Association of *VvmybA1* Gene Expression with Anthocyanin Production in Grape (*Vitis vinifera*) Skin-color Mutants

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Summary

By using white-skinned cultivars ('Italia' and 'Muscat of Alexandria') and putative red-skinned sports ('Ruby Okuyama' and 'Flame Muscat') from the white cultivars, we analyzed the expression and function of a *myb*-related gene, *VvmybA1*, involved in the regulation of anthocyanin biosynthesis in grapes. A simple sequence repeat (SSR) analysis showed that 'Ruby Okuyama' and 'Flame Muscat' are true derivatives by bud mutation from 'Italia' and 'Muscat of Alexandria', respectively. The *VvmybA1* transcript was detected in berry skins of 'Ruby Okuyama' after coloring had begun; it was not detectable in those of 'Italia'. Three *VvmybA* cDNAs (*VvmybA1*, *VvmybA2*, and *VvmybA3*) from 'Ruby Okuyama' under the control of the 35S promoter were introduced into somatic embryos of 'Kyoho' by particle bombardment. Upon the introduction of *VvmybA1*, red cells were induced in the embryos, whereas the introduction of *VvmybA2* or *VvmybA3* failed to do so. The *VvmybA1* cDNA was also shown to have the ability to induce the anthocyanin-producing cells in the skin tissues of 'Muscat of Alexandria'. The relationship between the skin-color mutations and the detailed structure of the *VvmybA1* gene is also described.

Key Words: anthocyanin, grape, *myb*, retrotransposon, solo LTR.

Introduction

The color of grape skins is determined by the accumulation of anthocyanins. Several genes for anthocyanin biosynthesis have been isolated from grape (Sparvoli et al., 1994), and the expression of the gene for UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UGT) has been shown to be critical for anthocyanin biosynthesis in grapes (Boss et al., 1996a, b; Kobayashi et al., 2001). Previously, we showed that ectopic expression of cDNAs (*VlmybA1-1*, *VlmybA1-2*, and *VlmybA2*) for *myb*-related genes was sufficient to induce pigmentation and expression of *UGT* gene in non-colored somatic embryos of 'Kyoho', a black-skinned cultivar of *Vitis labruscana* (Kobayashi et al., 2002). Furthermore, most recently, we showed that a homolog of *VlmybA1-1*, *VvmybA1*, in *V. vinifera* was associated with the bud-mutation from white to red in berry skins of grapes (Kobayashi et al., 2004), and that the expression of *VvmybA1* gene closely correlated with that of anthocyanin biosynthetic genes (Jeong et al., 2004). However, the function of *VvmybA1* gene has not yet been sufficiently characterized. In this paper, we describe in detail the function of the *VvmybA1* gene and

the relationship between skin-color mutations and the structure of the *VvmybA1* gene.

Materials and Methods

SSR analysis

DNA was extracted from the leaves of 'Italia', 'Ruby Okuyama', 'Muscat of Alexandria', and 'Flame Muscat' as described previously (Kobayashi et al., 1996) and was used for SSR analysis at 8 SSR loci: VVS1, VVS2, VVS3, VVS4, VVMD5, VVMD6, VVMD7, and VVMD8 (Bowers et al., 1996; Thomas and Scott, 1993). The analysis was performed as reported previously (Thomas et al., 1994) with some modifications. The PCR reaction that was carried out in 20 μ L, comprised of 20 ng DNA, 0.2 μ M of each primer (Bowers et al., 1996; Thomas and Scott, 1993), 200 μ M dNTPs, 2.5 mM MgCl₂, and 0.8 units of AmpliTaq Gold polymerase (Applied Biosystems). After pre-PCR at 95°C for 12 min, a reaction cycle of 94°C for 1 min, 51°C (except for VVS3, in which case, the temperature was changed to 49°C) for 1 min, and 73°C for 1 min was repeated 45 times, followed by 72°C for 10 min. One primer of each primer pair was labeled with fluorescent 6-FAM dye. Aliquots (0.5 μ L) of the PCR reaction products were mixed with 12 μ L of formamide and 0.5 μ L of a DNA size standard (Gene Scan-500 ROX, Applied

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Biosystems); then, 2 μ L of each mixture was loaded onto a DNA Sequencer Model 377 (Applied Biosystems) operated by a GENESCAN software.

Northern and Southern blot analyses

Skin samples of 'Ruby Okuyama' were taken at the following stages of development: a young stage (21 days after flowering (DAF)); just before veraison (45 DAF; in grapes, the onset of berry softening is called veraison, and occurs just before berries begin to color); when berries began to color (48 DAF); when berries were half-colored (52 DAF); and when berries were fully red and mature (77 DAF). Skins from 'Italia' were also sampled at the same developmental stages. The samples were immediately frozen in liquid nitrogen and stored at -80°C until use. RNA extracted from the samples was used for northern blot analysis as described by Kobayashi et al. (2002). A portion of the coding region of *VlmybA1-1* (DDBJ Accession No. AB073010) from 'Kyoho' grape was used as a probe. The probe was labeled with digoxigenin (DIG) using primers F1: 5'-GGTTCAGTTCCAGGAAGAAGG-3' and R1: 5'-CCCTCACCTCCCTGGATTTGT-3' and a PCR-DIG probe synthesis kit (Roche Diagnostics). Southern blot analysis was performed according to Kobayashi et al. (2002) by using DNA from the above 4 cultivars and DIG-labeled probes prepared from the coding region and 5'-flanking regions of the *VvmybA1* gene.

cDNA libraries

cDNA libraries made from mature berry skins of 'Italia', 'Ruby Okuyama', 'Muscat of Alexandria', and 'Flame Muscat' were screened by using the same coding region of *VlmybA1-1* as above but labeled with horseradish peroxidase. Probe labeling, screening and sequencing were performed as described by Kobayashi et al. (2002).

Transient expression assays

The full-length coding region of *VvmybA1*, *VvmybA2*, or *VvmybA3* from 'Ruby Okuyama' was integrated into p35S C1 (Goff et al., 1990), from which the cDNA for *C1* had been removed by *Xho*I and *Sac*I digestion according to Kobayashi et al. (2002). Bombardment of the resulting constructs into somatic embryos of 'Kyoho' and culture of the embryos were performed as described (Kobayashi et al., 2002). *VvmybA1* was also integrated into the binary vector pBI121 (Clontech) in place of the β -glucuronidase (GUS) gene sequence, and the chimeric gene was introduced into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating. Half-cut berries of 'Muscat of Alexandria', harvested at 48 DAF (just after the veraison) and surface-sterilized with 70% ethanol, were treated with *A. tumefaciens* suspensions (5×10^8 colony-forming units $\cdot \text{mL}^{-1}$) and then incubated on a hormone-free MS medium, containing acetosyringon (100

μM) for 4 days in the dark at 25°C . The berries were then transferred to a hormone-free MS medium that contained carbenicillin sodium ($250 \mu\text{g} \cdot \text{mL}^{-1}$) and incubated for another 10 days under the same conditions.

Genomic libraries

DNA samples, extracted from 'Italia' and 'Ruby Okuyama', were partially digested with *Sau*3AI and cloned into the λ DASH II vector (Stratagene). By using the same probe for screening the cDNA libraries, approximately 2×10^5 recombinant phages of the genomic libraries were screened. The positive clones from the first screening were picked, suspended in 100 μL SM (100 mM NaCl, 10 mM MgSO_4 , 0.01% gelatin, and 50 mM Tris-HCl, pH 7.5), and checked by PCR to determine whether they contained *VvmybA1* gene. The primers F1 and R1 described above were used for PCR detection. PCR reactions that were performed in 10 μL , consisted of 200 μM dNTPs, 0.2 μM of each primer, 0.2% Triton X-100, 0.2 units of Z-Taq polymerase (Takara), and 0.5 μL of phage suspension. After denaturing at 95°C for 5 min, a reaction cycle of 94°C for 4 s, 58°C for 4 s, and 72°C for 10 s was repeated 35 times, followed by 72°C for 10 min. After checking the size of the PCR products with agarose gel electrophoresis, clones, confirmed to contain *VvmybA1* gene, were then subjected to a second round of screening. From clones that passed the second screening, DNA was extracted, digested with *Not*I, and the fragments carrying the *VvmybA1* gene were integrated into the pYES2 vector (Invitrogen). To sequence the integrated DNA, an EZ::TN<KAN-2> transposon (Epicentre, Wisconsin, USA) was inserted into the pYES2 clones; the DNA, prepared from kanamycin-resistant transposon insertion clones, was then subjected to sequence analyses by using transposon-specific primers.

Results

SSR analysis on white cultivars and red-skinned sports

'Italia', a white-skinned cultivar of *V. vinifera*, is an offspring from a cross between a black-skinned 'Muscat Hamburg' and a white-skinned 'Bicane' (an old cultivar of unknown origin); one of the parents of 'Muscat Hamburg' is the white-skinned 'Muscat of Alexandria'. Two red-skinned cultivars of *V. vinifera*, 'Ruby Okuyama' and 'Flame Muscat', are thought to have arisen by bud-mutation from 'Italia' and 'Muscat of Alexandria', respectively. However, the origins of 'Ruby Okuyama' and 'Flame Muscat' have not yet been confirmed. SSR analyses, using 8 markers, revealed that the molecules amplified from 'Italia' and 'Muscat of Alexandria' coincided in size with those from 'Ruby Okuyama' and 'Flame Muscat', respectively, and verified the relationships among these cultivars (Table 1).

Table 1. Comparison of genotypes at 8 SSR loci between white cultivars and their red–skinned sports.

Cultivar	Locus															
	VVS1		VVS2		VVS3		VVS4		VVMD5		VVMD6		VVMD7		VVMD8	
Italia	160	188	129	146	216	216	167	174	228	234	205	207	241	245	134	134
Ruby Okuyama	160	188	129	146	216	216	167	174	228	234	205	207	241	245	134	134
M. Alexandria	178	178	129	146	216	216	167	174	224	228	188	207	247	249	134	134
Flame Muscat	178	178	129	146	216	216	167	174	224	228	188	207	247	249	134	134

The values in the table represent the sizes (in bp) of the amplification products at each locus.

Screening of *VlmybA1-1* homologs

In a previous report, *VlmybA* genes (*VlmybA1-1*, *VlmybA1-2*, and *VlmybA2*) were shown to play a critical role in the regulation of anthocyanin biosynthesis in the black–skinned ‘Kyoho’ grape (Kobayashi et al., 2002). The expression of homologs of the genes in the mature berry skins of white–skinned cultivars and their red–skinned sports was also examined by using *VlmybA1-1* from ‘Kyoho’ as a probe. In the white–skinned ‘Italia’ and ‘Muscat of Alexandria’, two transcripts with different molecular weights (1.3 kb and 0.8 kb) were detected, whereas an extra transcript with a different size (1.0 kb) in addition to the above two transcripts were detected in the red–skinned ‘Ruby Okuyama’ and ‘Flame Muscat’ (Kobayashi et al., 2004). Similarly, the extra transcript with 1.0 kb size was detected in berry skins of ‘Ruby Okuyama’ after coloring had begun, but not in those of ‘Italia’ (Fig. 1A). Since the extra transcript was detected exclusively in the red–skinned sports, we tried to isolate the corresponding cDNA.

Screening cDNA libraries, made from mature berry skins of ‘Italia’, ‘Ruby Okuyama’, ‘Muscat of Alexandria’, and ‘Flame Muscat’, led to the identification of about 20 positive clones, each from approximately 1×10^5 recombinant phages, which were then converted to pBluescript II phagemids. Sequencing of the above clones revealed that there were three different species (designated *VvmybA1*, *VvmybA2*, and *VvmybA3*) in the libraries of ‘Ruby Okuyama’ and ‘Flame Muscat’ (Fig. 1B), but only two (*VvmybA2* and *VvmybA3*) in the libraries of ‘Italia’ and ‘Muscat of Alexandria’. The cDNA clones with the same name had identical sequences in their coding regions without exception, even though they were isolated from different cultivars. These findings coincide with the results of northern blot analyses; *VvmybA1* (AB097923), *VvmybA2* (AB097924), and *VvmybA3* (AB097925) correspond to 1.0, 1.3, and 0.8 kb transcripts, respectively.

Previously, we isolated *VlmybA1-1*, *VlmybA1-2*, and *VlmybA2* from ‘Kyoho’ (Kobayashi et al., 2002). These three cDNA clones displayed similar sequences but differed in the occurrence of a particular sequence,

(A)

(B)

Fig. 1. Expression and structure of *VvmybA*s. (A): Northern blot analysis of RNA from ‘Italia’ and ‘Ruby Okuyama’ berry skins taken at several stages of development. Capital letters indicate the stages (Y, young; B, just before veraison; S, beginning to color in ‘Ruby Okuyama’; H, half-colored in ‘Ruby Okuyama’; M, mature). (B): Structures of *VvmybA* cDNAs. R2 and R3 represent repeat 2 and repeat 3 observed in the DNA-binding domain of plant *myb*-related genes. Z and Z' represent the duplicated region. X and Y indicate acidic regions.

X–Y, an acidic region located downstream from the DNA-binding domain. The X–Y region is present once in *VlmybA1-1* and *VlmybA1-2*, but twice in *VlmybA2* (Kobayashi et al., 2002). The *VvmybA1* exhibits a higher homology to *VlmybA1-1* (98.3% identity in their coding region); however, *VvmybA2* has a different multiple sequence (Z–Z') compared to *VlmybA2*, downstream from the DNA-binding domain (Fig. 1B). The sequences of the second repeated-region Z' (280 bp) are slightly different from those of the Z region (282

bp). This duplication generates a protein with altered amino acids in the Y region (the stop codon TAG appears at positions 800–802 of *VvmybA2*). In contrast, *VvmybA3* has a truncated Z region, in which 209 residues are missing. This deletion causes the loss of most of the X region and a subsequent frame shift; the frame shift also generates a protein with different amino acids in the Y region (the stop codon TAG appears at positions 479–481 of *VvmybA3*).

Transient expression assays of *VvmybAs*

When the three cDNAs from ‘Ruby Okuyama’ were introduced into somatic embryos by particle bombardment, *VvmybA1* induced red cells (Fig. 2A), but *VvmybA2* (Fig. 2B) and *VvmybA3* (data not shown) did not. Due to the difficulty of introducing *VvmybA1* into grape skins by bombardment, the cDNA was introduced into the skins by using *A. tumefaciens*. No pigmentation was observed in the skin tissues of ‘Muscat of Alexandria’ (Fig. 2C); however, red cells appeared in the skin tissues of ‘Flame Muscat’ (Fig. 2D). Introduction of *VvmybA1* into cut grapes of ‘Muscat of Alexandria’ with *A. tumefaciens*, likewise, resulted in red cells in the skin tissues (Figs. 2E, 2F). The induced red pigment was demonstrated to be anthocyanin, as it changed color from red to blue when the tissue was exposed to 0.1 M sodium hydroxide (Figs. 2G, 2H) as described (Uimari and Strommer, 1998).

Isolation of genomic clones for the *VvmybA1* gene

We next investigated how *VvmybA1* gene expression was turned “ON” in the red-skinned sports. Southern blot analysis, using part of the coding region of *VvmybA1* gene as a probe (probe 1), revealed different banding patterns between the white-skinned cultivars and the red-skinned sports (Fig. 3A). ‘Ruby Okuyama’ and ‘Flame Muscat’ had an extra 2.9 kb *DraI* band and an extra 4.9 kb *HindIII* band in addition to other bands that were present in all four cultivars. Since the molecular basis of these differences might provide key knowledge about how the sports arose, genomic clones for *VvmybA1* gene were isolated from ‘Italia’ and ‘Ruby Okuyama’ and sequenced.

Genomic clones for *VvmybA1* gene were isolated as previously reported (Kobayashi et al., 2004). Here we show the details of the structure of the clones (Fig. 4). Four clones (It22, It34, It62, and It74) from ‘Italia’ were identical within the sequences determined, which suggests that *VvmybA1* gene is homozygous in ‘Italia’ (a diploid). One of the clones, It22, was named *VvmybA1a* (AB111100). On the other hand, two kinds of clones were observed in ‘Ruby Okuyama’. Two clones (Ru45 and Ru76) had the same sequences as *VvmybA1a*, but two others (Ru11 and Ru21) had a different 5′-flanking region from *VvmybA1a*, to suggest that the latter clones represented a second allele of the gene. Ru21 was designated *VvmybA1b* (AB111101). No difference was

observed between *VvmybA1a* and *VvmybA1b* in their coding sequences. From the comparison of the sequences between the genomic and cDNA clones of *VvmybA1* gene, *VvmybA1* gene was found to contain 2 introns (15390–15476 and 15607–15725 on the *VvmybA1a* sequences).

As reported (Kobayashi et al., 2004), *VvmybA1a* contained a retrotransposon, *Gret1* (Grapevine retrotransposon 1), upstream from the *VvmybA1*-coding sequences (Fig. 4). *Gret1* is inserted 181 bp upstream from the translation start codon (ATG) of *VvmybA1* gene. The sequences of the two LTRs were almost identical, but showed differences at four nucleotides, suggesting that the insertion of *Gret1* occurred relatively recently in evolution. Direct repeats of a 5 bp sequence, ACACA, were observed on the outer side of each LTR, to indicate that *Gret1* generates a 5 bp duplication of a target sequence upon retroposition. Although no homology to any other plant retrotransposons was discovered in the LTR sequences of *Gret1*, the internal region of *Gret1* showed similarities to the *gag-pol* region (encoding *gag* protein, protease, reverse transcriptase, RNaseH, and integrase) of the Ty3-gypsy type retrotransposons *RetroSor1* (AF098806, Llaca et al., direct submission) from *Sorghum bicolor*, *RIRE2* (AB030283) from *Oryza sativa* (Ohtsubo et al., 1999), and *Cinful-1* (AF049110, SanMiguel et al., direct submission) from *Zea mays*. The BLAST score and BLAST *E* value for these amino acid sequences compared to the ORF2 of *Gret1* are as follows: *RetroSor1* (score=810; *E* value=0.0); ORF2 of *RIRE-2* (score=788; *E* value=0.0); and *Cinful-1* (score=783; *E* value=0.0). *Gret1* has a typical polypurine tract (PPT), AAAAAGGGGGG, upstream from the 3′-LTR, and a putative primer binding site (PBS), TGGCGCTGTCTGTGG, downstream from the 5′-LTR.

Plant retrotransposons are known to range from a few copies to more than 50,000 copies per haploid genome (Kumar and Bennetzen, 1999). The Southern blot analyses in this study indicate that *Gret1*-related retrotransposons are present in multiple copies in grapevine genomes (Figs. 3B, 3C). In the *VvmybA1b* allele, carried by the red-skinned sports, *Gret1* was missing, leaving behind its 3′-LTR flanked by 5 bp of a duplicated target site (Fig. 4). The restriction map of *VvmybA1b* revealed a *DraI* fragment and a *HindIII* fragment that matched the sizes of the extra fragments (2.9 kb and 4.9 kb, respectively) observed in ‘Ruby Okuyama’ and ‘Flame Muscat’ by Southern blot analysis (Figs. 3A, 4).

Discussion

SSR analysis has been demonstrated to be an effective technique for identifying grapevine cultivars and evaluating their genetic relationships (Bowers et al., 1996; Lamboy and Alpha, 1998; Thomas et al., 1994; Thomas and Scott, 1993). Twenty-six cultivars of *V. vinifera* were distinguished by their genotype at the 5 SSR loci

Fig. 2. Photographs illustrating the transient expression assays of VvmybAs. A, Red cells were induced in the embryos by the introduction of VvmybA1; B, The introduction of VvmybA2 failed to induce the red cells; C, Skin tissues of 'Muscat of Alexandria'; D, Skin tissues of 'Flame Muscat'; E and F, Red cells (arrows) were induced in the skin tissues of 'Muscat of Alexandria' by the introduction of VvmybA1; G and H, The red pigment changed color from red (arrow in G) to blue (arrow in H) by exposing the tissue to 0.1 M sodium hydroxide. Bars indicate 200 μ m (A, B) and 1 mm (C-H).

Fig. 3. Gel plates of Southern blot analyses of DNA from white-skinned cultivars and their red-skinned sports. (A): The membrane was hybridized with a DIG-labeled probe from the coding region of VvmybA1. The arrowheads with numerals indicate the sizes of the DNA fragments. (B): The membrane was hybridized with a DIG-labeled probe from the 5'-flanking region of VvmybA1a. (C): The membrane was hybridized with another DIG-labeled probe from the 5'-flanking region of VvmybA1a. Numerals on the right side indicate the sizes (bp) of the DNA size markers (λ EcoT14 I).

Fig. 4. Structures of genomic clones from 'Italia' and 'Ruby Okuyama'. Probes used for Southern blot analyses are indicated below the map of *VvmybA1a*. Restriction endonuclease sites are indicated above the map of *VvmybA1a* and *VvmybA1b*. TS, A duplicated target site.

(VVS1, VVS2, VVS3, VVS4, and VVS5), but the genotype of 'Sauvignon Rose' (a sport having rose-colored berries) was identical to that of 'Sauvignon Blanc' (with white berries), which suggests that the different berry color is due to the occurrence of a somatic mutation (Thomas and Scott, 1993). Similarly, 77 cultivars of *V. vinifera* were distinguished by the SSR analyses by using 4 SSR loci (VVMD5, VVMD6, VVMD7, and VVMD8) with the exception of the grapes considered to be somatic variants of a cultivar and those known or suggested to be synonymous (although one cultivar may or may not be synonymous) (Bowers et al., 1996). Furthermore, Lamboy and Alpha (1998) estimated the probability that two randomly selected grape germplasm accessions would have identical genotypes. Based on the data from 5 SSR loci (VVS2, VVS4, VVMD6, VVMD7, and VVMD8) by using 110 accessions of 25 grape taxa, they reported that the probability was about two chances in one hundred million. In the present study, SSR analyses were carried out by using more markers than in the above studies, and revealed that the genotype of 'Italia' and 'Muscat of Alexandria' was identical to that of 'Ruby Okuyama' and 'Flame Muscat', respectively. Southern blot analyses that used 5'-flanking regions of *VvmybA1a* (Fig. 4) as probes, also showed no differences in the banding patterns between the white cultivars and the red-skinned sports (Figs. 3B, 3C). Furthermore, we showed that there were no differences in *UFGT* gene sequences between 'Italia' and 'Ruby Okuyama' and between 'Muscat of Alexandria' and 'Flame Muscat' (Kobayashi

et al., 2001). These results and the morphological similarity between the cultivars, except for the berry skin color, indicate that 'Ruby Okuyama' and 'Flame Muscat' are truly derived by bud mutation from 'Italia' and 'Muscat of Alexandria', respectively.

Three kinds of *VlmybA*-related transcripts (for *VvmybA1*, *VvmybA2*, and *VvmybA3*) were detected in the mature berry skins of the red-skinned sports, but only *VvmybA2* and *VvmybA3* transcripts were detected in those of the white-skinned progenitors (Kobayashi et al., 2004). In our study, furthermore, *VvmybA1* was shown to have the ability to induce anthocyanin production in skin tissue cells of 'Muscat of Alexandria'. In a previous study of the black-skinned 'Kyoho' grape, we showed that the cDNAs *VlmybA1-1*, *VlmybA1-2*, and *VlmybA2* induced pigmentation and expression of *UFGT* gene in the non-colored somatic embryos of this grape, which suggests that these genes are involved in the regulation of anthocyanin biosynthesis via *UFGT* gene expression (Kobayashi et al., 2002). The three 'Kyoho' cDNAs and *VvmybA1* had a conserved acidic region, designated X-Y; however, this region was altered in *VvmybA2* and *VvmybA3* (Fig. 1B) and these two cDNAs did not function to induce pigmentation when introduced into the embryos (Fig. 2B). It has been shown that an acidic region at the carboxyl terminus of C1, a MYB protein of maize, can function as a transcriptional activation domain (Goff et al., 1991). Therefore, the X-Y region of the grape MYB proteins may form at least part of the transcriptional activation domain and may be necessary to produce functional proteins.

Only one allele for *VvmybA1* gene, *VvmybA1a*, was detected in a genomic library of 'Italia', whereas two alleles, *VvmybA1a* and *VvmybA1b*, were observed in that of 'Ruby Okuyama'. In *VvmybA1a*, a retrotransposon, *Gret1*, was inserted into the 5'-flanking region near the coding sequences of the gene. Since no expression of *VvmybA1* gene was detected in 'Italia', the insertion of *Gret1* must block the expression of *VvmybA1* gene and kept the skin color white. Retrotransposon insertion within or in proximity to a gene can result in gene inactivation or alterations in the expression pattern of the gene or in the structure of the encoded protein (Kumar and Bennetzen, 1999). In apples, apetalous mutants, lacking *MdPI* gene expression, have been induced by retrotransposon insertion into an intron of the gene (Yao et al., 2001), and an allele of *Md-ACSI*, showing very low expression, contains a SINE retroelement in the 5'-flanking region of the gene (Sunako et al., 1999). Although little information is available about retrotransposons in grapevines, a *copia*-like element, *Vine-1*, is present in multiple copies in grapevine genomes and is inserted into exon 4 of an alcohol dehydrogenase gene (Verries et al., 2000). As *Gret1*-related retrotransposons are also present in multiple copies in grapevine genomes, these retrotransposons may be involved in changing the structures and expression patterns of many grapevine genes. It is thought that cultivation of the grapevine began during the Neolithic era (6000–5000 BC) along the eastern shores of the Black Sea (Mullins et al., 1992). Because grapes in the wild usually have black or reddish-black skin, white-skinned grapes are thought to be derived from colored grapes through the loss in the ability to make anthocyanins (Slinkard and Singleton, 1984). Since the *VvmybA1a* allele is widely distributed among cultivars of *V. vinifera* and *V. labruscana*, we hypothesize that *Gret1* originally inserted upstream of one of the *VvmybA1*-coding sequences of a black-skinned ancestor and that, subsequently, a white-skinned grape was produced by spontaneous crossing (Kobayashi et al., 2004).

In the *VvmybA1b* allele, carried by the red-skinned sports, *Gret1* is missing, leaving behind its 3'-LTR flanked by 5 bp of a duplicated target site (Fig. 4). This structure is reminiscent of a reversion event previously documented in yeast. Roeder and Fink (1980) demonstrated that the *his4-912* mutation was the result of the insertion of a 6,200 bp transposable element that shared the homology to the Ty1 retrotransposon into a promoter region near the coding sequences of the *his4* gene of yeast, and that cold-sensitive His⁺ revertants derived from the *his4-912* mutant had only a single copy of LTR (solo LTR). This phenomenon was attributed to a reciprocal recombination between the two LTR sequences at opposite ends of Ty1, and resulted in excision of the internal region of the element and creation of a solo LTR. Excision of the retrotransposon restores

function to the regulatory region upstream from the *his4* gene. Similarly, we believe that 'Ruby Okuyama' is a revertant and that the solo LTR observed in *VvmybA1b* has been produced by recombination between the *Gret1* LTRs. The existence of the solo LTR in the genome has also been observed in some plants (Vicent et al., 1999; Shirasu et al., 2000), and the data presented here provide the experimental evidence for the generation of a solo LTR in a plant.

In this paper, we showed that *VvmybA1* gene plays an important role in the regulation of anthocyanin biosynthesis in grapes, and that the bud-mutation from white to red berry skins in grapes is caused by the deletion of a retrotransposon inserted in the *VvmybA1* gene.

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ブドウの果皮色変異体におけるアントシアニン合成と *VvmybA1* 遺伝子発現との関連

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摘 要

黄緑色品種(‘イタリア’および‘マスカット・オブ・アレキサンドリア’)と、これら品種から枝変わりにより生じたとされている赤色品種(‘ルビー・オクヤマ’および‘フレイム・マスカット’)を用いて、ブドウのアントシアニン合成の制御に関わる *myb* 様遺伝子(*VvmybA1*)の発現および機能を調べた。SSR分析により、赤色品種の‘ルビー・オクヤマ’および‘フレイム・マスカット’は、黄緑色品種の‘イタリア’および‘マスカット・オブ・アレキサンドリア’から枝変わりにより生じたものであることが確認された。*VvmybA1* 転写産物は‘ルビー・オクヤマ’の着色期以降の果皮で検出された

が、‘イタリア’の果皮ではいずれの時期においても検出されなかった。また、‘ルビー・オクヤマ’から単離された3種の *VvmybA* cDNAを‘巨峰’の体細胞胚に遺伝子銃により導入したところ、*VvmybA1*の導入により体細胞胚に赤色細胞が形成されたが、*VvmybA2*や*VvmybA3*の導入では赤色細胞は形成されなかった。さらに、*VvmybA1*には‘マスカット・オブ・アレキサンドリア’の果皮組織中にアントシアニンを産生する細胞を形成させる能力があることが示された。なお、果皮色変異と*VvmybA1*遺伝子の構造との関連についても検討した。