountain View, CA, USA) in place of the *β*-glucuronidase (*GUS*) gene sequence. The chimeric gene was introduced into *Agrobacterium tumefaciens* strain LBA4404 by means of triparental mating. Half-cut berries of 'Italia' (*V. vinifera*; white-skinned) were surface-sterilized with 70% ethanol, treated with the *A. tumefaciens* suspension, and then incubated in hormone-free MS medium containing acetosyringon (100 μM) for 4 days in the dark at 25°C. After that, the berries were transferred into hormone-free MS medium that contained carbenicillin sodium (250 μg/mL) and incubated for another 10 days under the same conditions. Leaf disks and petioles of kiwifruit (*Actinidia deliciosa* 'Hayward') were also infected with the *A. tumefaciens* suspension that contained *VlmybA1-3*, and were incubated under the same conditions as the half-cut grape berries to confirm that the kiwifruit could be transformed using the gene and that this transformation would lead to anthocyanin accumulation. The red cells in berries that had been cut in half were observed under a Leica MZ12 stereoscopic microscope. Red cells in the leaf disks and petioles of kiwifruit were observed under a Keyence VHX-900 digital microscope.

Investigation of skin color and PCR analysis of the haplotypes at the region of berry color loci

We used 3-year-old seedlings of an Iku71 (*V. labruscana*; red-skinned) × 626-84 (*V. labruscana*; red-skinned) cross to investigate the segregation of skin color ($n = 76$). The color of the seedlings was visually assessed as white or red/black at harvest time. The colors of 35 grape cultivars (*V. vinifera* and *V. labruscana*) were also investigated. gDNA was extracted from young leaves of the parents, all crossed seedlings, and of all 35 grape cultivars and were used as the template for PCR. The primers for *VvmybA1a* were F1 (5′-AAAAAGGGGGGCAATGTAGGGACCC-3′) and R1 (5′-GAACCTCCTTTTTGAAGTGGTGACT-3′) and those for *VvmybA1c* and *VlmybA1-3* were F2 (5′-GGACGTT AAAAAATGGTTGCACGTG-3′) and R1 (Kobayashi et al. 2004). The primers for *VlmybA1-2* were F3 (5′-CACC ACTTGAAAAAGAAGGTC-3′) and R2 (5′-TCTTGATC CAGCTCAGCTAAC-3′). PCR reactions were performed in a total volume of 10 μL, comprising 5 ng DNA, 200 μM dNTPs, 0.2 μM each primer, and 0.5 units of ExTaq polymerase (Takara). The PCR cycling conditions for *VvmybA1a*, *VvmybA1c*, and *VlmybA1-3* detection were an initial phase at 95°C for 3 min; 34 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* were an initial phase at 95°C for 3 min; 34 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. PCR products were purified using SUPREC-PCR

(Takara) and sequenced directly, as described above. In the segregation analysis of the crossed seedlings, we used chi-square tests to determine the agreement between the observed skin color and the expected haplotype ratios. The total anthocyanin content in the berry skin was analyzed according to Shiraishi et al. (2007). In summary, 1 g of berry skin from each of the crossed seedlings was macerated in 20 mL of 50% aqueous acetic acid (v/v) for 12 h at 4°C in the dark. We then diluted 1 mL of extracted material to 10 mL in 50% aqueous acetic acid, and measured the total anthocyanin content by reading the absorbance at 520 nm with a spectrophotometer (UV-260, Shimadzu, Kyoto, Japan). 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 haplotypes at a region of berry color loci and the total anthocyanin content was evaluated using Fisher's protected LSD test.

Results

Isolation and sequencing of VlmybA alleles

A novel putative *Myb*-related transcription factor sequence, *VlmybA1-3* (DDBJ accession number AB427165), was isolated from 'Concord' and 'Campbell Early' by the inverse PCR method. Nucleotide sequence analysis revealed that the predicted coding region of *VlmybA1-3* differed from those of other homologous genes (*VvmybA1*, *VvMYBA2r*, *VvMYBA2w*, *VlmybA1-1*, *VlmybA1-2*, *VlmybA2*, *VvmybA1*^{SUB}) that have been isolated from grapes thus far. Although the number of deduced amino acid residues of VlMYBA1-3 was the same as those of VvMYBA1, VlMYBA1-1, and VvMYBA1^{SUB}, the sequence of its residues was slightly different (Fig. 1). In addition, two insertions (44 and 111 bp) compared with *VvmybA1c* were observed within the upstream part of the *VlmybA1-3* coding region (Fig. S1, Fig. 2). Lijavetzky et al. (2006) reported that the *VvmybA1*^{SUB} (GenBank accession number DQ345539) that had been isolated from 'Sultanina' (white-skinned, *V. vinifera*) also contained 44 and 111-bp insertions in the upstream part of the coding region. However, the sequences of the two insertions were slightly different from those in *VlmybA1-3*. Comparison of these sequences revealed many single-nucleotide polymorphisms (SNPs) in their exons and two indels (insertion/deletions, 33 and 3 bp) in their second intron (Fig. S1). Moreover, the coding and upstream regions of *VlmybA1-2*, for which only cDNA had previously been isolated (Kobayashi et al. 2002), were isolated from 'Concord' and 'Campbell Early' (DDBJ accession number AB427164). The 44-bp insertion is present in the upstream part of the *VlmybA1-2* coding region, and the sequence coincided completely with the 44-bp insertion in

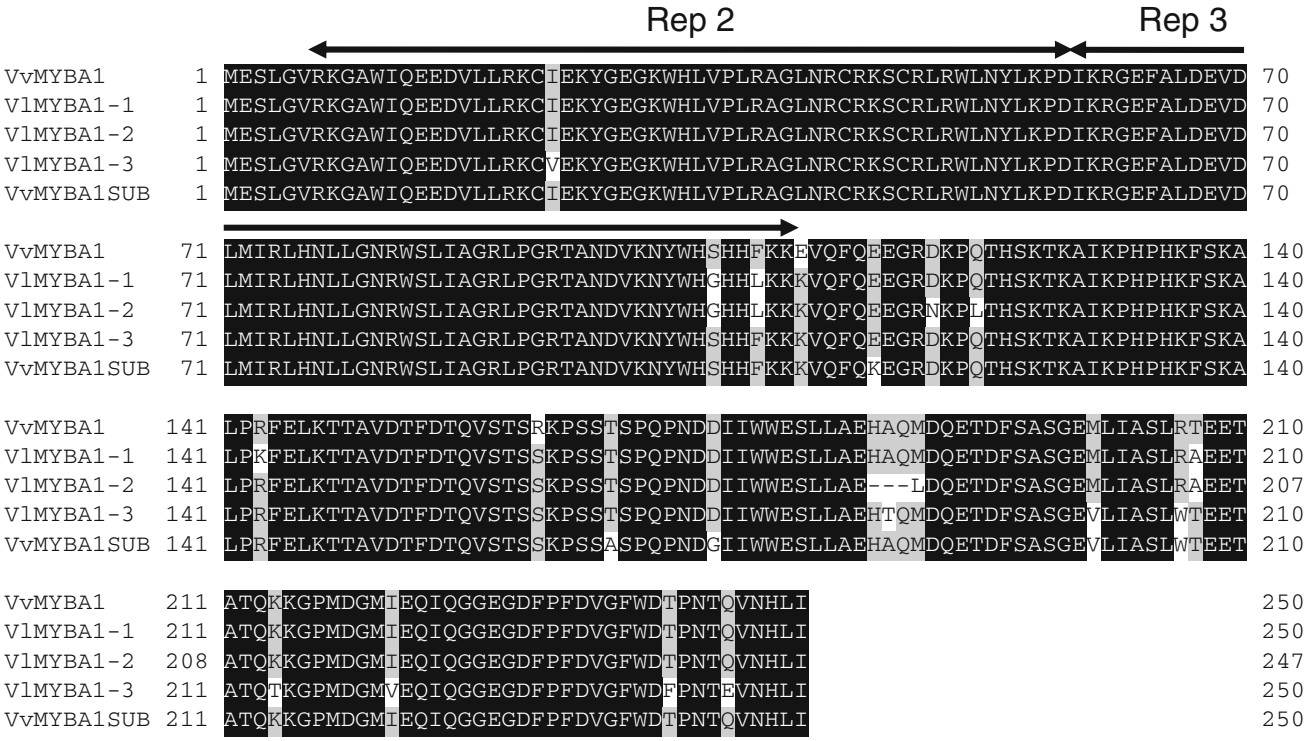

Fig. 1 Deduced amino acid sequences of VvMYBA1, VlMYBA1-1, VlMYBA1-2, VlMYBA1-3, and VvMYBA1^{SUB}. Rep2 and Rep3 represent repeats 2 and 3, respectively, observed in the DNA-binding domain of plant MYB-related proteins

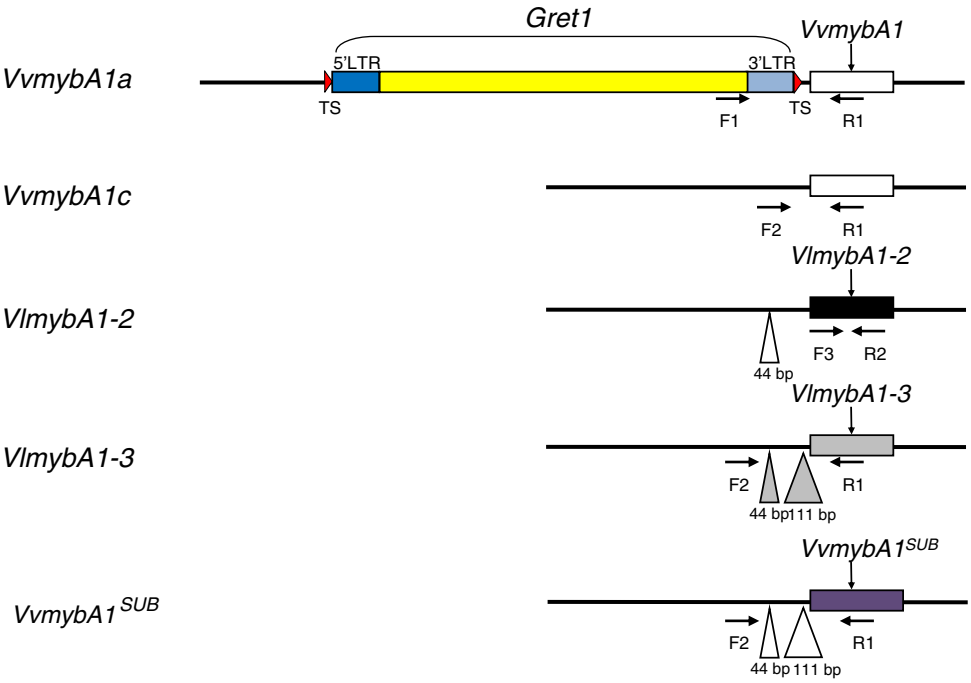

Fig. 2 Structures of *VvmybA1a*, *VvmybA1c*, *VlmybA1-2*, *VlmybA1-3*, and *VvmybA1*^{SUB}. Primer positions are indicated below the maps. *F1–F3* forward primers. *R1, R2* reverse primers. *Gret1* grapevine retrotransposon 1. *LTR* long terminal repeat. *TS* duplicated target site for the *Gret1* insertion

VvmybA1^{SUB} (Fig. S1, Fig. 2). However, the 111-bp insertion was not found, and the coding sequence differed from that of *VvmybA1*^{SUB}.

Vector construction and transient gene expression assays

We performed transient gene expression assays using *A. tumefaciens* to clarify whether *VlmybA1-3* regulates anthocyanin biosynthesis. After approximately 2 weeks of culture, red cells appeared at the cut ends of the half-cut grape berries (Fig. 3a). The red cells were also observed at the cut ends of the leaf disks and petioles of kiwifruit (Fig. 3b). The induced red pigment was confirmed to be anthocyanin, as it changed color from red to blue when the tissue was exposed to 0.1 M sodium hydroxide (data not shown), as described by Uimari and Strommer (1998). Thus, *VlmybA1-3* appears to regulate anthocyanin biosynthesis in grape skin and in the leaf and petiole of kiwifruit.

Investigation of the skin color of seedlings and the haplotypes at the region of berry color loci

We investigated the relationship between the segregation of skin color and the haplotypes at the region of berry color loci using the Iku71 (red-skinned) × 626-84 (red-skinned) cross. The skin color of the seedlings segregated to 57 red/black-skinned and 19 white-skinned (Table 1). Walker et al. (2007) reported that *VvMYBA1w* (equivalent to *VvmybA1a*) and *VvMYBA2w*, and *VvMYBA1r* (equivalent to *VvmybA1c*) and *VvMYBA2r*, were closely linked on the same chromosome in the examined grape accessions, and named them the white and red alleles, respectively. Using their findings, we determined the haplotypes at the region by means of PCR. Haplotype A (HapA, equivalent to the white allele) and haplotype C (HapC, equivalent to the red allele) were identified by the detection of *VvmybA1a* and *VvmybA1c*, respectively (Fig. 4). PCR analysis revealed that Iku71 contained both HapA and HapC, whereas 626-84 contained HapA, *VlmybA1-3*, and *VlmybA1-2* (Fig. 5). In the Iku71 × 626-84 cross, all white-skinned seedlings contained only HapA; 19 of the red/black-skinned seedlings contained HapA and HapC; 12 red/black-skinned seedlings contained HapA, *VlmybA1-3*, and *VlmybA1-2*; and 26 red/black-skinned seedlings contained HapC, *VlmybA1-3*, and *VlmybA1-2*. Interestingly, *VlmybA1-2* was detected in all the seedlings containing *VlmybA1-3*, but was not detected in any of the seedlings containing HapA/HapA or HapA/HapC. Therefore, *VlmybA1-3* and *VlmybA1-2* appear to sit at nearby positions on the same chromosome. From these results, we named the haplotype containing the alleles *VlmybA1-3* and *VlmybA1-2* at two nearby *MybA* loci as Haplotype E (HapE; Fig. 4). There was a strong association between berry pigmentation and the presence of HapC or HapE. Our expectation that the seedlings from the HapA/HapC × HapA/HapE cross would be segregated as 1 HapA/HapA:1 HapA/HapC:1HapA/HapE:1 HapC/HapE was verified by the chi-square test (Table 1).

We also investigated the relationship between the haplotypes at the region of berry color loci and total anthocyanin content in the grape berry skin. The total anthocyanin content in HapC/HapE (two functional haplotypes) was significantly higher than that in HapA/HapC and HapA/HapE

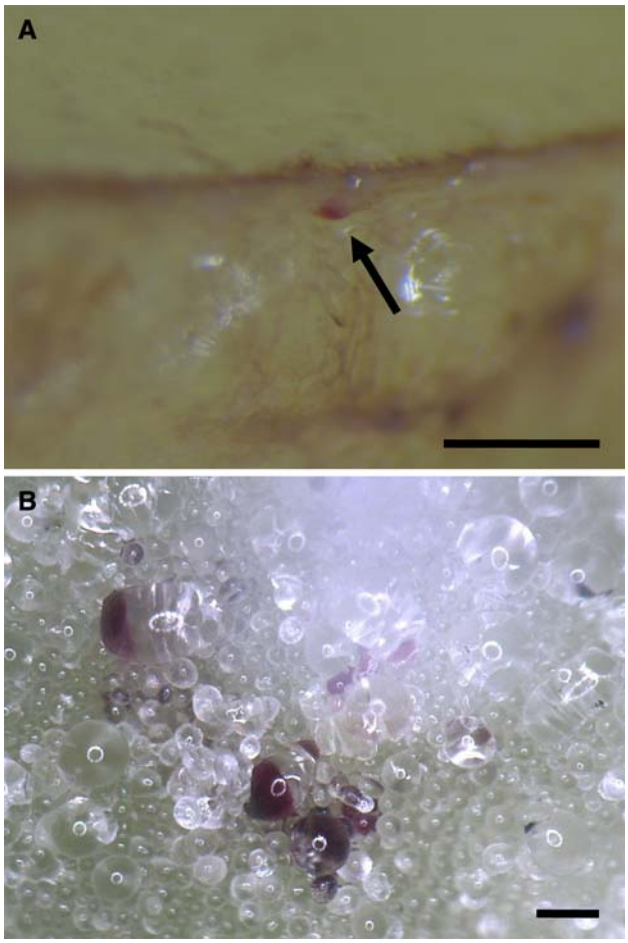

Fig. 3 Results of transient expression assays for *VlmybA1-3*. Anthocyanin-producing cells (*arrow*) were induced in **a** the skin tissues of 'Italia' and **b** the petiole tissues of kiwifruit by the introduction of *VlmybA1-3*. *Scale bars* indicate **a** 1 mm and **b** 200 μm

(Fig. 6); the two latter haplotypes did not differ significantly. Although the seedlings with two functional haplotypes tended to have increased anthocyanin accumulation in the grape berry skin, total anthocyanin content in the individual seedlings differed greatly even within the same haplotypes at the region of berry color loci. This result indicates that in addition to the haplotypes, various other factors are involved in determining the final anthocyanin content in the skin.

Haplotyping of the 35 grape cultivars

We analyzed 35 grape (*V. vinifera* and *V. labruscana*) cultivars to determine their haplotypes at the region of berry color loci using PCR (Table 2). Four out of six white-skinned cultivars of *V. vinifera* contained HapA only, but 'Sultanina' and 'Niunai' (in the *V. vinifera orientalis* group) contained both HapA and $VvmybA1^{SUB}$. Most color-skinned cultivars of *V. vinifera* contained HapA/HapC, but 'Koshu' and 'Ryugan' (in the *V. vinifera orientalis* group) also contained HapA and $VvmybA1^{SUB}$. We named the haplotype containing the $VvmybA1^{SUB}$ allele Haplotype F (described in more detail in the Discussion section). Only 'Ruby Okuyama' contained HapA and *VvmybA1b*. We predict that *VvMYBA2w* exists at the *VvmybA2* locus in 'Ruby Okuyama' because *VvmybA1b* originated from *VvmybA1a*. Therefore, we named the haplotype containing the alleles *VvmybA1b* and *VvMYBA2w* at the region of berry color loci Haplotype B (HapB; Fig. 4). No *V. vinifera* cultivar contained HapE. Both of the white-skinned cultivars of *V. labruscana* we examined contained HapA only. In the color-skinned cultivars of *V. labruscana*, we detected four haplotypes: HapA/HapE, HapA/HapC, HapE only and HapC only. 'Pinot Blanc' would contain HapA and *VvmybA1d* (a null allele at the *VvmybA1* locus, reported by Yakushiji et al. 2006), but we could not construct the primers for *VvmybA1d*. Walker et al. (2006) hypothesized that 'Pinot Blanc' has a deletion of the *VvmybA1c* and *VvMYB2r* alleles in HapC. From these findings, we named the haplotype containing two null alleles at the *VvmybA1* and *VvmybA2* loci Haplotype D (HapD; Fig. 4).

Discussion

In this study, we isolated the novel *Myb*-related allele *VlmybA1-3* from color-skinned cultivars of *V. labruscana*.

Table 1 Skin color segregation in the seedlings of the Iku71 × 626-84 cross

Cross[a]	Total number of progeny	Number of progeny				Expected ratio	χ^2 value	*P* value[c]
		Red/black			White			
		A/C	A/E	C/E	A/A			
Iku71 (A/C[b]) × 626-84 (A/E)	76	19	12	26	19	1:1:1:1	5.16	0.16

[a] Iku71 red-skinned *V. labruscana*, 626-84 red-skinned *V. labruscana*

[b] A/C Haplotype A/Haplotype C, A/E Haplotype A/Haplotype E, C/E Haplotype C/Haplotype E, A/A Haplotype A/Haplotype A. Haplotype E contained both *VlmybA1-3* and *VlmybA1-2*

[c] The *P* value represents the significance of the difference between the actual segregation results and the expected ratio (chi-square test)

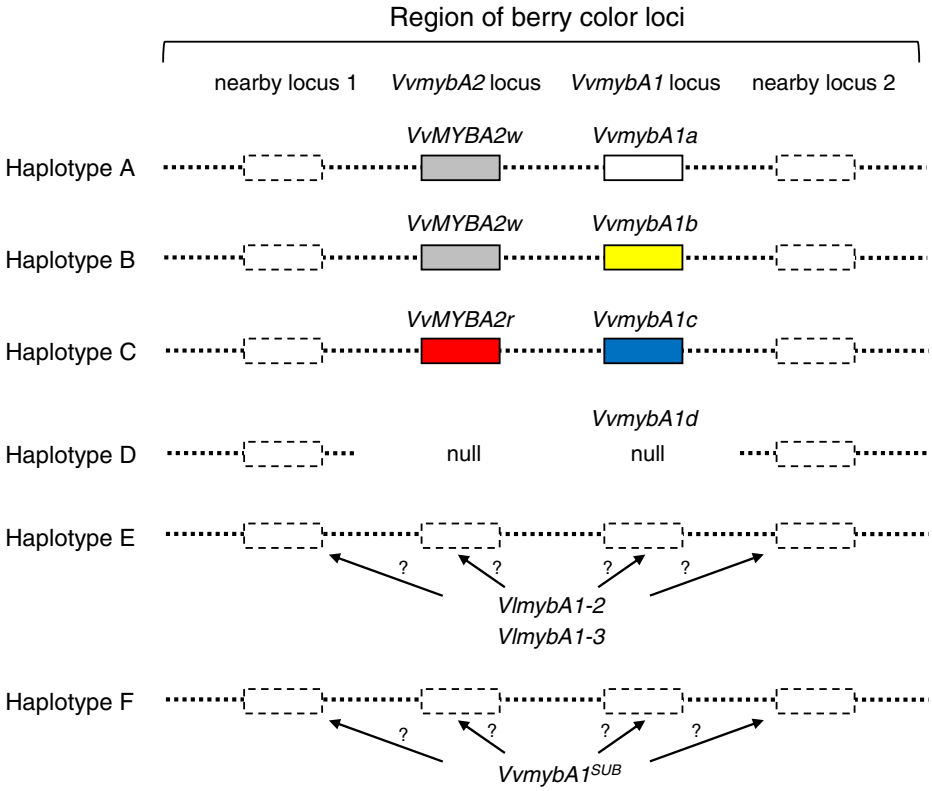

Fig. 4 Haplotypes at the region of berry color loci. Haplotypes A and C are equivalent to the white and red alleles, respectively, described by Walker et al. (2007). *Dotted lines* indicate unidentified alleles. Positions of the loci of *VlmybA1-2*, *VlmybA1-3*, and *VvmybA1*^{SUB} were not clarified

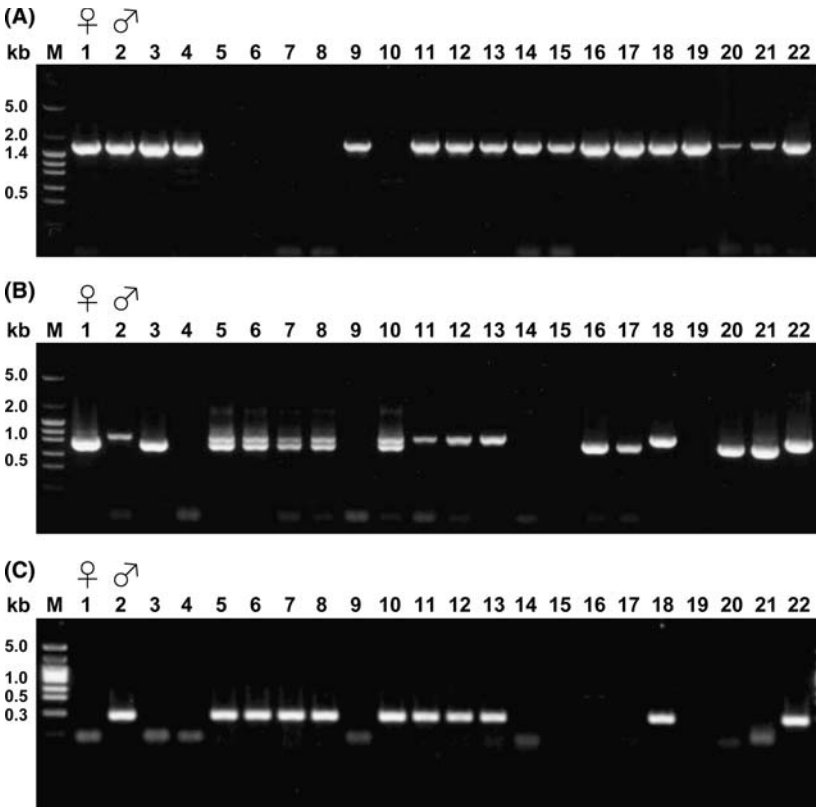

Fig. 5 PCR analysis of the *MybA* alleles in seedlings of the Iku71 × 626-84 cross. **a** PCR analysis of *VvmybA1a*. **b** PCR analysis of *VvmybA1c* and *VlmybA1-3*. Larger fragment, *VlmybA1-3*; smaller fragment, *VvmybA1c*. **c** PCR analysis of *VlmybA1-2*. *Lane 1* Iku71 (red-skinned), *lane 2* 626-84 (red-skinned), *lanes 4*, *9*, *14*, *15*, and *19* white-skinned seedlings, *lanes 3*, *5–8*, *10–13*, *16–18*, and *20–22* red/black-skinned seedlings, *M* size markers. Twenty out of 76 samples were loaded into the well

In *VlmybA1-3*, we found 111 and 44-bp insertions in the upstream part of its coding region compared with *VvmybA1c*, and the sequence of its coding region differed from those of other *VvmybA*s and *VlmybA*s. Interestingly, Lijavetzky et al. (2006) and This et al. (2007) also reported the presence of 111 and 44-bp insertions within the upstream part of the coding region of *VvmybA1*^{SUB}, but the sequences of these insertions and of the coding region of

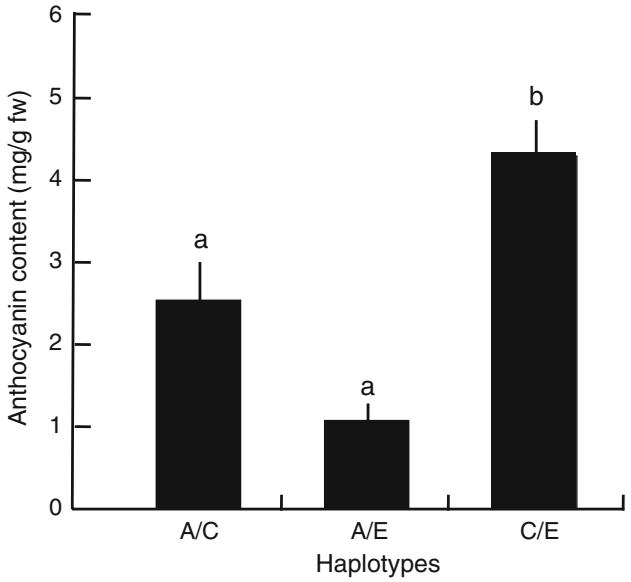

Fig. 6 Relationship between the haplotypes at the region of berry color loci and the total anthocyanin content in grape skins of seedlings from the Iku71 × 626-84 cross. A/C, Haplotype A/Haplotype C; A/E, Haplotype A/Haplotype E; C/E, Haplotype C/Haplotype E. *Bars* labeled with the same *letter* did not differ significantly ($P > 0.05$, Fisher's protected LSD test)

VlmybA1-3 differed from those of *VvmybA1*^{SUB}. We also found a 44-bp insertion in the upstream part of the coding region of *VlmybA1-2*, and the sequence coincided completely with the 44-bp insertion in *VvmybA1*^{SUB}. The homologies between these insertion sequences suggest a genetic relationship among these three alleles. Previously, the cDNAs of *VlmybA1-1* and *VlmybA2* had been isolated from the mature berries of tetraploid 'Kyoho' (*V. labruscana*) (Kobayashi et al. 2002). Therefore, these two *Myb*-related genes might have originated in *V. labrusca*. Unfortunately, we have been unable to design primers capable of isolating the coding and upstream regions of *VlmybA1-1* and *VlmybA2*. Further analyses are thus needed to clarify the genetic relationships between the *VvmybA* and *VlmybA* alleles.

Several *Myb*-related genes (*VvmybA1*, *VvMYBA2r*, *VlmybA1-1*, *VlmybA1-2*, and *VlmybA2*) induced the production of red cells in the embryos and skin tissues of white-skinned grapes when introduced into these tissues by means of particle bombardment or *A. tumefaciens* (Kobayashi et al. 2002, 2005; Walker et al. 2007). In the present study, we demonstrated that *VlmybA1-3* also regulated anthocyanin biosynthesis in the skin of white-skinned grapes when it was introduced into this tissue by means of *A. tumefaciens*. Moreover, it induced the production of anthocyanins at the cut ends of leaf disks and petioles of kiwifruit. Recently, Geekiyanage et al. (2007) and Koshita et al. (2008) reported that *VlmybA2* and *VlmybA1-2* produce anthocyanins in several dicot plants, including tobacco (*Nicotiana tabacum*), *Arabidopsis*, kiwifruit, tomato (*Lycopersicon esculentum*), and eggplant (*Solanum melongena*). Therefore, the expression of *VlmybA1-3* may also be able to produce anthocyanin pigments in dicot plants other than grape and kiwifruit, and the function of *Myb*-related genes would thus be highly conserved in this taxon.

Previously, we reported that the *VvmybA1* genotype (consisting of *VvmybA1a* and *VvmybA1c*) determines the presence or absence of anthocyanin, and that *VvmybA1* is the major gene responsible for the determination of grape skin color (Azuma et al. 2007). We also suggested that *VvmybA1a* and *VvmybA1c* are related alleles. Walker et al. (2006) reported that the berry color locus in 'Cabernet Sauvignon' (*V. vinifera*) is composed of two very similar and adjacent genes (*VvMYBA1* and *VvMYBA2*), either of which could control berry color. They isolated the red and white berry-color alleles from a BAC library of 'Cabernet Sauvignon', and revealed that the red allele was not present in any white cultivar of *V. vinifera* that they examined. In their study, the red grapes contained *VvMYBA1r* (functional, equivalent to *VvmybA1c*) and *VvMYBA2r* (functional), whereas the white grapes contained *VvMYBA1w* (not functional, equivalent to *VvmybA1a*) and *VvMYBA2w* (not functional, equivalent to *VvmybA2*). *VlmybA1-3* and *VlmybA1-2* would thus exist at nearby positions on the same chromosome, because the PCR products of both were either present or absent in all cultivars; we named this combination Haplotype E (HapE; Fig. 4). We confirmed that all crossed seedlings that contained HapE had colored skin. However, it is not clear whether *VlmybA1-3* or *VlmybA1-2* exists at the *VvmybA1* locus or other nearby loci (Fig. 4). Nevertheless, we confirmed that the presence or absence of anthocyanins in grape skin is determined by haplotypes at the region of berry color loci (HapC and HapE are dominant over HapA). Although *VlmybA1-1* and *VlmybA2*, isolated from *V. labruscana* 'Kyoho' by Kobayashi et al. (2002), might also occur as HapE or other haplotypes, we have not yet constructed specific PCR primers to detect them. Further studies are thus needed to confirm whether *VlmybA1-1* and *VlmybA2* exist in HapE.

We investigated the relationship between the haplotypes and total anthocyanin content using crossed seedlings, and confirmed that seedlings with two functional haplotypes (HapC/HapE) had a higher anthocyanin content than seedlings with only a single functional haplotype (HapA/HapC, HapA/HapE). Recently, Lijavetzky et al. (2006) also reported that seedlings homozygous for the functional *VvmybA1*^{ALF} allele (*ALF/ALF*) showed slightly higher color index for red grapes (CIRG) values than heterozygous (*ITA/ALF*) seedlings. They also reported that all cultivars carrying two functional *VvmybA1* alleles, including 'Black Seedless', 'Almeria Nera', 'Dattier Noir', and 'Negra Tardia', are known by names that allude to their dark skin

Table 2 Haplotypes of 35 grape cultivars at the region of berry color loci

Cultivar name	Skin color	Species[a]	Haplotype					
			A[b]	B	C	E		F
			Vva[c]	Vvb	Vvc	Vl1-2	Vl1-3	SUB
Black Hamburg	Black	*V. vinifera*	+	−	+	−	−	−
Cabernet Sauvignon	Black	*V. vinifera*	+	−	+	−	−	−
Chardonnay	White	*V. vinifera*	+	−	−	−	−	−
Emperor	Red	*V. vinifera*	+	−	+	−	−	−
Flame Tokay	Pink	*V. vinifera*	+	−	+	−	−	−
Hiro Hamburg	Black	*V. vinifera*	+	−	+	−	−	−
Italia	White	*V. vinifera*	+	−	−	−	−	−
Kaiji	Red	*V. vinifera*	+	−	+	−	−	−
Koshu	Pink	*V. vinifera*	+	−	−	−	−	+
Niunai	White	*V. vinifera*	+	−	−	−	−	+
Muscat Hamburg	Black	*V. vinifera*	+	−	+	−	−	−
Pinot Blanc	White	*V. vinifera*	+	−	−	−	−	−
Pinot Gris	Grey	*V. vinifera*	+	−	+	−	−	−
Pinot Noir	Black	*V. vinifera*	+	−	+	−	−	−
Queen	Red	*V. vinifera*	+	−	+	−	−	−
Ryugan	Pink	*V. vinifera*	+	−	−	−	−	+
Riesling Lion	White	*V. vinifera*	+	−	−	−	−	−
Rizamat	Pink	*V. vinifera*	+	−	+	−	−	−
Ruby Okuyama	Red	*V. vinifera*	+	+	−	−	−	−
Sekirei	Red	*V. vinifera*	+	−	+	−	−	−
Sultanina	White	*V. vinifera*	+	−	−	−	−	+
Aki Queen	Red	*V. labruscana*	+	−	−	+	+	−
Benizuiho	Red	*V. labruscana*	+	−	−	+	+	−
Buffalo	Black	*V. labruscana*	−	−	+	−	−	−
Campbell Early	Black	*V. labruscana*	−	−	−	+	+	−
Concord	Black	*V. labruscana*	+	−	−	+	+	−
Golden Muscat	White	*V. labruscana*	+	−	−	−	−	−
Honey Black	Black	*V. labruscana*	+	−	−	+	+	−
Kyoho	Black	*V. labruscana*	+	−	−	+	+	−
North Red	Red	*V. labruscana*	+	−	−	+	+	−
Ryuho	Red	*V. labruscana*	+	−	−	+	+	−
Schuyler	Black	*V. labruscana*	+	−	+	−	−	−
Seneca	White	*V. labruscana*	+	−	−	−	−	−
Steuben	Black	*V. labruscana*	−	−	+	−	−	−
Sunny Rouge	Red	*V. labruscana*	+	−	−	+	+	−

[a] *V. labruscana Vitis vinifera* × *Vitis labrusca*

[b] *A* Haplotype A, *B* Haplotype B, *C* Haplotype C, *E* Haplotype E, *F* Haplotype F

[c] *Vva VvmybA1a*, *Vvb VvmybA1b*, *Vvc VvmybA1c*, *Vl1-2 VlmybA1-2*, *Vl1-3 VlmybA1-3*, *SUB VvmybA1*^{SUB}

+ detected, − not detected

color. 'Steuben', 'Buffalo', and 'Campbell Early' which have a high anthocyanin content in the berry skin, were also homozygous for HapC or HapE. In grapes, there are many *Myb*-related genes that function in anthocyanin biosynthesis, such as *VvmybA1*, *VvMYBA2r*, *VlmybA1-1*, *VlmybA1-2, VlmybA1-3*, and *VlmybA2*. It is possible that the total anthocyanin content is affected by additive or synergistic effects of these genes, as shown by the observed variation in the colors of grape skins within a haplotype. However, even if a seedling were homozygous for functional haplotypes, some seedlings contained quite little anthocyanin content. This result suggests that the final anthocyanin content is a quantitative trait, and results from the sum of the expression of many enzymes related to anthocyanin biosynthesis as well as other enzymes. The final anthocyanin content and composition in berry skins of the same individual would thus vary in response to internal and external factors 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; Mori et al. 2007; Yamane et al. 2006). Therefore, even if a seedling has genes that could potentially cause it to accumulate a high anthocyanin content, other factors might affect the final anthocyanin content and composition through their effects on the transcription levels of *Myb*-related genes and other genes in the anthocyanin biosynthesis pathway. Thus far, many *Myb*-related genes have been isolated, but it is unclear whether their responsiveness to internal and external factors is the same. Further studies are thus needed to elucidate the characteristics of each *Myb*-related gene as a function of various internal and external factors. Nevertheless, our findings suggest that selecting for seedlings homozygous in functional haplotypes would be more likely to produce seedlings with a high anthocyanin content in breeding programs for wine and table grapes.

We detected several kinds of haplotypes at the region of berry color loci among the various *V. vinifera* and *V. labruscana* cultivars that we tested. The *V. vinifera* haplotypes formed four groups: HapA/HapA, HapA/HapB, HapA/HapC, and HapA/*VvmybA1*^{SUB}. On the other hand, HapE was detected only in *V. labruscana* cultivars. These findings suggest that HapE originated in *V. labrusca*. Among the cultivars we examined, we detected *VvmybA1*^{SUB} (isolated from 'Sultanina' by Lijavetzky et al. 2006) in 'Sultanina', 'Koshu', 'Ryugan', and 'Niunai'. We did not detect functional alleles of *VvmybA* (*VvmybA1b* or *VvmybA1c*) or *VlmybA* (*VlmybA1-3* or *VlmybA1-2*) in these cultivars. From these results, we predicted that *VvmybA1*^{SUB} might be a member of an independent haplotype, and named the putative haplotype containing *VvmybA1*^{SUB} Haplotype F (HapF; Fig. 4). Cultivars of *V. vinifera* have been eco-geographically classified into three proles (convar.): *pontica*, *occidentalis*, and *orientalis* (Negrul 1938). In addition, microsatellite analysis of the *occidentalis* and *orientalis* cultivars of *V. vinifera* showed a clear separation in a dendrogram based on phenetic distances (Goto-Yamamoto et al. 2006). In the present study, we showed that HapF was present only in the *orientalis* cultivars 'Sultanina', 'Koshu', 'Ryugan', and 'Niunai'. These results suggest that HapF differentiated in the *orientalis* cultivars. Thus far, it has not been determined whether *VvmybA1*^{SUB} regulates anthocyanin biosynthesis, because *VvmybA1*^{SUB} has been detected in both white-skinned cultivars ('Niunai' and Sultanina') and color-skinned cultivars ('Koshu' and 'Ryugan'). Lijavetzky et al. (2006) reported that *VvmybA1* was expressed in two color-skinned somatic variants from 'Sultanina' ('Sultanine Rosee' and 'Sultanine Rouge'), but not in 'Sultanina'. They compared the *VvmybA1*^{SUB} sequences of these accessions and showed that they were identical, indicating that the color difference could not be explained by variation within the coding part of the sequence. These results suggest the existence of either a transposon insertion in the upstream promoter region of *VvmybA1*^{SUB} or of a second regulatory locus that controls berry skin color. It is necessary to confirm whether the structural gene of *VvmybA1*^{SUB} functions in anthocyanin accumulation, and to compare the uncovered upstream region of *VvmybA1*^{SUB} in white-skinned cultivars with that in red-skinned mutants. In addition to the *VvmybA* and *VlmybA* alleles, some paralogous genes might exist at nearby positions in *VvmybA1*^{SUB} locus, and might regulate anthocyanin biosynthesis. Walker et al. (2006) hypothesized that there might be many more types of functional alleles than non-functional alleles at the berry color loci. Therefore, undiscovered functional alleles and haplotypes may exist in various *Vitis* species. Further studies with a broader range of accessions, including native American and East Asian wild grapes, are needed to develop a full understanding of these alleles and haplotypes. Detailed analysis of the diversity of alleles and haplotypes at the region of berry color loci in future studies would contribute to elucidating the origins and evolution of *Vitis* species.

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