Biosci. Biotechnol. Biochem., **70** (6), 1506–1508, 2006

Note

A Skin Color Mutation of Grapevine, from Black-Skinned Pinot Noir to White-Skinned Pinot Blanc, Is Caused by Deletion of the Functional *VvmybA1* Allele

Hiroshi YAKUSHIJI,[1] Shozo KOBAYASHI,[1,†] Nami GOTO-YAMAMOTO,[2] Soek TAE JEONG,[2] Tamotsu SUETA,[3] Nobuhito MITANI,[1] and Akifumi AZUMA[1]

[1]*Grape and Persimmon Research Station, National Institute of Fruit Tree Science, Akitsu, Higashi-Hiroshima, Hiroshima 729-2494, Japan*
[2]*National Research Institute of Brewing, Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan*
[3]*Tamba Wine, Toyoda-Toriino, Kyotamba, Kyoto 622-0231, Japan*

Received December 5, 2005; Accepted January 16, 2006; Online Publication, June 23, 2006
[doi:10.1271/bbb.50647]

A white-wine grape, Pinot Blanc, is thought to be a white-skinned mutant of a red-wine grape, Pinot Noir. Pinot Noir was heterozygous for *VvmybA1*. One allele was the non-functional *VvmybA1a*, and the other was the functional *VvmybA1c*. In Pinot Blanc, however, only *VvmybA1a* was observed, and the amount of *VvmybA1* DNA in Pinot Blanc was half that in Pinot Noir. These findings suggest that deletion of *VvmybA1c* from Pinot Noir resulted in Pinot Blanc.

Key words: anthocyanin; *myb*; null allele; retrotransposon; *Vitis vinifera*

In grapes (*Vitis vinifera* L.), black and red cultivars accumulate anthocyanin in their skins, but white cultivars do not synthesize them.[1)] Expression of the gene for UFGT is critical for anthocyanin biosynthesis in grapes.[1–3)] Kobayashi *et al.*[4–6)] indicated that *Myb*-related genes such as *VvmybA1* (DDBJ accession no. AB097923) regulate anthocyanin biosynthesis in grapes *via Ufgt* gene expression. In addition, Jeong *et al.*[7)] reported that expression of *VvmybA1* coincided with that of the genes for anthocyanin biosynthetic enzymes, including *Ufgt*, and the accumulation of anthocyanin in grape skins. We also found that bud mutation from white-skinned Italia to red-skinned Ruby Okuyama is caused by the deletion of a retrotransposon inserted in *VvmybA1*.[5,6)] There are also many other skin-color mutations in grapes. For example, white-skinned Pinot Blanc is thought to have arisen by bud mutation from black-skinned Pinot Noir.[8)] In this study, we investigated the mechanism of that skin color mutation. Regner *et al.*[9)] reported that Pinot Noir and Pinot Blanc did not show any SSR polymorphism at any of the 34 SSR loci tested. We have confirmed that our Pinot Noir and Pinot Blanc samples showed identical SSR profiles using 8 loci: VVS1, VVS2, VVS3, VVS4, VVMD5, VVMD6, VVMD7, and VVMD8[10,11)] (data not shown).

Southern blot analysis. Total DNA was extracted from the leaves of both cultivars by the CTAB method (Rogers and Bendich[12)]), with slight modifications. One g of young leaves was ground into a fine powder with liquid nitrogen and a pestle in a mortar. Ten ml of washing buffer (100 mM HEPES [pH 8.0], 0.1% polyvinylpyrrolidone [soluble], and 10 mM 2-mercaptoethanol) was added, and mixed, then the homogenate was centrifuged 3–4 times at 15,000 × *g* for 5 min at 4 °C to remove polysaccharides. DNA was extracted from the pellet, and the extracted DNA was purified by the PEG precipitation method (1/4 volume of 4 M NaCl and 1 volume of 13% PEG were added to the DNA solution, and DNA was precipitated by centrifugation at 20,000 × *g* for 15 min at 4 °C after keeping the mixture for 1 h on ice, then the DNA pellet was washed with 70% ethanol and finally resolved in TE buffer). Southern blot analysis was carried out according to Kobayashi *et al.*[6)] using the DNAs and a DIG-labeled probe prepared from the coding region of *VvmybA1*. DNAs (2.0 µg each) were digested with endonucleases (*Apa*I, *Eco*RV, *Hin*dIII, *Dra*I, *Rsa*I, *Msp*I, *Pst*I, and *Pvu*II), separated by electrophoresis in 1% agarose gel in buffer (40 mM Tris•acetate [pH 8.0], 1 mM EDTA), and transferred to a nylon membrane. Southern blot analysis showed different banding patterns between cultivars (Fig. 1). Some DNA fragments (asterisks) that appeared in Pinot Noir were missing from Pinot Blanc. This finding suggests that Pinot Blanc might have lost a DNA region, including *VvmybA1*, from that of Pinot Noir.

† To whom correspondence should be addressed. Tel: +81-846-45-4719; Fax: +81-846-45-5370; E-mail: skobaya@affrc.go.jp

Abbreviations: CHS, chalcone synthase; CTAB, cetyltrimethylammonium bromide; DIG, digoxigenin; LTR, long terminal repeat; PCR, polymerase chain reaction; PEG, polyethylene glycol; SSR, simple sequence repeat; UFGT, UDP–glucose: flavonoid 3-*O*-glucosyltransferase

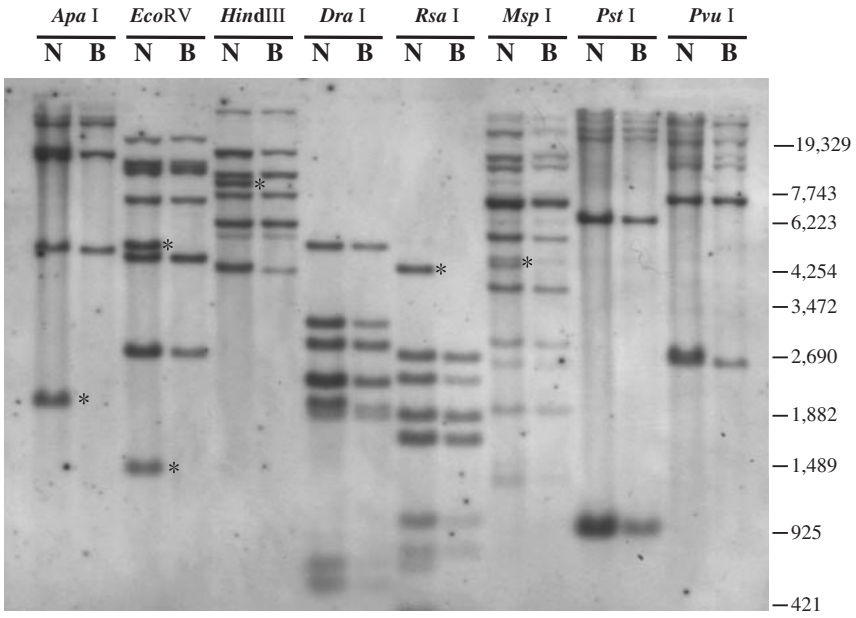

Fig. 1. Southern Blot Analysis of Genomic DNA from Pinot Noir (N) and Pinot Blanc (B).

DNAs (2.0 μg each) were digested with *Apa*I, *Eco*RV, *Hin*dIII, *Dra*I, *Rsa*I, *Msp*I, *Pst*I, and *Pvu*II, and separated by electrophoresis. A DIG-labeled probe was prepared from the coding region of *VvmybA1* (the position of the probe is indicated in Fig. 2). Asterisks show DNA fragments that appeared in Pinot Noir but were missing in Pinot Blanc. Numerals on the right side indicate the sizes (bp) of the DNA size markers (λ*Eco*T14I).

Quantitative real-time PCR. The relative amount of DNA sequences of *VvmybA1* in the genome of each cultivar was determined by quantitative real-time PCR using their genomic DNAs as templates. Similarly, we determined those of *Chs3* (AB066274) and *Ufgt* (AF000372), genes that play important roles in anthocyanin biosynthesis, as controls. For real-time PCR, we used a GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA) and the Quanti-Tect SYBR Green PCR Kit (Qiagen, Hilden, Germany), as described in the manufacturers' manuals. The sequences and positions of the three primer sets are described in Jeong *et al.*[7] The final primer concentrations were 0.25 μM for *Ufgt* and *VvmybA1* and 0.5 μM for *Chs3*. The final concentration of the template DNA was 0.5 ng/μl. A standard DNA for a calibration curve was prepared as a five-times dilution series of the genomic DNA of Pinot Noir to cover the template concentration. Real-time PCR was performed under the following conditions: 95 °C for 15 min, followed by 35–40 cycles of denaturing at 95 °C for 15 s, annealing for 20 s at 52 °C for *VvmybA1* and at 56 °C for *Ufgt* and *Chs3*, and extension at 72 °C for 20 s. Real-time PCR was carried out with at least three replicates per sample, and the average data were expressed as amounts of Pinot Blanc DNA relative to those of Pinot Noir. As shown in Table 1, the amount of *VvmybA1* in the Pinot Blanc genome was half that in the Pinot Noir genome. In contrast, there were no statistical differences in the amounts of *Chs*3 and *Ufgt* sequences between genomes.

Genomic clones of VvmybA1. DNA samples extracted from Pinot Noir and Pinot Blanc were partly digested with *Sau*3AI and cloned into the λ DASH II vector (Stratagene, La Jolla, CA), and genomic clones of *VvmybA1* were isolated as previously reported.[6] Five clones (PB32, PB41, PB53, PB81, and PB82) from Pinot Blanc were identical within the sequences determined, and had the same sequences as *VvmybA1a* (AB111100) from Italia. We named PB81 *VvmybA1a-PB* (AB242300). In *VvmybA1a*, a retrotransposon, *Gret1*, is inserted in the 5′-flanking region near the coding sequences of *VvmybA1*, and the insertion very likely blocks the expression of *VvmybA1*.[5] In a previous study of white cultivars, we found only the *VvmybA1a* allele of *VvmybA1* (as shown in Fig. S1 of Kobayashi *et al.*[5]), and did not detect *VvmybA1* transcripts in any cultivars, including Pinot Blanc (as shown in Fig. 1B, lane 1, of Kobayashi *et al.*[5]), while the *VvmybA1* transcript was detected in all the colored cultivars examined (a perfect correlation was observed between the expression of *VvmybA1* and skin color.[5]).

Table 1. Relative Amounts of DNA Sequences of *Chs3*, *Ufgt*, and *VvmybA1* in Pinot Noir and Pinot Blanc Genomes

Cultivar	Relative amount of DNA		
	Chs3	*Ufgt*	*VvmybA1*
Pinot Noir	1.00 ± 0.03 a	1.00 ± 0.12 a	1.00 ± 0.06 b
Pinot Blanc	0.98 ± 0.05 a	0.92 ± 0.07 a	0.51 ± 0.05 a

Values are means ± SD. Different letters indicate significant differences ($P < 0.01$) between means (*t* test).

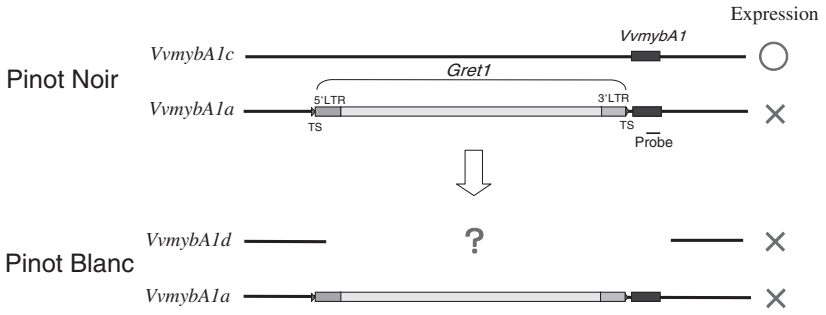

Fig. 2. Structures of Genomic Clones from Pinot Noir and Pinot Blanc.
VvmybA1a contained *Gret1*, but *VvmybA1c* did not. The probe used for Southern blot analysis is indicated below the map of *VvmybA1a*. TS and LTR represent a duplicated target site and a long terminal repeat respectively.

On the other hand, we observed two kinds of clone in Pinot Noir. One clone (PN45) had the same sequences as *VvmybA1a*; we named it *VvmybA1a-PN* (AB242301). But three others (PN21, PN57, and PN64) were different from *VvmybA1a*; we named PN64 *VvmybA1c-PN* (AB242302). *VvmybA1a-PN* and *VvmybA1c-PN* differed in their 5′-flanking region of *VvmybA1*, but were identical in their coding sequences (Fig. 2). The 5′-flanking region of *VvmybA1c-PN* was also different from that of *VvmybA1b* (AB111101) from Ruby Okuyama. *VvmybA1b* has a solo LTR of *Gret1*,[5] but *VvmybA1c-PN* does not. This finding indicates that *VvmybA1c* is most likely the original sequence of *VvmybA1* before the insertion of *Gret1*. Pinot Noir is heterozygous for the *VvmybA1* gene, which does not show any contradiction to the previous result obtained by PCR (Fig. S1, lane 13 of Kobayashi *et al.*[5]). As previously reported, the *VvmybA1* transcript was detected in Pinot Noir (Fig. 1B, lane 9 of Kobayashi *et al.*[5]). Thus *VvmybA1c* must be functional and must have the potential to induce anthocyanin biosynthesis in berry skins. Pinot Blanc had half the dosage of the *VvmybA1* gene as Pinot Noir (Table 1), suggesting that a genomic DNA region including the functional *VvmybA1c* allele was deleted from Pinot Noir, resulting in Pinot Blanc, which has a non-functional *VvmybA1a* and a null allele (Fig. 2). The null allele was tentatively named *VvmybA1d*, and isolation of it is in progress.

In conclusion, the skin-color mutation from black-skinned Pinot Noir to white-skinned Pinot Blanc was caused by deletion of the functional *VvmybA1c* allele of Pinot Noir, and thus anthocyanin biosynthesis in Pinot Blanc is inhibited by its inability to express *VvmybA1*.

Acknowledgments

We thank Dr. R. Nakaune of the Grape and Persimmon Research Station, National Institute of Fruit Tree Science, for helpful advice, and T. Nakasumi of the same station for technical assistance.

References

1) Boss, P. K., Davies, C., and Robinson, S. P., Expression of anthocyanin biosynthesis pathway genes in red and white grapes. *Plant Mol. Biol.*, **32**, 565–569 (1996).
2) Boss, P. K., Davies, C., and Robinson, S. P., Anthocyanin composition and anthocyanin pathway gene expression in grapevine sports differing in berry skin colour. *Aust. J. Grape Wine Res.*, **2**, 163–170 (1996).
3) Kobayashi, S., Ishimaru, M., Ding, C. K., and Goto, N., Comparison of UDP–glucose: flavonoid 3-*O*-glucosyltranserase (UFGT) gene sequences between white grapes (*Vitis vinifera*) and their sports with red skin. *Plant Sci.*, **160**, 543–550 (2001).
4) Kobayashi, S., Ishimaru, M., Hiraoka, K., and Honda, C., *Myb*-related genes of the Kyoho (*Vitis labruscana*) regulate anthocyanin biosynthesis. *Planta*, **215**, 924–933 (2002).
5) Kobayashi, S., Goto-Yamamoto, N., and Hirochika, H., Retrotransposon-induced mutations in grape skin color. *Science*, **304**, 982 (2004).
6) Kobayashi, S., Goto-Yamamoto, N., and Hirochika, H., Association of *VvmybA1* gene expression with anthocyanin production in grape (*Vitis vinifera*) skin-color mutants. *J. Jpn. Soc. Hort. Sci.*, **74**, 196–203 (2005).
7) Jeong, S. T., Goto-Yamamoto, N., Kobayashi, S., and Esaka, M., Effects of plant hormones and shading on accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. *Plant Sci.*, **167**, 247–252 (2004).
8) Galet, P., "Grape Varieties and Rootstock Varieties," Oenoplurimedia, Chaintre, p. 116 (1998).
9) Regner, F., Stadlbauer, A., Eisenheld, C., and Kaserer, H., Genetic relationships among Pinots and related cultivars. *Am. J. Enol. Vitic.*, **51**, 7–14 (2000).
10) Bowers, J. E., Dangl, G. S., Vignani, R., and Meredith, C. P., Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). *Genome*, **39**, 628–633 (1996).
11) Thomas, M. R., and Scott, N. S., Microsatellite repeats in grapevine reveal DNA polymorphisms when analyzed as sequence-tagged sites (STSs). *Theor. Appl. Genet.*, **86**, 985–990 (1993).
12) Rogers, S. O., and Bendich, A. J., Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.*, **5**, 69–76 (1985).