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Spontaneous variation regarding grape berry skin color: A comprehensive study of berry development by means of biochemical and molecular markers

Vanessa Ferreira[a,b,*], Fátima Fernandes[c], David Carrasco[b], Marivel Gonzalez Hernandez[d], Olinda Pinto-Carnide[a], Rosa Arroyo-García[b], Paula Andrade[c], Patrícia Valentão[c], Virgílio Falco[e], Isaura Castro[a]

[a] *Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas (CITAB), Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal*
[b] *Centro de Biotecnología y Genómica de Plantas (CBGP), Campus de Montegancedo, Autovía M40, km38, 28223, Pozuelo de Alarcón, Madrid, Spain*
[c] *REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, R. Jorge Viterbo Ferreira, no 228, 4050-313 Porto, Portugal*
[d] *Ciencias Química y Sensorial Enológica, Instituto de Ciencias de la Vid y el Vino, Universidad de La Rioja, Complejo Científico Tecnológico - Madre de Dios 51, 26006 Logroño, Spain*
[e] *Centro de Química de Vila Real (CQ-VR), Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal*

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ABSTRACT

Understanding grape berry development and the metabolism of different classes of compounds responsible for traits like berry's color is imperative to control and improve quality aspects of grapes. A colorimetric, biochemical and molecular characterization allowed the comprehensive description of the pigment-related characteristics of nine berry skin color somatic variants, belonging to four different varieties. Although the observed berry skin color variability was not fully explained by *MybA* locus, the phenolic profiles allowed inferring about specific interferences among the biosynthetic pathways. Data were consistent concerning that grapes showing cyanidin-3-*O*-glucoside as the major anthocyanin and flavonols with two substituent groups in the lateral B-ring are generally originated by a white ancestor. After retro-mutation, these grapes seem to keep the dysfunction on flavonoid hydroxylases enzymes, which negatively affect the synthesis of both flavonols and anthocyanins with three substituent groups in the lateral B-ring. Overall, the obtained results indicate that the color differences observed between somatic variants are not solely the result of the total amount of compounds synthesized, but rather reflect a different dynamics of the phenolic pathway among the different color variants of the same variety.

Chemical compounds: Gallic acid (PubChem CID: 370); Caftaric acid (PubChem CID: 6,440,397); Catechin (PubChem CID: 73,160); Epigallocatechin gallate (PubChem CID: 65,064); Quercetin-3-*O*-galactoside (PubChem CID: 5,281,643); Quercetin-3-*O*-glucoside (PubChem CID: 25,203,368); Malvidin-3-*O*-glucoside (PubChem CID: 443,652); Peonidin-3-*O*-*p*-coumaroylglucoside (PubChem CID: 44,256,849); Malvidin-3-*O*-*p*-coumaroylglucoside (PubChem CID: 44,256,988); Resveratrol-3-*O*-glucoside (PubChem CID: 25,579,167).

1. Introduction

Grapevine berries are complex organs formed by diverse tissues that follow a development pattern typical of non-climacteric species. As a non-climacteric fruit, grape berry displays a double sigmoidal growth curve with two rapid growth periods separated by a lag phase (Castellarin et al., 2015). The onset of ripening is a short period known as *véraison* that marks the boundary between the lag phase and the second growth period after which growth declines. *Véraison* is generally characterized by several processes, such as berry softening, acids decrease, sugar accumulation, loss of photosynthetic capacity and initiation of color development (Robinson & Davies, 2000).

Since the beginning of grapevine domestication, berry skin color has been used for cultivar characterization and became greatly diversified. Variants with different skin color, including black, red, pink, grey and white (yellow–green) have arisen as a result of natural hybridization and human selection over millennia (Alcalde-Eon, García-Estévez, Martín-Baz, Rivas-Gonzalo, & Escribano-Bailón, 2014; Azuma et al., 2008; Rustioni, De Lorenzis, Hârţa, & Failla, 2016). This morpho-physiological diversity regarding grape berry skin color could be

* Corresponding author at: Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas (CITAB), Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal.
E-mail address: vanessa.cm.ferreira29@gmail.com (V. Ferreira).

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spontaneously generated by genetic alterations, namely somatic polymorphisms during plant growth, which can include duplications, insertions or minor changes, such as SNPs (Torregrosa et al., 2011), mainly caused by the activity of transposable elements (Carrier et al., 2012). In fact, there are many examples of other spontaneous variations including flavor, early or late ripening, size and compactness of bunches, canopy growth or yield that have been identified in multiple varieties (Torregrosa et al., 2011). Ultimately, grapevine somatic variants could be useful to investigate gene biological function, because they result from the effect of single mutation events in a given genetic background. This kind of knowledge could also avoid undesirable changes, such as morphological mutations that can occur. Therefore, somatic variants are an unique resource for both functional genomics and breeding and should be considered a possible solution to ethical problems surrounding genetically modified organisms or interspecific grapevine crosses (Torregrosa et al., 2011). In several countries, some grapevine somatic mutants are more widespread than the corresponding wild type variety, namely Pinot Gris, Pinot Meunier or the berry color somatic variants of Cabernet Sauvignon, which give the opportunity to produce some unique wines (Migliaro et al., 2014; Torregrosa et al., 2011).

At the molecular level, the most well-documented polymorphisms leading to various phenotypes within varieties are those that affect berry color. These color differences are determined by the composition and quantity of phenolic compounds, particularly anthocyanins, which are one of the most important plant pigments (Azuma et al., 2008). Anthocyanins start to be synthesized during the onset of ripening and are gradually accumulated in the berry skin throughout grape ripening, although their concentration may decrease slightly just before harvest and/or during over-maturing (Robinson & Davies, 2000). These pigments' profile and concentration largely vary, depending on the grapevine cultivar (Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006), and are not even synthesized by white-berried ones (Boss, Davies, & Robinson, 1996). The main anthocyanins found in grapes are derived from cyanidin, peonidin, delphinidin, petunidin and malvidin. They generally occur as glycosides and acylglycosides, being malvidin-3-*O*-glucoside the most abundant in almost all colored grape cultivars (Fraige, Pereira-Filho, & Carrilho, 2014). Anthocyanins synthesis follow the same multi-branched of phenylpropanoid biosynthetic pathway and UDP glucose-flavonoid 3-O– glucosyltransferase (UFGT) represents the key enzyme of this pathway, which is regulated by two Myb-related genes (Boss et al., 1996; S. K. Kobayashi, Ishimaru, Hiraoka, & Honda, 2002). It was shown that the insertion of *Gret1* retrotransposon in *VvMybA1* promotor gene and single nucleotide polymorphisms (SNP) in *VvMybA2* gene are associated with the loss of pigmentation in white-skinned cultivars of *Vitis vinifera* (S. Kobayashi, Goto-Yamamoto, & Hirochika, 2004; Walker et al., 2007).

The aim of this work was to characterize, through the analysis of grape development, a set of several grape berry skin color somatic variants (previously confirmed by microsatellites), comprising different groups of colored and non-colored related cultivars, that show clear differences in the phenolic biosynthesis. To highlight the changes that occur during berry development, particularly regarding berry color, between the skin color somatic variants analyzed, a surface color analysis by CIELab colorimetric measurement was applied. A detailed HPLC-DAD analysis was also performed to study the phenolic profile, as well as, a molecular characterization of the genetic structure of berry color locus (*VvMybA1* and *VvMybA2* genes).

2. Material and methods

2.1. Grape cultivars and sampling

Vines of *Vitis vinifera* L. belonging to four different varieties (Malvasia Fina, Moscatel Galego, Pinot and Pique-poul), each one comprising related white [Malvasia Fina (MF), Moscatel Galego Branco (MGB), Pinot Blanc (PB)] and colored-skin berried cultivars [Malvasia Fina Roxo (MFR), Moscatel Galego Roxo (MGR), Pinot Gris (PG), Pinot Noir (PN), Pique-poul Gris (PPG) and Pique-poul Noir (PPN)], were grown in the same plot, in the experimental vineyard of the University of Trás-os-Montes and Alto Douro, Vila Real (41°19′ N, 7°44′ W, 500 m above mean sea level), Baixo Corgo sub-region of the Demarcated Douro Region, northern Portugal. This vineyard was installed in 1995 and the distance between the plants and rows was 1.8 m and 1.2 m, respectively. Each cultivar was represented by six plants. All vines were grown under the same cultivation practices (spraying of crop protectants, weed control, shoot guiding). The identity of these cultivars as true berry skin color somatic variants was previously confirmed through the analysis of nuclear microsatellite markers (SSRs) (Ferreira et al., 2016).

Considering the limited number of vines for each cultivar and to assure bunches in perfect conditions until the end of maturation, one bunch was collected by cultivar in each sampling date of 2013 season, comprising four developmental stages: green (G), *véraison* (V), ripe (R) and harvest (H). Subsequent samplings were performed on a different vine. Small portions of each bunch were uniformly separated for the analysis of the several parameters (contents of sugars, organic acids, metals and phenolic compounds). The samples were properly separated and labeled in plastic bags onto ice and kept frozen at − 20 °C until use.

For molecular analyses, young leaves were collected from each skin color somatic variant, properly labeled and conserved at − 80 °C until use.

2.2. Chemical analyses

Oenological properties, such as berry weight and pH were evaluated on 30 berries simultaneously. Individual berry weight was then measured as the average of the total weight obtained for the 30 berries. Contents of sugars, organic acids, metals and phenolic compounds were also determined for the four developmental stages analyzed.

2.2.1. Sugars and organic acids

D-glucose and D-fructose concentration was determined by an enzymatic procedure (D-glucose and D-fructose test kit AK00041; NZYTech, Lisbon, Portugal).

Tartaric acid was determined by the metavanadate colorimetric procedure. Tartaric acid reacted with ammonium metavanadate in a 30% *v*/v acetic acid solution to yield an orange-yellow color. Absorbance measurements were performed at 500 nm on a Hitachi U-2000 Double Beam Spectrophotometer. The calibration curve was established by reading the absorbance of standard solutions of tartaric acid (1–5 g/L) in the same spectrophotometer. Moreover, L-Malic acid was determined by an enzymatic procedure (L-malic acid test kit AK00011; NZYTech, Lisbon, Portugal).

2.2.2. Calcium, magnesium and potassium

Calcium (Ca), magnesium (Mg) and potassium (K) were determined by atomic absorption spectrophotometry in a Thermo iCE 3300 apparatus (Thermo Fisher Scientific, Cambridge, UK). Potassium and magnesium were measured at 766.5 nm and 285.2 nm, respectively, in a dilution of 1:200, and calcium was measured at 422.7 nm in a dilution of 1:40. Cesium chloride (0.1%) was used as ionization suppressor to potassium determination, and strontium chloride (1.0%) was used to minimize interference by phosphates in calcium determination. Calibration curves were established by reading the absorbance of standard solutions of each element (K: 2–20 mg/L Mg: 0.1–1 mg/L Ca: 1–10 mg/L).

2.2.3. Phenolic compounds

Healthy berries from each sample were lyophilized (Virtis SP Scientific Sentry 2.0 Apparatus, Gardiner, NY, USA) and then powdered in an appliance mill (model A327R1, Moulinex, Spain). The powdered

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