

Article

GRETI RETROTRANSPOSON AND VVMYBA1 GENE SEQUENCES IN SOMATIC MUTANTS OF NEW TABLE GRAPE VARIETIES ‘BRASIL’ AND ‘BLACK STAR’ (VITIS VINIFERA L.)

RETROTRANSPOSON GRETI E SEQUÊNCIAS DO GENE VVMYBA1 EM MUTANTES SOMÁTICOS DE NOVAS VARIEDADES DE UVAS DE MESA ‘BRASIL’ E ‘BLACK STAR’ (VITIS VINIFERA L.)

Danuza Kelly Strioto¹, Claudete Aparecida Mangolin², Sandra Aparecida de Oliveira Collet², Andrea Florindo das Neves¹, Liriana Belizario Cantagalli², Maria de Fátima P.S. Machado^{2*}

¹ Postgraduate Program in Genetics and Breeding, State University of Maringá, 87020-900 Maringá, PR, Brazil.

² Department of Biotechnology, Genetics and Cell Biology, State University of Maringá, 87020-900 Maringá, PR, Brazil.

* Corresponding author: Tel.: +55 44 3011 4681; e-mail: mfpsmachado@uem.br

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SUMMARY

Somatic mutations in grapes are relatively frequent and associated with diversity in grape skin color and berry morphology. Mutations that occur on a side branch of the ‘Benitaka’ cultivar with rosy-red berry skin color generated the ‘Brasil’ cultivar, and mutations that occurred on a side branch of ‘Brasil’ generated the ‘Black Star’ cultivar, both showing a black color in the berry skin. Therefore, genetic characterization of the *Gret1* retrotransposon and the *VvmybA1* gene in ‘Italia’, ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star’ was started to find whether the altered coloration of berries in ‘Brasil’ and ‘Black Star’ is a product of different mutation patterns in the investigated sequences. Six primer combinations were used for the amplification of different sequences of the *Gret1* retrotransposon and *VvmybA1* gene of the five cultivars. Polymerase chain reaction (PCR) of the *Gret1* retrotransposon and the *VvmybA1* gene and sequencing of the amplified products using six primer combinations showed no different alleles or different nucleotide sequences in ‘Brasil’ and ‘Black Star’. The sequencing of the *VvmybA1* gene in the present study showed that the mutations that occurred in the cultivar ‘Italia’ for generating the ‘Benitaka’ cultivar persisted in the ‘Brasil’ and ‘Black Star’ cultivars.

RESUMO

As mutações somáticas em uvas são eventos relativamente frequentes e estão associados à diversidade de cor da película e da morfologia dos bagos. Mutações que ocorreram em ramos da cultivar ‘Benitaka’ com a cor da película dos bagos rosa-avermelhada originaram a cultivar ‘Brasil’, e mutações que ocorreram em ramos da cultivar ‘Brasil’ originaram a cultivar ‘Black Star’, ambas com bagos de coloração azul negra. Portanto, uma caracterização genética do retrotransposon *Gret1* e do gene *VvmybA1* em ‘Italia’, ‘Rubi’, ‘Benitaka’, ‘Brasil’ e ‘Black Star’ foi iniciada para verificar se a coloração alterada dos bagos nas cultivares ‘Brasil’ e ‘Black Star’ é produto de diferentes padrões de mutação nas sequências investigadas. Seis combinações de *primers* foram utilizadas para amplificar diferentes sequências do retrotransposon *Gret1* e do gene *VvmybA1* das cinco cultivares. A amplificação por reação em cadeia da polimerase (PCR) do retrotransposon *Gret1* e do gene *VvmybA1*, assim como a sequenciação dos produtos amplificados pelas seis combinações de *primers* não mostrou alelos diferentes ou sequências nucleotídicas diferentes em ‘Brasil’ e ‘Black Star’. A sequenciação do gene *VvmybA1* neste estudo mostrou que as mutações que ocorreram na cultivar ‘Italia’ e que originaram a cultivar ‘Benitaka’ persistiram nas cultivares ‘Brasil’ e ‘Black Star’.

Keywords: grapes, clonal propagation, somatic mutations, ‘Brasil’ cultivar, ‘Black Star’ cultivar.

Palavras-chave: uvas, propagação clonal, mutações somáticas, cultivar ‘Brasil’, cultivar ‘Black Star’.

INTRODUCTION

The diversity of grape skin color is relevant to the preference and marketing of table grapes and wine grapes. Somatic mutations in grapes are relatively

frequent events associated with the diversity of grape skin color and berry morphology, which are economically important characteristics. Somatic mutations in the ‘Italia’ cultivar [Piróvano 65 (VIVC

5582: *Vitis* International Variety Catalogue (www.vivc.de) database] and its somatic mutants have been the biological source for at least five new varieties of table grape. ‘Italia’ (white berries) is a hybrid of the cross between ‘Bicane’ (white berries; VIVC n° 1341) and ‘Muscat Hamburg’ (black berries; VIVC n° 8226) bred by Angelo Pirovano in Italy. Independent mutations that occurred on side branches of the ‘Italia’ cultivar generated the ‘Rubi’ (VIVC n° 22689; Kishino and Mashima, 1980), ‘Benitaka’ (VIVC n° 19816; Sousa, 1996), and ‘Redimeire’ (Pires *et al.*, 2003) cultivars. Mutations that occurred on side branches of the ‘Benitaka’ cultivar generated the ‘Brasil’ cultivar (VIVC n° 19817; Gonçalves, 1995), and mutations that occurred on side branches of the ‘Brasil’ cultivar generated the ‘Black Star’ cultivar (Roberto *et al.*, 2012). The light rosy-red color of the berry skin and a more intense and uniform rosy-red color are observed in the ‘Rubi’ and ‘Benitaka’ cultivars, respectively, while black and red colors have been observed for the berry skin and pulp, respectively, of the ‘Brasil’ cultivar. The most striking feature that distinguishes the ‘Black Star’ grape as a new variety from the ‘Brasil’ cultivar is the ellipsoid and elongated shape of its berries.

Several biochemical and molecular markers have been used to assess the genetic divergence between the ‘Italia’ cultivar, showing the green color of the berry skin and its colored somatic mutants (Oliveira-Collet *et al.*, 2005; Orasmo *et al.*, 2007; Maia *et al.*, 2009, 2018; Strioto *et al.*, 2019a,b). However, the biochemical and molecular divergence analysis could not explain the differences between the colors and shapes of the grape berries of the different cultivars. Berry skin color differences may reflect the polymorphism of genes controlling pigment distribution in them but not of genes associated with intermediary metabolism or with determined DNA segments distributed in the genome of the cultivars. However, Xia *et al.* (2021) have suggested that the methylation levels of the *VvmybA1* promoter play a crucial role in regulating grape skin coloration. The high methylation level of the *VvmybA1* promoter was positively correlated with low concentration of anthocyanins. The color of red and black grapes may result from the formation, accumulation, and distribution of anthocyanins, which exist in the berry skin. Expression of the gene encoding UDP-glucose: flavonoid 3-O-glucosyltransferase (UGFT) is essential for anthocyanin synthesis in grapes (Boss *et al.*, 1996a,b; Ford *et al.*, 1998). *UGFT* is expressed in all colored cultivars but is not expressed in white cultivars (Kobayashi *et al.*, 2001). *UGFT* transcription is regulated by two MybA transcription factors that are encoded by the genes *VvmybA1* and *VvmybA2*, organized in a cluster on chromosome 2 (Kobayashi *et al.*, 2002) while a set of three functional genes (*VvmybA1*, *VvmybA2*, and *VvmybA3*) for *UGFT* transcription factors are

associated with the grape berry pigmentation pathway (Walker *et al.*, 2007).

Kobayashi *et al.* (2005) showed that *VvmybA1* plays an important role in regulating anthocyanin biosynthesis in grapes. The emergence of the white berry genotype is a result of silencing the *VvmybA1* gene (Kobayashi *et al.*, 2005; Walker *et al.*, 2007) because of the insertion of the retrotransposon *Gret1* into its promoter (Lijavetzky *et al.*, 2006; This *et al.*, 2006; Azuma *et al.*, 2009). The bud-mutation from white to red berry skin in grapes is caused by the deletion of the retrotransposon inserted into *VvmybA1*. Cultivars with *Gret1* inserted into the promoter region of the *VvmybA1* gene (*VvmybA1a* allele) in homozygosity do not produce anthocyanins, as in the case of the white cultivar ‘Italia’, whereas colored cultivars have at least one functional *VvmybA1* allele (*VvmybA1b* or *VvmybA1c*). An intra-long terminal repeat (LTR) recombination between the 5’-LTR and 3’-LTR in the *Gret1* gene led to the loss of an internal part of the retrotransposon and caused the recovery of *VvmybA1* expression (allele *VvmybA1b*) and anthocyanins production in the colored berries of ‘Ruby Okuyama’ (Kobayashi *et al.*, 2004, 2005). Kobayashi *et al.* (2004) showed that the cultivar ‘Ruby Okuyama’ is heterozygous for the *VvmybA1a* and *VvmybA1b* alleles. However, Azuma *et al.* (2009) reported that the recovery of the pink skin color in ‘Benitaka’ was determined by the occurrence of the functional allele *VvmybA1^{BEN}* at the *VvmybA1* locus, supposedly produced by homologous recombination between *VvmybA1a* and *VvmybA3*. ‘Benitaka’ has the *VvmybA1* locus in heterozygous with the *VvmybA1a* allele (non-functional) and a new *VvmybA1^{BEN}* allele that restores the transcription of the *VvmybA1* gene.

The events involved in restoring the transcriptional activity of the *VvmybA1* gene have been reported for the ‘Ruby Okuyama’ and ‘Benitaka’ cultivars. However, reports that address any changes in the *VvmybA1* gene related to the change in color from black detected in the berry skin of the ‘Brasil’ and ‘Black Star’ cultivars were not found in the literature. Black grape cultivars stand out for their high content of phenolic compounds and antioxidant properties (Hadidi *et al.*, 2014). Furthermore, the antibacterial properties (Georgiev *et al.*, 2014) and the highest nutraceutical properties (Crupi *et al.*, 2015) reported for black grapes encourage researchers to investigate and understand their generation. The current study investigates sequences of the *Gret1* retrotransposon and the *VvmybA1* gene in the ‘Italia’, ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars to find if the altered coloration of berries in the ‘Brasil’ and ‘Black Star’ cultivars is a product of different mutation patterns in the *Gret1* retrotransposon and *VvmybA1* sequences.

MATERIALS AND METHODS

Polymerase chain reaction (PCR) amplification of the *Gret1* retrotransposon and the *VvmybA1* gene in cultivars

The origin of the ‘Brasil’ and ‘Black Star’ cultivars and striking features of the shape and color of the berries that distinguish the ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star’ grapes as new varieties from the ‘Italia’ cultivar are shown in Figure 1. Partially expanded contaminant-free leaves were collected from four plants of each vineyard of ‘Italia’, ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars, established at 23° 30' 56" S/51° 47' 57" W, in Marialva, Paraná state, Brazil. Samples were individually stored in labeled plastic screen bags to avoid mixing of grape varieties, maintained on ice (at

4 °C), transferred to the laboratory, frozen in liquid nitrogen, and stored at -80 °C until DNA extraction. DNA was extracted from leaf tissues following Thomas *et al.* (1993), with minor modifications reported by Strioto *et al.* (2019a), which specified using 100 mg leaves from individual plants instead of 2.0 g. Leaf samples from each plant were centrifuged for 10 min at 3000 rpm at room temperature (~22 °C). DNA quantity was determined using a Picodrop Spectrophotometer (Pico 100; version 4.0/21/03/11). The average concentration of DNA was between 33 ng/μL and 450 ng/μL per sample. After quantification, DNA samples were diluted to a final concentration of 10 ng/L until use. Amplification reactions were performed using DNA extracted from 20 young leaves obtained from five cultivars, and the “F” (forward) and “R” (reverse) primers a, d3, b, e1, c, and d (Table I).

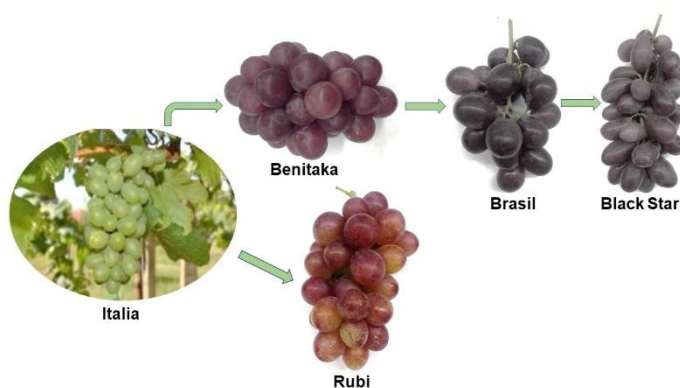


Figure 1. Origin of the ‘Brasil’ and ‘Black Star’ cultivars, and striking features of the shape and color of their berries that distinguishes the five grape cultivars.

Combinations of primers a^(F)-d3^(R), b^(F)-d3^(R), b^(F)-e1^(R) (Lijavetzky *et al.*, 2006), a^(F)-c^(R), b^(F)-c^(R) (Kobayashi *et al.*, 2004; Azuma *et al.*, 2007), and b^(F)-d^(R) (Mitani *et al.*, 2009) were used for

amplification of different sequences of the *Gret1* retrotransposon and the *VvmybA1* gene in the ‘Italia’, ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars (Figure 2).

Table I

Sequences of primers used for amplifying different sequences of the *Gret1* retrotransposon and the *VvmybA1* gene

Primer name	Gene	Primer sequence
a	<i>VvmybA1</i>	AAAAAGGGGGCAATGTAGGGACCC ^(F)
d3	<i>VvmybA1</i>	CCTGCAGCTTTTCGGCATCT ^(R)
b	<i>VvmybA1</i>	GGACGTTAAAAAATGGTTGCACGTG ^(F)
e1	<i>VvmybA1</i>	GTCTTTCGCTTGCCAAGTGT ^(R)
c	<i>VvmybA1</i>	GAACCTCCTTTTGAAGTGGTGACT ^(R)
d	<i>VvmybA1</i>	CACAGACAGCGCCAATGTTGT ^(R)

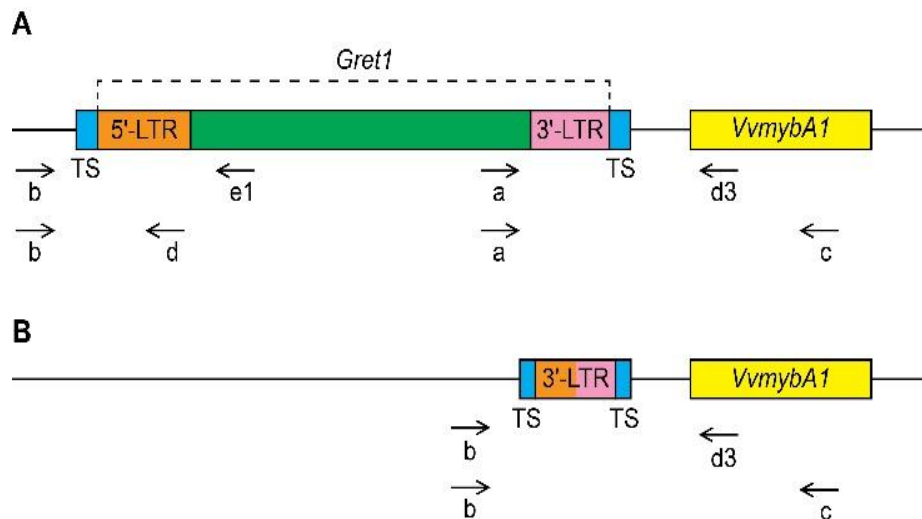


Figure 2. Combinations of primers used for amplifying different sequences of the *Gret1* retrotransposon and the *VvmybA1* gene. In A, the *Gret1* retrotransposon is inserted in the promoter of the *VvmybA1* gene in ‘Italia’. B shows an intra-LTR recombination between the 5′-LTR and 3′-LTR in the *Gret1* gene in ‘Ruby Okuyama’ (according to Kobayashi *et al.*, 2004).

PCR was performed with 20 μ L, containing 20 ng of genomic DNA, 1 \times buffer (10 mM Tris–HCl pH 8.8), 2.0 mM of $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, 0.3 μ M of each primer, one unit of Taq polymerase platinum (Invitrogen; Lonza Bioscience, Morrisville, NC, USA), and Milli-Q water to bring the reaction to the final volume. PCR was performed on a Veriti thermal cycler (Applied Biosystems), and the cycling conditions were as follows: initial denaturation at 95 $^{\circ}C$ for 3 min, followed by 34 cycles at 94 $^{\circ}C$ for 30 s, annealing at 60–65 $^{\circ}C$ for 30 s, and extension at 72 $^{\circ}C$ for 60 s. A final extension step was performed at 72 $^{\circ}C$ for 5 min. After DNA amplification, 4 μ L loading buffer (0.25% bromophenol blue and 30% glycerol) was added to each amplification product, and electrophoresis was performed on a 1.2% agarose gel with 0.5 \times TBE buffer (44.5 mM Tris-borate and 1 mM EDTA) at 60 V for 4 h. After electrophoresis, the gels were stained with ethidium bromide at 0.5 μ g/mL, and images were acquired with a Molecular Image LOCCUS L-PIX-HE system using Picasa 3. The size of the PCR products was determined using a 1-kb DNA ladder (Invitrogen).

Sequencing of the amplified products with primers specific for the *Gret1* retrotransposon and the *VvmybA1* gene

One DNA sample of each cultivar (‘Italia’, ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star’) was amplified using primer combinations $a^{(F)}$ - $d3^{(R)}$, $b^{(F)}$ - $d3^{(R)}$, $b^{(F)}$ - $e1^{(R)}$, $a^{(F)}$ - $c^{(R)}$, $b^{(F)}$ - $c^{(R)}$, and $b^{(F)}$ - $d^{(R)}$ and outsourced to Biotecnologia Pesquisa e Inovação (BPI), Technology, Research, and Innovation in Botucatu, São Paulo, Brazil for sequencing. The BigDye® Terminator v3.1 Cycle Sequencing Kit

(Applied Biosystems) was utilized for sequencing, and ethanol/EDTA/sodium acetate was used for the precipitation reaction, according to the protocol suggested by the manufacturer. Sequencing was performed by capillary electrophoresis using an ABI 3730 Genetic Analyzer (Applied Biosystems). Chromas software (Technelysium DNA sequencing software; <http://technelysium.com.au/wp/>) was used to identify the amplified nucleotide sequences in the five cultivars.

Basic local alignment search tool (Altschul *et al.*, 1990) was used to compare the sequences obtained with the sequences available in the NCBI database. The AB111100.1 sequence of the ‘Italia’, which is 20,286-bp long comprising the *Gret1* retrotransposon and the *VvmybA1* gene, was used to align the sequences, amplified using the six primer combinations. BioEdit (Hall, 1999) was used to align the sequencing results with the AB111100.1 sequence available in the database (<https://www.ncbi.nlm.nih.gov/nuccore/AB111100.1>).

RESULTS AND DISCUSSION

Using the primer combination $a^{(F)}$ - $d3^{(R)}$ for amplifying the 3′-LTR of the *Gret1* retrotransposon and the beginning of the *VvmybA1* gene (Lijavetzky *et al.*, 2006) revealed an allele of 1,200 bp in ‘Italia’ (*VvmybA1*^{ITA}), ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star.’ An additional allele of 1,600 bp was observed in ‘Benitaka’, ‘Brasil’, and ‘Black Star.’ The three cultivars ‘Benitaka’, ‘Brasil’, and ‘Black Star’ are heterozygous (*VvmybA1*^{ITA}/*VvmybA1*^{BEN}) at the *VvmybA1* locus (Figure 3A). The primer combination $b^{(F)}$ - $d3^{(R)}$ was also used to amplify the 3′-LTR of *Gret1* retrotransposon and the beginning

of the *VvmybA1* gene (Lijavetzky *et al.*, 2006); using this combination revealed an allele of 600 bp in the five cultivars and an allele of 1,400 bp in the ‘Rubi’ cultivar (*VvmybA1*^{RUB}) (Figure 3B).

The primer combination b^(F)-e1^(R) used to amplify the 5'-LTR of the *Gret1* retrotransposon (Lijavetzky *et al.*, 2006) revealed an allele of 1,100 bp in the five cultivars, showing that ‘Italia’, ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars have the *Gret1* retrotransposon inserted into the promoter of the *VvmybA1* gene at one of the homologous chromosomes (Figure 3C). The primer combination b^(F)-d^(R) used to amplify the 5'-LTR of *Gret1* retrotransposon (Mitani *et al.*, 2009) revealed an allele of 1,000 bp in the five cultivars, supporting the evidence that the ‘Italia’, ‘Rubi’, ‘Benitaka’,

‘Brasil’, and ‘Black Star’ cultivars have the *Gret1* retrotransposon inserted into the promoter of the *VvmybA1* gene at one of the homologous chromosomes (Figure 3D).

The a^(F)-c^(R) primer combination used to amplify the *VvmybA1* locus and the 3'-LTR region of the *Gret1* retrotransposon (Azuma *et al.*, 2007) revealed an allele of 1,600 bp in the five cultivars and an allele of 2,000 bp in the ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars (Figure 3E). The b^(F)-c^(R) primer combination used to amplify the *VvmybA1* locus and 3'-LTR of the *Gret1* retrotransposon (Azuma *et al.*, 2007) revealed an allele of 900 bp in the five cultivars and an allele of 1,600 bp in the ‘Rubi’ cultivar (Figure 3F).

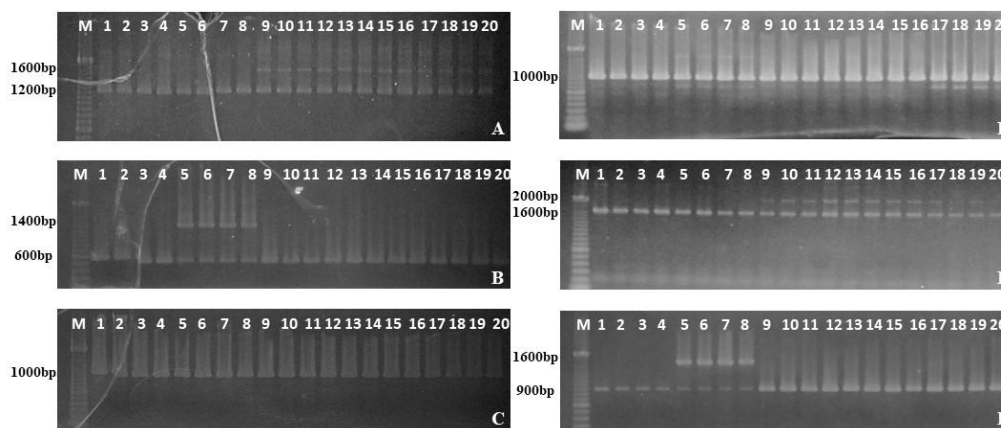


Figure 3. PCR products of specific primer combinations are shown: amplified sequences of the *Gret1* retrotransposon and the *VvmybA1* gene in ‘Italia’ (lanes 1–4), ‘Rubi’ (lanes 5–8), ‘Benitaka’ (lanes 9–12), ‘Brasil’ (lanes 13–16), and ‘Black Star’ (lanes 17–20). The a^(F)-d3^(R) combination evidenced an allele with 1,200 bp in ‘Italia’ (*VvmybA1*^{ITA}) and also in ‘Rubi’, ‘Benitaka’, ‘Brasil’ and ‘Black Star’, and an allele with 1,600 bp was observed in ‘Benitaka’, ‘Brasil’ and ‘Black Star’ (A); the b^(F)-d3^(R) combination evidenced an allele with 600 bp in the five cultivars and an allele with 1,400 bp in the ‘Rubi’ (B); the b^(F)-e1^(R) combination evidenced an allele with 1,100 bp in the five cultivars (C); the b^(F)-d^(R) combination evidenced an allele with 1,000 bp in the five cultivars (D); the a^(F)-c^(R) combination identified an allele with 1,600 bp in the five cultivars and an allele with 2,000 bp in the ‘Benitaka’, ‘Brasil’ and ‘Black Star’ cultivars (E) and the b^(F)-c^(R) primers identified an allele with 900 bp in the five cultivars and an allele with 1,600 bp in the ‘Rubi’ (F). M represents the lane loaded with a 1 kb DNA ladder.

The use of the six primer combinations to amplify specific sequences of the *Gret1* retrotransposon and the *VvmybA1* gene in the ‘Italia’, ‘Rubi’, ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars showed that the ‘Italia’ cultivar is homozygous at the *VvmybA1* gene (*VvmybA1*^{ITA}/*VvmybA1*^{ITA}) and the ‘Rubi’ and ‘Benitaka’ cultivars with the *VvmybA1* gene in heterozygous (*VvmybA1*^{ITA}/*VvmybA1*^{RUB} and *VvmybA1*^{ITA}/*VvmybA1*^{BEN}, respectively); this is consistent with the findings of Kobayashi *et al.* (2004) and Azuma *et al.* (2009). The six primer combinations also showed the

VvmybA1^{ITA}/*VvmybA1*^{BEN} genotype in the ‘Brasil’ and ‘Black Star’ cultivars. No additional alleles were observed in the new cultivars as ‘Brasil’ generated from a somatic mutation in ‘Benitaka’, and ‘Black Star’ generated from the ‘Brasil’ cultivar.

The six PCR products obtained using the b^(F)-c^(R) primer combination were sequenced to identify mutation sites in the nucleotide sequences of the *VvmybA1* gene. Sequences amplified by the b^(F)-c^(R) primer combination showed changes in nucleotides at three positions in the first intron of *VvmybA1*. Sequences amplified using the b^(F)-c^(R) primer

combination also showed changes in the nucleotides in the first and third exons of *VvmybA1*. The ATC codon in the first exon of the *VvmybA1* gene encoding the amino acid isoleucine in the ‘Italia’ and ‘Rubi’ cultivars was replaced by ACC encoding the amino acid threonine in the ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars (Figure 4A).

The dinucleotide “TA” corresponding to the 15,437th and 15,438th positions in the ‘Italia’ and ‘Rubi’ cultivars was replaced by the dinucleotide “AG” in

the ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars (Figure 4B). The “A” nucleotide at position 15,477 of the first intron of *VvmybA1* in ‘Italia’ and ‘Rubi’ was replaced by “G” in the ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars. The deletion of “T” was observed in the third exon of *VvmybA1* in the ‘Brasil’ cultivar (Figure 4C). The replacement of “T” of the ATC codon in the ‘Italia’ cultivar by “C” in ‘Benitaka’, conferring the exchange of the codon for ACC in the first exon of *VvmybA1*, has also been reported by Azuma *et al.* (2009).

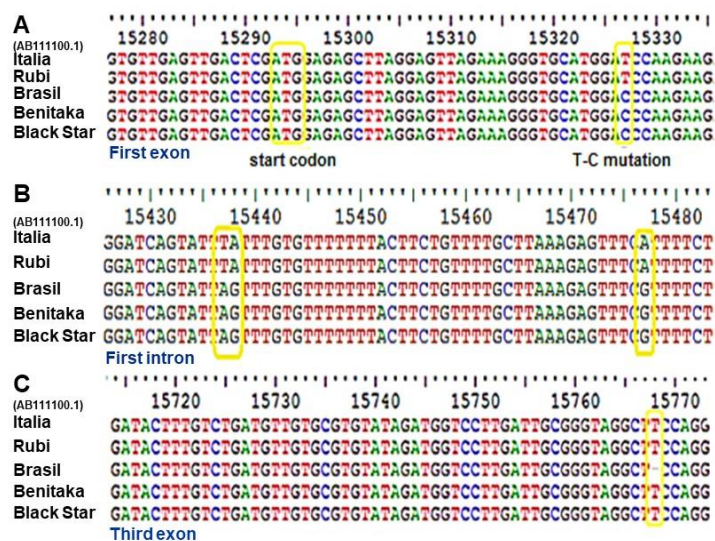


Figure 4. Sequences amplified using the b^(F)-c[®] primer combination shows changes of nucleotides in three positions of the first intron of *VvmybA1*: the ATC codon in the first exon of *VvmybA1* encoding the amino acid isoleucine in the ‘Italia’ and ‘Rubi’ cultivars was replaced by ACC at position 15327 encoding the amino acid threonine in the ‘Benitaka’, ‘Brasil’, and ‘Black Star’ cultivars (A); the dinucleotide “TA” at the 15437th and 15438th positions in ‘Italia’ and ‘Rubi’ was replaced by the dinucleotide “AG” in ‘Benitaka’, ‘Brasil’, and ‘Black Star’, and nucleotide “A” at position 15477 of *VvmybA1* in the ‘Italia’ and ‘Rubi’ was replaced by “G” in ‘Benitaka’, ‘Brasil’, and ‘Black Star’ (B); deletion of a nucleotide “T” is evident in the third exon of *VvmybA1* in ‘Brasil’ (C).

PCR amplification of the *Gret1* retrotransposon and the *VvmybA1* gene showed no different alleles in ‘Benitaka’, ‘Brasil’, and ‘Black Star’. Except for the deletion of a nucleotide “T” at position 15,768 of the *VvmybA1* gene in the ‘Brasil’ cultivar that was present in ‘Black Star’, the sequencing of the amplified products using the six primer combinations showed no difference in the nucleotide sequences in ‘Benitaka’, ‘Brasil’, and ‘Black Star’. The deletion of “T” at position 15,768 of the *VvmybA1* gene in the ‘Brasil’ cultivar that was present in the ‘Black Star’ cultivar may be due to a somatic mutation that occurred in the ‘Brasil’ cultivar after the generation of ‘Black Star’. It seems that this phenomenon was not associated with the diversity of grape skin color and berry morphology. Somatic mutations in grapes appear to be relatively frequent events that may or may not be associated

with the diversity in grape skin color and berry morphology. A higher number of anthocyanins has been reported in black-berried grape cultivars (Conner and Maclean, 2013; Fan *et al.*, 2018). Röckel *et al.* (2020) showed that repetitive DNA elements (grapevine color enhancer, GCE) in the promoter of *VvmybA1* were strongly associated with an increase in the expression of *VvmybA1* itself, the *VvUGT* gene regulated by it, and the anthocyanins content. However, anthocyanins profile variations may be due to the differential accumulation of anthocyanins types (He *et al.*, 2010). High levels of cyanidin-based (red) or delphinidin-based anthocyanins (blue to purple) may be determinants of berry skin color (Mu *et al.*, 2014; Azuma *et al.*, 2015). Thus, the transcript levels of flavonoid 3'-hydroxylase and flavonoid 3', 5'-hydroxylase may be involved in the biosynthesis of cyanidin-based

and delphinidin-based anthocyanins. Furthermore, several alleles of the *VvmybA1* and *VvmybA2* genes are associated with the regulation of anthocyanins and tannins biosynthesis in grapes (Jiu *et al.*, 2021). A detailed functional characterization of the *VvmybA2r* and *VvmybA2w* genes as well as their potential regulatory networks involved in the formation of berry color has been reported by Jiu *et al.* (2021). Investigation of the *VvmybA2* gene and the epigenetic events in the promoter of the *VvmybA1* gene, as suggested by Xia *et al.* (2021), are potential focus areas for future studies to identify events related to changes in color to black, detected in the berry skin of the 'Brasil' and 'Black Star' cultivars.

CONCLUSIONS

Changes detected in the *VvmybA1* gene, which plays an important role in regulating anthocyanins biosynthesis in grapes, did not explain the color change to black in the berries of the 'Brasil' and 'Black Star' cultivars. The sequencing of the *VvmybA1* gene in the present study showed that the mutations that occurred in the cultivar 'Italia' for generating the cultivar 'Benitaka' (replacement of "TA" by "AG" at the 15,437 and 15,438 positions, "A" by "G" nucleotides at position 15,477 of the first intron, and "ATC" by "ACC" codon in the first exon of the *VvmybA1* gene) persisted in the 'Brasil' and 'Black Star' cultivars. Despite the detected differences in the *VvmybA1* gene, specific mutations that occurred only in the 'Brasil' and 'Black Star' cultivars with black berries could not be determined.

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