Scientia Horticulturae 244 (2019) 88–93

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Genetics and expression of anthocyanin pathway genes in the major skin-pigmented Portuguese cultivar 'Vinhão' developing berries

Vanessa Ferreira[a,b,*], Isaura Castro[a], David Carrasco[b], Olinda Pinto-Carnide[a], Rosa Arroyo-García[b]

[a] Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro, 5000-801 Vila Real, Portugal

[b] Centre for Plant Biotechnology and Genomics (UPM-INIA, CBGP), Campus de Montegancedo, Autovía M40 km. 38, 28223 Pozuelo de Alarcón, Madrid, Spain

ARTICLE INFO

Keywords:
Vinhão
Berry skin color
MYBA1/A2
Gene expression

ABSTRACT

'Vinhão' is an autochthonous Portuguese cultivar with an intense black-bluish skin color, highly appreciated due to this feature. This study aimed to give the first insights into the genetic background that may be responsible for the skin color properties of cv. 'Vinhão'. For this purpose, the allelic composition of *MYBA1* and *MYBA2* genes was investigated, along with quantification of the expression levels of structural and regulatory genes involved in the anthocyanin biosynthetic pathway via qRT-PCR. The molecular characterization of *MYBA1* and *MYBA2* loci revealed that cv. 'Vinhão' is homozygous for the functional allele in both genes, corresponding to the most ancestral haplotype, which is consistent with the high colored phenotype that characterizes this cultivar. There were no differences in the DNA sequence of the *MYBA1* promoter region between cv. 'Vinhão' and the grapevine reference genome Pinot Noir. The expression patterns of genes playing key functional roles in anthocyanin biosynthesis was analyzed in four developmental stages. The dynamics occurring throughout grape berry development revealed the involvement of these genes in the progression of key development events, mainly from veraison to mature berries. These findings provide the first molecular characterization focused on the skin color feature of cv. 'Vinhão' to improve our understanding of the genetics behind its intense skin pigmentation.

1. Introduction

'Vinhão' is a high quality autochthonous cultivar and represents the most used cultivar in Vinhos Verdes and Lafões Portuguese DOC regions for red wine production. In Portugal, it is also known as Sousão in Douro DOC region, which corresponds to the main national synonym for this red cultivar. In Spain, cv. 'Vinhão' is cultivated in Galicia region under the name 'Sousón' (Castro et al., 2012, 2011; Martín et al., 2006). As reported by Fonseca (1791), the possible expansion of cv. 'Vinhão' through the provinces of Minho and Douro in Portugal and Galicia in Spain, could have occured during the middle of the 19th century by Galician people that worked in Douro viticulture due to the demand for workers generated by the increasing exportation of Porto wine to England. Although this red cultivar has been neglected either by producers or by the wine consumers in the past, nowadays, has gain some expression in the current market mainly due its color properties (intense black-bluish skin color), which are highly appreciated once adds intense color to the wines.

In grapevine, the berry color is mostly determined by the presence or absence of anthocyanins, usually in the epidermal and hypodermal layers of the berry, being colorless in the flesh. However, anthocyanin synthesis is also activated in the flesh of some grapevine cultivars, which are called *teinturier* (dyer) cultivars (Falginella et al., 2012), such as cv. 'Alicante Bouschet'. Anthocyanin synthesis starts during veraison and is part of the flavonoid pathway that also produces flavonols, catechins, and proanthocyanidins through specific enzymes that use the same metabolic intermediates.

The main anthocyanidins synthesized in grapes are cyanidin and peonidin (di-substituted in the lateral B-ring) and delphinidin, petunidin and malvidin (tri-substituted), which are synthesized via different branches of the pathway. The flavonoid 3′,5′-hydroxylase (F3′5′H) enzymes compete for substrates with the similar enzyme flavonoid 3′-hydroxylase (F3′H). If F3′5′H activity prevails over F3′H, the products of flavonoid hydroxylases are predominately driven into the branch of the pathway that leads to the synthesis of delphinidin (blue purple derivatives) at the expense of those channeled into the synthesis of

* Corresponding author at: Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro, 5000-801 Vila Real, Portugal.

E-mail address: vanessa.cm.ferreira29@gmail.com (V. Ferreira).

https://doi.org/10.1016/j.scienta.2018.09.023
Received 1 May 2018; Received in revised form 25 August 2018; Accepted 11 September 2018

cyanidin (red derivatives) (De Lorenzis et al., 2016; Falginella et al., 2012).

Extensive molecular studies have been focused on grape color variation through the analysis of several genes of the anthocyanin biosynthetic pathway, including transcription factors with regulatory function on structural genes of the pathway. Among the structural genes, *V. vinifera* UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*VvUFGT*) revealed to be a master switch in the control of berry color, strictly regulated by two very similar adjacent transcription factors, *MYBA1* and *MYBA2* (Ageorges et al., 2006; Kobayashi et al., 2004; Lijavetzky et al., 2006; This et al., 2007; Walker et al., 2007). These genes are inherited together and are often considered as part of a single large locus called by 'berry color locus' (Carrasco et al., 2015). The occurrence of different mutations that leads to the disruption of these two genes conducted to several different phenotypes, namely to the unpigmented phenotype of most white-skinned cultivars, but also other color shades of grey, pink, red or black. *MYBA1* gene inactivation occurs through the insertion of the *Gret1* retrotransposon in its promotor region and different alleles have been described: *VvmybA1a* (non-functional allele) containing the complete *Gret1* retrotransposon inserted upstream of the *MYBA1* coding sequence; *VvmybA1b* (functional allele) harboring a single copy of the *Gret1* 3′-LTR region (solo 3′-LTR) in the 5′-flanking region near the coding sequences of *MYBA1* as a consequence of the retrotransposon partial excision, which allows the gene expression; *VvmybA1c* that completely lacks the *Gret1* retrotransposon insertion is considered the wild-type functional allele, prior to the insertion of *Gret1*; *VvmybA1d*, the null allele, which arises from the complete deletion of the *MYBA1* gene region (Azuma et al., 2007; Kobayashi et al., 2004; Yakushiji et al., 2006). *MYBA2* gene is inactivated by two non-conservative mutations: i) SNP at position VvMYBA2R44 (or K980) in the coding sequence (T instead of G) that leads to an amino acid substitution (change of arginine at in the red allele to leucine in the white allele), rendering non-function; ii) frame shift resulting in a smaller protein (dinucleotide deletion altering the reading frame at the amino acid position 258) (Walker et al., 2007). Moreover, the altered pigmentation of berries' skin can be affected by different mutation patterns on the skin cell layers of the shoot apical meristem (Ferreira et al., 2018; Migliaro et al., 2017; Vezzulli et al., 2012).

'Vinhão' is a well-defined cultivar from the ampelographic and chemical point of view, with a clearly visible accumulation of anthocyanins in its berry skin, which significantly affect wine quality (Dopico-García et al., 2008; Teixeira et al., 2016). However, the molecular causes of the berry skin color trait on cv. 'Vinhão', with an extraordinary capacity to extract color to must, dying like no other Portuguese cultivar, have never been investigated before. The present work aims to characterize cv. 'Vinhão' at different genetic levels related with berry skin color: *MYBA1* and *MYBA2* genes polymorphisms and transcriptional regulation during berry development of genes involved in the anthocyanin biosynthetic pathway, intending to add more value to this extremely pigmented cultivar both from a historical and a scientific point of view.

2. Material and methods

2.1. Berry sampling

Grapevine berries of *Vitis vinifera* L. cv. 'Vinhão' (clone VN0249) were sampled in a grapevine germplasm collection from North of Portugal at 2015, the ampelographic collection of the 'Vinhos Verdes Region Viticulture Comission' (CVRVV) 'Estação Vitivinícola Amândio Galhano' in Arcos de Valdevez (41°81′N,8°41′W), inside 'Vinhos Verdes' DOC Region. Three replications were collected during fruit development, in four sampling dates, corresponding to the following developmental stages: green soft (berries beginning to touch), veraison (berries changes color), advanced ripening berries and full maturation (berries ripe for harvest). Each replication contained at least 15 berries randomly picked from 10 vines. Berries were immediately frozen in liquid nitrogen in the vineyard and then stored at −80 °C until being processed.

2.2. Varietal identity through microsatellite analysis

Genomic DNA was isolated from young leaves using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Twelve nuclear microsatellite markers were studied using two multiplex PCRs involving VVS2, VVMD5 andVVMD7 for set A, and VVMD27, ssrVrZAG62 and ssrVrZAG79 for set B. The remaining six microsatellites were amplified by individual PCR (VVMD28, VVMD32, VVIv37, VVIv67, VVIp31 and VMC4f3), according to the method of Castro et al. (2011).

2.3. PCR analysis of MYBA1 gene structure and sequencing

Two PCR assays were used in order to determine the presence or absence of the *Gret1* retrotransposon in the *MYBA1* promoter region. The presence of *Gret1* retrotransposon in the *MYBA1* promoter region (*VvmybA1a* allele, non-functional) was detected using the primers F1 (5′- AAAAAGGGGGGCAATGTAGGGACCC- 3′) and d3 (5′−CCTGCAG CTTTTTCGGCATCT- 3′) and PCR amplifications were performed as reported in Lijavetzky et al. (2006). PCR reactions to amplify putative functional alleles were assessed with FD2 (5′- TAGCTGCTGCCACTGC ATAG- 3′) and R1 (5′-GAACCTCCTTTTTGAAGTGGTGACT- 3′) primers, as recommended by Azuma et al. (2008). PCR fragments were separated by electrophoresis in 1.5% (w/v) agarose gel in TBE buffer, stained with ethidium bromide and photographed under UV light.

PCR amplification to isolate *MYBA1* promotor region and part of the coding sequence was performed using the FD2 and R1 primers as described above. PCR fragments were separated as previously described and purified using the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel - Fischer Scientific, Düren, Germany). PCR amplicons resulting from amplification were directly sequenced at STABVIDA company (http://www.stabvida.com). Sequence analysis and alignment were performed using the SnapGene® software v.2.7.3 (GSL Biotech, Chicago, IL, USA; http://snapgene.com). The alignments of reverse and forward sequences were applied to produce consensus sequences. The amplicon sequences were searched by BLAST-N against the genome assembly.

2.4. MYBA2 polymorphism through SNaPshot assay

For *MYBA2* gene, the single nucleotide polymorphism (SNP), VvMYBA2R44 (K980) was investigated by a SNaPshot assay, according to the protocol reported in the ABI PRISM SNaPshot Multiplex kit (Life Technology Corporation, Foster City, CA, USA). PCR fragments were separated by electrophoresis in 0.8% (w/v) agarose gel in TBE buffer, stained with ethidium bromide, and purified by QIAquick Gel Extraction Kit (QIAGEN, Düren, Germany) according to the manufacturer's instructions. The SNaPshot PCR products were enzymatically treated with 1 U each of calf intestinal alkaline phosphatase (CIP; New England Biolabs, Beverly, MA, USA) to degrade excess PCR primers and dNTPs and incubated at 37 °C for 1 h, followed by 15 min at 75 °C to inactivate the enzyme. The purified SNaPshot PCR products were detected on capillary electrophoresis instrument ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) at the Genomics Unit of the Madrid Science Park (fpcm.es/en/servicios-cientificos) and data analysis was performed by Peak ScannerTM Software v1.0 (Applied Biosystems, Foster City, CA, USA).

2.5. RNA isolation and complementary DNA synthesis

For gene expression studies, total RNA was extracted from berry skins according to the procedures described by Reid et al. (2006).