Ciência Téc. Vitiv. 33(2) 184-190. 2018

MOLECULAR CHARACTERIZATION OF BERRY COLOR LOCUS ON THE PORTUGUESE CV. 'FERNÃO PIRES' AND CV. 'VERDELHO' AND THEIR RED-BERRIED SOMATIC VARIANT CULTIVARS

CARATERIZAÇÃO MOLECULAR DO LOCUS DA COR DO BAGO NOS CULTIVARES PORTUGUESES 'FERNÃO PIRES' E 'VERDELHO' E SEUS CULTIVARES VARIANTES SOMÁTICOS ROSADOS

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(Received 26.01.2018. Accepted 21.12.2018)

SUMMARY

Genotyping studies are increasing the knowledge on grapevine biodiversity, particularly regarding grape berry skin color somatic variants, supporting the research on the color trait. This study aimed to evaluate the effect of the berry color locus, and its surrounding genomic region, on the color variation of the Portuguese white-skinned cultivars 'Fernão Pires' and 'Verdelho' and its derived red-berried somatic variants cv. 'Fernão Pires Rosado' and cv. 'Verdelho Roxo', respectively.
The analysis of *Gret1* insertion within the *VvMYBA1* gene revealed no polymorphism responsible for white-to-red shift of the red-skinned cv. 'Fernão Pires Rosado' and cv. 'Verdelho Roxo'. Moreover, *VvMYBA2* showed an important role regarding the phenotypic variation of cv. 'Fernão Pires', through the recovery of the functional allele G on cv. 'Fernão Pires Rosado'. Regarding the data obtained for cv. 'Verdelho' and cv. 'Verdelho Roxo', both cultivars showed *Gret1* insertion on *VvMYBA1* and non-functional T allele on *VvMYBA2* in homozygosity for both cell layers of shoot apical meristem, suggesting the occurrence of other mutational events responsible for the color gain.

RESUMO

Os estudos de genotipagem têm contribuído para o aumento do conhecimento acerca da biodiversidade de videira, em particular no que se refere a variantes somáticos para a cor do bago, cimentando a investigação sobre a caraterística da cor. Este estudo teve como objetivo avaliar o efeito do locus da cor do bago e região genómica adjacente na variação da cor de cultivares portuguesas de cor branca, 'Fernão Pires' e 'Verdelho', e os seus variantes somáticos rosados, cv. 'Fernão Pires Rosado' e cv. 'Verdelho Roxo', respetivamente.
A análise da inserção do retransposão *Gret1* no gene *VvMYBA1* não revelou nenhum polimorfismo responsável pela alteração de cor branca para rosada nos cultivares 'Fernão Pires Rosado' e 'Verdelho Roxo'. Além disso, o gene *VvMYBA2* revelou possuir um papel importante relativamente à variação fenotípica no cultivar 'Fernão Pires', através da recuperação do alelo funcional G no cultivar 'Fernão Pires Rosado'. Em relação aos dados obtidos para os cultivares 'Verdelho' e 'Verdelho Roxo', ambos apresentaram a inserção do *Gret1* no gene *VvMYBA1* e o alelo T não funcional no gene *VvMYBA2* em homozigotia para ambas as camadas celulares do meristema apical, o que sugere que a recuperação de cor se deve à ocorrência de outros eventos mutacionais

Key words: somatic variants, color locus, *MYBA1-2,* SSRs, cell layer.
Palavras-chave: variantes somáticos, locus da cor, *MYBA1-2,* SSRs, camada celular.

Somatic variation plays a crucial role in intravarietal grapevine diversity, generating novel interesting phenotypes. Due to the layered structure of the shoot apical meristem (SAM), a somatic mutation can spread throughout the entire SAM or remain restricted to the cell layer in which it occurred, giving rise to chimeras. Chimeras are thus composed by two genetically distinct tissue layers placed adjacent to one another and are usually selected for their distinguished phenotype (Einset and Pratt, 1954; Thompson and Olmo, 1963).

Among the spontaneous somatic mutations occurring in grapevine, those affecting the berry color locus are the most well studied at molecular level since they are relatively frequent events and occurred long ago in several cultivars, such as Pinot Noir and Cabernet Sauvignon (Walker *et al.*, 2006; Yakushiji *et al.*, 2006) and more recently in others such as Alfrocheiro Preto (Zanol *et al.*, 2011), Muscat of Alexandria (De Lorenzis *et al.*, 2015) and Tempranillo Tinto (Carbonell-Bejerano *et al.*, 2017).

Grape skin color results from the accumulation of anthocyanins both in epidermal and subepidermal cell layers and is genetically regulated by a major locus on chromosome 2. This berry color locus comprises two functional transcription factors, *VvMYBA1* and *VvMYBA2*, that induce the transcription of *VvUFGT* gene, a key point in the anthocyanins' biosynthetic pathway, and represents the major determinant for setting of berry skin color (Walker *et al.*, 2007; Fournier-Level *et al.*, 2010; Pelsy, 2010; Ferreira *et al.*, 2018b).

VvMYBbA1 gene silencing results from a *Gret1* retrotransposon insertion in its promotor region. The presence of *Gret1* retrotransposon in the promoter of the *VvMYBA1* gene was firstly described and associated with a loss-of-function in 'Italia' and 'Muscat of Alexandria' cultivars, being called VvmybA1a non-functional allele (Kobayashi *et al.*, 2004). The most likely original sequence of *VvMYBA1*, before the *Gret1* retrotransposon insertion, corresponds to the allele VvmybA1c, described as wild-type allele, that lacks *Gret1* completely. Regarding *VvMYBA2*, a non-synonymous single nucleotide polymorphism in the *VvMYBA2* coding region (VvMYBA2R44) leads to an amino acid substitution (change of arginine residue at position 44 in the red allele [G] altered to leucine in the white allele [T]), leading to a non-functional allele (Walker *et al.*, 2007). Other relatively frequent genetic event has been described for loss of pigmentation, which is a large deletion removing both *VvMYBA1* and *VvMYBA2* genes (Walker *et al.*, 2006; Yakushiji *et al.*, 2006; Vezzulli *et al.*, 2012; Migliaro *et al.*, 2017). Color recovery also results from different genetic alterations, namely by the partial excision of *Gret1* or by homologous recombination between *VvMYBA1* and *VvMYBA3* genes (This *et al.*, 2007; Azuma *et al.*, 2009)

Recently, several grapevine genotypes have been characterized at the color locus using a layer-specific approach and different evolutionary models have been established for the origin of berry skin color mutants (Vezzulli *et al.*, 2012; Migliaro *et al.*, 2017; Ferreira *et al.*, 2018a). The origin of a colorless berry skin mutant derived from a colored ancestor can be ascribed to two distinct models, based on the difference in the size of the chromosome deletion: a) the sequential model, named 'Cabernet Sauvignon' like (Walker *et al.*, 2006), and b) the parallel model, named 'Pinot' like (Vezzulli *et al.*, 2012). On the other hand, the 'Revertant' model has been described for the color gain, where the main mechanism involves the partial excision of the *Gret1* retrotransposon from the *VvMYBA1* promotor (Azuma *et al.*, 2009; De Lorenzis *et al.*, 2015).

Portugal has a long tradition on grapevine cultivation and even nowadays keep a great diversity of grapevine cultivars, with 343 cultivars legally accepted for wine production (MAMAOT, 2012), being 240 considered autochthonous (Cunha *et al.*, 2016). A molecular characterization using six nuclear microsatellite loci was performed by Veloso *et al.* (2010) on 313 accessions of grapevine from the Portuguese National Ampelographic Collection (CAN). This study allowed to identify eleven different sets of accessions with identical SSR allele patterns, which were identified as berry color somatic variants. The genetic background of berry skin color was previously evaluated in several of these skin color somatic variants (Malvasia Fina, Moscatel Galego and Pinot) through the analysis of *VvMYBA1* allelic variation, examining the presence, absence or excision of *Gret1* retrotransposon in the promotor region of the gene and through the SNP detection in the coding region of *VvMYBA2* gene (Ferreira *et al.*, 2017).

The objective of the present study was to evaluate, using a layer-specific approach, the effect of the genotype of the berry color locus and its surrounding genomic region on the color variation of four cultivars 'Fernão Pires' and 'Verdelho' and their derived colored cultivars 'Fernão Pires Rosado' and 'Verdelho'. 'Fernão' Pires is the most used green-yellow cultivar in Portugal. It is an old Portuguese cultivar mentioned in manuscripts before the XVIII century and has a recognized synonym, Maria Gomes. It has a long history of use in the Ribatejo

Portuguese DOC Region, but has a greater morphological diversity in the northern region of Bairrada suggesting that its cultivation started earlier there (Robinson *et al.*, 2012); it is currently also cultivated worldwide, namely in Australia, New Zealand, California and South Africa. Verdelho is a cultivar with no expression in Portugal mainland but is one of the most important cultivars in the Portuguese Atlantic islands. It is thought that Verdelho has been brought to Madeira from the Mediterranean island of Crete (region of Candia, the modern Heraklion). Being historically described with an excellent reputation due to its aromatic and fresh wines, it is nowadays grown in vineyards of New Zealand, USA, Argentina, South Africa and Romania, and mainly in Australia where was introduced from Madeira (OIV 2010).

MATERIAL AND METHODS

Plant material

The molecular mechanisms of color variation in grapevine somatic variants for berry skin color were determined in seven *Vitis vinifera sativa* cultivars, 'Fernão Pires', 'Verdelho' and 'Pinot Noir'and their derived somatic variants cv. 'Fernão Pires Rosado', cv. 'Verdelho Roxo', cv. 'Pinot Gris' and cv. 'Pinot Blanc', being the 'Pinot' group used as reference. Cultivar names, berry color and location are shown in Table I. According to their ancestry, cultivars were divided into two groups: (A) less pigmented/ unpigmented cultivars derived from a pigmented ancestor cultivar, and (B) pigmented cultivars derived from an unpigmented ancestor cultivar (Table I).

Genomic DNA extraction

The shoot apical meristem (SAM) of grapevine is composed by two different cell layers, L1 forming the epidermis and L2 making up most of the other parts of the plant, including mesophyll cells and gametes (Figure 1) (Vezzulli *et al.*, 2012; Migliaro *et al.*, 2014; Ferreira *et al.*, 2018a). Therefore, a layer-specific approach was performed in order to establish the molecular mechanisms behind color reversions. For that, two genomic DNA samples were isolated from each cultivar, namely from 100-200 mg of young leaf (L1+L2) and from 200-300 mg of woody shoot (L2). Leaf and pith woody shoot material were grounded using a TissueLyser II (Qiagen, Hilden, Germany) and DNA extraction was performed using the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. In each analysis, the genetic difference between the L1+L2 (leaf) and L2 (woody shoot pith) corresponds to the make-up of L1 cell layer.

Table I

List of plant material divided in two groups (A and B) based on the berry skin color of the ancestor cultivar

Lista do material vegetal dividido em dois grupos (A e B) com base na cor da película do bago do cultivar ancestral

Cultivar	Berry color[1]	Code	Repository#	Accession number
Group A				
Pinot Noir (ancestor)	B	PN	UTAD	F2.13.1
Pinot Blanc	W	PB	UTAD	F2.2.3
Pinot Gris	G	PG	UTAD	F2.13.2
Group B				
Fernão Pires (ancestor)	W	FP	INIAV/CAN	52810
Fernão Pires Rosado	R	FPR	INIAV/CAN	52815
Verdelho (ancestor)	W	V	UTAD	F2.10.2
Verdelho Roxo	R	VR	INIAV/CAN	51513

[1] B – Black; G – Grey; R – Red; W – White
UTAD - Universidade de Trás-os-Montes e Alto Douro (Vila Real, Portugal); INIAV - Instituto Nacional de Investigação Agrária e Veterinária/ CAN - Coleção Ampelográfica Nacional (Dois Portos, Portugal)

Molecular analyses

In order to confirm the trueness-to-type of the plant material, all cultivars were genotyped with a set of nine nuclear microsatellite markers (SSRs), including those recommended by OIV for the identification of grape varieties: VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79, VVMD28, VVMD32 and VVIv67. The amplification reactions were set up as multiplex PCRs allowing to detect the amplicons of the nine SSR loci in two single capillary electrophoresis, according to Castro *et al.* (2011).

From the genetic point of view, *VvMYBA1* and *VvMYBA2* gene polymorphisms were investigated to understand the effect of the berry color locus genotype on the color variation in cv. 'Fernão Pires', cv. 'Verdelho' and cv. 'Pinot' (as reference). The detection of functional and non-functional alleles for *VvMYBA1* (*Gret1* insertion and other length polymorphisms) and gene polymorphism (R44 SNP) for *VvMYBA2* was performed according to Carrasco *et al.* (2015).

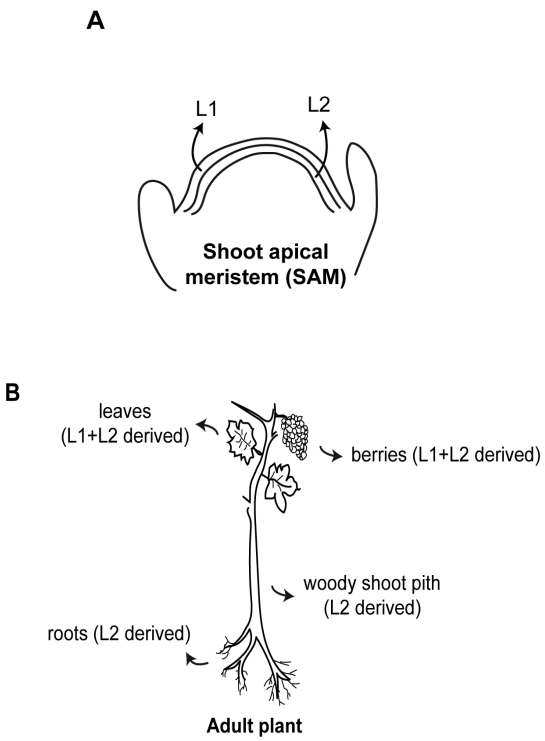

Figure 1. Schematic representation of the shoot apical meristem (SAM) organization in the context of tissue differentiation. A) Cells from the SAM of grapevine are arranged in layers. L1 and L2 represent two clonally distinct cell groups. B) Cartoon depicting the different plant tissues derived from their respective cell layers.

Representação esquemática da organização do meristema apical no contexto da diferenciação dos tecidos. A) As células do meristema apical em videira estão organizadas em camadas. L1 e L2 representam dois grupos de células distintos. B) Esboço com representação de diferentes tecidos vegetais derivados das suas respetivas camadas celulares.

Another set of 10 SSR markers (SC8_0146_010, SC8_0146_026, VVNTM1, VVNTM2, VVNTM3, VVNTM4, VVNMT5, VVNTM6, VVIU20, VMC7G3) surrounding the berry color locus (*VvMYBA1* and *VvMYBA2*) and distributed along the distal arm of chromosome 2 was also used to detect possible polymorphisms in this region (Migliaro *et al.*, 2017). The PCR conditions were employed as reported by Vezzulli *et al.* (2012). Capillary electrophoresis was carried out on ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA) at the Genomics Unit of the Parque Científico de Madrid (Madrid, Spain) and the fragments were sized with Peak Scanner V1.0 software (PE Applied Biosystems, CA, USA) using GeneScan 500 LIZ size standard as an internal ladder (Life Technologies).

RESULTS AND DISCUSSION

Genotyping by SSR markers

Nine SSR loci were used to genotype seven cultivars, cv. 'Fernão Pires', cv. 'Verdelho' and cv. 'Pinot Noir' and their derived somatic variant cv. 'Fernão Pires Rosado', cv. 'Verdelho Roxo', cv. 'Pinot Gris' and cv. 'Pinot Blanc', in order to ascertain the genetic identity of each cultivar and confirm their trueness-to-type.

The allelic profiles in the nine SSR loci amplified were the same for cv. 'Fernão Pires' and cv. 'Fernão Pires Rosado', cv. 'Verdelho' and cv. 'Verdelho Roxo' and among cv. 'Pinot Blanc', cv. 'Pinot Gris' and cv. 'Pinot Noir', confirming that the somatic variant cultivars and their relative ancestor cultivar share the same genetic profile (Supplementary Table I). In order to improve the management of the Portuguese Grapevine National Collection and update the list of cultivars officially authorized for wine production in Portugal, Veloso *et al.* (2010) performed a molecular characterization of 313 grapevine accessions. Among these accessions, 'Fernão Pires' and 'Fernão Pires Rosado', 'Verdelho' and 'Verdelho Roxo', 'Pinot Blanc', 'Pinot Gris' and 'Pinot Noir' were considered as distinct cultivars based on their berry color skin, although each set of accessions displayed identical SSR profiles. Nuclear SSR analysis has been used as an effective technique to identify grapevine varieties and evaluate genetic relationships. However, the reference SSR markers commonly used for grape varietal identification are not helpful to perform an accurate molecular characterization of berry skin color somatic variants once they are genetically identical to their original cultivars, which lead to the development of other molecular tools to distinguish them (Giannetto *et al.*, 2008; Vezzulli *et al.*, 2012). Therefore, on this study, a layer-specific genotyping system focused on berry color locus and surrounding genomic region was conducted for the molecular discrimination of the studied ancestor cultivars and their derived somatic variant cultivars.

Layer-specific genotyping at the berry color locus

Based on the analysis of *VvMYBA1* gene for the detection of the non-functional allele (with *Gret1* insertion, called VvmybA1a) and putative functional alleles, both white-skinned Portuguese cultivars, 'Fernão Pires' and 'Verdelho', and the white-skinned reference cv. 'Pinot Blanc', contained only the non-functional allele (VvmybA1a). The pigmented-skinned reference cultivars, 'Pinot Gris' and 'Pinot Noir' revealed both non-functional (VvmybA1a) and

functional (VvmybA1c) alleles, while the colored-skinned cultivars 'Fernão Pires Rosado' and 'Verdelho Roxo' showed only the non-functional allele (VvmybA1a) (Table II).

To further understand the genetic basis of cv. 'Fernão Pires Rosado' and cv. 'Verdelho Roxo' berry skin color, *VvMYBA2* SNP polymorphisms were also examined. Like what happened for *VvMYBA1* gene, all the white-skinned ancestor cultivars (cv. 'Fernão Pires' and cv. 'Verdelho') showed only the non-functional allele that, in the case of the *VvMYBA2* gene, corresponds to the SNP T at the VvMYBA2R44 position. The presence of the functional allele G at VvMYBA2R44 position detected in the reference cultivars 'Pinot Noir' and 'Pinot Gris', was also detected in the cv. 'Fernão Pires Rosado', allowing to explain the color gain of this red-berried somatic variant cultivar, derived from its white-skinned ancestor cultivar 'Fernão Pires' (Table II).

Table II

VvMYBA1 and *VvMYBA2* allelic polymorphisms

Polimorfismos alélicos dos genes VvMYBA1 e VvMYBA2

Group	Cultivar code	Berry color[1]	*VvMYBA1*		*VvMYBA2*
			VvmybA1a Gret1	*VvmybA1c No Gret1*	*VvMYBA2R44*
A	PN	B	x	x	T/G
	PG	G	x	x	T/G
	PB	W	x	-	T/T
B	FPR	R	x	-	T/G
	FP	W	x	-	T/T
	VR	R	x	-	T/T
	V	W	x	-	T/T

[1] B – Black; G – Grey; R – Red; W – White

Considering both genes (*VvMYBA1* and *VvMYBA2*) Fournier-Level *et al.* (2010) define three different haplotypes, considering the presence or absence of the *Gret1* insertion in the promotor region of *VvMYBA1*, and the presence of a functional G allele or a mutated/ non-functional T allele in VvMYBA2R44 position: haplotype 'colored' (N), 'altered color' (Rs) or 'white' (B). The control colored cultivars, 'Pinot Noir' and 'Pinot Gris', both hold the *Gret1* insertion and a putative functional allele at *VvMYBA1* (VvmybA1c) and a functional G allele at VvMYBA2R44, corresponding to the haplotype Rs. The profile of the cv. 'Fernão Pires Rosado' revealed a low frequency haplotype, recombined "white" (Rec), holding the *Gret1* insertion and a functional G allele at VvMYBA2R44, resulting in an altered color (red), as previously described for the cv. 'Malvasia Fina Roxo' by Ferreira *et al.* (2017). The white-skinned Portuguese cultivars, 'Fernão Pires' and 'Verdelho', as well as the white-skinned reference cultivar 'Pinot Blanc', were consistent with the haplotype 'B'. Surprisingly, also the red-berried cultivar 'Verdelho Roxo', revealed this 'white' haplotype.

Since grapevine berry develops from different layers of the apical meristem, a layer-specific approach was applied in order to determine the genetic background of skin color somatic variant cultivars, as well as, understand the evolutionary events leading to their origin. This approach allowed the identification of deletions with different length and position between the reference cultivars of 'Pinot' group. These deletions affected only the inner cell layer in the less pigmented derived somatic variant cv. 'Pinot Gris', and both cell layers in the unpigmented derived somatic variant cv. 'Pinot Blanc' (Table III), as previously described for the first time by Vezzulli *et al.* (2012), which lead to the description of the Parallel evolutionary model, where the grey and white-skinned derived somatic variant cultivars of a specific pigmented ancestor cultivar arose independently. However, the layer-specific approach applied does not discriminate the Portuguese cultivars 'Verdelho' and 'Verdelho Roxo'. No genetic difference was found when L1 + L2- and L2-derived tissues were compared between the unpigmented ancestor cultivar 'Verdelho' and its red-berried derived somatic variant cv. 'Verdelho Roxo', as it has been previously described for the cv. 'Chasselas Violet' and cv. 'Sauvignon Rouge' and their unpigmented ancestor cultivars, 'Chasselas Blanc' and 'Sauvignon Blanc' (Migliaro *et al.*, 2017). These results might suggest the existence of additional loci controlling grape berry skin color or the occurrence of different mutational events responsible for the color gain. Additionally, the loci were homozygous and monomorphic along an extensive genomic region on the distal arm of chromosome 2 (Table III). This homozygosity has been previously described for other skin color somatic variants derived from an unpigmented ancestor, which was associated with a selective sweep of this genomic region (Migliaro *et al.*, 2017).

Table III

Genetic profile of berry color locus and its surrounding genomic region of group A and B cultivars. The grey background indicates the deleted region and the pink one indicates the putatively homozygous regions. he – heterozygous; ho – homozygous; *Gret1* – non-functional allele; ? – undetected functional allele; Non-*Gret1* – functional allele.

Perfil genético do locus da cor do bago e região genómica adjacente nos cultivares do grupo A e B. O fundo cinzento indica a região deletada e o rosa, regiões putativamente homozigóticas. he – heterozigótico; ho – homozigótico; *Gret1* – alelo não funcional; ? – alelo funcional não detetado; Non-*Gret1* – alelo funcional.

				Molecular marker and genomic coordinate on chromosome 2 (in Mb)													
	Cultivar code	Layer	Berry color[1]	SC8_010 12674	SC8_026 12970	VVNTM1 14149	VVNTM2 14151	VvMYBA2 14181		VvMYBA1 14248		VVNTM3 14288	VVNTM5 14325	VVNTM6 14330	VVNTM4 14384	VVIU20 16539	VMC7G3 18270
Group A	PN	L1+L2	B	he	ho	he	he	T	G	*Gret1*	Non-*Gret1*	he	he	ho	ho	he	he
		L2		he	ho	he	he	T	G	*Gret1*	Non-*Gret1*	he	he	ho	ho	he	he
	PG	L1+L2	G	he	ho	he	he	T	G	*Gret1*	Non-*Gret1*	he	he	ho	ho	he	he
		L2		he	ho	ho	ho	T	T	*Gret1*	*Gret1*	ho	ho	ho	ho	ho	ho
	PB	L1+L2	W	he	ho	ho	ho	T	T	*Gret1*	*Gret1*	ho	ho	ho	ho	he	he
		L2		he	ho	ho	ho	T	T	*Gret1*	*Gret1*	ho	ho	ho	ho	ho	he
Group B	FPR	L1+L2	R	ho	ho	ho	ho	T	G	*Gret1*	?	ho	ho	ho	ho	ho	he
		L2		ho	ho	ho	ho	T	G	*Gret1*	?	ho	ho	ho	ho	ho	he
	FP	L1+L2	W	ho	ho	ho	ho	T	T	*Gret1*	*Gret1*	ho	ho	ho	ho	ho	he
		L2		ho	ho	ho	ho	T	T	*Gret1*	*Gret1*	ho	ho	ho	ho	ho	he
	VR	L1+L2	R	he	he	ho	ho	T	T	*Gret1*	?	ho	ho	ho	ho	ho	ho
		L2		he	he	ho	ho	T	T	*Gret1*	?	ho	ho	ho	ho	ho	ho
	V	L1+L2	W	he	he	ho	ho	T	T	*Gret1*	*Gret1*	ho	ho	ho	ho	ho	ho
		L2		he	he	ho	ho	T	T	*Gret1*	*Gret1*	ho	ho	ho	ho	ho	ho

[1] B – Black; G – Grey; R – Red; W – White

CONCLUSIONS

The experimental data obtained suggest that *VvMYBA2* gene might be playing a fundamental role for color gain in cv. 'Fernão Pires Rosado' through the recovery of functional allele G on VvMYBA2R44 position in relation to its white-berried ancestor cultivar 'Fernão Pires'. On the other hand, *VvMYBA1* and *VvMYBA2* solely do not explain the pigmented phenotype of cv. 'Verdelho Roxo' and further experiments should be done in order to understand the origin behind this phenotypic somatic variant.

ACKNOWLEDGMENTS

This work was supported by European Investment Funds by FEDER/COMPETE/POCI – Operational Competitiveness and Internationalization Programme, under Project POCI-01-0145-FEDER-006958, and National Funds by FCT - Portuguese Foundation for Science and Technology, under the project UID/AGR/04033/2013. V. Ferreira is also indebted to FCT for her grant (SFRH/BD/96400/2013). Work in R. Arroyo-García lab is funded by the Project Grants RTA2014-00029-C03-01. The authors would like to thank to INIAV (Instituto Nacional de Investigação Agrária e Veterinária) and UTAD (Universidade de Trás-os-Montes e Alto Douro) for kindly providing the plant material.

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