Pink-Colored Grape Berry Is the Result of Short Insertion in Intron of Color Regulatory Gene

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Abstract

We report here that pink grape berries were obtained by a short insertion in the intron of the MybA1 gene, a gene that regulates grape berry color. Genetic variation was detected among the MybA1 genes from grapes cultivated worldwide. PCR analysis of the MybA1 gene demonstrated that the size of the MybA1 gene in the red allele differs among grapes. Oriental V. vinifera bearing pink berries has the longest MybA1 gene among grapes, whereas the shortest MybA1 gene was detected in occidental V. vinifera grapes. The nucleotide sequences of the MybA1 genes demonstrated that oriental V. vinifera has two additional gene fragments (44 bp and 111 bp) in the promoter region of the MybA1 gene in the red allele and another 33 bp fragment in the second intron of the MybA1 gene in the red allele. The short insertion in the intron decreased the transcriptional activity in the model system and retained MybA1 transcripts with unspliced intron in the total RNA. From the experiments using deletion mutants of the 33 bp short insertion, 16 bp of the 3’ end in the insertion is a key structure for a defect in splicing of MybA1 transcripts. Thus, a weakly colored grape berry might be a result of the short insertion in the intron of a color regulatory gene. This is new evidence concerning the molecular mechanism of the fates of grape berry color. These findings are expected to contribute to the further understanding of the color variation in grape berries, which is correlated with the evolutionary events occurring in the MybA1 gene of grapes.

Introduction

Wine grapes belong to the species Vitis vinifera, which is classified into three groups: occidentalis, pontica, and orientalis [1]. The East Asian V. vinifera, including the Japanese and Chinese cultivars, is grouped in orientalis as well as the West Asian V. vinifera cultivars, including Muscat of Alexandria and Sultanina [2]. The Japanese and Chinese V. vinifera cultivars have unique characteristics that distinguish them from occidental V. vinifera. For example, the cluster and berry sizes of the indigenous Japanese V. vinifera cultivar Koshu and the indigenous Chinese V. vinifera cultivar Ryugan are two to three times larger than those of the major occidental cultivars, such as Cabernet Sauvignon, Pinot Noir, and Chardonnay. One of unique phenomena in those berries is that their skin colors are confined to green-yellow or pink. Fundamental questions associated with skin color deficiency in Asian V. vinifera remain unanswered, although the pink-colored berry skin of Koshu is due to the low accumulation of anthocyanins in the skin [3].

Black/red berries are the result of anthocyanin accumulation in the skins. UDP-glucose:flavonoid 3-o-glucosyltransferase (UGFT), which glycosylates anthocyanidins, is the key enzyme responsible for the accumulation of anthocyanins in grape berry skins [4] (Figure S1). Its expression is transcriptionally regulated by MybA transcription factors [5,6]. The MybA genes are closely clustered in a single locus, referred to as the berry color locus [6]. The insertion of the retrotransposon Gret1 into the promoter region of the MybA1 gene induced the inactivation of the promoter, resulting in the white berry allele [5]. On the other hand, nucleotide mutations in the MybA2 gene produced the white berry allele of the MybA2 gene [6]. A large deletion of the MybA genes in the red allele at the berry color locus also induced the white mutation of berry skins [7–9]. Thus, the mutation in the MybA genes is a good basis upon which to understand the genetic modifications that cause phenotypic mutations in berry color. Although the precise molecular mechanism of the somatic mutation at the red color locus remains unclear, the MybA cluster may be preferentially fragile in the grape genomic sequence and the somatic mutations have frequently occurred, resulting in frequent color mutation.

To determine why Japanese and Chinese grape berries accumulate less anthocyanin in their skins than black/red occidental grape berries, we performed a comparative genomic analysis among Vitis species. In the present study, we identified three distinctive fragments in the MybAI genes of oriental V. vinifera. One fragment inserted in the second intron of Koshu MybAI gene decreased MybAI transcription. Consequently, we propose a new hypothesis concerning the molecular mechanism of the fates of grape berry color.

Results

Anthocyanin accumulation in Koshu grape berry

The biosynthetic pathway of anthocyanins in grapes is shown in Figure S1. The concentrations of anthocyanins in the skins of
black grape berries, Cabernet Sauvignon and Merlot, were higher than those in the skins of pink grape berry, Koshu, and white grape berries, Riesling and Chardonnay, correlating with berry color (Figure 1).

Low MybA1 gene expression is associated with pink color of Koshu grape berry

MybA1 regulates anthocyanin accumulation in grape berry skin via UFGT gene expression (Figure S1). Koshu berry skin expressed low levels of both UFGT and MybA1 genes at harvest compared with Cabernet Sauvignon berry skin (Figure 2). These findings suggest that the low expression of the MybA1 gene in Koshu berry skin is associated with the pink color of the skin.

Analysis of MybA1 gene structure in Koshu

PCR primer set a and c was used to amplify the region between the MybA1 coding region and Gret1 in the white allele, and primer set b and c was used to amplify the region between the MybA1 coding region and its promoter in the red allele [5] (Figure 3A). White alleles were detected in black, pink, and white V. vinifera (Figure 3B), while the red allele was not detected in white V. vinifera cvs. Riesling and Chardonnay (Figure 3C, Rie and Cha). These results coincide with those of a previous study [5] and suggest that black/red cultivars have red and white alleles in their genome whereas white cultivars have only white alleles in their genome. Pink Koshu had the largest MybA1 gene among the V. vinifera species (Figure 3C, KS). Interestingly, the red allele of oriental V. vinifera cvs. Ryugan and Huotianhong also had a large MybA1 gene (Figure 3C, Rhu and HU). The PCR product amplified from the red allele of oriental V. vinifera was approximately 200 bp larger than those of the occidental V. vinifera cultivars (Figure 3C, Table S1). DNA sequence analysis of the MybA1 genes demonstrated that Koshu had three additional gene fragments (44 bp, 111 bp, and 33 bp) in the red allele of the MybA1 gene relative to the sequence of Cabernet Sauvignon (Figure S2A). Two of the fragments (44 bp and 111 bp) occur were found in the promoter region of the MybA1 gene and the other 33 bp fragment was found in the second intron of the MybA1 gene (Figure 4, Figure S2A). Without exception, oriental V. vinifera bearing pink berries had the fragments in the MybA1 gene of the red allele (Figure 4, Figure S2A). Meanwhile, the DNA sequence of the MybA1 gene of the white allele was identical between Koshu and Cabernet Sauvignon (Figure S2B). These results suggest that the MybA1 gene in the red allele of V. vinifera orientalis is structurally different from that of V. vinifera occidentalis.

Analysis of the MybA1 gene structure in grapes

The Gret1-inserted MybA1 white allele was also detected in V. labrusca and V. labruscana (Figure 3B, Vlcon and Vldela). In contrast, the North American rootstock Vitis species, V. rupestris and V. riparia, and the East Asian Vitis species, V. flexuosa, V. shiranii, V. saccharifera, and V. amurensis, did not contain the Gret1 insertion in the MybA1 gene (Figure 3B). These results are supported by a previous report [10]. North American Vitis species generated a single amplified product on PCR with the primer set b and c, although the sizes of the products differed among the Vitis species (Figure 3C, Vru, Vri, Vlcon, and Vldela). This primer set amplified two or three products from the East Asian Vitis species, except V. flexuosa (Figure 3C). Together with the fact that the East Asian Vitis...
Figure 2. UFGT and MybA1 gene expression in Koshu grape berry skin. (A) UFGT. (B) MybA1. CS, Cabernet Sauvignon. KS, Koshu. *p<0.01 as compared with CS.
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Figure 3. Analysis of MybA1 gene structure in grapes. (A) Primer maps used for PCR analysis. (B) PCR analysis of white alleles in Vitis species using primer set a and c. (C) PCR analysis of red alleles in Vitis species using primer set b and c. Numbers on the right indicate the positions of the molecular size markers. These results represent reproducible data from three independent experiments. CS, Cabernet Sauvignon. Me, Merlot. Syr, Syrah. PN, Pinot Noir. KS, Koshu. Rie, Riesling. Cha, Chardonnay. Ryu, Ryugan. HU, Huotianhong. Vru, V. rupestris. Vri, V. riparia. Vicon, V. labrusca cv. Concord. Vdel, V. labruscana cv. Delaware. Vfi, V. ficifolia. Vco, V. coignetiae. Vfl, V. flexuosa. Vshi, V. shiragai. Vsa, V. saccharifera. Vamu, V. amurensis.
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species have no white-allele-containing Gt1 retrotransposon, these results suggest that the East Asian Vitis species contain only the red MybA1 alleles.

Two fragments inserted into the promoter region of the MybA1 gene of V. vinifera orientalis were also detected in the MybA1 genes of the North American and East Asian Vitis species (Figures 4, Figure S3). In contrast, the 33 bp fragment in the second intron was distinctive of V. vinifera orientalis, such as Koshu, Ryugan, and Huotianhong, all of which bear pink berries (Figures 4, Figure S3).

Cluster analysis of the MybA1 genes among grapes demonstrated that V. vinifera orientalis cultivars were far away from V. vinifera occidentalis cultivars (Figure S4). Again, the MybA1 gene in the red allele of V. vinifera orientalis is genetically different from that of V. vinifera occidentalis.

The 33 bp short insertion decreases the amount of transcripts

Gene fragments between the second exon and the third exon, including the second intron, of Cabernet Sauvignon or Koshu MybA1 gene were inserted in front of the GUS reporter gene (Figure 5A). Also, to determine whether the 33 bp short insertion

![Figure 4. Comparison of MybA1 gene structure in Vitis grapes.](https://doi.org/10.1371/journal.pone.0021308.g004)
has any sequence-specific effects in the second intron, we made two deletion mutants (pBI/KS17 and pBI/KS16) and an antisense mutant (pBI/KS33R) of the 33 bp short insertion (Figure 5B). GUS activity of BY-2 cells transformed by pBI/KSmybA1 was less than one-third of BY-2 cells transformed by pBI/CSmybA1 (Figure 5C). BY-2 cells transformed by an antisense mutant pBI/KS33R showed high GUS activity. A deletion mutant pBI/KS16 decreased GUS activity similarly to pBI/KSmybA1, while GUS activity of BY-2 cells transformed by pBI/KS17 was similar to that of pBI/CSmybA1 (Figure 5C). Although MybA1-GUS mRNA was detected in both transformants by RT-PCR analysis, BY-2 cells transformed by pBI/CSmybA1 expressed the transcripts more abundantly than those transformed by pBI/KSmybA1 (Figure 5D).

Taken together, these results suggest that the 33 bp short insertion in the second intron of the Koshu MybA1 gene decreases the amount of transcripts of the reporter gene, and that 16 bp of the 3′ end of the 33 bp short insertion is essential sequences for a defect in splicing of MybA1 transcripts in Koshu grape, but not that the length of the short insertion.

Prediction of RNA secondary structure of second intron of Koshu MybA1 gene

The second intron of the Koshu MybA1 gene is 152 bp, whereas that of Cabernet Sauvignon MybA1 gene is 119 bp. To determine the effect of the 33 bp short insertion on the splicing efficiency of the second intron in the MybA1 pre-matured transcripts in Koshu and Cabernet Sauvignon berry skins, two primer sets for intron splicing assay were used (Figure 6A). The unspliced second intron of MybA1 in total RNA was detected by more than three to four times in Koshu skin compared with that in Cabernet Sauvignon skin (Figure 6B). Considering that Koshu berry skin expressed low levels of MybA1 at harvest compared with Cabernet Sauvignon berry skin (Figure 2), this result suggests that the 33 bp short insertion in the second intron of the Koshu MybA1 gene may generate and accumulate frequently unspliced MybA1 mRNA with the second intron in Koshu berry skin, but not affect transcription activity.

Discussion

Sequence polymorphisms, including 44 bp and 111 bp insertions, in the promoter of VvmybA1 have been reported by other groups [11–13]. Although the gene structure of MybA1 is a major determinant of grape berry color [11], these insertions in the MybA1 promoter seem to be not closely related to grape berry color. Although the 44 bp insertion is associated with red or pink grape berries in V. vinifera [12], other Vitis species, such as V. labruscana [13], have black berries irrespective of the 44 bp insertion. This study also demonstrated that the 44 bp and 111 bp insertions were spread to black Vitis species, namely, V. labruscana, V. ursinus, V. riparia, V. couguerianae, and V. amurensis (Figure 4). Therefore, both insertions do not function as the determinants for the color variation of grape berries. This result suggests that the 33 bp short insertion in the second intron of the color regulatory gene, because unspliced MybA1 mRNA was accumulated in Koshu berry skin (Figure 6B). In maize, the insertion of non-intronic sequences into introns interfered with mRNA splicing, resulting in a reduction in the mRNA content [14]. Although the actual level of spliced MybA1 transcripts was not measured in this study, unspliced MybA1 mRNA is generated by more than three to four times in Koshu berry skin compared with that in Cabernet Sauvignon berry skin (Figure 6B). In addition, the length of the short insertion is not essential for a defect in splicing of MybA1 transcripts. The experiments using deletion mutants of the 33 bp short insertion demonstrated that 16 bp of the 3′ end of the 33 bp short insertion is a key sequence. Consequently, unspliced MybA1 may be degraded by an unknown mechanism, resulting in the suppression of anthocyanin synthesis due to less MybA1 protein in the skin.

Where did the 33 bp short insertion in the MybA1 second intron come from? One possibility is that a transposable element was involved in the insertion. Both the copy number and the distribution of retrotransposons in grape genome are one of the signposts for grape evolution and domestication [5,15,16]. However, so far, we could not find any nucleotide sequences corresponding to the 33 bp short insertion from the grapevine genome and the EST sequences in NCBI databases and grape Massively Parallel Signature Sequencing (MPSS) database (http://mpss.udel.edu/grape/). As the databases are constructed from the sequences of occidental V. vinifera cultivars, further investigations are necessary to elucidate where the 33 bp short insertion came from and how it was inserted in the MybA1 second intron.

From the above study, we hypothesize that pink grape berry is the result of the short insertion in the intron of the color regulatory gene MybA1. This is a new hypothesis concerning the molecular mechanism underlying the fate of grape berry skin color, although we could not demonstrate the direct evidence that unspliced MybA1 transcripts does not function to induce anthocyanin biosynthesis in plants. Future studies to understand the function of spliced and unspliced MybA1 transcripts isolated from pink-colored grape would prove our hypothesis.

Methods

Plant materials

Young leaves of Vitis vinifera cvs. Cabernet Sauvignon, Merlot, Syrah, Pinot Noir, Koshu, Riesling, and Chardonnay; V. labruscana cv. Concord; V. labruscana cv. Delaware; V. riparia cv. Glorie de
Montpellier; and *V. rupestris* cv. St. George were collected from an experimental vineyard at the University of Yamanashi. Young leaves of *V. vinifera* cvs. Ryugan and Huotianhong were supplied by the National Research Institute of Brewing, Japan. Young leaves of *V. coignetiae* Pulliat, *V. ficifolia* var. Lobata Nakai, *V. saccharifera* Makino, *V. flexuosa* Thunb., *V. shiragai* Makino, and *V. amurensis* Rupr. were obtained from the University Farm of Kyoto Prefectural University, Japan.

**Measurement of anthocyanin composition**

Anthocyanin (cyanidin, peonidin, delphinidin, petunidin, and malvidin) composition in berry skins was measured using reversed-phase high performance liquid chromatography as described previously [3]. Berries were collected at 19 weeks post flowering.

**RNA isolation**

RNA was isolated from plant materials as described by Furiya et al. [9].

**Real-time PCR**

First-strand cDNA was synthesized from total RNA using a PrimeScript RT Reagent Kit (Takara, Otsu, Japan) and real-time PCR was performed using an SYBR Premix Ex Taq II (Takara), according to the manufacturer’s instructions. Nucleotide sequenc-

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**Figure 5. Effect of 33 bp short insertion on the transcription of a reporter gene.** (A) Transformation constructs. Gene fragments from the second exon to the third exon, including the second intron, of Cabernet Sauvignon or Koshu *MybA1* gene were inserted in front of the GUS reporter gene, resulting in pBI/CsmybA1 or pBI/KsmybA1 plant expression plasmids, respectively. Red boxes indicate the 33 bp short insertion in the second intron of Koshu *MybA1*. Yellow boxes indicate the ATG start codon. 35S pro, 35S promoter. Nos-ter, Nos terminator. (B) A schematic representation of the deletion (pBI/KS17 and pBI/KS16) and antisense (pBI/KS33R) mutants for 33 bp short insertion. The plant expression vectors having these mutants were constructed by procedures detailed in Methods. (C) GUS activity. BY-2 cells transformed by the plant expression vectors were used. GUS activity in the graph is expressed as the amount of 4-MU produced in one minute by one mg of protein extracted from the cells. a, p<0.01 as compared with pBI/CsmybA1. b, p<0.05 as compared with non-transformant cells (NC). (D) RT-PCR analysis. MybA1-GUS transcripts were detected by RT-PCR analysis using MGfw and MGrv primers. CS, pBI/CsmybA1. KS, pBI/KsmybA1. Myb-GUS, MybA1-GUS mRNA. Actin, an internal control.

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es of the primers used in this study were as follows: UFGT primers (5'-CCCTTACGTTAGCAGGATTGAG-3' and 5'-TGGGTGACAGAAGGCTGTTTAG-3', corresponding to bases 554-576 and 684-663 of V. vinifera UFGT, GenBank accession no. AF000372, respectively), VmmybA1 primers (5'-GGAAGGCTCGAGCAGAAAAG-3' and 5'-ATCCCGAGAACGCACATCCA-3', corresponding to bases 607–627 and 720–701 of V. vinifera VmmybA1, GenBank accession no. AB111101 CDS region, respectively), and β-actin primers (5'-CAAGAGCTGAAACTGCTGAG-3' and 5'-AATGGAGATGGCTGGAAGAGG-3', corresponding to bases 554–576 and 607–627 of V. vinifera β-actin, GenBank accession no. AF369524, respectively). Primers for intron splicing assay were e2 (5'-TCAAGAGCTGAAACTGCTGAG-3') and e2r (5'-GGAAGGCTCGAGCAGAAAAG-3'). β-Actin was used for normalization and expression levels were expressed as relative values.

Reverse transcription-PCR
Reverse transcription-PCR (RT-PCR) was performed according to a previously published method [9]. Nucleotide sequences of the primers used in this study were as follows: MGwv, 5'-CTGTTCATGGCTGTTATGGAC-3' and MGrv, 5'-GGGCTCAATGGGCGTTATACG-3' (Figure 5A). PCR conditions were as follows: after incubation at 95°C for 5 min, PCR amplification was performed for 25 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C for 10 min. The PCR products were separated on a 2.0% agarose gel and visualized by ethidium bromide staining under ultraviolet illumination.

DNA isolation
Genomic DNA was isolated from young leaves using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Analysis of MybA1 gene structure by PCR
PCR primers a, b, and c, designed by Kobayashi et al. [5], were used to amplify the 5' flanking region and the coding region of the MybA1 genes from white and red alleles (Figure 3A). Briefly, PCR was performed with 50 ng of genomic DNA using the following PCR cycling conditions: 95°C for 3 min, 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1.5 min, followed by a final extension step at 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining under ultraviolet illumination. PCR products amplified with primers b and c or a and c, corresponding to the red or white alleles of the MybA1 gene, respectively, were sequenced with a dye-terminator cycle-sequencing reaction (GeneRacer Japan, Tokyo, Japan). Alignment analysis of the nucleotide sequences of the MybA1 genes was performed with ClustalW version 1.83.

Cluster analysis of the MybA1 genes was performed using the neighbor-joining (NJ) method with bootstrap analysis using Molecular Evolutionary Genetics Analysis software (www.megasoftware.net).

Plasmid construction for intron functional analysis
Gene fragments from the second exon to the third exon, including the second intron, were amplified from the genomic DNA of Cabernet Sauvignon and Koshu by PCR using primers 5'-CGTGTGACAGAAGGCTGTTTAG-3' containing an XbaI site (underline) and ATG start codon (italic) and 5'-CTGGGATCCCATGTTACTGACTG-3' containing a BamHI site (underline) (Figure 5A). The PCR product was digested by XbaI and BamHI and ligated into the XbaI and BamHI sites of binary vector pBI121 (Clontech), resulting in a pBI/CSmybA1 plasmid from Cabernet Sauvignon or a pBI/KSmybA1 plasmid from Koshu, respectively (Figure 5A).

To create two deletion and an antisense mutants of 33 bp short insertion, inverse PCR was performed from pBI/CSmybA1 using the following primers; 5'-TTGTGTAAGTCTCCAGACTCACAGA-3' and 5'-TTGAAAGCCCATCAGACAGG-3'. PCR cycling conditions: 95°C for 30 sec, 65°C for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C for 10 min. The PCR products were self-ligated, resulting in pBI/CSmybA1 plasmid from Cabernet Sauvignon or a pBI/KSmybA1 plasmid from Koshu, respectively (Figure 5A).

Transformation
Tobacco BY-2 cells were transformed using pBI/CSmybA1 or pBI/KSmybA1 plasmids according to previously described methods [17].

β-Glucuronidase reporter assay
GUS activity of the transformed BY-2 cells was also determined using a FluorAce β-glucuronidase reporter assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. GUS activity was expressed as the amount of 4-methyl umbelliferone (4-MU) produced in one minute by one mg of protein. The experiments for GUS staining and GUS activity were carried out using three independent transformants.

Statistics
Data are presented as means ± standard deviations. Statistical analysis was performed using the Student’s t-test and ANOVA (analysis of variance) using Excel statistics software (Social Survey Research Information, Tokyo, Japan).
Supporting Information

Figure S1 Pathway leading to the synthesis of anthocyanins. MybA1 is a transcription factor that regulates the transcription of the UFGT gene. PAL, phenylalanine ammonia lyase. C4H, cinnamate 4-hydroxylase. 4CL, 4-coumarate ligase. C3H, coumarate-3-hydroxylase. STS, stilbene synthase. CHS, chalcone synthase. CHI, chalcone isomerase. F3’H, flavonoid 3’-hydroxylase. F3’,5’H, flavonoid 3’,5’-hydroxylase. F3H, flavonone-3-hydroxylase. DFR, dihydroflavonol 4-reductase. LDOX, leucoanthocyanidin dioxygenase. UFGT, UDP-glucose:flavonoid 3-O-glucosyltransferase. (PDF)

Figure S2 Alignment of the nucleotide sequences of the MybA1 gene between Koshu and Cabernet Sauvignon. (A) Promoter and coding regions of MybA1 in the red allele. (B) MybA1 coding regions in the white allele. Koshu has three additional gene fragments (44 bp, 111 bp, and 33 bp, shaded black) in the red allele of the MybA1 gene relative to the sequence of Cabernet Sauvignon. The DNA sequence of the MybA1 gene of the white allele is identical between Koshu and Cabernet Sauvignon. CS, Cabernet Sauvignon. KS, Koshu. (PDF)

Figure S3 Alignment of nucleotide sequences of MybA1 genes of the red allele among grapes. The 33 bp short fragments in the second intron of the MybA1 gene of oriental V. vinifera cultivars are shaded black. Unique nucleotide sequences are indicated by asterisks. Vl, V. flexuosa. KS, Koshu. RU, Ryugan. CU, Cuvee Sauvignon. ME, Merlot. SYR, Syrah. PN, Pinot Noir. SYR, Syrah. K, Koshu. RU, Ryugan. H, Huotianhong. (PDF)

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Table S1 Size of MybA1 PCR products amplified from red alleles in Vitis species. (PDF)