Universidade de Trás-os-Montes e Alto Douro

Grape berry color variation: genomic and metabolomic analysis

Tese de Doutoramento em Genética Molecular Comparativa

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GRAPE BERRY COLOR VARIATION: GENOMIC AND METABOLOMIC ANALYSIS

The present PhD Thesis was carried out expressly for obtaining a PhD in "Genética Molecular Comparativa" at University of Trás-os-Montes and Alto Douro, in accordance with the provisions of Decree-Law No. 74/2006, of March 24, altered by Decrees-Law No. 107/2008, of June 25, and 230/2009, of September 14, rectified by the declaration No. 81/2009, of October 27; according to the Dispatch No. 5505/2013.

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UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

GRAPE BERRY COLOR VARIATION:

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PEER REVIEWED PUBLICATIONS

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The PhD candidate was involved on the study design and sampling of the plant material, performed the experiments, analyzed and interpreted the data, as well as, on writing the scientific articles included in the Thesis as chapters. The candidate was also author of the bibliographic introductory review and of the final chapter where the main conclusions and outlook of the study are summarized.

To accomplish with the main and specific objectives projected for this PhD Thesis, the following articles (published or submitted for publication) have been included:

***Chapter 1*: Vanessa Ferreira**, Olinda Pinto-Carnide, Rosa Arroyo-García, Isaura Castro. Berry color variation in grapevine as a source of diversity. *Plant Physiology and Biochemistry*. 2018; 132:696-707 (DOI: 10.1016/ j.plaphy.2018.08.021).

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***Chapter 4*: <u>Vanessa Ferreira</u>**, José Tomás Matus, Olinda Pinto-Carnide, David Carrasco, Rosa Arroyo-García, Isaura Castro. Genetic analysis of a white-*to*-red berry skin color reversion and its transcriptomic and metabolic consequences in grapevine (*Vitis vinifera* cv. 'Moscatel Galego'). *BMC Genomics* (Submitted for publication – Under review).

RESUMO

A videira (*Vitis vinifera* L.) é uma das mais antigas culturas perenes domesticadas no mundo e tem sido extensamente cultivada e apreciada tanto pelos seus frutos como pelo vinho. Ao longo do processo de domesticação de *Vitis vinifera* subsp. *sylvestris* para *Vitis vinifera* subsp. *sativa*, um processo evolutivo complexo e de longa duração levou a alterações dramáticas na biologia da uva. Desde cedo, os vitivinicultores selecionaram os fenótipos de videira capazes de assegurar uma maior e mais regular produção e qualidade do fruto, mantendo-os através de propagação vegetativa, multiplicando assim, genótipos altamente desejáveis. No entanto, também ocorreram cruzamentos naturais entre as novas cultivares introduzidas e o germoplama local, juntamente com o aparecimento de eventos somáticos.

Uma das maiores contribuições para a diversidade existente na videira cultivada foi o aparecimento de mutações somáticas que afetam a cor do bago levando a vários fenótipos. De facto, esta característica tem sido usada como base de seleção em programas de melhoramento devido à sua influência sobre os vitivinicultores, enólogos e consumidores, representando um importante fator económico nesta cultura.

As uvas não são obviamente só tintas ou brancas, em vez disso apresentam uma enorme seleção de cores variando desde uvas esbranquiçadas, amarelas, verdes, a rosadas, rosa-pálido, e até vermelhas mais escuras, roxas e pretas. Este grande intervalo de cor não pode ser explicado pela simples presença de um determinado grupo de moléculas, assim, o presente estudo destinou-se a aprofundar o conhecimento atual sobre como a variação da cor do bago é afetada pela síntese de compostos fenólicos e os seus fatores genéticos subjacentes.

Primeiramente, foi efetuada a identificação de mutantes para a cor do bago num conjunto de germoplasma contendo vinte e cinco acessos de videira através da genotipagem com doze *loci* de microssatélites. Entre os onze grupos de putativos mutantes para a cor do bago genotipados, nove acessos, agrupados em quatro famílias diferentes, foram identificados como verdadeiros mutantes para a cor, incluindo variantes com película de cor tinta, roxa ou rosada e branca derivados de uma única variedade. O perfil fenólico dos mutantes para a cor do bago confirmados revelou que estes podem ser distinguidos de acordo com a sua composição em compostos não-corados e antocianinas. Além disso, este

trabalho beneficiou do uso complementar de técnicas moleculares e químicas para a correta identificação dos mutantes para a cor do bago estudados.

A alteração da cor do bago, de verde para branco/amarelo em cultivares não-corados ou de verde para rosa-avermelhado/preto-azulado em cultivares corados devido à síntese e acumulação de antocianinas ocorre no início do amadurecimento (pintor). Com base nestes factos, foi realizada uma primeira tentativa de caracterizar estas mudanças recorrendo a uma abordagem integrada combinando dados colorimétricos (medição CIELab), metabólicos (perfil fenólico através de HPLC-DAD) e genotípicos (composição alélica dos genes *MYBA1* e *MYBA2*). Este estudo focou-se nas alterações ocorridas durante o desenvolvimento do bago, de forma a melhorar o conhecimento acerca da diversidade da cor do bago em videira utilizando variantes somáticos para a cor do bago. No geral, o processo de biossíntese/ acumulação de antocianinas demonstrou uma correlação com os parâmetros colorimétricos analisados. Apesar da variabilidade da cor do bago observada entre os variantes somáticos analisados não ser totalmente explicada pelo genótipo do *locus* da cor do bago, os perfis fenólicos permitiram inferir sobre interferências específicas, nomeadamente sobre possíveis disfunções a diversos níveis da via biossintética, que poderão estar por detrás da variação de cor observada.

Adicionalmente, um estudo de caso focado num cultivar Português extremamente pigmentado (cv. 'Vinhão') foi conduzido durante o desenvolvimento do bago, fornecendo as primeiras descobertas sobre o fundo genético e transcritómico que poderá ser responsável pelas propriedades colorimétricas deste cultivar.

Vários tipos de mutações têm sido identificados no *locus* da cor do bago como sendo responsáveis por reversões de cor em videira. Através de uma abordagem específica em camadas, os mecanismos moleculares responsáveis por reversões de cor foram determinados num conjunto de variantes somáticos para a cor da película do bago, nunca estudados anteriormente, através da caracterização genética do *locus* da cor do bago e da região genómica adjacente. Além da observação e descrição dos modelos e mecanismos responsáveis pela reversão de cor mais conhecidos, foi também proposto um novo mecanismo responsável pela composição genética de variantes pouco pigmentados descendentes de um ancestral não pigmentado, no qual o ganho de cor parece resultar da recuperação do alelo funcional G no gene *MYBA2*. Além disso, foi observado que os eventos mutacionais responsáveis pelo ganho/ recuperação de cor são menos compreendidos e diferentes dos descritos para a perda de cor. Desta forma, foi realizado o estudo de um caso

de reversão de cor, de branco para rosado, de forma a melhor compreender as suas consequências a nível transcritómico e metabólico em videira, especificamente no cv. 'Moscatel Galego'. Os resultados obtidos demonstraram que a coloração do variante roxo foi recuperada a partir do fenótipo branco do cv. 'Moscatel Galego Branco' através da ativação parcial do *locus* da cor do bago. A coloração roxa do cv. 'Moscatel Galego Roxo' foi também associada à reduzida atividade da sub-via dos flavonoides tri-hidroxilados e à diminuição da metilação/ acilação das antocianinas.

Palavras-chave: *Vitis vinifera* L.; Variação espontânea; Cor do bago; Compostos fenólicos; Genes *MYB*

ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the oldest perennial domesticated fruit crops in the world and has been widely cultivated and appreciated both for its fruit and wine. During the domestication process of the wild *Vitis vinifera* subsp. *sylvestris* to *Vitis vinifera* subsp. *sativa*, a complex and long-term evolutionary process led to dramatic changes on grape biology. Since early, vine growers selected the grapevine phenotypes capable of ensuring a greater and regular fruit production and quality, maintaining them through vegetative propagation, thus multiplying highly desirable genotypes. However, natural crossings between newly introduced cultivars and the local germplasm also occurred, alongside with the emergence of somatic events.

One of the major contributors for the existing diversity in cultivated grapevine has been the appearance of somatic mutations that affect berry skin color leading to various phenotypes. Indeed, this feature has been used as basis for selection on breeding programs due to its influence on vine growers, winemakers and consumers, representing an important economic factor on this crop.

Grapes are not obviously only red or white, instead they provide a huge assortment of colors ranging from whitish, yellow, green, to pink, grey, and to darker red, purple and black berries. This broad range of color cannot be explained by the simple presence of a specific group of molecules, thus, the present study intended to deepen the current knowledge about how grape berry skin color variation is affected by the synthesis of phenolic compounds and their underlying genetic factors.

Primarily, the identification of grape berry skin color mutants was performed by genotyping a germplasm set of twenty-five grapevine accessions with twelve microsatellite loci. Among the eleven groups of putative berry skin color mutants genotyped, nine accessions, which were grouped in four different families, were identified as true color mutants, including related black, grey or red and white-skinned variants derived from a single variety. The phenolic profile of the confirmed berry skin color mutants revealed that they could be distinguished according to their non-colored compounds and anthocyanins composition. Moreover, this work benefits from the complementary use of molecular and chemical approaches for the correct identification of the berry skin color mutants studied.

The change of berry skin color, from green to white/yellow in non-colored cultivars or from green to pink-red/blue-black in colored cultivars due to anthocyanin synthesis and

accumulation occurs during the onset of ripening (veraison). Based on these facts, a first attempt to characterize these changes by means of an integrative approach combining colorimetric (CIELab measurement), metabolic (phenolic profile by HPLC-DAD) and genotypic (allelic composition of *MYBA1* and *MYBA2* genes) data was performed. This study was focused on the changes that occur during berry development, to improve the knowledge regarding grape berry skin color diversity using somatic variants for berry skin color. Overall, the process of anthocyanin biosynthesis/ accumulation showed a correlation with the colorimetric parameters analysed. Despite the berry skin color variability observed among the somatic variants analyzed was not fully explained by the berry color locus genotype, the phenolic profiles allowed to infer about specific interferences, namely some possible dysfunctions at different levels of the biosynthetic pathway, which could be behind the color variation observed.

Additionally, a case study focused on an extremely skin-pigmented Portuguese cultivar (cv. 'Vinhão') was conducted throughout berry development, providing the first insights into the genetic and transcriptomic background that may be responsible for the skin color properties of this cultivar.

Several types of mutations have been identified at the berry color locus as being responsible for color reversions in grapevine. Through a layer-specific approach, the molecular mechanisms responsible for berry skin color reversion were determined on a subset of somatic variants for berry skin color never investigated before, by the genetic characterization of the berry color locus and its surrounding genomic region. In addition to the observation and description of the most well-known models and mechanisms behind berry skin color reversions, a novel mechanism for the genetic make-up of less-pigmented variants evolving from an unpigmented ancestor was also proposed, in which color gain seems to result from the recovery of the functional G allele on *MYBA2*. Moreover, it was observed that the mutational events responsible for color gain/ recovery are less understood and different from those described for color loss. On this way, a case study of a white-*to*-red berry skin color reversion was also performed, in order to better understand its transcriptomic and metabolic consequences in grapevine, specifically in the cv. 'Moscatel Galego'. The results obtained showed that the coloration of the red-skinned variant was recovered from the white-skinned phenotype of cv. 'Moscatel Galego Branco' by the partial activation of the berry color locus. The red-skinned coloration in cv. 'Moscatel Galego Roxo'

was also associated with the reduced activity of the flavonoid trihydroxylated sub-branch and decreased anthocyanins methylation/acylation.

Keywords: *Vitis vinifera* L.; Spontaneous variation; Berry color; Phenolic compounds; *MYB* genes

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LIST OF SYMBOLS AND ABBREVIATIONS

% – Percentage

× g – Acceleration relative to gravity

Å – Ångström

ºC – Degree Celsius

AcNaOH – Sodium acetate

ANR – Anthocyanidin reductase

ANS – Anthocyanidin synthase

AOMT – Anthocyanin *O*-methyltransferase

AT – Acyl-transferase

BC – Before Christ

bHLH – Basic helix-loop-helix

C^*_{ab} – Chroma

C4H – Cinnamate-4-hydroxylase

CAN – *Coleção Ampelográfica Nacional* (Portuguese National Amplelographic Collection)

cDNA – Complementary deoxyribonucleic acid

CGGR – Centre of Grapevine Genetic Resources

CHI – Chalcone isomerase

CHS – Chalcone synthase

CIEL*a*b* – *Commission Internationale de L'éclairage* (International Commission of Lighting); L*, lightness; a*, redness; b*, yelloness

CIP – Alkaline phosphatase, calf intestinal

4CL – 4-coumaroyl: CoA-ligase

Ct – Threshold cycle

CTAB – Cetyltrimethyl ammonium bromide

cv – Cultivar

CVRVV – *Comissão Vitivinícola da Região dos Vinhos Verdes* (Vinhos Verdes Region Viticulture Commission)

DEGs – Diferentially expressed genes

DEPC – Diethyl pyrocarbonate

DFR – Dihydroflavonol 4-reductase

DNA – Deoxyribonucleic acid

dNTPs – Deoxyribonucleotides triphosphate

DOC – *Denominação de Origem Controlada* (Controlled Designation of Origin)

EDTA – Ethylenediamine tetraacetic acid

EF1α – Elongation factor 1 alpha

ESI – Electrospray ionization

EtOH – Ethanol

EVAG – *Estação Vitivinícola Amândio Galhano* (Amândio Galhano Viticulture Station)

F3'5'H – Flavonoid 3',5'-hydroxylase

F3'H – Flavonoid 3'-hydroxylase

F3H – Flavanone 3-hydroxylase

FAM – Fluorescein amidite

FCT – *Fundação para a Ciência e a Tenologia* (Portuguese Foundation for Science and Technology)

FDR – False discovery rate

FLS – Flavonol synthase

FPKM – Fragments per kilobase of transcript per million mapped fragments

GAI – Gibberellic acid-insensitive

GO – Gene ontology

GOEA – Gene ontology enrichment analysis

Gret1 – Grape retrotransposon 1

GST – Glutathione *S*-transferase

GT - UDP-glucuronic acid:flavonol-3-*O*-glucuronosyltransferase

h_{ab} – Hue angle

Hap – Haplotype

HEX – Hexachlorofluorescein succinimidyl ester

HPLC-DAD – High-performance liquid chromatography with diode-array detection

HPLC-MS – High-performance liquid chromatography coupled to mass spectrometry

INDEL – Insertion or deletion

INIAV – *Instituto Nacional de Investigação Agrária e Veterinária* (Portuguese National Institute for Agricultural and Veterinary Research)

INRA – *Institut National de la Recherche Agronomique* (French National Institute for Agricultural Research)

LAR – Leucoanthocyanidin reductase

LDOX – Leucoanthocyanidin dioxygenase

LTR – Long terminal repeat

MeOH – Methanol

min – Minute; s – second

mRNA – Messenger ribonucleic acid

MYB – Myeloblastosis

N – North

NEC – Non-endcapped

OIV – *Organisation Internationale de la Vigne et du Vin* (International Organisation of Vine and Wine)

OMT – *O*-methyltransferase

PAL – Phenylalanine ammonia lyase

PC – Principal component

PCA – Principal component analysis

PCR – Polymerase chain reaction

PDA – Photodiode array detector

PP – Phenylpropanoid

PVP – Polyvinylpyrrolidone

qRT-PCR – Quantitative reverse transcription polymerase chain reaction

RhaT - Rhamnosyltransferase

RNA – Ribonucleic acid

rpm – Revolutions per minute

SAM – Shoot apical meristem

siRNA – Small interfering ribonucleic acid

SNP – Single-nucleotide polymosphism

SNV – Single-nucleotide variation

SPE – Solid-phase extraction

SRA – Sequence read archive

SSR – Simple sequence repeat

STS – Stilbene synthase

SV – Strutural variation

Taq DNA polymerase – *Thermus aquaticus* DNA polymerase

TBE – Tris-borate-EDTA

TE – Tris-EDTA

TET – Tetrachlorofluorescein succinimidyl ester

TF – Transcription factor

Tris – Tris(hydroxymethyl)aminomethane

U – Enzyme unit

UDP – Uridine diphosphate

UFGT – UDP-glucose: flavonoid 3-*O*-glucosyltransferase

UTAD – *Universidade de Trás-os-Montes e Alto Douro* (University of Trás-os-Montes and Alto Douro)

UTR – Untranslated region

UV – Ultraviolet

V – Volts

v – Volume

VIVC – *Vitis* International Variety Catalogue

w – Weight

W – West

WAV – Weeks after veraison

WDR or WD40 – Tryptophan-aspartic acid (WD) repeat protein

WRKY – Tryptophan-arginine-lysine-tyrosine (WRKY) protein domain

THESIS OUTLINE

Grapevine (*Vitis vinifera* L.) is one of the oldest perennial domesticated fruit crops in the world; it has been widely cultivated and is highly appreciated both for its fruit and wine.

Berry skin is an important phenotypic trait when considering table and wine grapes quality. It is a determinant fruit-specific trait either for consumers or winemakers and, consequently, berry skin color is a key economic factor on this crop. On this way, the main objective of this work was to deepen the current knowledge about the metabolomics and genetics behind grapes berry skin color trait, studying the molecular mechanisms contributing for the existing natural variation in the cultivated grapevine (*Vitis vinifera* L.). In order to achieve this main goal, more specific objectives were defined:

i) Identify the most determining phenolic compounds underlying berry skin color variation;
ii) Highlight the changes that occur during berry development, particularly regarding berry skin color;
iii) Uncover the molecular mechanisms responsible for the skin color phenotype reversions.

Chapter 1 comprises a review about the general state of the art on *Vitis vinifera* L. origin, domestication and diversity, particularly regarding berry skin color variation as a source of diversity. The purpose of this chapter is to briefly introduce the current knowledge and up-to-date literature on grapevine diversity, mostly related with the phenolic composition and genetic factors underlying berry skin color variation. In this context, and considering both wine consumers and producers interests, but also the fundamental biology behind this quality trait, this review supports the importance of berry skin color in cultivated grapevine.

Grapevine shows a great diversity of berry skin colors, ranging from whitish, yellow, green, to pink, grey, and to darker red, purple and black berries. Thus, in ***Chapter 2*** the identification of the most determining phenolic compounds underlying berry skin color variation at harvest time in the cultivated grapevine was performed by a detailed HPLC-DAD analysis, through the study of different groups of color and non-colored related grapevine cultivars derived from single varieties identified and selected by Simple Sequence Repeat (SSR) molecular markers. Herein, molecular and chemical approaches complemented each other in the correct identification of grape berry skin color mutants.

An integrative approach combining colorimetric, metabolic and genotypic data is presented on ***Chapter 3***, focusing on the changes that occur during berry development, particularly regarding berry skin color. This strategy aimed to improve the knowledge regarding grape berry skin color diversity using nine skin color somatic variants, belonging to four different varieties, which showed clear differences in the phenolic biosynthesis. This characterization was performed through: a) surface color analysis by CIELab colorimetric measurement; b) HPLC-DAD analysis to study the phenolic profile; and c) molecular characterization of the genetic structure of the berry color locus (*MYBA1* and *MYBA2* genes). Overall, the results showed that the berry skin color variability was not fully explained by the berry color locus. However, the phenolic profiles allowed to infer about specific interferences among the biosynthetic pathways during berry development.

Taking into consideration the colorimetric features of an extremely skin pigmented Portuguese cultivar 'Vinhão', dying like no other Portuguese cultivar, a molecular characterization at different genetic levels related with berry skin color was also described in this chapter. Analysis of *MYBA1* and *MYBA2* gene polymorphisms and transcriptional regulation during berry development of several genes involved in the anthocyanin biosynthetic pathway were performed to clarify the biological meaning for its great capacity to extract color and, consequently, add more value to this autochthonous Portuguese cultivar. The findings obtained suggest that the berry color locus (*MYBA1/A2* genes) may be playing a major role on the skin color feature of the cv. 'Vinhão'.

At the molecular level, the most well-documented polymorphisms leading to various phenotypes within varieties are those that affect berry skin color. The studies presented on ***Chapter 4*** took advantage of a well-established layer-specific approach to genetically characterize the berry color locus and its surrounding genomic region. The developed work aimed to uncover the molecular mechanisms responsible for the skin color phenotype reversion on a set of skin color somatic variants, that have never been investigated before through this approach, including the red-skinned variants cv. 'Fernão Pires Rosado' and cv 'Verdelho Roxo' of the Portuguese white-skinned cultivars 'Fernão Pires' and 'Verdelho'. The results obtained provide additional information about the molecular mechanisms responsible for berry skin color reversions.

Additionally, a study of a white-*to*-red berry skin color reversion and its transcriptomic and metabolic consequences were assessed on the white-skinned cv. 'Moscatel Galego Branco' and its red-skinned reverted cv. 'Moscatel Galego Roxo', that belong to the large family of Muscats. The described findings allowed to hypothesize that the main reason behind the pigmentation of the red-skinned variant cv. 'Moscatel Galego Roxo' is probably through a deficient activation of the trihydroxylated flavonoid sub-branch. A predictive model based on the partial activation of the berry color locus, was also proposed for the white-*to*-red color reversion in grapevine, specifically in cv. 'Moscatel Galego'.

In ***Chapter 5***, the main outcomes and outlook of the described work are presented and summarised, bringing together Chapters 2, 3 and 4, considering the objectives initially proposed.

CHAPTER 1 | STATE OF THE ART

Berry color variation in grapevine as a source of diversity

Vanessa Ferreira, Olinda Pinto-Carnide, Rosa Arroyo-García, Isaura Castro. Berry color variation in grapevine as a source of diversity. *Plant Physiology and Biochemistry*. 2018; 132:696-707 (DOI: 10.1016/ j.plaphy.2018.08.021).

Abstract

Even though it is one of the oldest perennial domesticated fruit crops in the world, grapevine (*Vitis vinifera* L.) cultivation today is the result of both conventional breeding practices (i.e. hybridizations adopted during the last century) and vegetative propagation. Human-assisted asexual propagation has allowed the maintenance of desired traits but has largely impacted the frequency of spontaneous somatic mutations observed in the field. Consequently, many grapevine fruit attributes to date have been artificially selected, including: fruit yield, compactness, size and composition, the latter being greatly diversified in the pursuit of altering berry skin coloration. The present review provides an overview of various aspects related to grapevine diversity, with a special emphasis on grape berry skin color variation and will discuss the current knowledge of how grape skin color variation is affected by the synthesis of phenolic compounds, particularly anthocyanins and their underlying genetic factors. We hope this knowledge will be useful in supporting the importance of the berry color trait diversity in cultivated grapevines, which is used as basis for selection during breeding programs because of its application for vine growers, winemakers and consumers.

1.1 INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most valuable agricultural crops worldwide and has been an integral part of human history since its domestication about 6,000–8,000 years ago (McGovern et al., 2017). This species is a member of the Vitaceae family, with *Vitis* being the only species of important economic interest, due mainly to its use as table grapes or for processing into wine (Galet, 1988; Levadoux et al., 1962; This et al., 2006).

During the domestication process of the wild *Vitis vinifera* subsp. *sylvestris* to *Vitis vinifera* subsp. *sativa*, a complex and long-term evolutionary process led to dramatic changes in grape morphology and physiology. Consequently, the resulting variability in several important traits has contributed to the large phenotypic diversity that is found in the cultivated grapevine nowadays (Terral et al., 2010; This et al., 2006). In response to different selective pressures, cultivar diversification has clearly occurred in several fruit traits (color, flavor, seedlessness). However, for some of these traits, the biological relevance and/ or understood meaning of this variability still remains unclear (Cabezas et al., 2006; Emanuelli et al., 2010; Fournier-Level et al., 2010; This et al., 2006).

From early times, vine growers have selected the grapevine phenotypes capable of ensuring a greater and more regular fruit production and quality, maintaining them through vegetative propagation (Bacilieri et al., 2013). Although vegetative propagation has been used as a strategy for multiplication of plants with desired features, natural crossings between the newly introduced cultivars and the local germplasm also occurred along with the emergence of somatic mutation events (Pelsy, 2010). Somatic mutations that affect berry skin color leading to various phenotypes have been one of the major contributors for the current diversity in cultivated grapevines. Indeed, several pigmented cultivars with certified clones with different color shades are currently known, such as, cv. ‘Aramon’, cv. ‘Grenache’, cv. ‘Pinot’ and cv. ‘Terret’. Moreover, white-skinned cultivars, such as cv. ‘Savagnin’, cv. ‘Chardonnay’ and cv. ‘Chasselas’ also comprise pigmented clones (Pelsy et al., 2015).

Research on grape berry color demonstrates that in white-skinned cultivars the absence of anthocyanins is related with the insertion of the *Gret1*, a long retrotransposon, in the promotor region of *MYBA1* gene, combined with two non-conservative mutations in the coding sequence of *MYBA2* gene (Fournier-Level et al., 2010; Lijavetzky et al., 2006). Additionally, several molecular and cellular mechanisms have been described as

being behind berry skin color reversions occurring on grape cultivars, which is linked to the phenolic profiles, specifically with the anthocyanin profile of the cultivars and ultimately with the skin color phenotype diversity.

The present review provides a general overview of grapevine diversity, with an emphasis on the berry skin color phenotype as a source of diversity, highlighting the phenolic compounds that determine this trait in grapevines and their underlying genetic factors.

1.2 GRAPEVINE ORIGIN AND DOMESTICATION

Vitis vinifera L. (2n = 38) belongs to the family Vitaceae, which comprises about 60 inter-fertile wild *Vitis* species distributed in Asia, North America and Europe under subtropical, Mediterranean and continental temperate climatic conditions (Galet, 1988; Levadoux et al., 1962). It is the single *Vitis* species that has acquired significant economic interest over time; some other species, for example the North American *V. rupestris*, *V. riparia* or *V. berlandieri*, are used as breeding rootstock due to their resistance against grapevine pathogens such as phylloxera [*Daktulosphaira vitifoliae* (Fitch)], oidium [*Uncinula necator* (Schw.) Burr] and mildews. Indeed, a great majority of the cultivars widely cultivated for fruit, juice and mainly for wine, classified as *Vitis vinifera* L. subsp. *vinifera* (or *sativa*), derive from wild forms [*Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi] (This et al., 2006), an event estimated to have occurred approximately 22,000 years ago. Since then, the cultivated form has suffered a steady decline in the effective population size, probably resulting from low-intensity management by humans before the domestication (Zhou et al., 2017). Data based on morphological differentiation and genetic relationships between wild and cultivated grapes has suggested the existence of at least two origins for the cultivated germplasm, one in the Near East and another in the western Mediterranean region (Arroyo-García et al., 2006). Thus, it is accepted that during the process of grapevine dissemination, multiple events of domestication and spontaneous hybridizations appeared among selected individuals and local wild populations, revealing an important contribution of *Vitis vinifera* subsp. *sylvestris* from both the Near East and Eastern Europe to crop diversity.

Archeological and historical findings suggest that the primary domestication event, originating the cultivated grapevine from its wild ancestor, could have occurred around

4000-6000 BC in the Near-East region between the Black and Caspian seas (Myles et al., 2011; Terral et al., 2010; This et al., 2006). The first evidence of wine production is found in the northern Zagros mountains of Iran, dating around 5000-5400 BC (McGovern, 2003; McGovern et al., 1996).

From the primary domestication region, humans gradually spread the initial cultivars to adjacent regions in the central and southern Zagros mountains, Jordan Valley and Egypt (Myles et al., 2011; This et al., 2006). Further dispersal occurred, following the main Mediterranean civilizations, which conducted grapevine expansion to more distant Mediterranean regions like Crete, southern Greece, both coasts of the Italian and Iberian peninsulas and the north of Africa. Under the influence of the Roman Empire this culture was expanded inland, mainly through the main trade routes of the Rhine, Rhone, Danube and Garonne rivers and reached other European temperate regions (This et al., 2006). In the middle ages, the Catholic Church had a significant role in disseminating grape cultivation mainly through the crusades that aimed to spread their religion to new regions (Sefc et al., 2003; This et al., 2006). Moreover, the extension of Islam also contributed to the diffusion of grape viticulture (particularly table grape cultivars) to North of Africa, Iberian Peninsula and Middle East (Zinelabidine et al., 2010).

During the 16th century, missionaries introduced the European grapevines to America, with seeds and cuttings from their places of origin, such as France, Germany, Spain, Italy and Eastern Europe. Later, during the 19th century, cuttings were also imported to South Africa, Australia and New Zealand. After millennia of global expansion, in the mid nineteenth-century, different disease-causing agents brought from America reached to Europe, particularly the phylloxera aphid, leading to a drastic change in the genetic diversity in the European vineyards with impacts to the present days. European viticulture was saved from extinction through the introduction of some non-vinifera species as rootstocks (indigenous from Eastern North America), which have a natural resistance to phylloxera and other soil-born problems that are not present in the European grapevines (This et al., 2006). In the 60's and 70's of 20th century, French grapevine varieties, such as cv. 'Chardonnay', cv. 'Cabernet Sauvignon', cv. 'Syrah' or cv. 'Merlot', were introduced in large numbers in the viticulture of the new world countries due to the perception of wine quality associated with French wine denominations (This et al., 2006). A recent study focused on the basis of grapevine genotype × environment (G×E) interactions showed that, for example, the cv. 'Cabernet Sauvignon' was less dependent on growth conditions and that its transcriptome remained

more stable across vintages and locations, suggesting that the limited plasticity of this French cultivar may underpin its success in many different parts of the globe (Dal Santo et al., 2018; Paim Pinto et al., 2016).

1.3 SPONTANEOUS VARIATION IN GRAPEVINE

1.3.1 The role of naturally occurring mutations and human selection on grapevine diversity

During the domestication and selection processes the biology of grapes underwent several dramatic changes that led to a significant divergence in important traits, contributing to the large phenotypic diversity found nowadays in cultivated grapevines. One of the most important events during domestication was the appearance of hermaphrodite flowers, removing the necessity to maintain both male and female wild plants (This et al., 2006). Additionally, cultivar diversification as a response to different selective pressures was clearly observed for berry color (Fournier-Level et al., 2010), Muscat flavor (Emanuelli et al., 2010) or table grape seedlessness (Cabezas et al., 2006). Other traits also appear to have been modified through domestication, such as seed size, leaf size and morphology, although their biological significance is not completely understood (This et al., 2006).

A few decades ago the number of different cultivars held in germplasm collections around the world was estimated at approximately 10.000 (Alleweldt and Dettweiler, 1994), a large number that included several synonyms (different names for the same cultivar) and homonyms (identical name for different cultivars). The current estimation of the number of different grapevine cultivars is around 5.000, being many of them closely related. Information on the origin, main use and pedigree of these cultivars can be found on the Vitis International Catalogue (VIVC, http://www.vivc.de), which is a valuable tool to easily compare molecular fingerprints.

Genetic diversity has long been studied in grapevine with different sets of molecular markers (Castro et al., 2016, 2012, 2011; Ferreira et al., 2015), which has provided partial views of the genetic relationships among cultivated and wild germplasm, as well as on the expansion and evolution of domesticates (This et al., 2006). Although self-pollination is the major mating system in grapevine (Jackson, 2008), a primary conclusion of genetic diversity studies found that some cultivars were derived by spontaneous hybridizations

from other existing cultivars. The first demonstrated case was cv. ‘Cabernet Sauvignon’, shown to be a spontaneous hybrid from the cross between cv. ‘Cabernet Franc’ and cv. ‘Sauvignon Blanc’ (Bowers and Meredith, 1997). In fact, the probable reason why cultivars from a given region seem to be more closely related than to cultivars from other regions (Sefc et al., 2000) is because they have close family relationships.

Moreover, the current globalization of wine markets has focused only on a small number of highly appreciated cultivars of both white (cv. ‘Chardonnay’, cv. ‘Sauvignon Blanc’) and red cultivars (cv. ‘Cabernet Sauvignon’, cv. ‘Syrah’ and cv. ‘Merlot’), and the demand for healthy disease-free plant material has led to a drastic reduction of diversity in the cultivated grapevine. Consequently, most of the traditional and local cultivars have almost disappeared, and some of those cultivars are only found in germplasm collections (This et al., 2006).

As stated before, human activity has played a pivotal role in shaping the existing diversity of grapes. In fact, most of the today’s cultivars do not have a deliberate origin as they are the result of different processes of selection on certain grapevine genotypes that spontaneously occurred in nature. Since early times, vine growers have selected grapevine genotypes capable of ensuring a greater and more regular fruit production and quality, and maintained them through vegetative propagation – a process which is believed to have been used since the beginning of the domestication process (Bacilieri et al., 2013).

Despite the importance of vegetative propagation as a conservation strategy to maintain desired phenotypes as genetically identical clones of the original donor plant (i.e. true-to-type), somatic mutations may naturally occur during plant growth and accumulate over time. Consequently, this somatic mutation might lead to a slightly different genotype and eventually to a different phenotype on a specific plant of the same cultivar, leading to clonal polymorphism. The new generated clone can be vegetatively propagated retaining the selected genetic and morpho-physiological difference, giving rise to a new cultivar (Pelsy, 2010). From the start of grapevine domestication, vegetative propagation combined with somatic variation have been widely exploited by vine growers to improve the phenotypic features of classic cultivars, representing a valuable source of diversity (This et al., 2006).

Higher plants have stratified apical meristems with layers of dividing cells that are characterized by different ontologies. Each cell layer develops independently from the adjacent layers, leading to the different plant tissues within an organ (Neilson-Jones,

1969). Thompson and Olmo (1963) proposed that the shoot apical meristem (SAM) of grapevine is composed of 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.

Somatic variation results from mutational events during plant development in a single cell that belongs to a specific shoot apical meristem cell layer. This mutated cell can then propagate by mitotic division, leading to the emergence of a mutated section and giving rise to a chimera. Such structures are specific types of genetic mosaic in which one or two entire cell layers of the apical meristem are genetically distinct from the others and remain developmentally independent from the adjacent layers. According to their spatial patterning, chimeras can be classified in the following three types: mericlinal, sectorial and periclinal. Mericlinal chimeras comprise a mutation in just a part of one tissue layer. Sectorial chimeras contain a mutation in a section of several layers and periclinal chimeras have a mutation in one or more entire layers (Figure 1.1) (Dermen, 1960).

Briefly, somatic mutations can be propagated to the whole plant by a two-step process. First, a somatic mutation emerges in one cell of the shoot apical meristem, and is then propagated by cell division to an entire cell layer, generating a stable chimeric structure. Afterwards, the replacement of one cell layer by another can lead to a homogenization of the genotype of the whole plant, representing the second step of the process (Hocquigny et al., 2004). Due to the low level of organization of cell division in the inner layers, the most common cellular rearrangement observed is the invasion of the outer L1 layer by cells from the inner layer. Although rare, the opposite phenomenon, L1 cell invasion of the inner layer, is also accepted (Pelsy, 2010).

In grapevine, somatic variation occurs with relative frequency and is described as quite a common phenomenon. Despite the fact that mutation frequencies and rates are still relatively unknown, the reversion from the chimeric to the original status seems to occur more frequently than the opposite, maybe due to the unstable nature of the chimeras (reviewed in Pelsy, 2010; Torregrosa et al., 2011). Indeed, several variant traits have been described, including for berry color or flavor, date of ripening, canopy growth, size and compactness of bunches or productivity (Figure 1.1, Table 1.1).

Some cultivars are known to be more prone to exhibit somatic polymorphisms than others. The basis for the wide range of clones in some cultivars could be genetic, due to a more unstable genetic background, but also the reflex of a longer history of cultivation or growth at a large extension, in which the age of the genotype should be considered, as well as the total area planted in different vineyards around the world to produce different

wines with specific oenological characteristics (Pelsy et al., 2015; Torregrosa et al., 2011).

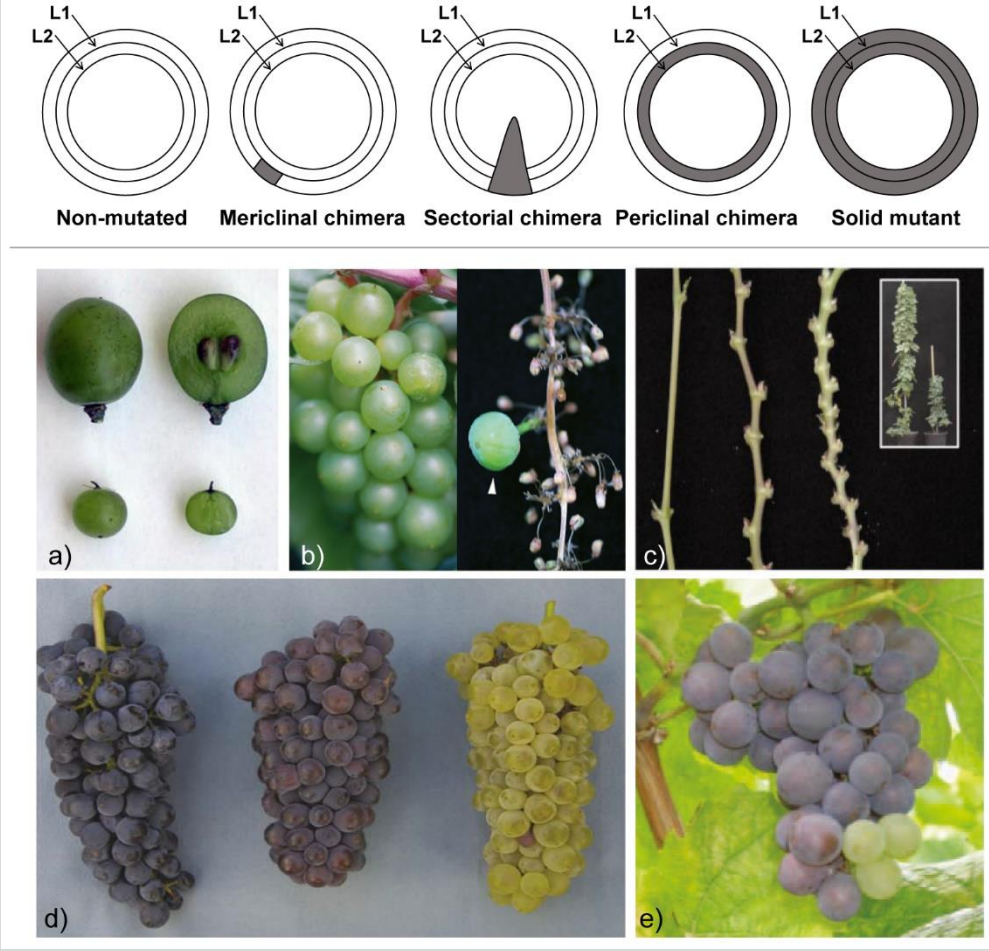

Figure 1.1 – Somatic variation in grapevine. **Top:** Diagram representing the three types of chimeras (Mericlinal, Sectorial and Periclinal) classified according to their spatial patterning [Adapted from Torregrosa et al., (2011)]. **Bottom:** Examples of mutant phenotypes of different varieties. a) 'Corinto Bianco' and its seedless mutant; b) cv. 'Ugni Blanc' and its fleshless mutant; c) Dwarfism phenotype of cv. 'Pinot Meunier'; d) cv. 'Pinot Noir' and its grey and white-skinned color variants; e) White-skinned berries variegation in cv. 'Pinot Gris' bunch [Adapted from Boss and Thomas (2002); This et al., (2006); Vargas et al., (2007); Pelsy, (2010)].

As an example, the 'Pinot' group typifies clonal phenotypic diversity, being one of the most ancient grapevine varieties made up of a large panel of clones (Pelsy et al., 2015). This great diversity of clones, which is due to spontaneous somatic mutations that have occurred over time is manifested in traits such as berry skin color (cv. 'Pinot Noir', cv. 'Pinot Gris' and cv. 'Pinot Blanc'), flesh pigmentation (cv. 'Pinot Teinturier'), yield level (cv. 'Pinot Fin' versus cv. 'Pinot Moyen'), leaf morphology (cv. 'Pinot Meunier'), canopy growth (cv. 'Pinot Detroit') and absence of wax on berries (cv. 'Pinot Moure').

At the genetic level, clonal polymorphism can result from single-nucleotide polymorphisms (SNPs), variations of SSR (Simple Sequence Repeat) length and insertion or deletion (INDELs) events.

The cultivar 'Pinot Meunier', a periclinal somatic variant of the cv. 'Pinot Noir', shows an increase of trichomes on the leaf surface, which could be the result of an ancient mutation occurring only in the L1 epidermal layer (Franks et al., 2002). Besides inflorescences instead of tendrils, an extreme dwarfism was also observed by the same authors in plants regenerated from cv. 'Pinot Meunier' L1 cell layer, whereas plants regenerated from L2 cell layer were tall and with normal glabrous leaves, similar to cv. 'Pinot Noir' phenotype. These variations were found to be genetically heritable and associated with a point mutation in *GAI1* gene involved in the gibberellic acid signaling (Boss and Thomas, 2002). This mutation in the DELLA domain converts a leucine residue into a histidine, which seems to alter the gibberellin-response properties of the protein, resulting in a critical 'Pinot' L1 mutant phenotype.

The cv. 'Ugni Blanc' is another case of somatic variation that had a major importance on the understanding of the molecular mechanisms behind fleshy fruit morphogenesis. A fleshless berry (*Flb*) somatic variant was described for this cultivar. Fernandez et al. (2013) showed that the fleshless phenotype is caused by an inverted-repeat transposable element in the promoter region of the PISTILLATA-like (*PI*) gene, a gene that was shown to be drastically repressed after flower fertilization in previous grapevine studies (Dauelsberg et al., 2011; Poupin et al., 2007). The same authors also concluded that *PI* alteration causes its ectopic expression and that this effect is cell layer-specific. The abnormal *PI* expression after fertilization in the L2 cell layer of the *Flb* somatic variant and derived *Flb* mutant plants abrogates flesh development by preventing the differentiation of the highly vacuolated cells that are characteristic of this mesocarp tissue. Moreover, when the mutation is also present in the L1 cell layer, fruit development cannot proceed normally, being blocked at fruit set.

Table 1.1 – Described variants for some of the most well-known international grapevine varieties [Adapted from Pelsy (2010)].

Varietal group	Variants	Phenotype	Clonality ascertaining	References
Cabernet Sauvignon	Cabernet Sauvignon	Black-skinned berries		
	Malian	Bronze-skinned berries	Bud sport of Cabernet Sauvignon	Boss et al. (1996); Walker et al. (2006)
	Shalistin	White-skinned berries	Bud sport of Malian	
Chardonnay	Chardonnay Blanc	White-skinned berries/ neutral aroma		
	Chardonnay Muscaté	Rosy-skinned berries/ aromatic	16 SSR	Duchêne et al. (2009)
	Chardonnay Rose	Rosy-skinned berries/ neutral aroma	Bud sport of Chardonnay Blanc	This et al. (2007)
Italia	Italia	Green-skinned berries		
	Ruby Okuyama	Light-rosy-skinned berries	Bud sport of Italia	Kobayashi et al. (2004)
	Rubi	Light-rosy-skinned berries	Bud sport of Italia	de Oliveira Collet et al. (2005)
	Benitaka	Rosy-skinned berries	Bud sport of Italia	Azuma et al. (2009)
	Brasil	Black-skinned and red-fleshed berries	Bud sport of Benitaka	de Oliveira Collet et al. (2005)
Muscat d'Alexandria	Muscat d'Alexandria	White-skinned berries		
	Flame Muscat	Red-skinned berries	Bud sport of Muscat d'Alexandria	Kobayashi et al. (2004)
	Zibibbo Nero	Red-skinned berries	Bud sport of Muscat d'Alexandria	De Lorenzis et al. (2015)
Pinot	Pinot Noir	Glabrous leaves/ black waxed berries		
	Pinot Moure	No wax berries	50 SSR	Hocquigny et al. (2004)
	Pinot Meunier	Hairy leaves and dwarfism	50 SSR	Franks et al. (2002)
	Pinot Gris	Red-grey-skinned berries	Bud sport of Pinot Noir	Vezzulli et al. (2012)
	Pinot Blanc	White-skinned berries	Bud sport of Pinot Noir	Vezzulli et al. (2012)
Savagnin	Savagnin blanc	White-skinned berries/ neutral aroma		
	Savagnin Rose	Rosy-skinned berries/ neutral aroma	16 SSR	Duchêne et al. (2009)
	Gewurztraminer	Rosy-skinned berries/ aromatic	16 SSR	
Ugni Blanc	Ugni Blanc	Fleshy berries		
	Fleshless mutant	Fleshless berries	Bud sport of Ugni blanc	Fernandez et al. (2006)
Pedro Ximenes	Pedro Ximenes	Seeded berries		
	Corinto Bianco	Seedless berries	20 SSR	Vargas et al. (2007)

1.3.2 Color variation as a consequence of chimerism

The clonal polymorphism affecting berry skin color is the most well-studied polymorphism leading to various phenotypes, having been intensively investigated in different grapevine varieties (Ferreira et al., 2018, 2016; Giannetto et al., 2008; Migliaro et al., 2014).

The occurrence of chimeric structures (with three or four-allele genotypes) has been detected by using microsatellite markers in a wide range of varieties, including cv. 'Primitivo' (Franks et al., 2002), cv. 'Greco di Tufo' and cv. 'Corvina Veronese' (Crespan, 2004), cv. 'Cabernet Sauvignon' (Moncada et al., 2006) or cv. 'Pinot' (Hocquigny et al., 2004). A certain locus presenting three alleles in one individual can be explained by a chimeric structure consisting of two cell layers with genotypes combining one common and one specific allele of each layer, whereas a four-allele locus is due to two completely different genotypes in each layer.

The certified clone PG52 of cv. 'Pinot Gris' showed a triallelic profile at VVS2 microsatellite loci (126:134:148) (Hocquigny et al., 2004). Self-progenies of this clone produced only white-berried plants carrying two alleles (126:134), whereas plants regenerated from the L1 cell layer produced black berries with a different genotype (134:148). Thus, the grey-colored berry phenotype of PG52 clone could result from a chimeric structure, comprised of a colored L1 epidermis and L2 cells with a mutation that prevents anthocyanin synthesis, where the [134] allele at locus VVS2 is the common allele of both cell layers and the [148] and [126] alleles are specific to the L1 and L2 cell layers, respectively. Moreover, PG52 clone occasionally produces colored berries with white variegations. The colored skin sector was found to have the tri-allele profile, identical to the leaf at the VVS2 locus, whereas the white skin sector showed the diallelic combination transmitted to the self-progenies. A displacement from the L2 cell layer to the L1 could be responsible for generating the white-berried sector on cv. 'Pinot Gris' berries. Such displacements can affect a few berries or a whole bunch on a cane, according to the development stage during which the displacement occurs (Pelsy, 2010).

Moreover, the lack of anthocyanin pigments in the white-skinned phenotype has been associated with the insertion of *Gret1* retrotransposon in the promoter region of *MYBA1* gene combined with two non-conservative mutations in the coding sequence of *MYBA2*, a point mutation and a dinucleotide deletion (Kobayashi et al., 2004; Walker et

al., 2007), both regulating the transcription of UFGT (UDP-glucose: flavonoid 3-*O*-glucosyltransferase), a key point in the anthocyanin pathway.

It is now well established that the clonal difference between the black-skinned cv. 'Pinot Noir' and the green-yellow cv. 'Pinot Blanc' was caused by a large deletion, which removed the functional colored alleles of both *MYB* genes that regulate pigment production. This mutation led to the emergence of the colorless phenotype observed in cv. 'Pinot Blanc'. In turn, the grey-skinned phenotype of cv. 'Pinot Gris' results from a chimeric structure generated by the two-step process described above, composed of a colored L1 epidermis and L2 cells with a mutation that prevents anthocyanin synthesis (Hocquigny et al., 2004; Walker et al., 2006; Yakushiji et al., 2006). More recently, through a structural dynamics study along chromosome 2, comprising 29 'Pinot' clones, Vezzulli et al. (2012) concluded that cv. 'Pinot blanc' is not a bud sport of cv. 'Pinot Gris', since deletions comprising *MYB* genes have different lengths, ranging from 100 kb to 179 kb for cv. 'Pinot Blanc' clones and from 4,202 kb to 4350 kb in the L2 cell layer of cv. 'Pinot Gris'. Consequently, a 'Parallel Model' was proposed to explain the evolutionary events that led to the formation of 'Pinot' berry color somatic variants, where the black-skinned ancestor cv. 'Pinot Noir' gave rise to the grey-skinned (cv. 'Pinot Gris') and the white-skinned (cv. 'Pinot Blanc') berry variants independently (Figure 1.2-A). This model was an important advance in the knowledge on the emergence of somatic color mutants within a variety. Before that the prevailing idea was that these variants arise sequentially, the so called 'Sequential Model', proposed by Walker et al. (2006), in which the black-skinned berry ancestor (cv. 'Cabernet Sauvignon') gave rise to a periclinal chimera, the bronze-skinned phenotype of cv. 'Malian', through a deletion encompassing the functional alleles of *MYBA1* and *MYBA2* in the L2 cells, which in turn gave rise to the white-skinned phenotype of cv. 'Shalistin', as the result of a cellular rearrangement (or displacement) in cv. 'Malian' whereby the L2 cell layer (unpigmented phenotype) replaced the L1 cells (pigmented phenotype) (Figure 1.2-B).

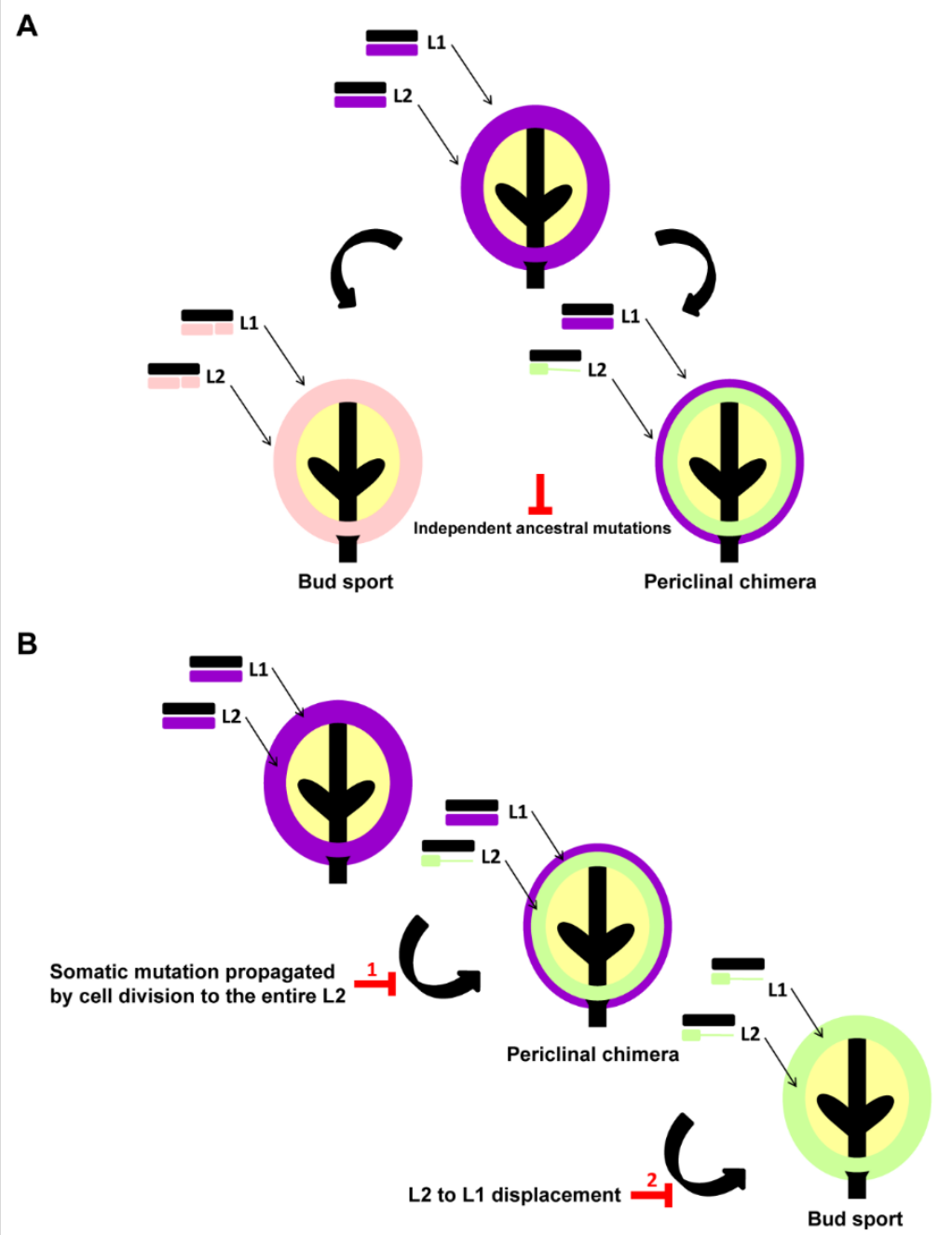

Figure 1.2 – Schematic representation of the evolutionary models that explain the development of somatic berry skin color mutants within a variety: Parallel model (A) and the Sequential model (B) [Adapted from Ferreira et al. (2018)].

1.4 GRAPEVINE BERRY SKIN COLOR: AN IMPORTANT QUALITY TRAIT

1.4.1 Biochemical and genetic factors underlying berry skin color

Grapevine berries are complex organs composed of three clearly distinguished parts, the exocarp (skin) and mesocarp (flesh), which together constitute the pericarp, and

the seeds. Berry development follows a double sigmoid growth pattern with three distinct phases, two growth periods separated by a lag phase. The onset of ripening occurring at the end of the lag phase (a short period known as veraison) represents a key point in berry development in which several important changes occur, including the initiation of sugar accumulation, berry softening, berry pigmentation driven by the synthesis of anthocyanins in red cultivars, catabolism of organic acids and flavor maturation (Hardie et al., 1996; Robinson and Davies, 2000).

In this sense, several compounds accumulate during grape development in pericarp, and their presence is responsible for many relevant agronomic traits for table and wine grape quality, namely berry crispness and firmness, aroma and taste-related traits. For instance, the ratio between sugar (glucose and fructose) and acid (malic and tartaric) content at harvest is important for the flavor in table grapes and for the production of quality wines (Lijavetzky et al., 2012). Moreover, berry coloration is also an important trait quality that is used as the basis for selection during breeding programs. The color description in grape is an important factor for market acceptance and is crucial for two main reasons: 1) impact on consumers of table grapes, since they generally prefer well pigmented grapes and 2) for winemakers in the management of product transformation. Especially for the latter, the evaluation of the phenolic compounds content, particularly anthocyanins, is extremely relevant (Rustioni et al., 2013).

Considering that color is such an important quality parameter in table grapes and wines, it is imperative to understand how berry coloration is affected by genetic factors that regulate phenolic compounds biosynthesis, particularly flavonoids and anthocyanins, as this knowledge will contribute to a more stable production of high-quality pigmented grapes (Azuma, 2018).

Phenolic compounds are synthesized from the amino acid phenylalanine through the phenylpropanoid pathway (Figure 1.3), which is one of the most well-studied pathways in the plant kingdom, both at the enzymatic and regulatory levels. In grapes the synthesis of these compounds responds to specific developmental cues and to environmental factors, such as low temperature and light, which have a synergistic effect on their accumulation. Additionally, recent studies also make reference that this response seems to be cultivar-specific, namely regarding the effect of solar radiation on phenolic compounds accumulation. Since phenolic compounds also influence organoleptic properties of the berries (such as taste and color) and are associated with several beneficial attributes for human health, this highlights the importance of adding new knowledge on

secondary metabolites in grapevine to help vine growers to develop new cultivation techniques to modify and improve grape quality and adapt to a changing climate (reviewed by Azuma, 2018; Flamini et al., 2013; Matus, 2016; Wong and Matus, 2017).

Among the different phenolic compounds that influence the quality of grapes and wines, flavonoids (C6–C3–C6 skeleton structure) and stilbenes (C6-C2-C6) are the most important ones. Flavonoids are one of the most abundant groups of compounds in grape berry and consist of a mixture of different glycosylated and acylated derivatives of flavonols, flavan-3-ols [when organized into polymers are called proanthocyanidins (PAs) or condensed tannins] and anthocyanins. Among these classes of flavonoids, flavonols and anthocyanins are the main pigments that determine the berry skin color in grapevine.

Flavonols are yellow pigments that contribute directly to the color of white wines, being masked by anthocyanins in the red wines, whereas anthocyanins are pigments responsible for the orange, pink, red, blue and purple color of the berry skin in colored grapes. However, there are also a few cultivars, called teinturier or dyed cultivars, that can accumulate anthocyanins in their flesh in parallel with the accumulation in the berry skin, such as cv. 'Alicante Bouschet', also called cv. 'Garnacha Tintorera' (He et al., 2010; Teixeira et al., 2013). Although the profile of these compounds is commonly used as a fingerprint for cultivar identification, different clones of the same variety may present different anthocyanin contents, which is related with the level of intravarietal diversity of each grape variety (Van Leeuwen et al., 2013).

Usually, the main representatives of flavonols in red grapes are quercetin-3-*O*-glucosides (dihydroxylated) followed by myricetin (trihydroxylated), whereas quercetin and kaempferol (monohydroxylated) derivatives constitute the major flavonol compounds in white grapes (Castillo-Muñoz et al., 2007; Mattivi et al., 2006). Moreover, other less abundant flavonols can be synthesized; isorhamnetin is the methylated form of quercetin, and laricitrin and syringetin are the methylated forms of myricetin (Czemmel et al., 2017; Flamini et al., 2013; Teixeira et al., 2013). Regarding the quality of red wines, flavonols influence their color by means of copigmentation, a phenomenon that occurs due to molecular associations between anthocyanins and flavonols or other non-colored phenolic compounds (Castillo-Muñoz et al., 2010; Liang et al., 2011).

The genes coding for the enzymes of the flavonoid branch within the phenylpropanoid pathway are transcriptionally regulated by the combinatorial interaction between three classes of transcription factors (TFs): myeloblastosis (R2R3-MYB), basic

helix-loop-helix (bHLH) and tryptophan-aspartic acid repeat protein (WDR or WD40) (Ramsay and Glover, 2005). A fourth class of TFs (WRKY) has been recently characterized in grapevine by Amato et al. (2017), who proposed *WRKY26* as a putative regulator of vacuolar transport and acidification. Additionally, it was found that *WRKY26* is also involved in the flavonoid pathway, showing a particular role in proanthocyanidin biosynthesis and deposition. In grapevine, the R2R3-MYB proteins family is currently understood to be composed of 134 genes (Wong et al., 2016) and is characterized as two highly conserved DNA binding domains and a variant number of C-terminal motifs (Dubos et al., 2010). These genes have been related to phenylpropanoid regulation and are thought to have expanded by gene duplication (Matus et al., 2008).

Although the initial regulatory mechanism of the first flavonol branch target gene flavonol synthase 1 (FLS1 or FLS4) by the R2R3-MYB transcription factor *MYBF1* is well-established, it is unclear how the high diversity of flavonol compounds found in grapes is achieved. Until recently, only two structural genes had been identified as involved in flavonol modification, namely *GT5* and *GT6*, which encode a UDP-glucuronic acid: flavonol-3-*O*-glucuronosyltransferase and a double functional UDP-glucose/UDP-galactose: flavonol-3-*O*-glucosyltransferase/ galactosyltransferase, respectively (Ono et al., 2010). However, Czemmel et al. (2017) identified other promising novel flavonol biosynthetic genes, such as a flavonol glucosyltransferase (GT3) and a rhamnosyltransferase (RhaT1).

In addition, the anthocyanin content and composition, which are greatly affected by cultivar type and viticultural/ environmental conditions, are also an important factor with a direct impact on color variation of grape berry skin, providing a huge assortment of colors ranging from whitish, yellow, green, to pink, grey, and to darker red, purple and black berries. These different color shades and hues are mainly influenced by the presence of five major anthocyanins in grape, which differ from each other in the number and positions of the hydroxyl and methoxyl groups on the B-ring (reviewed by He et al., 2010). Cyanidin and peonidin are dihydroxylated precursors of red anthocyanins, whereas delphinidin, petunidin and malvidin are trihydroxylated precursors of blue and purple (Figure 1.3). In *Vitis vinifera*, anthocyanins are mainly present as 3-*O*-monoglucoside derivatives due to the presence of two disruptive mutations in the anthocyanin 5-*O*-glucosyltransferase gene, which produces a non-functional form of the enzyme. Additionally, anthocyanins can also be acylated with aromatic and aliphatic acids as acetylmonoglucoside and p-coumaroylmonoglucoside derivatives.

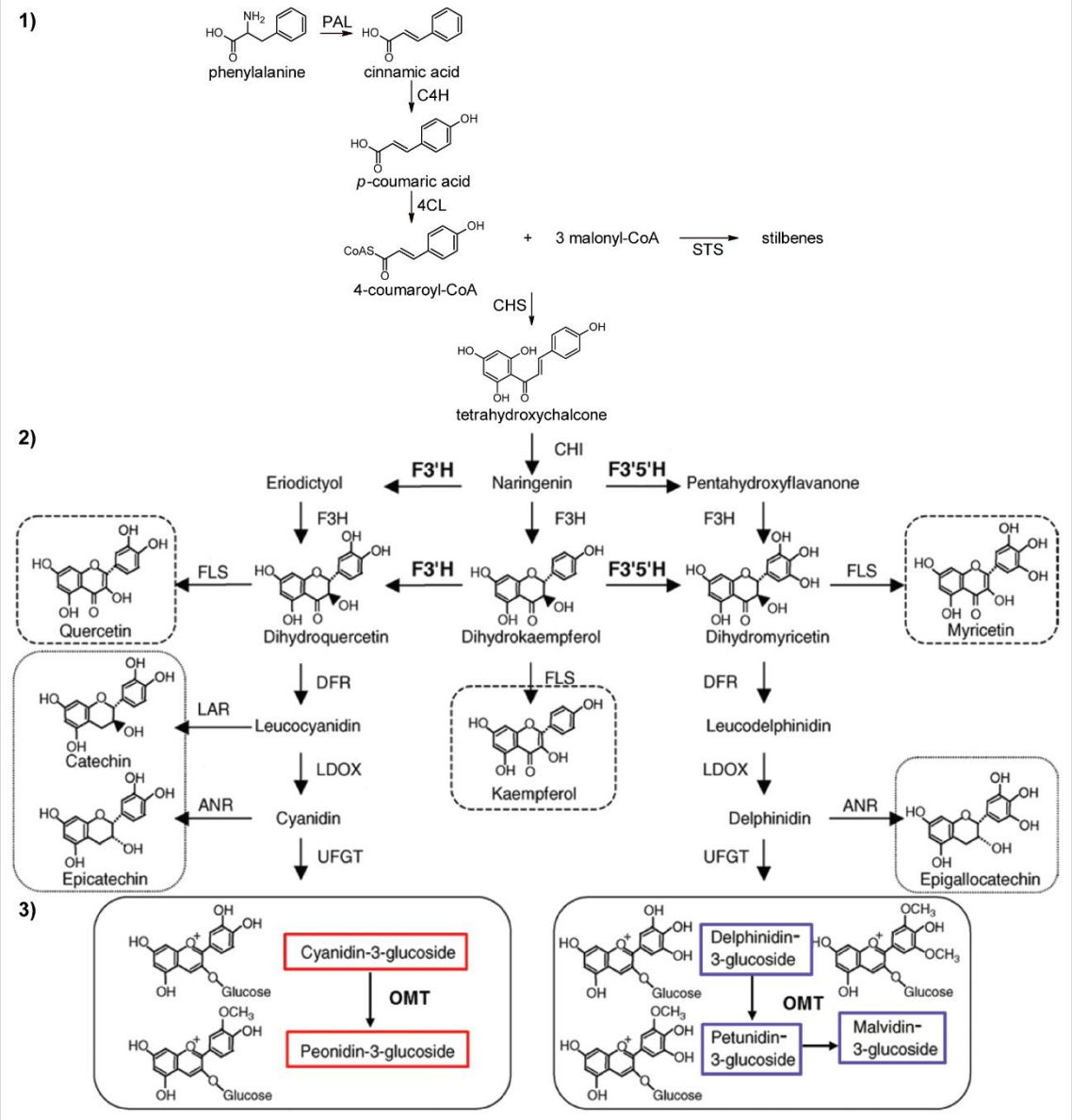

Figure 1.3 – Schematic representation of the main pathways involved in flavonoid biosynthesis in grapes. 1) general biosynthetic phenylpropanoid pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl: CoA-ligase; CHS, chalcone synthase; STS, stilbene synthase. 2) basic upstream flavonoid pathway leading to the biosynthesis of colored anthocyanidins. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase, ANR, anthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase. 3) Specific branch for the anthocyanin modification of free anthocyanidins. Colored boxes indicate the skin color produced by the five major anthocyanins (red boxes represent the dihydroxylated anthocyanins and purple boxes the trihydroxylated anthocyanins). UFGT, UDP-glucose: flavonoid 3-*O*-glucosyltransferase; OMT, *O*-methyltransferase [Adapted from Flamini et al. (2013) and Kuhn et al. (2014)].

The anthocyanin synthesis regulation is directly related with the activity of different R2R3-MYB transcription factors, some of which are located in two well-described grape color loci. The recently identified 'vegetative color locus' (Matus et al., 2017) harbors *MYBA5/6* and *MYBA7* genes (Chr. 14), while the 'berry color locus' comprises *MYBA1* and *MYBA2* genes (Chr. 2) (Walker et al., 2007), two essential genes that determine berry skin color variation (Fournier-Level et al., 2010). Both loci share the regulation of late biosynthetic and modification/ transport-related genes, such as UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) and anthocyanin 3-*O*-glucoside-6''-*O*-acyltransferase (3AT) (Matus et al., 2017; Rinaldo et al., 2015; Walker et al., 2007). However, the synthesis of specific anthocyanins is also affected by the expression of early biosynthetic genes that encode flavonoid 3'-hydroxylase (F3'H) and flavonoid 3', 5'-hydroxylase (F3'5'H). These enzymes competitively control the synthesis of di- and trihydroxylated anthocyanins, respectively, whereas anthocyanin *O*-methyltransferase (AOMT) methylates anthocyanins of both groups. Usually F3'5'H activity prevails over F3'H, and the products of flavonoid hydroxylases are predominately channeled into the branch of the pathway involved in the biosynthesis of delphinidin (blue purplish derivatives) at the expense of those involved in the synthesis of cyanidin (reddish derivatives) (Castellarin et al., 2006; Castellarin and Di Gaspero, 2007; Jeong et al., 2006). In contrast with the well-characterized *MYBA1* gene, which promotes the synthesis of larger quantities of trihydroxylated anthocyanins, the vegetative color locus genes (*MYBA6/7*) are not able to activate the trihydroxylated branch due to the lack of a C-terminal protein motif that is present on *MYBA1* and related genes of the berry color locus. Consequently, only dihydroxylated anthocyanins with a reddish hue are almost exclusively accumulated on the vegetative organs, both in black- and white-skinned cultivars (Matus et al., 2017). Additionally, anthocyanin synthesis is also induced by light and high sucrose levels (Yamakawa et al., 1983). More specifically, it has been shown that the *DFR* gene expression, which is higher after ripening onset (Boss et al., 1996), is induced by sucrose. Behind this regulation is the presence of two sucrose boxes on the gene promoter to which sugar can bind and activate gene expression (Gollop et al., 2002).

In summary, several members of the R2R3-MYB family have been characterized, many of which act as positive regulators of the synthesis of anthocyanins (*MYBA1/A2*, *MYB5B, MYBA6* and *MYBA7*), proanthocyanidins (*MYBPA1/PA2/PAR*) and flavonols (*MYBF1*). Along with its role as an activator of flavonoid pathway structural genes (*CHI*, *F3'5'H, ANS, LAR1 and ANR*), *MYB5B* also regulates other processes such as vacuolar

acidification and membrane remodeling (Cavallini et al., 2015). This is of particular relevance since, in other genera like *Petunia*, it has been shown that vacuolar pH affects the coloration of the synthesized anthocyanins. An increase in pH shifts the color from purple to blue in plant lines that synthesize malvidin or petunidin derivatives, or from red to a dull gray in a cyanidin background (Tornielli et al., 2009).

More recently, repressors of the accumulation of small weight phenolic acids and flavonoids (*MYB4A*) (Cavallini et al., 2015) and positive regulators of stilbenoid accumulation (*MYB15* and *MYB14*) (Holl et al., 2013) have also been identified. The differential activation or repression of different steps of the pathway is responsible for restricting the anthocyanins accumulation to specific developmental stages and also in response to different environmental factors and agricultural practices (reviewed by Matus, 2016).

1.4.2 The berry color locus: master regulator of berry skin color

Skin color in grapes has become greatly diversified, showing different color shades as a result of hybridization and human selection. As stated above, the color of berry skin is mainly associated with the content and composition of anthocyanins, which is determined by the allelic status of a major locus that spans over a 200-kb region on chromosome 2 (Azuma et al., 2009; Fournier-Level et al., 2009; Matus et al., 2008). This berry color locus comprises a cluster of closely related R2R3-MYB genes and pseudogenes, among which the functional *MYBA1* and *MYBA2* genes, two adjacent transcription factors that regulate the transcription of UFGT (UDP-glucose:flavonoid 3-*O*-glucosyltransferase), represent the major genetic determinant of skin color variation. A third gene (*MYBA3*) has also been functionally tested but does not activate anthocyanin synthesis and the less related gene *MYBA4* gene also does not seem to play a role in anthocyanin biosynthesis in grape berries (Figure 1.4) (Walker et al., 2007).

The emergence of the colorless phenotype observed on the white-skinned cultivars, a derived state that arose from the highly colored (less diverse and homozygous) wild ancestor. This resulted from the simultaneous occurrence of mutations in *MYBA1* and *MYBA2* genes, which cause the loss of transcription factor expression and, consequently, abrogate anthocyanin biosynthesis. Different combinatorial mutations on these two genes led to the emergence of not only the white-skinned phenotypes, but also to a range of pale-phenotypes, largely assessed in different genotypes and somatic

variants (Ferreira et al., 2018, 2017; Lijavetzky et al., 2006; Migliaro et al., 2014). These were selected during the domestication process, favoring a rapid diffusion of the less colored phenotypes, leading to the diversification of berry color. Nowadays, both vine growers and winemakers take advantage of this color diversity to address consumer tastes in table grapes and wine.

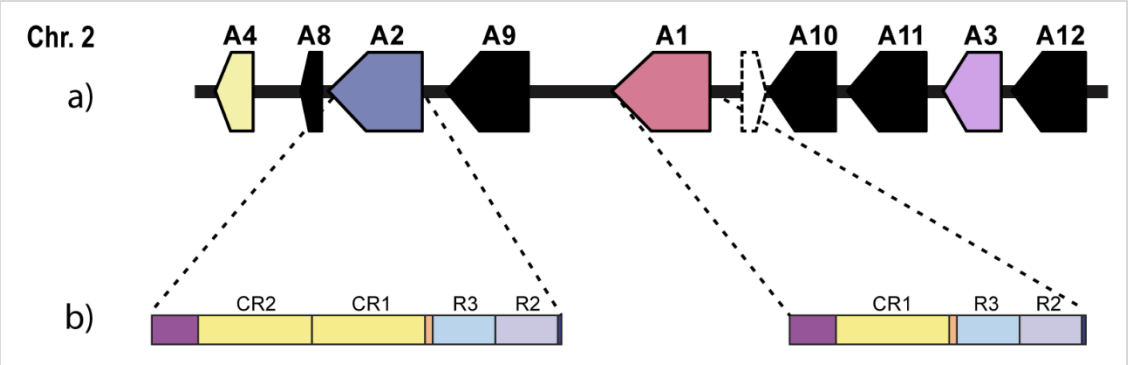

Figure 1.4 – The berry color locus on chromosome 2. a) Schematic representation of the anthocyanin-related *MYBA* genes as present in the grapevine genome of cv. 'Pinot Noir' PN 40024. All genes are displayed in the same orientation, mostly positioned in tandem, with the exception of *MYBA1*/ *MYBA10* that share the presence of a non-*MYB* gene between them. Gene sizes are represented to scale, but intergenic regions are not; b) Genomic organization of MYBA1 and MYBA2 protein variants. Each domain is shown with a specific color box, indicating that the MYB repeats are identical between the proteins, R2 and R3 DNA binding domains and the C-terminal domain, which is repeated in VvMYBA2 (CR1 and CR2) [Adapted from Matus et al. (2017) and Walker et al. (2007)].

The mutations on *MYBA1* and *MYBA2* genes can be grouped as summarized by Matus et al. (2017): (i) inactivation of *MYBA1* through the insertion of the *Gret1* retrotransposon in its promoter/ 5' untranslated region (5'-UTR) (Kobayashi et al., 2005, 2004); (ii) non-synonymous single-nucleotide polymorphism and a frame-shift mutation in the *MYBA2* coding region (Walker et al., 2007); or (iii) a large deletion removing both *MYBA1* and *MYBA2* from the berry color locus (Walker et al., 2006; Yakushiji et al., 2006).

In more detail, the absence of anthocyanins in white-skinned cultivars has been largely associated with the insertion of the *Gret1*, a 10,422 bp long gypsy-type retrotransposon, in the promoter region of *MYBA1* gene, which leads to transcriptional inactivation. The presence of *Gret1* retrotransposon in the promoter of the *MYBA1* gene was first described and associated with a loss-of-function in cv. 'Italia' and cv. 'Muscat of Alexandria' cultivars resulting in the non-functional allele *VvmybA1*^{ITA}, also called *VvmybA1a* allele (Kobayashi et al., 2004). On the other hand, the most likely original sequence of *MYBA1*, before the *Gret1* retrotransposon insertion, corresponds to the

functional wild-type allele *VvmybA1*^{AFL}, commonly known as *VvmybA1c* allele, which completely lacks *Gret1* (Yakushiji et al., 2006). Moreover, other *MYBA1* functional alleles have been described, mainly associated with reversions from mutated-to-functional allelic versions. The partial *Gret1* retrotransposon excision from the *MYBA1* promoter leaving behind its solo-3'LTR region constitutes the *VvmybA1*^{RUO} allele, also named *VvmybA1b*. This allele has been firstly described on cv. 'Ruby Okuyama', which may have occurred as a result of intra-recombination between the 5'LTR and 3'LTR of *Gret1* in the 5'-flanking region near the coding region of *MYBA1*, and its functional allele (Kobayashi et al., 2005, 2004). Since then it has been identified in several red-skinned somatic variants derived from white-skinned cultivars, and described as the main mechanism responsible for color recovery on white-skinned cultivars (Ferreira et al., 2018; Migliaro et al., 2014).

In addition, the rosy-skinned somatic variant cv. 'Benitaka' derived from the white-skinned cv. 'Italia' is caused by the appearance of the functional allele *VvmybA1*^{BEN}, which was reported as a result of homologous recombination between the promoter regions of the non-functional allele of *MYBA1* (*Vvmyb*^{ITA}) and *MYBA3*, resulting in the restoration of *MYBA1* transcripts on cv. 'Benitaka' (Azuma et al., 2009). Furthermore, a less frequent functional allele, called *VvmybA1*^{SUB}, was also observed in some cultivars, as the result of three short insertions, two in the 5'-UTR region (111- and 44-bp) and one in the second intron (33-bp) (Lijavetzky et al., 2006; This et al., 2007).

Regarding *MYBA2* gene polymorphisms, Walker et al. (2007) reported that *MYBA2* can be inactivated by two non-conservative mutations, one leading to an amino acid substitution (change of arginine residue at position 44 in the red allele [G] to leucine in the white allele [T], within the first MYB repeat) and the other to a frame shift resulting in a smaller protein (a 2-bp deletion [CA] was found altering the reading frame at the amino acid position 258). The non-functional allele of this gene was named *VvmybA2w*, while the functional allele was called *VvmybA2r*.

Walker et al. (2007)) reported that *Vvmyb1w* (equivalent to *VvmybA1*^{ITA}) and *VvmybA2w*, and *VvmybA1r* (equivalent to *VvmybA1*^{AFL}) and *VvmybA2r*, were closely linked on the same chromosome, and named them the white and red alleles, respectively. Since these two adjacent *MYB* alleles in the berry color locus are inherited together, it is helpful to consider them as part of a single *MYB* haplotype (Azuma et al., 2009, 2008). Based on the detection of *VvmybA1*^{ITA} and *VvmybA1*^{AFL}, it was first thought that haplotype A (Hap A, equivalent to the white allele) corresponds to a non-functional 'white'

haplotype, containing both non-functional alleles *VvmybA1*^{ITA} and *VvmybA2w* and that haplotype C (Hap C, equivalent to the red allele) corresponds to the 'colored' haplotype. Later on, Fournier- Level et al. (2010) reported that Hap C could be divided into two subgroups: Hap C-N and Hap C-Rs. Hap C-N is presumed to be the ancestral MYB haplotype, containing the functional *VvmybA1*^{AFL} and *VvmybA2r* alleles and Hap C-Rs contains the functional *VvmybA1*^{AFL} and the non-functional *VvmybA2w* (Figure 1.5).

Taking into account this haplotype structure, it seems that the *Gret1* insertion in the *MYBA1* promoter region occurred after the emergence of *VvmybA2w*. Other *MYB* haplotypes have been identified, namely by Walker et al. (2006) and Yakushiji et al. (2006) who showed that the skin color mutation responsible for changing black-skinned cv. 'Pinot Noir' to white-skinned cv. 'Pinot Blanc' is caused by deletion of the *VvmybA1*^{AFL} and *VvmybA2r* alleles in Hap C-N, resulting in the non-functional Hap D, which contains null alleles both at the *MYBA1* (called *VvmybA1*^{PNB} allele) and *MYBA2* loci (Figure 5) (Azuma et al., 2008). Furthermore, Hap B seems to be originated from Hap A since it contains the alleles *VvmybA1*^{RUO} (resulting from intra-recombination between the 5'LTR and 3'LTR of *Gret1* in the 5'-flanking region near the coding region of *MYBA1*) and *VvmybA2w* (Figure 1.5).

The color recovery in rosy-skinned cv. 'Benitaka', a bud sport of the white-skinned cv. 'Italia' (Hap A/Hap A), is caused by the appearance of the functional *VvmybBA1*^{BEN} allele at the *MYBA1* locus, due to homologous recombination between the promoter regions of the non-functional allele of *MYBA1* and *MYBA3*, as mentioned earlier (Azuma et al., 2009). The occurrence of this allelic combination, *VvmybA1*^{BEN} and *VvmybA2w*, at the color locus gave rise to a new functional haplotype called Hap G (Figure 1.5) (Azuma, 2018).

Several genetic studies have revealed that white-skinned cultivars are homozygous for non-functional Hap A (Hap A/ Hap A), whereas color-skinned cultivars contain at least one functional haplotype (Azuma et al., 2007; Kobayashi et al., 2004; Lijavetzky et al., 2006; This et al., 2007). Furthermore, it was also found that the *MYB* haplotype is a major genetic determinant of anthocyanin content in grape berry skin and several findings indicate that the combination of functional haplotypes at the color locus affects the quantity of anthocyanins. Thus, grape cultivars with two functional haplotypes have a higher anthocyanin content than those with only a single functional haplotype (Azuma et al., 2011, 2008; Ban et al., 2014; Bayo-Canha et al., 2012; Song et al., 2014). A similar conclusion was made by Carrasco et al. (2015) which, despite having found

new fucntional alleles in wild accessions, never observed in cultivated grapevine, showed that wild accessions carrying two functional haplotypes had higher anthocyanin levels than accessions with a single functional haplotype. Consequently, the difference in potential anthocyanin accumulation between Hap C-N and Hap C-Rs grapes can be explained by the number of functional *MYB* alleles in each haplotype. Hap C-N has two functional alleles (*VvmybA1*^{AFL} and *VvmybA2r*), whereas Hap C-Rs has only one (*VvmybA1*^{AFL}).

The *MYB* haplotype composition at the berry color locus is also a major genetic factor that determines the anthocyanin composition, since it has been demonstrated that the number and kind of functional *MYB* haplotypes affects the ratios of tri- to dihydroxylated anthocyanins and of methylated to non-methylated anthocyanins (Azuma, 2018). This has been demonstrated by Azuma et al. (2009), who showed that the two colored bud sports of the white-skinned cv. 'Italia' (Hap A/ Hap A), the red-skinned cv. 'Ruby Okuyama' (Hap A/ Hap B) and the rosy-skinned cv. 'Benitaka' (Hap A/ Hap G) in addition to having different *MYB* haplotype composition also have different anthocyanins content and composition. The cv. 'Ruby Okuyama' has predominantly dihydroxylated non-methylated anthocyanins, whereas cv. 'Benitaka' has predominantly methylated anthocyanins and a moderate amount of trihydroxylated anthocyanins, which is also reflected by a *F3'5'H/ F3'H* expression ratio and much higher A*OMT* expression in cv. 'Benitaka' than in cv. 'Ruby Okuyama'.

1.5 CONCLUSIONS

Grapevine has a highly diversified phenotypic variation and some key features affect desired attributes for grape growers, not only in regard to crop yield, but also quality-related features, such as the color of the berry skin. Berry skin color is one of the most important evolutionary traits and a prime factor contributing to grape quality and subsequent market value. This major quality-trait is mainly determined by the quantity and composition of anthocyanins, and decreased grape quality has recently been associated with the poor coloration of red-berried cultivars. Considering the worldwide commercial importance of grapes, this review provides a useful overview of the current knowledge about the genetic control of anthocyanin biosynthesis, contributing to a more

comprehensive understanding of the metabolic and genetic factors that control grape skin color.

During the last decade, several studies related with grape skin color have shown that R2R3-MYB gene structure and composition at the color locus is the major genetic determinant of the anthocyanin content and composition in grape berry skin. However, these genes might be single parts of a highly complex network. In this sense, the multi-omics data integration (genomics, transcriptomics, proteomics, metabolomics) allows to building complex maps of molecular regulation and interaction. In grapevine, network analyses have mostly been adopted to infer about gene function and coordinated biological processes related to plant metabolism, namely the regulatory mechanisms that control berry composition (reviewed in Wong and Matus, 2017). Recently, Vannozzi et al. (2018) took advantage of the integrated network analysis and identified transcription factors of different families, including WRKYs that are putatively involved in the regulation of the grapevine STS multigenic family, along with the R2R3-MYB family (*MYB13*, *MYB14* and *MYB15*) (Wong et al., 2016). In this way, these studies could bring knowledge regarding new anthocyanin regulators, which may act as singular effectors in the activation of structural genes or through a combinatorial effect and play a major role in the synthesis of these compounds.

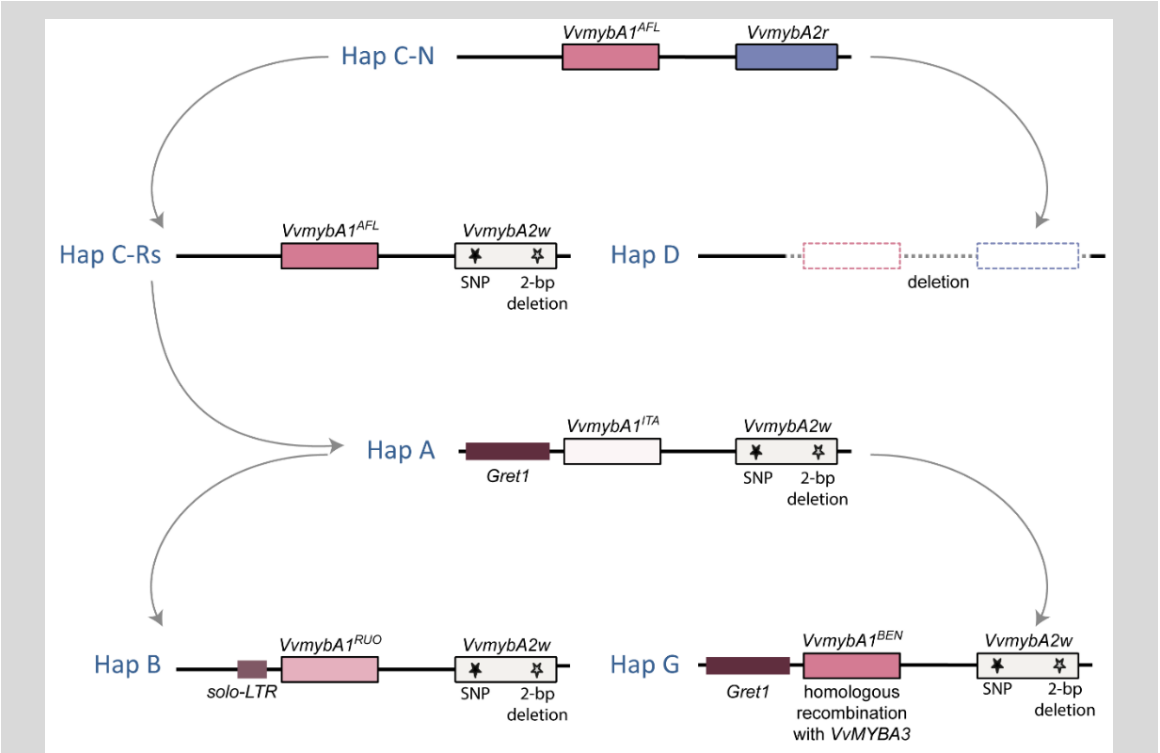

Figure 1.5 – Schematic representation of the evolutionary differentiation of the main *MYB* haplotypes at the berry color locus in grapevine [Adapted from Lacombe (2012) and Azuma (2018)].

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CHAPTER 2 | GRAPE BERRY SKIN COLOR VARIATION

Identification of *Vitis vinifera* L. grape berry skin color mutants and polyphenolic profile

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Abstract

A germplasm set of twenty-five grapevine accessions, forming eleven groups of possible berry skin color mutants, were genotyped with twelve microsatellite loci, being eleven of them identified as true color mutants. The polyphenolic profiling of the confirmed mutant cultivars revealed a total of twenty-four polyphenols, comprising non-colored compounds (phenolic acids, flavan-3-ols, flavonols and a stilbene) and anthocyanins. Results showed differences in the contribution of malvidin-3-*O*-glucoside to the characteristic anthocyanins profile of the cv. 'Pinot Noir'. Regarding the two 'Pique-Poul' colored variants, the lighter variant was richer than the darker one in all classes of compounds, excepting anthocyanins. In cv. 'Moscatel Galego Roxo' the F3'H branch pathway seems to be more active than F3'5'H, resulting in higher amounts of cyanidin, precursor of the cyanidin derivatives. As far as we are aware, this is the first time that a relationship between the content of polyphenolic compounds is established in groups of grape berry skin color mutant cultivars.

2.1 INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most cultivated fruit plants and an economically important crop worldwide. Grapes, consumed either as fresh fruit or as products derived from them (wine, juice and others), are a rich source of polyphenolic compounds (Fraige et al., 2014). These compounds are one of the main quality factors of grapes and wine, due to their contribution to wine color, oxidation reactions, interactions with proteins, ageing behavior of wines and sensorial characteristics, such as bitterness and astringency (Figueiredo-González et al., 2012a). In red wines, the perception of astringency has been mainly attributed to proanthocyanidins, also known as condensed tannins, which are involved in copigmentation processes with the anthocyanins and the formation of new pigments, which contribute to the stability and definition of red wine color (Quijada-Morín et al., 2012).

In addition, polyphenols are confirmed to have a variety of effects on human health, such as anti-inflammatory, antimicrobial and anti-aging, and also to play a preventing role against cardiovascular diseases (Ivanova et al., 2011). They are considered to be the key compounds responsible for the antioxidant potential of grapes and wine (Burns et al., 2000).

Polyphenolic compounds are mainly present in skin and seeds of grape berries and can be classified in two groups, flavonoids and non-flavonoids, based on the primary chemical structures of hydroxybenzenes (Gómez Gallego et al., 2011; Liang et al., 2011). The majority of the flavonoids found in grapes include flavan-3-ols, flavonols and anthocyanins, three structural classes divided according to the oxidation degree of their heterocyclic ring. Non-flavonoids are mainly hydroxycinnamic and hydroxybenzoic acids, stilbenes and volatile phenols (Figueiredo-González et al., 2012a; Liang et al., 2011; Teixeira et al., 2013).

Among these classes of polyphenolic compounds, hydroxycinnamic and hydroxybenzoic acids play critical roles in developing the bitterness and astringency properties of wine. The synthesis of hydroxycinnamic acids occurs mainly before veraison and they are commonly accumulated in berry skin and flesh of white and red varieties. Their concentration decreases with the increase of fruit size and dilution solutes during ripening. Particularly in white wines, hydroxycinnamic acids contribute to color browning under oxidation with non-phenolic molecules. Compared to the amount of

hydroxycinnamic acids, hydroxybenzoic derivatives levels are commonly low in wine (Teixeira et al., 2013).

Anthocyanins are the most abundant polyphenolic compounds in colored grapes, being responsible for red, purple and blue pigmentation of the grape berries and, consequently, of the red wine. The second most abundant class of flavonoids is flavan-3-ols. In grape, flavan-3-ols exist as monomers or linked forming condensed tannins. Flavan-3-ols have a direct impact on the complexity of wine taste and mouthfeel, being responsible for bitterness of wine and also associated with astringency (Liang et al., 2011; Teixeira et al., 2013). Flavonols are found in grapes and wine as glycosides. Flavonols are yellow pigments that directly contribute to the color of white wines, being masked by anthocyanins in red wines. However, flavonols are also important cofactors for color enhancement, affecting red wine color by means of copigmentation (Castillo-Muñoz et al., 2010; Liang et al., 2011).

Because of the role of polyphenols in the overall quality and, therefore, the market value of grapes and grape products, there has been considerable interest and research in determining the composition and contents of polyphenolic compounds in grape cultivars (Liang et al., 2011) and wines, particularly by relating the polyphenolic content and profile with specific features, such as color, astringency and sweetness (Figueiredo-González et al., 2014b, 2014a, 2013; Quijada-Morín et al., 2012). Although polyphenolic compounds biosynthesis in *V. vinifera* L. grapes is under genetic control and can be affected by several factors, as grape variety, ripening stage, climate, soil, light, place of growing and vine cultivation, the differences among grape cultivars are sometimes enough to make possible to use grape polyphenolic composition as a tool for cultivar authenticity and differentiation (Castillo-Muñoz et al., 2010).

Grape variety identification can be achieved by accurate genetic methods (Polymerase Chain Reaction – PCR), usually molecular markers. However, such methods are not currently available for grape berry color mutant cultivars discrimination. As so, the aim of this study was to provide data about the characteristic profiles of polyphenolic compounds of ripe grapes, by determining the compounds on a group of color and non-color related berried grapevine cultivars, derived from single varieties identified and selected by Simple Sequence Repeat (SSR) molecular markers.

2.2 MATERIAL AND METHODS

2.2.1 Standards and reagents

Acetic acid, acetonitrile, methanol (MeOH), ethyl acetate, formic acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany) and hydrochloric acid from Pronalab (Lisboa, Portugal). The polyphenolic compounds used as reference were purchased from Sigma-Aldrich (Steinheim, Germany) (caftaric acid, gallic acid, *p*-coumaric acid, quercetin-3-*O*-glucoside, epigallocatechin gallate, resveratrol-3-*O*-glucoside and syringic acid) and Extrasynthése (Genay, France) (catechin, epigallocatechin, cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, (−)-epicatechin, epicatechin gallate, isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside and syringetin-3-*O*-glucoside).

The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Solid-phase extraction (SPE) was performed with Chromabond C18 non-endcapped (NEC) columns (50 μm particle size, 60 Å porosity; 10 g sorbent mass/ 70 mL reservoir volume) from Macherey-Nagel (Düren, Germany).

2.2.2 Grape samples

Twenty-five grapevine accessions of different cultivars were harvested in 2011 in the same plot, in the experimental vineyard of the University of Trás-os-Montes and Alto Douro, Vila Real (41º19'N, 7º 44' W, 500 m above mean sea level), Baixo Corgo sub-region of the Demarcated Douro Region, northern Portugal. The cultivation practices followed (spraying of crop protectants, weed control, shoot guiding) were the same for all vines. The mean annual temperature in the region was 13.3 ºC; and total annual rainfall was 721 mm (IPMA, 2015).

Sampling was performed by picking young leaves and optimum ripeness berries randomly distributed throughout each plant. Samples represent a putative berry color mutant pool, which includes samples with similar designation, differing only in relation to the name of its berry skin color.

After harvest, the entire grapes were stored at -20 ºC and freeze-dried in a Labconco Freezone 4.5 apparatus (Kansas City, MO, USA). The lyophilized samples were then

powdered in an appliance mill (model A327R1, Moulinex, Spain). The powdered material was kept in a desiccator, in the dark, until analysis.

Voucher specimens were deposited at Laboratório de Farmacognosia, Faculdade de Farmácia, Universidade do Porto, and at Laboratório de Marcadores Moleculares, University of Trás-os-Montes and Alto Douro under the following designations: AL092011_B ('Alvarelhão'), ALB092011_W ('Alvarelhão Branco'), B092011_B ('Bastardo'), BR092011_R ('Bastardo Roxo'), BB092011_W ('Bastardo Branco'), CB092011_W ('Carrega Branco'), CT092011_B ('Carrega Tinto'), MF092011_W ('Malvasia Fina'), MFR092011_R ('Malvasia Fina Roxo'), MR092011_W ('Malvasia Rei'), MRT092011_B ('Malvasia Rei Tinto'), MGB092011_W ('Moscatel Galego Branco'), MGR092011_R ('Moscatel Galego Roxo'), MGT092011_B ('Moscatel Galego Tinto'), M092011_B ('Mourisco'), MB092011_W ('Mourisco Branco'), PB092011_W ('Pinot Blanc'), PG092011_R ('Pinot Gris'), PN092011_B ('Pinot Noir'), PPG092011_R ('Pique-poul Gris'), PPN092011_B ('Pique-poul Noir'), TB092011_W ('Touriga Branco'), TT092011_B ('Touriga Tinto'), G092011_W ('Gouveio'), GR092011_R ('Gouveio Roxo').

2.2.3 DNA extraction and nuclear microsatellite amplification

Accessions were genotyped by amplifying a set of twelve microsatellite markers, including the *Organisation Internationale de la Vigne et du Vin* (OIV) core set: VVS2, VVMD5, VVMD7, VVMD27, ssrVrZAG62, ssrVrZAG79, that correspond to OIV801 to OIV806 descriptors (OIV, 2009), established by the European Project GENRES # 81 for the identification of grapevine cultivars and part of the OIV 'Descriptor List for Grapevine Varieties and *Vitis* Species' along with VVMD28, VVMD32 (Bowers, Dangl, & Meredith, 1999), VVIv37, VVIv67, VVIp31 (Merdinoglu et al., 2005) and VMC4f3 (Di Gaspero et al., 2000).

Young leaves genomic DNA was extracted using DNeasy® Plant Mini Kit (QIAGEN, Düren, Germany) purification kit, according to the manufacturer's instructions. Extracted genomic DNA was quantified using a UV spectrometer (Nanodrop® ND-1000, Fisher Scientific, Wilmington, Delaware, USA), followed by quality check in a 1.0 % (w/v) agarose gel electrophoresis. Necessary dilutions were done (approximately 10 ng/µL) and kept at 4 ºC for further utilization.

Each 20 µL PCR mixture contained 0.2 mM of deoxynucleotide triphosphate (dNTP), 2 mM of $MgCl_2$, 10 ng of template DNA, various concentrations of primer and 1 U of *Taq* DNA polymerase in the manufacturer's buffer. One primer of each pair was fluorescently labeled with 6-fluorescein amidite (FAM) (blue), tetrachlorofluorescein succinimidyl ester (TET) (green) or hexachlorofluorescein succinimidyl ester (HEX) (yellow). PCR amplifications were performed in a T-100^{TM} Thermal Cycler (BIORAD). The program comprised an initial denaturation step (95 ºC/ 5 min), followed by 40 cycles of 94 ºC/ 45 s, 50 ºC/ 60 s and 72 ºC/ 90 s.

Two multiplex PCRs were carried out with the OIV Simple Sequence Repeat (SSR) core set, the first one involving VVS2, VVMD5 and VVMD7 (set A), and the second VVMD27, ssrVrZAG62 and ssrVrZAG79 (set B).

Set A multiplex reactions contained 0.2 µM of VVS2, 0.5 µM of VVMD5 and 0.25 µM of the VVMD7 primer pairs. Set B reactions included 0.5 µM of VVMD27 and of ssrVrZAG79, and 0.1 µM of ssrVrZAG62 primer pairs. Individual reactions were performed with the remaining six primer pairs (VVMD28, VVMD32, VVIv37, VVIv67, VVIp31 and VMC4f3), with a primer concentration of 0.5 µM.

The amplicons were separated in 3 % (w/v) agarose gel electrophoresis in Tris-Borate-EDTA (TBE) buffer, for 2 h at a constant voltage of 120 V, followed by ethidium bromide staining to verify the existence of amplicons, and then by capillary electrophoresis (ABI PRISM model 310, PE Applied Biosystems, CA, U.S.A). GENESCAN-350 TAMRA (PE Applied Biosystems, CA, USA) was included as internal sizing standard and labeled products were analyzed and sized using Peak Scanner V1.0 software (PE Applied Biosystems, CA, U.S.A).

2.2.4 Extraction of polyphenolic compounds

Healthy berries from each accession (ca. 5 g) were extracted with 100 mL of 80 % (v/v) MeOH for 2 h, under stirring (300 rpm) after flushing with nitrogen in order to prevent oxidations during extraction. The extract was centrifuged (10 min, 4000 rpm) and the material was re-extracted with 100 mL of 80 % (v/v) MeOH (15 min). The combined supernatants were evaporated to dryness under reduced pressure, at 30 ºC. The residue was dissolved in 50 mL of deionized water and applied on the SPE cartridge, preconditioned with 20 mL of ethyl acetate, 20 mL of methanol and 20 mL of 0.01 M HCl. Non-colored phenolics (fraction I) and anthocyanins (fraction II) were eluted with

20 mL of ethyl acetate and 40 mL of methanol containing 0.1 % HCl, respectively. The eluates were concentrated under reduced pressure and the residues obtained were redissolved in appropriate volume of methanol (fraction I) and acidified water (pH 3.0) (fraction II), membrane-filtered (0.45 µm) and an aliquot of 20 µL was injected into an HPLC-DAD system.

2.2.5 HPLC-DAD analysis of polyphenolic compounds

Non-colored phenolic compounds and anthocyanins were analyzed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 column (25.0 cm×0.46 cm, 5 µm particle size; Waters, Milford, MA, USA).

2.2.5.1 Non-colored compounds

The mobile phase solvents consisted of 2 % (v/v) acetic acid in water (eluent A) and 0.5 % (v/v) acetic acid in water and acetonitrile (50:50, v/v, eluent B) using a gradient program as follows: from 10 to 24 % B (20 min), from 24 to 30 % B (20 min), from 30 to 55 % B (20 min), from 55 to 70 % B (5 min), from 70 to 80 % B (5 min), from 80 to 100 % (5 min), 100 % B isocratic (5 min). Flow rate was 1.0 mL/min. Chromatograms were registered at 280, 320 and 350 nm. Compounds were identified by comparing their retention times and UV spectra with those of authentic standards and with literature data (Dopico-García et al., 2008). Quantification was performed by external standard method. Flavan-3-ols, syringic and gallic acids were determined at 280 nm, hydroxycinnamic derivatives and resveratrol-3-*O*-glucoside were quantified at 320 nm and flavonols at 350 nm. Coutaric acid was determined as *p*-coumaric acid and the other compounds as themselves. Standards and samples were analyzed in triplicate.

2.2.5.2 Anthocyanins

The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v, eluent A; 40:10:50, v/v/v; eluent B) using a gradient program as follows: from 10 to 25 % B (10 min), from 25 to 31 % B (5 min), from 31 to 40 % (5 min), from 40 to 50 % B (10 min), from 50 to 100 % B (10 min). Flow rate was 0.8 mL/min. Detection was performed at 500 nm. Compounds were identified by comparing their chromatographic behavior and UV spectra with those of authentic standards and with literature data (Dopico-García et al., 2008). Quantification was performed by external standard method. Petunidin-3-*O*-*p*-

coumaroylglucoside and petunidin-3-*O*-glucoside were quantified as petunidin; peonidin-3-*O*-*p*-coumaroylglucoside and malvidin-3-*O*-*p*-coumaroylglucoside were determined as peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside, respectively. The other compounds were determined as themselves. Standards and samples were analyzed in triplicate.

2.2.6 Statistical analysis

Principal component analysis (PCA) was carried out using SPSS® 21.0 software (IBM, NY, USA). PCA was applied for reducing the number of variables (24 variables corresponding to each identified phenolic compound: gallic acid, caftaric acid, coutaric acid, catechin, syringic acid, epicatechin, epigallocatechin gallate, epicatechin gallate, resveratrol-3-*O*-glucoside, myricetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, syringetin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, petunidin-3-*O*-*p*-coumaroylglucoside, peonidin-3-*O*-*p*-coumaroylglucoside and malvidin-3-*O*-*p*-coumaroylglucoside) to a smaller number of the new derived variables (principal components, PCs) that adequately summarize the original information, i.e., the phenolic composition of the grape berry skin color mutant cultivars. PCA method shows similarities between samples projected on a plan and makes it possible to identify which variables determine these similarities and in what way.

2.3 RESULTS AND DISCUSSION

2.3.1 Microsatellite analysis for grape berry color mutants identification

In the last years, developments in DNA analysis for varieties discrimination through microsatellite fingerprinting in viticulture has become the technique of choice for grape varietal identification and distinction (Bowers, Dangl, Vignani, & Meredith, 1996; Sefc, Regner, Turetschek, Glossl, & Steinkellner, 1999). Several grapevine varieties develop color mutants, originating new cultivars, in which phenotypic identification can be difficult before the fruit setting. However, these cultivars have the same profile than the original variety if they are analyzed by using microsatellite markers, thus facilitating the identification of the true grape berry color mutants. The European project GENRES#081 established a set of six microsatellite markers, which was included in the 'Descriptor List

for Grapevine Varieties and *Vitis* Species' and used in this study (OIV, 2009). Although these six microsatellite loci are considered as the minimal standard marker set for grapevine cultivar identification, one other group of six microsatellite loci were amplified in order to proceed to a more accurate grape cultivar authentication (Table 2.1).

In this study, eleven groups of two or three accessions, with names suggesting the existence of berry skin color mutation were characterized with microsatellites. By comparison with published profiles, identification of most of the varieties was confirmed, and six new genotypes were detected (Table 2.1).

As a consequence of the microsatellite analysis, eleven of the twenty-five accessions were selected and identified as true berry skin color mutants, belonging to five distinct families: 'Malvasia Fina', 'Gouveio', 'Moscatel Galego', 'Pinot', and' Pique-poul' (shaded in grey in Table 2.1), which were used for polyphenols composition analysis. The remaining cases, 'Alvarelhão', 'Carrega', 'Mourisco', and 'Touriga', the supposed color mutation were discarded.

The accessions 'Bastardo Branco', 'Moscatel Galego Tinto', the two 'Malvasia Fina' and 'Gouveio', and the three 'Pinot' profiles were confirmed by comparison with previously published results (Pinto-Carnide et al., 2003; Veloso et al., 2010) (Table 2.1). The accessions 'Carrega Tinto' and 'Touriga Tinto' were identified as the cv. 'Tinta Grossa' (Veloso et al., 2010) and cv. 'Touriga Franca' (Martín et al., 2006), respectively (Table 2.1). Furthermore, 'Mourisco' was identified as the cv. 'Marufo' (Castro et al., 2011).

Moreover, synonymies with Spanish cultivars were also confirmed, namely 'Alvarelhão' with the cv. 'Brancellao'; 'Alvarelhão Branco' with the cv. 'Prieto Picudo Blanco I'; 'Gouveio' and 'Gouveio Roxo' with the cv. 'Godello'; 'Moscatel Galego Branco' and 'Moscatel Galego Roxo' with the cv. 'Moscatel de Grano Menudo' (Martín, Borrego, Cabello, & Ortiz, 2003), 'Malvasia Rei' with the cv. 'Palomino' Fino (EU-VITIS, 2015) and 'Pique-poul Gris' and 'Pique-poul Noir' with the cv. 'Picapoll Negro' (Cabello et al., 2012) (Table 2.1). As it can be observed, microsatellites profiles were the same for color mutants (Table 2.1).

The three 'Bastardo' accessions showed different profiles although Bastardo and 'Bastardo Branco' had always at least one allele of each loci in common, indicating a possible parentage relationship among them. The same occurred with 'Malvasia Rei' and 'Malvasia Rei Tinto', and also with 'Moscatel Galego Tinto' and 'Moscatel Galego Branco'.

Table 2.1 – Results of the analysis with twelve SSR loci in eleven groups of *V. vinifera* L. accessions, each group including two or three accessions that presumably could be berry skin color mutants.

Cultivar name	Berry color[1]	SSR loci																						Identified SSR profile
		VVS2		VVMD5		VVMD7		VVMD27		VrZAG62		VrZAG79		VMC4f3		VVMD28		VVMD32		VVIp31		VVIv37		VVIv67		
Alvarelhão	B	130	150	218	222	237	237	181	185	187	193	249	257	171	177	231	255	237	253	186	190	163	173	355	360	Brancellao (a)
Alvarelhão Branco	W	140	156	232	236	237	237	175	181	185	185	249	249	181	185	233	255	249	259	174	188	155	155	353	360	Prieto Picudo Blanco I (a)
Bastardo	B	140	148	222	234	255	255	171	185	187	187	243	245	165	177	231	245	237	253	178	188	159	167	368	371	New genotype
Bastardo Roxo	R	130	140	224	232	245	251	175	181	187	193	243	249	171	187	233	255	253	259	180	194	159	173	NA	NA	New genotype
Bastardo Branco	W	140	148	222	234	255	255	171	185	187	187	243	249	177	177	231	231	237	253	188	190	159	161	371	371	Bastardo (b)
Carrega Branco	W	132	142	224	232	245	245	182	181	191	193	237	249	163	171	231	243	249	259	192	194	167	173	360	368	New genotype
Carrega Tinto	B	140	148	230	234	237	251	175	191	187	199	249	255	165	204	245	255	249	269	174	178	157	159	361	368	Tinta Grossa (b)
Gouveio	W	150	156	222	234	237	241	181	185	185	187	249	249	177	185	231	255	249	269	178	188	159	167	363	368	Gouveio, Godello (a, b)
Gouveio Roxo	R	150	156	222	234	237	241	181	185	185	187	249	249	177	185	231	255	249	269	178	188	159	167	363	368	Gouveio, Godello (a, b)
Malvasia Fina	W	140	142	222	236	237	255	175	191	187	187	245	249	165	204	231	233	249	253	188	188	157	159	368	371	Malvasia Fina (b, c)
Malvasia Fina Roxo	R	140	142	222	236	237	255	175	191	187	187	245	249	165	204	231	233	249	253	188	188	157	159	368	371	Malvasia Fina (b, c)
Malvasia Rei	W	140	142	224	236	237	247	181	191	187	193	249	255	173	204	233	245	253	255	186	188	159	163	360	361	Malvasia Rei, Palomino Fino (d)
Malvasia Rei Tinto	B	130	142	224	236	237	247	181	185	187	193	241	249	171	173	231	245	253	269	188	190	159	163	353	361	New genotype
Moscatel Galego Branco	W	130	130	224	232	231	247	175	191	185	195	249	253	165	204	243	265	261	269	182	186	159	161	360	371	Moscatel de Grano menudo (a)
Moscatel Galego Roxo	R	130	130	224	232	231	247	175	191	185	195	249	253	165	204	243	265	261	269	182	186	159	161	360	371	Moscatel de Grano menudo (a)
Moscatel Galego Tinto	B	130	148	222	224	237	247	175	185	185	187	249	253	171	204	243	255	237	261	182	186	159	173	360	371	Moscatel Galego Tinto (b)

Table 2.1
(continued)

Mourisco	B	140	142	224	228	237	241	179	191	187	191	245	255	173	181	241	251	237	249	174	190	155	159	353	361	Marufo (e)
Mourisco Branco	W	130	140	222	222	NA	NA	179	185	187	103	245	249	177	181	225	245	237	249	178	186	159	163	360	371	New genotype
Pinot Blanc	W	134	148	224	234	237	241	181	185	187	193	237	243	171	177	215	233	237	269	178	182	149	159	360	368	Pinot (b)
Pinot Gris	R	134	148	224	234	237	241	181	185	187	193	237	243	171	177	215	233	237	269	178	182	149	159	360	368	Pinot (b)
Pinot Noir	B	134	148	224	234	237	241	181	185	187	193	237	243	171	177	215	233	237	269	178	182	149	159	360	368	Pinot (b)
Pique-poul Gris	R	130	130	222	228	237	241	175	185	187	187	249	249	171	204	231	233	237	259	178	182	159	161	358	360	Picapoll Negro (f)
Pique-poul Noir	B	130	130	222	228	237	241	175	185	187	187	249	249	171	204	231	233	237	259	178	182	159	161	358	360	Picapoll Negro (f)
Touriga Branco	W	132	142	224	232	245	245	181	181	191	193	237	249	163	171	231	243	249	253	192	194	167	173	360	368	New genotype
Touriga Tinto	B	140	150	222	224	237	241	177	179	191	193	243	245	173	204	231	251	237	269	174	182	155	159	353	363	Touriga Franca (g)

[1] B=black; W=white; R=red.
Grey-shaded groups are considered as true skin color mutants, based on the coincidence of microsatellite profiles. Right hand column identifies the cultivars based on the references: a) (Martín et al., 2003); b) (Veloso et al., 2010); c) (Pinto-Carnide et al., 2003); d) http://www.eu-vitis.de; e) (Castro et al., 2011); f) (Cabello et al., 2012); g) (Martín et al., 2006). NA = not amplified.

2.3.2 Polyphenolic compounds

Twenty-four polyphenolic compounds, distributed by colored and non-colored compounds, were identified (Figure 2.1 and Figure 2.2) and quantified (Table 2.2) in all berry skin color mutants selected by SSRs.

2.3.2.1 Non-colored compounds

Among non-colored polyphenolic compounds, four phenolic acids, eleven flavonoids and one stilbene were identified (Table 2.2).

2.3.2.1.1. Phenolic acids

Gallic (**1**), syringic (**5**), caftaric (**2**) and coutaric (**3**) acids were the four phenolic acids identified in all studied mutants (Figure 2.1, Table 2.2). Phenolic acids constituted one of the less represented group of non-colored compounds, both in colored and non-colored variants (Table 2.2), as also previously observed by Liang et al., (2011).

Among hydroxybenzoic acids, with exception of the cv. 'Pinot Gris', the content of gallic acid (**1**) was higher than that of syringic acid (**5**). Caftaric acid (**2**) was the main hydroxycinnamic acid.

The cv. 'Pinot Noir' revealed the highest amount of both hydroxybenzoic (ca. 71 mg/kg dry berry) and hydroxycinnamic acids (ca. 58 mg/kg dry berry) (Table 2.2).

2.3.2.1.2. Flavan-3-ols

Four flavan-3-ols were identified: catechin (**4**), epicatechin (**6**), epigallocatechin gallate (**7**) and epicatechin gallate (**8**) (Figure 2.1, Table 2.2). Epigallocatechin gallate (7), which is negatively correlated with astringency (Quijada-Morín et al., 2012), was not detected in five of the berry skin color mutants (Table 2.2), namely in the black-skinned mutant variants, cv. 'Pinot Noir' and cv. 'Pique-poul Noir' and also in cv. 'Pinot Gris', cv. 'Gouveio' and cv. 'Gouveio Roxo' (Table 2.2). Despite, this compound was detected in the remaining mutants, in general, its representativeness was reduced when compared with the other three flavan-3-ols. Catechin (**4**) gave the highest contribution for the amount of this class of flavonoids, representing more than 62 % of total flavan-3-ols in all berry skin color mutants (Table 2.2). The highest concentrations of flavan-3-ols are generally found in green grapes and decrease during ripening (Mulinacci et al., 2008). Although these compounds are located in both grapes seed and skin, concentrations are

much lower in the last. In addition, their composition is also different, skin containing both flavan-3-ols and their galloylated forms, whereas seed presents mainly the first (González-Manzano et al., 2004). Catechin usually is the main flavanol in both skin and seed, although epicatechin is also well represented; however, in some grape cultivars, these monomers are found at similar levels or the amount of epicatechin is higher (Dopico-García et al., 2008; Escribano-Bailón et al., 1995; Santos-Buelga et al., 1995).

The cv. 'Pinot Gris' was the richest mutant in flavan-3-ols (ca. 2127 mg/ kg dry berry), showing even higher quantities than its black-skinned variant, cv. 'Pinot Noir' (1415 mg/ kg dry berry) (Table 2.2). Among white mutants, the one corresponding to 'Pinot' variety (cv. 'Pinot Blanc') also revealed high content of flavan-3-ols (ca. 871 mg/ kg dry berry). In opposition, the cv. 'Malvasia Fina' was the poorest in this kind of compounds, considering both its white and red-skinned variants (ca. 121 mg/kg of dry berry and ca. 132 mg/kg of dry berry, respectively) (Table 2.2).

2.3.2.1.3. Flavonols

Several flavonols, including 3-*O*-glycosides of myricetin (**10**), quercetin (**11-13**), kaempferol (**14**), isorhamnetin (**15**) and syringetin (**16**), were found (Table 2.2). Myricetin-3-*O*-glucoside (**10**) was only detected in the cv. 'Pinot Noir' (Figure 2.1, Table 2.2). Moreover, cv. 'Moscatel Galego Roxo', cv. ' Pinot Gris'' and cv. 'Pinot Noir' were the only mutants that presented syringetin-3-*O*-glucoside (**16**).

The cv. 'Pique-poul Gris' revealed the highest flavonols content (ca. 258 mg/kg dry berry), quercetin-3-*O*-galactoside (**11**) accounting for 39 % of the flavonols determined in this skin color mutant variant (Table 2.2). In fact, quercetin derivatives were the most represented flavonols in all mutant variants, ranging between 66 and 98 % of the compounds in cv. 'Gouveio' and cv. 'Pique-poul Noir', the poorest and richest mutant variants, respectively.

2.3.2.1.4. Stilbenes

Only one stilbene, resveratrol-3-*O*-glucoside (**9**), was found, being present in all berry skin color mutants (Figure 2.1, Table 2.2). With the exception of the cv. 'Pinot Noir', that was the richest mutant in this stilbene (Table 2.2), red berried grapes showed higher resveratrol-3-*O*-glucoside levels than their white and black berried grape variants. However, resveratrol-3-*O*-glucoside (**9**) did not represent more than 4 % of total non-colored compounds content in all berry skin color mutants.

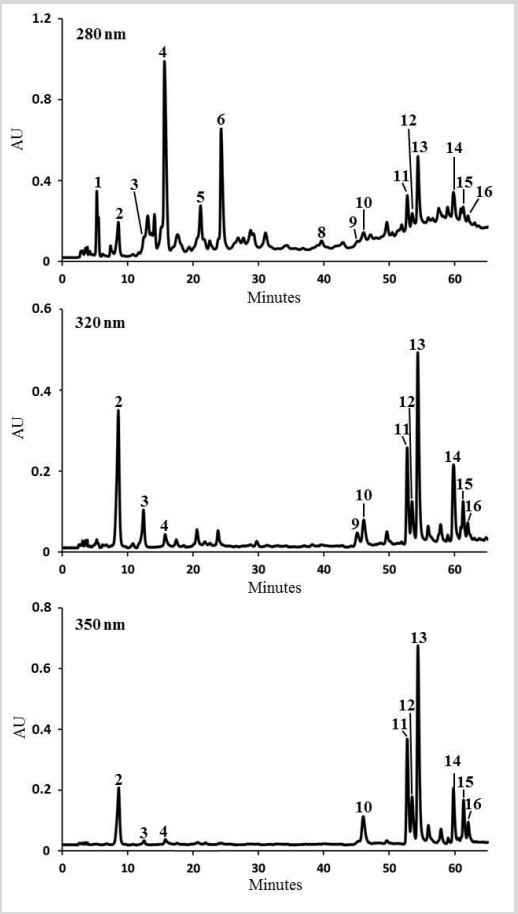

Figure 2.1 – HPLC DAD chromatogram of non-colored phenolics of *V. vinifera* cv. 'Pinot Noir' grapes hydromethanolic extract. (1) Gallic acid; (2) Caftaric acid; (3) Coutaric acid; (4) Catechin; (5) Syringic acid; (6) Epicatechin; (7) Epigallocatechin gallate; (8) Epicatechin gallate; (9) Resveratrol-3-*O*-glucoside; (10) Myricetin-3-*O*-glucoside; (11) Quercetin-3-*O*-galactoside; (12) Quercetin-3-*O*-rutinoside; (13) Quercetin-3-*O*-glucoside; (14) Kaempferol-3-*O*-glucoside; (15) Isorhamnetin-3-*O*-glucoside; (16) Syringetin-3-*O*-glucoside.

2.3.2.2 Anthocyanins

In this study, eight anthocyanins were identified (Figure 2.1, Table 2.1). The detected compounds were monoglucoside derivatives of five anthocyanidins, [delphinidin (**17**), cyanidin (**18**), petunidin (**19** and **22**), peonidin (**20** and **23**) and malvidin (**21** and **24**)], some of them being also acyl derivatives (compounds **22**, **23** and **24**).

Anthocyanins are synthesized in grape cells at the cytosolic surface of the endoplasmic reticulum, by a multienzyme complex *via* the flavonoid pathway (Boss et al., 1996). The genes encoding the early steps enzymes of the polyphenolic biosynthetic pathway, namely chalcone synthase, chalcone isomerase and flavanone-3-hydroxylase, belong to multicopy families and the different number gene copies may have temporal

and spatial partitioned expression profiles that sometimes coincide with the biosynthesis of a particular flavonoid (Kuhn et al., 2014).

As expected, these colored polyphenols were only found in black and red-skinned mutant variants. However, two exceptions were observed, namely in cv. 'Malvasia Fina Roxo' and cv. 'Gouveio Roxo'. Despite the cv. 'Malvasia Fina Roxo' revealed some red pigmentation in its skin berry, it did not present detectable amounts of anthocyanins. The anthocyanins composition is affected by the expression of flavonoid 3'5'-hydroxylase (F3'5'H) and flavonoid 3'-hydroxylase (F3'H) genes. *F3'5'H* genes are present in highly redundant copy numbers and only two copies of *F3'H* genes are found in the grape genome, being one copy expressed and the other transcriptionally silent (Kuhn et al., 2014). The prevalence of *F3'5'H* over *F3'H* in black cultivars would lead to the presence of more delphinidin, the precursor of blue/purple petunidin and malvidin derivatives, and, in contrast, it would yield less cyanidin, the precursor of the red peonidin derivatives (Kuhn et al., 2014). On this way, the lack of detectable amounts of anthocyanins in 'Malvasia Fina Roxo' suggests a lack of activity of both *F3'5'H* and *F3'H* genes. Another possibility that can influence and be behind the color observed in the cv. 'Malvasia Fina Roxo' is the synthesis of other compounds, such as carotenoids, which are lipid soluble pigments found in many vegetable crops (Jackson, 2008) usually masked by the presence of anthocyanins that donates the dominant color. Moreover, in the field no detectable skin color development was observed for the cv. 'Gouveio Roxo' during ripening, which suggests that these compounds were not being synthesized. The variation of anthocyanin composition and concentration in grapes is a consequence of many factors, such as cultivar, climate (like sunlight exposure, UV radiation, temperature), canopy management, fertilizers and water regimes, affecting both the expression of the structural and regulatory genes (Downey, Dokoozlian, & Krstic, 2006; He et al., 2010). Recently, some studies addressed the effects of anti-fungal treatments on the color and phenolic profile of red wines, concluding that, in general, the anti-fungal substances had different effects depending on the cultivar and on the phenolic compound analyzed , usually resulting in less colorful wines (Briz-Cid et al., 2015, 2014). Therefore, the accession analyzed and designated as cv. 'Gouveio Roxo', despite presenting the profile of 'Gouveio' variety, as confirmed by the molecular analysis (Table 2.1), it does not correspond to a red-skinned variant of 'Gouveio' evidenced by the lack of anthocyanins.

Qualitative differences among berry skin color mutants of the same variety were also seen (Table 2.2). The cv. 'Pinot Gris' did not show the three acylated anthocyanins (compounds **22**, **23** and **24**) present in the cv. 'Pinot Noir' (Table 2.2).

Delphinidin-3-*O*-glucoside (**17**), petunidin-3-*O*-glucoside (**19**) and petunidin-3-*O*-*p*-coumaroylglucoside (**22**) were not detected in the cv. 'Pique-poul Gris', but were found in the other red mutant variants, as well as in its black-skinned variant cv. 'Pique-poul Noir'. However, the cv. 'Pique-poul Gris' presented peonidin-3-*O*-*p*-coumaroylglucoside (**23**) and malvidin-3-*O*-*p*-coumaroylglucoside (**24**) that were not detected in the remaining red berried mutant variants (Table 2.2). As so, the presence or absence of these compounds could be a specific feature of the cv. 'Pique-poul Gris', allowing to easily distinguish this red color variant from the other red berried variants studied.

Quantitatively, the cv. 'Pinot Noir' was clearly the mutant with the highest amount of anthocyanins (ca. 723 mg/kg dry berry), about eight times more than its red-skinned variant, cv. 'Pinot Gris' (ca. 91 mg/kg dry berry) (Table 2.2**)**. Liang et al. (2011) described that during the whole course of maturation, the skin cells of colored grapes accumulate anthocyanins and, consequently, the color of the berry is progressively darkened.

Malvidin-3-*O*-*p*-glucoside (**21**) was the major compound in the black-skinned mutant variants and in the cv. 'Pinot Gris', although at notably higher concentration in the cv. 'Pinot Noir' (ca. 449 mg/kg dry berry) (Table 2.2). The cv. 'Pinot Noir' is an international cultivar, being much studied concerning to its anthocyanin composition. Dimitrovska, Bocevska, Dimitrovski and Murkovic (2011) and Mattivi, Guzzon, Vrhovse, Stefanini and Velasco (2006) reported the anthocyanin profile of the cv. 'Pinot Noir', both showing malvidin-3-*O*-*p*-glucoside as the main anthocyanin. However, our results also showed the presence of three acylated derivatives (Figure 2.2, Table 2.2), petunidin-3-*O*-*p*-coumaroylglucoside, peonidin-3-*O*-*p*-coumaroylglucoside and malvidin-3-*O*-*p*-coumaroylglucoside (compounds **22**, **23** and **24**), which, as far as we know, are reported for the first time in this cultivar. This observation provides evidence about the influence of the environmental factors on the anthocyanin pattern.

Our results suggest that different proportions of individual anthocyanins, in addition to their total amount, can affect the color types of grape berry skin color mutants. Considering the cv. 'Pinot Noir', besides malvidin-3-*O*-glucoside (**21**), other anthocyanins, namely delphinidin-3-*O*-glucoside (**17**), petunidin-3-*O*-glucoside (**19)** and

peonidin-3-*O*-glucoside (**20**), should be highlighted because of their contribution to the total anthocyanins content of this grape (Table 2.2).

Several authors reported cyanidin derivatives as the minor group of anthocyanins in colored cultivars (Figueiredo-González et al., 2012b; Núñez et al., 2004). Furthermore, taking into account that cyanidin is the precursor of others anthocyanins, it is usual to find low concentrations of its derivatives in red colored grapes (Núñez et al., 2004). Nevertheless, this was not observed in the cv. 'Moscatel Galego Roxo', suggesting that the F3'H pathway is more active than F3'5'H, resulting in higher amounts of cyanidin-3-*O*-glucoside.

Previous studies described the UDPglucose:flavonoid-3-*O*-glucosyltransferase (UFGT) activity as critical for anthocyanins biosynthesis (Boss et al., 1996; Zheng et al., 2013). The control of the biosynthetic step mediated by UFGT in the anthocyanin pathway is mainly affected by *MYB* genes family. The presence of *Gret1* retrotransposon, in the promotor region of *MYBA1*, is associated with white-fruited cultivars when present in a homozygous state (Kobayashi et al., 2004). Additional polymorphisms in this gene are also strongly associated with a red or pink-fruited phenotype (This et al., 2007), which can possibly explain some of the color differences among the different sets of berry color mutants studied.

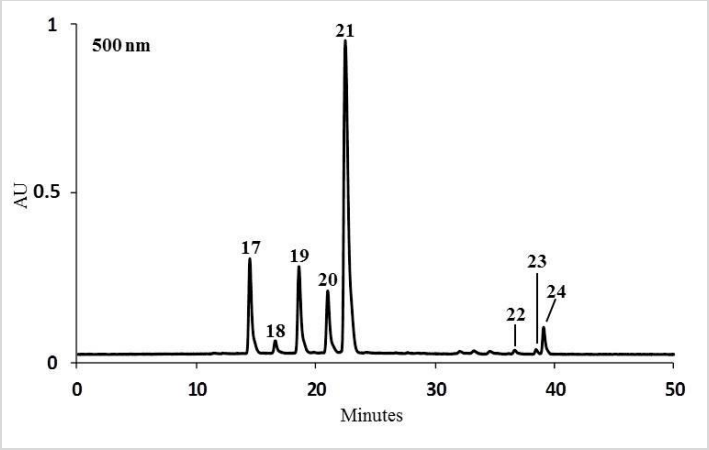

Figure 2.2 – HPLC DAD chromatogram of anthocyanins from *V. vinifera* cv. 'Pinot Noir' grapes hydromethanolic extract. (17) Delphinidin-3-*O*-glucoside; (18) Cyanidin-3-*O*-glucoside; (19) Petunidin-3-*O*-glucoside; (20) Peonidin-3-*O*-glucoside; (21) Malvidin-3-*O*-glucoside; (22) Petunidin-3-*O*-*p*-coumaroylglucoside; (23) Peonidin-3-*O*-*p*-coumaroylglucoside; (24) Malvidin-3-*O*-*p*-coumaroylglucoside.

Table 2.2 – Quantification of polyphenolic compounds in hydromethanolic extracts of 11 skin color mutant cultivars (mg/kg berry, dry basis)[a]. Within parenthesis standard deviations of three determinations. MF – 'Malvasia Fina'; G – 'Gouveio'; MGB – 'Moscatel Galego Branco'; PB – 'Pinot Blanc'; MFR – 'Malvasia Fina Roxo'; GR – 'Gouveio Roxo'; MGR – 'Moscatel Galego Roxo'; PPG – 'Pique-poul Gris'; PG – 'Pinot Gris'; PPN –'Pique-poul Noir'; PN – 'Pinot Noir'.

	White cultivars				Red cultivars					Black cultivars	
	MF	**G**	**MGB**	**PB**	**MFR**	**GR**	**MGR**	**PPG**	**PG**	**PPN**	**PN**
PHENOLIC ACIDS											
Hydroxybenzoic acids											
(**1**) Gallic acid	5.7 (0.1)	8.1 (0.1)	19.2 (0.5)	18.3 (0.0)	11.6 (0.6)	9.8 (0.2)	19.6 (0.1)	24.4 (0.3)	16.1 (0.3)	15.1 (0.3)	38.4 (0.0)
(**5**) Syringic acid	5.5 (0.0)	7.0 (0.1)	11.4 (0.3)	17.2 (0.1)	9.7 (0.8)	7.0 (0.0)	5.7 (0.0)	10.8 (0.9)	20.2 (1.1)	6.8 (0.1)	32.8 (2.0)
Hydroxycinnamic acids											
(**2**) Caftaric acid	22.8 (0.2)	10.5 (0.2)	24.0 (0.3)	28.1 (0.2)	15.8 (0.1)	14.9 (0.1)	45.4 (0.5)	44.2 (0.3)	12.1 (0.4)	10.1 (0.1)	53.9 (0.2)
(**3**) Coutaric acid	2.1 (0.1)	0.7 (0.1)	4.2 (0.0)	2.7 (0.1)	2.2 (0.0)	3.0 (0.0)	5.7 (0.0)	4.7 (0.0)	3.3 (0.0)	1.1 (0.0)	4.6 (0.1)
Σ	**36.1 (0.4)**	**26.3 (0.5)**	**58.8 (1.1)**	**66.3 (0.4)**	**39.3 (1.5)**	**34.7 (0.3)**	**76.4 (0.6)**	**84.1 (1.5)**	**51.7 (1.8)**	**33.1 (0.5)**	**129.7 (2.3)**
FLAVONOIDS											
Flavan-3-ols											
(**4**) Catechin	88.5 (1.1)	391.1 (19.7)	408.1 (7.2)	589.8 (2.5)	83.8 (5.5)	341.7 (3.1)	379.8 (10.2)	261.6 (1.7)	1615.5 (34.2)	212.5 (1.0)	977.0 (4.5)
(**6**) Epicatechin	24.0 (0.0)	133.6 (2.1)	206.0 (3.1)	254.2 (4.6)	36.2 (0.7)	118.9 (0.2)	130.0 (3.2)	104.0 (0.7)	474.8 (9.5)	119.2 (6.7)	423.6 (2.9)
(**7**) Epigallocatechin gallate	5.0 (0.1)	-	9.0 (0.6)	12.7 (2.1)	7.2 (0.5)	-	5.3 (0.0)	6.9 (0.1)	-	-	-
(**8**) Epicatechin gallate	3.3 (0.0)	10.3 (0.1)	19.9 (0.2)	14.4 (0.2)	4.4 (0.7)	13.9 (0.0)	11.5 (0.1)	23.4 (1.7)	36.4 (5.0)	11.7 (0.0)	14.4 (0.1)
Σ	**120.8 (1.2)**	**535.0 (21.9)**	**643.0 (11.1)**	**871.1 (9.4)**	**131.6 (7.4)**	**474.5 (3.3)**	**526.6 (13.5)**	**395.9 (4.2)**	**2126.7 (48.7)**	**343.4 (7.7)**	**1415.0 (7.5)**
Flavonols											
(**10**) Myricetin-3-*O*-glucoside	-	-	-	-	-	-	-	-	-	-	20.3 (0.8)
(**11**) Quercetin-3-*O*-galactoside	44.6 (0.3)	52.4 (0.4)	68.2 (1.7)	19.7 (0.6)	59.7 (0.7)	45.9 (1.2)	82.2 (0.7)	100.6 (1.5)	86.8 (1.2)	41.9 (0.1)	33.8 (0.4)
(**12**) Quercetin-3-*O*-rutinoside	21.6 (0.4)	30.6 (0.1)	23.5 (0.5)	3.1 (0.0)	28.0 (0.7)	20.1 (0.1)	24.8 (0.2)	27.6 (0.3)	25.8 (0.1)	16.0 (0.2)	30.5 (0.0)
(**13**) Quercetin-3-*O*-glucoside	48.4 (0.3)	81.3 (0.5)	51.6 (1.1)	5.9 (0.0)	70.7 (1.2)	54.4 (0.7)	52.7 (0.6)	82.3 (1.7)	63.8 (0.7)	39.3 (0.2)	67.2 (1.0)
(**14**) Kaempferol-3-*O*-glucoside	34.9 (0.6)	78.6 (0.2)	66.8 (1.4)	4.5 (0.2)	37.0 (1.1)	46.5 (0.2)	60.3 (0.9)	47.4 (1.0)	32.6 (0.7)	19.4 (0.2)	25.8 (0.3)
(**15**) Isorhamnetin-3-*O*-glucoside	1.4 (0.1)	4.4 (0.2)	4.2 (0.1)	1.5 (0.1)	1.6 (0.1)	5.4 (0.1)	2.9 (0.0)*	-	9.2 (0.1)	1.8 (0.1)	13.0 (0.0)
(**16**) Syringetin-3-*O*-glucoside	-	-	-	-	-	-		-	3.2 (0.3)	-	8.6 (0.2)
Σ	**150.9 (1.7)**	**247.3 (1.4)**	**214.3 (4.8)**	**34.7 (0.9)**	**197.0 (3.8)**	**172.3 (2.3)**	**222.9 (2.4)**	**257.9 (4.5)**	**221.4 (3.1)**	**118.4 (0.8)**	**199.2 (2.7)**

Table 2.2

(continued)

	White cultivars				Red cultivars					Black cultivars	
	MF	**G**	**MGB**	**PB**	**MFR**	**GR**	**MGR**	**PPG**	**PG**	**PPN**	**PN**
Anthocyanins											
(**17**) Delphinidin-3-*O*-glucoside	-	-	-	-	-	-	1.6 (0.1)	-	0.6 (0.0)	1.5 (0.1)	81.9 (0.6)
(**18**) Cyanidin-3-*O*-glucoside	-	-	-	-	-	-	5.1 (0.1)	0.7 (0.1)	0.1 (0.0)	4.3 (0.3)	9.9 (0.4)
(**19**) Petunidin-3-*O*-glucoside	-	-	-	-	-	-	0.4 (0.0)	-	2.8 (0.4)	2.7 (0.0)	94.8 (0.5)
(**20**) Peonidin-3-*O*-glucoside	-	-	-	-	-	-	1.2 (0.2)	0.6 (0.0)	7.8 (0.2)	13.4 (0.1)	52.2 (0.5)
(**21**) Malvidin-3-*O*-glucoside	-	-	-	-	-	-	0.9 (0.1)	0.7 (0.2)	79.5 (0.1)	25.7 (0.8)	448.7 (1.5)
(**22**) Petunidin-3-*O*-*p*-coumaroylglucoside	-	-	-	-	-	-	-	-	-	1.4 (0.4)	4.3 (0.1)
(**23**) Peonidin-3-*O*-*p*-coumaroylglucoside	-	-	-	-	-	-	-	0.3 (0.0)	-	2.9 (0.1)	3.6 (0.0)
(**24**) Malvidin-3-*O*-*p*-coumaroylglucoside	-	-	-	-	-	-	-	0.3 (0.0)	-	3.7 (0.1)	27.4 (0.1)
∑	-	-	-	-	-	-	**9.2 (0.5)**	**2.6 (0.3)**	**90.8 (0.7)**	**55.6 (1.9)**	**722.8 (3.2)**
STILBENES											
(**9**) Resveratrol-3-*O*-glucoside	1.4 (0.0)	1.1 (0.0)	0.9 (0.0)	1.7 (0.0)	1.9 (0.0)	2.0 (0.1)	1.2 (0.0)	3.7 (0.0)	2.1 (0.1)	0.7 (0.0)	5.5 (0.1)
TOTAL PHENOLS	**309.2 (3.3)**	**809.7 (23.8)**	**917.0 (17.0)**	**973.8 (10.7)**	**369.8 (12.7)**	**683.5 (6.0)**	**836.3 (17.0)**	**744.2 (10.5)**	**2492.7 (54.4)**	**551.2 (10.9)**	**2472.2 (15.8)**

[a] ∑: sum of the identified polyphenolic compounds; "-": not detected. *Isorhamnetin-3-*O*-glucoside and syringetin-3-*O*-glucoside were quantified together.

2.3.3 Principal Components Analysis (PCA)

To study the relationship between colored and non-colored related berried cultivars and their polyphenolic composition, PCA was applied to the content (mg/kg dry grape) of non-colored compounds (Figure 2.3 A–B) and anthocyanins (Figure 2.3 C–D).

PCA of normalized non-colored dataset explained 62.52 % of total variations, PC1 accounting for 43.10 % of the variance and PC2 for 19.42 % (Figure 2.3 A–B). As shown in Figure 2.3 A, three groups could be clearly distinguished (Figure 2.3 A–B). One group (G1) includes the color-related berried cv. 'Pinot Gris' and cv. 'Pinot Noir'. These variants appeared in the positive plan of PC1, due to the absence of epigallocatechin gallate, but highest content in flavano-3-ols (ca. 2127 and 1415 mg/kg dry grape, respectively), namely in catechin, as well as the high content of phenolic acids, namely syringic acid. Despite being included in the same group, the cv. 'Pinot Gris' appeared in the positive plan of the PC2 and the cv. 'Pinot Noir' in the negative one due to the presence of myricetin-3-*O*-glucoside in the last one. Group G2 included the white-skinned variants, cv. 'Gouveio' and cv. 'Moscatel Galego Branco' and the red-skinned variants, cv.'Malvasia Fina Roxo', cv. 'Moscatel Galego Roxo' and cv. 'Pique-poul Gris' due to their high content in glucoside derivatives of kaempferol and quercetin, with particular relevance for the cv. 'Pique-poul Gris', the richest one. The low amount of flavonols and phenolic acids in the cv. 'Malvasia Fina', cv. 'Gouveio Roxo' and cv. 'Pique-poul Noir' led to the inclusion of them in another group (G3), in the negative parts of PC1 and PC2. The cv. 'Pinot Blanc' was clearly separated from the other cultivars due to its low levels in flavonols (Figure 2.3 A–B).

PCA of anthocyanins explained 93.16 % of total variation, where PC1 accounts for 84.17 % of the variance and PC2 for 8.99 % (Figure 2.3 C–D). Due to the absence of anthocyanins in their composition, white-skinned variants such as the cv. 'Malvasia Fina', cv. 'Gouveio', cv. 'Moscatel Galego Branco' and 'cv. Pinot Blanc', were grouped together (G1) in the negative part of PC1 (Figure 2.3 C–D).

PCA confirmed that the anthocyanin profile was related to the grape skin color. Among color-berried mutants, two subgroups were established (Figure 2.3 C–D). One group (G2a) that appears in the positive plan of PC1 and PC2 includes the black-skinned variants cv. 'Pique-poul Noir' and cv. 'Pinot Noir' for their content in *p*-coumaroyl derivatives of petunidin, peonidin and malvidin. Another group (G2b) included the cv. 'Moscatel Galego Roxo' and cv. 'Pinot Gris', in which no *p*-coumaroyl derivatives were

identified. The red-skinned variant cv. 'Pique-poul Gris', in which the content of non-acylated anthocyanins was higher than that of acylated ones, was the poorest mutant regarding anthocyanins, appearing in the positive part of PC2, close to the group of white-skinned variants (G1).

2.4 CONCLUSIONS

The variation of polyphenolic compounds in groups of grape berry skin color mutants, including related black, red and white-berried mutant variants derived from a single variety identified by nuclear microsatellite analysis, was investigated, for the first time. The grape berry skin color mutants were distinguished according to their phenolic acids, flavan-3-ols, flavonols and anthocyanins composition. Molecular and chemical approaches complemented each other in the correct identification of the grape berry skin color mutants.

As expected, anthocyanins were the main group of compounds that allowed a clear division among color and non-colored related mutant variants. The results also revealed differences in the contribution of different anthocyanins to distinguish berry skin color mutants.

The observed chemical richness and differences among related mutant cultivars derived from a single variety encourage the use of berry skin color somatic variants, not only for the development of new cultivars with interesting characteristics, namely concerning the color feature, but also to improve knowledge on colored and non-colored cultivars and understanding the evolutionary events behind their origin.

The study of genes involved in the polyphenolic biosynthesis may help elucidating the genetics behind grape berry skin color and understand how this kind of compounds affected the skin pigmentation of the studied grape berry skin color mutants.

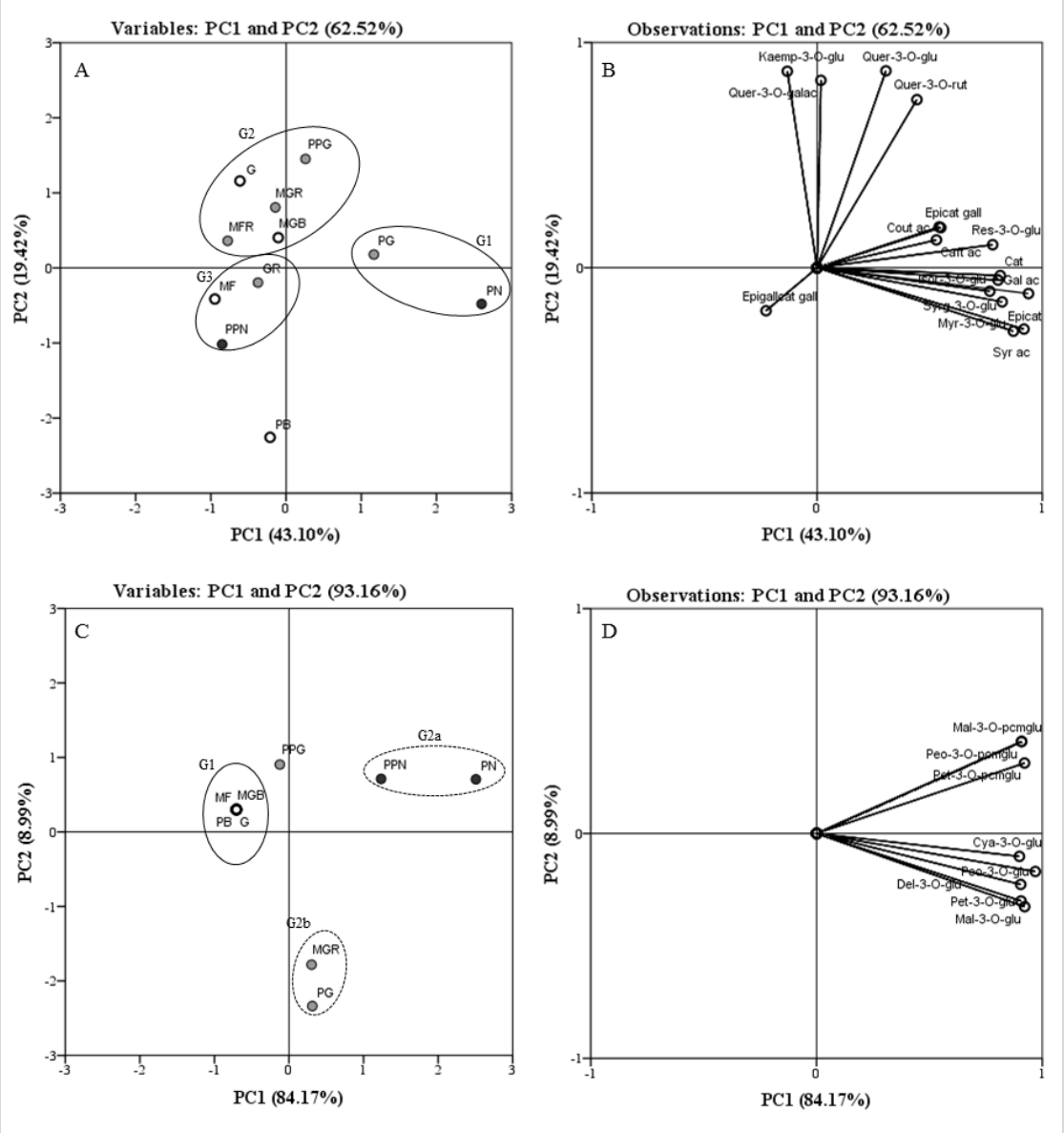

Figure 2.3 – Projection of grape berry skin color mutants (**A** and **C**) [variables: 'Malvasia Fina' (MF); 'Gouveio' (G); 'Moscatel Galego Branco' (MGB); 'Pinot Blanc'' (PB); 'Malvasia Fina Roxo (MFR); 'Gouveio Roxo' (GR); 'Moscatel Galego Roxo' (MGR); 'Pique-poul Gris' (PPG); 'Pinot Gris' (PG); 'Pique-poul Noir' (PPN); 'Pinot Noir' (PN)] and loadings by (**B)** non-colored polyphenols and (**D**) anthocyanins composition [variables: gallic acid (Gal ac); caftaric acid (Caft ac); coutaric acid (Cout ac); catechin (Cat); syringic acid (Syr ac); epicatechin (Epicat); epigallocatechin gallate (Epigallcat gall); epicatechin gallate (Epicat gall); resveratrol-3-*O*-glucoside (Res-3-*O*-glu); myricetin-3-*O*-glucoside (Myr-3-*O*-glu); quercetin-3-*O*-galactoside (Quer-3-*O*-galac); quercetin-3-*O*-rutinoside (Quer-3-*O*-rut); quercetin-3-*O*-glucoside (Quer-3-*O*-glu); kaempferol-3-*O*-glucoside (Kaemp-3-*O*-glu); isorhamnetin-3-*O*-glucoside (Isor-3-*O*-glu); syringetin-3-*O*-glucoside (Syr-3-*O*-glu); delphinidin-3-*O*-glucoside (Del-3-*O*-glu); cyanidin-3-*O*-glucoside (Cya-3-*O*-glu); petunidin-3-*O*-glucoside (Pet-3-*O*-glu); peonidin-3-*O*-glucoside (Peo-3-*O*-glu); malvidin-3-*O*-glucoside (Mal-3-*O*-glu); petunidin-3-*O*-*p*-coumaroylglucoside (pet-3-*O*-pcmglu), peonidin-3-*O*-*p*-coumaroylglucoside (peon-3-*O*-pcmglu), malvidin-3-*O*-*p*-coumaroylglucoside (pet-3-*O*-pcmglu)] into the plane composed by the principal components PC1 and PC2 containing 62.52 % and 93.16 % of the total variance for non-colored polyphenols and anthocyanins composition, respectively.

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CHAPTER 3 | GRAPE BERRY DEVELOPMENT

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

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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 the berry color 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.

3.1 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 veraison that marks the boundary between the lag phase and the second growth period after which growth declines. Veraison is generally characterized by several processes, such as berry softening, acids decrease, sugar accumulation, loss of photosynthetic capacity and initiation of color development (Robinson and 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 et al., 2014; Azuma et al., 2008; Rustioni et al., 2016). This morpho-physiological diversity regarding grape berry skin color could be 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 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 cv. ‘Pinot Gris’, cv. ‘Pinot Meunier’ or the berry color somatic variants of cv. ‘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 and Davies, 2000). These pigments' profile and concentration largely vary, depending on the grapevine cultivar (Mattivi et al., 2006), and are not even synthesized by white-berried ones (Boss et al., 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 et al., 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; Kobayashi et al., 2002). It was shown that the insertion of *Gret1* retrotransposon in *MYBA1* promotor gene and a single nucleotide polymorphisms (SNP) in *MYBA2* gene are associated with the loss of pigmentation in white-skinned cultivars of *Vitis vinifera* (Kobayashi et al., 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 (*MYBA1* and *MYBA2* genes).

3.2 MATERIAL AND METHODS

3.2.1 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 (41º19' N, 7º 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 be necessary, one bunch was collected by cultivar in each sampling date of 2013 season, comprising four developmental stages: green (G), veraison (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 ºC until use.

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

3.2.2 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.

3.2.3 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).

3.2.4 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).

3.2.5 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 material was kept in a desiccator, in the dark, until analysis.

For phenolic compounds extraction, ca. 5 g of each dry sample was extracted with 100 mL of 80 % MeOH (v/v), as previously reported (Dopico-García et al., 2008; Ferreira et al., 2016). Non-colored phenolics and anthocyanins fractions were after separated using SPE cartridge and each fraction was redissolved in appropriate volume of methanol (non-colored phenolics) and acidified water (pH 3.0) (anthocyanins), membrane-filtered (0.45 µm). An aliquot of 20 µL of each fraction was posteriorly injected into an HPLC-DAD

system equipped with a Spherisorb ODS2 column (25.0 cm×0.46 cm, 5 µm particle size; Waters, Milford, MA, USA).

The chromatographic analysis of the non-colored compounds was performed using the same analytical conditions that were previously reported (Dopico-García et al., 2008; Ferreira et al., 2016). Compounds were identified by comparing their retention times and UV spectra with those of authentic standards and with literature data (Dopico-García et al., 2008; Ferreira et al., 2016). Chromatograms were registered at 280 nm (for flavan-3-ols, syringic and gallic acids), 320 nm (for hydroxycinnamic acids and resveratrol-3-*O*-glucoside) and 350 nm (for flavonols). Quantification was performed by external standard method. Coutaric acid was determined as *p*-coumaric acid and the other compounds as themselves. Triplicate analyses were performed.

Anthocyanins analysis was performed as before (Dopico-García et al., 2008; Ferreira et al., 2016). Chromatograms were registered at 500 nm. Compounds were identified by comparing their chromatographic behavior and UV spectra with those of authentic standards and with literature data (Dopico-García et al., 2008; Ferreira et al., 2016). Quantification was performed by external standard method. Petunidin-3-*O*-*p*-coumaroylglucoside and petunidin-3-*O*-glucoside were quantified as petunidin; peonidin-3-*O*-*p*-coumaroylglucoside and malvidin-3-*O*-*p*-coumaroylglucoside were determined as peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside, respectively; the other compounds were determined as themselves. Triplicate analyses were performed.

3.2.6 Colorimetric measurements

The berry skin color was measured with a Minolta CR-300 tristimulus colorimeter (Minolta Co. Ltd., Osaka, Japan) having an 8-mm-diameter viewing area. For each cultivar and sampling stage, ten berries were randomly chosen for measuring CIELab coordinates; every berry was measured four times in opposite positions. Values of L^*, a^*, and b^* were determined to describe a three-dimensional color space. The vertical axis L^* is a measure of lightness, the values ranging from darkest black (0) to brightest white (100); a^* is a measure of redness (or $7a^*$ of greenness) and b^* of yellowness (or $7b^*$ of blueness) on the hue-circle (Gonçalves et al., 2007; Voss, 1992). The average of measurements of 10 berries *pe*r replication was used. The hue angle (h_{ab}) or tonality, expressing the color nuance and chroma, a measure of chromaticity (C^*_{ab}), indicating the

purity or saturation of the color, were calculated using the following equations: h_{ab}= tan^{-1}(b^*/a^*) and C^*ab= (a^{*2}+b^{*2})$^{1/2}$, respectively (Voss, 1992).

3.2.7 Statistical analyses

Principal component analysis (PCA) was carried out using SPSS® 21.0 software (IBM, NY, USA). PCA was applied for reducing the number of variables (23 variables corresponding to each identified phenolic compound to a smaller number of the new derived variables (principal components, PCs) that adequately summarize the original information, i.e., the phenolic composition of the grape berry skin color somatic variants at four different developmental stages.

Pearson correlation analysis was performed to corroborate relationships among color parameters and also between color parameters and phenolic profiles (significant correlations were set at $P < 0.05$), with Prism 6.02 (GraphPad Software, La Jolla, California, USA).

3.2.8 Molecular analyses

Genomic DNA was extracted from young leaves collected from each skin color somatic variant using DNeasy® Plant Mini Kit (QIAGEN, Düren, Germany) purification kit, according to the manufacturer's instructions. Extracted genomic DNA was quantified using a UV spectrometer (Nanodrop® ND-1000, Fisher Scientific, Delaware, USA), followed by quality check in a 1.0 % (w/v) agarose gel electrophoresis with ethidium bromide staining and photographed under UV light. Necessary dilutions were done (approximately 10 ng/µL) and kept at 4 ºC for further use.

MYBA1 and *MYBA2* gene polymorphisms (detection of functional and non-functional alleles) were investigated to understand the evolutionary events that gave origin to the color mutation of each cultivar studied.

The presence of *Gret1* retroelement in the *MYBA1* promoter region (*VvmybA1a* allele) was performed using the primers *a* and *d3* and PCR amplifications were performed as reported in Lijavetzky et al. (2006). In order to detect the functional alleles, *F2* and *R1* primers were used, 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.

For *MYBA2* gene, two point mutations (SNP) related to berry color, VvMYBA2R44 (Walker et al., 2007) and VvMYBA2C22 (Carrasco et al., 2015), were investigated by a SNaPshot approach, according to the protocol provided in the ABI PRISM SNaPshot Multiplex kit (Life Technology Corporation, California, 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 alkaline phosphatase, calf intestinal (CIP; New England Biolabs, Massachusetts, USA) to degrade excess PCR primers and dNTPs and incubated at 37 ºC for 1 h, followed by 15 min at 75 ºC to inactivate the enzyme. The purified SNaPshot PCR products were detected on capillary electrophoresis instrument ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA) and data analysis was performed by Peak ScannerTM Software v1.0 software.

3.3 RESULTS

3.3.1 Evaluation of grape berry skin color somatic variants development

In order to monitor the evolution of berry development, physical and biochemical parameters were analyzed at four different developmental stages (green, veraison, ripe and harvest) of grape berry skin somatic variants.

Berry weight generally followed the common pattern of increasing up to harvest (Figure 3.1 A–D). Two red-skinned cultivars, 'Moscatel Galego Roxo' and 'Malvasia Fina Roxo', and one black-skinned, 'Pique-poul Noir', were the exceptions: cv. 'Malvasia Fina Roxo' decreased in weight after veraison and cv. 'Moscatel Galego Roxo' and cv. 'Pique-poul Noir' decreased after ripe stage.

A similar trend was observed for pH, increasing up to harvest, with a more marked increase observed between the green and veraison stages (Figure 3.1 E–H).

Sugars (glucose and fructose) and organic acids (malic and tartaric acids) showed opposite behaviors. Malic and tartaric acids concentration decreased throughout maturation, equally showing a more pronounced decrease between green and veraison stages (Figure 3.1 I–L). On the other hand, glucose and fructose concentration increased along the four stages, especially between green and veraison stages (Figure 3.1 M–P). Nevertheless, in two black-skinned cultivars (cv. 'Pinot Noir' and cv. 'Pique-poul Noir')

and in the red-skinned cultivar ‘Malvasia Fina Roxo’, the glucose concentration slightly decreased between ripe and harvest stages.

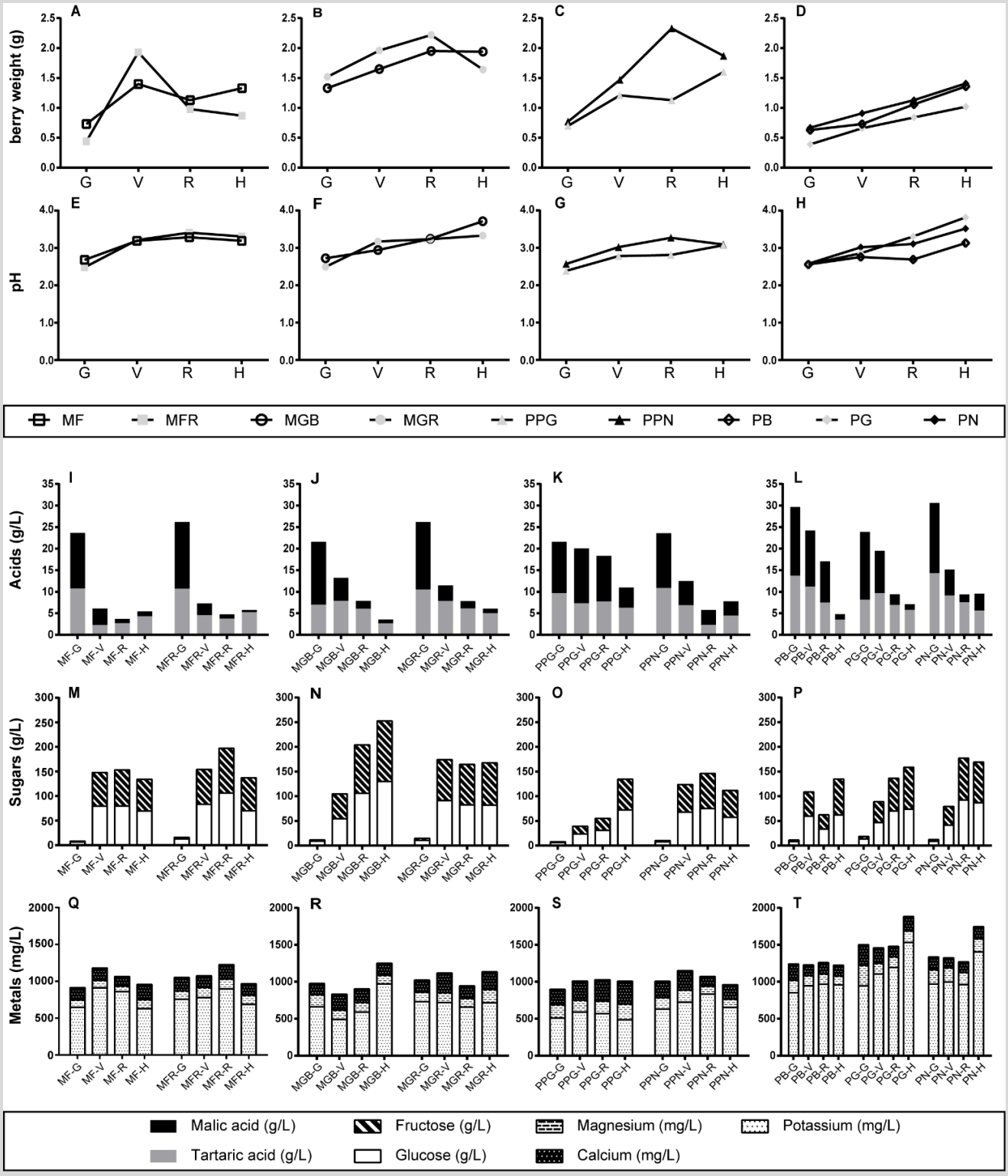

Figure 3.1 – Graphical representation of the different biochemical parameters evaluated during berry development (MF – ‘Malvasia Fina’; MFR – ‘Malvasia Fina Roxo’; MGB – ‘Moscatel Galego Branco’; MGR – ‘Moscatel Galego Roxo’; PPG – ‘Pique-poul Gris’; PPN – ‘Pique-poul Noir’; PB – ‘Pinot Blanc’; PG – ‘Pinot Gris’; PN – ‘Pinot Noir’; G – Green; V- Veraison; R – Ripe; and H – Harvest). A – D Berry weight; E – H pH; I – L Organic acids; M – P Sugars; Q – T Metals. Bars represent the standard deviation.

The monitoring of metals concentration did not reveal a uniform trend along the four developmental stages. Nevertheless, higher potassium concentration was observed for all somatic variants in the four developmental stages analyzed, comparing with the remaining metals (Figure 3.1 Q–T).

3.3.2 Phenolic compounds

As the major secondary metabolites contributing to grape berry color, phenolic compounds can be used as an effective fingerprinting tool in differentiating similar cultivars (Mattivi et al., 2006). As so, the qualitative and quantitative composition of the skin color somatic variants, regarding these secondary metabolites, was determined.

Twenty-three different compounds were identified, including two hydroxybenzoic acids, two hydroxycinnamic acids, four flavan-3-ols, seven flavonols, seven anthocyanins and one stilbene (Table 3.1). The two hydroxybenzoic acids identified in this study were gallic and syringic acids, the last being exclusive of the red-skinned 'Pinot Gris' cultivar in harvest stage. The two hydroxycinnamic acids were caftaric and coutaric acids, the first being the major compound in this group. Although hydroxycinnamic acids were more abundant than hydroxybenzoic acids, both followed the same pattern, consisting in increasing levels until veraison and then a decline in the later stages. However, all skin color somatic variants analyzed revealed high levels of phenolic acids, which were an appreciable component in non-colored cultivars.

Flavan-3-ols are another important group of phenols, although they were underrepresented in the red and black-skinned cultivars 'Moscatel Galego Roxo' and 'Pique-poul Noir', respectively (Table 3.1). Moreover, the cv. 'Pinot Noir' revealed the highest content of flavan-3-ols (2873.6 mg/ kg dried berry). Among the four flavan-3-ols identified catechin was the main one in the cv. 'Malvasia Fina Roxo' (892.9 mg/ kg dried berry), cv. 'Pique-poul Noir' (167.7 mg/ kg dried berry) and cv. 'Pinot Gris' (98.7 mg/ kg dried berry), with a higher concentration in the green stage. Epicatechin revealed to be the major flavan-3-ol in the cv. 'Malvasia Fina' (201.9 mg/ kg dried berry) and cv. 'Pinot Blanc' (76.8 mg/ kg dried berry) on harvest and ripe stages, respectively (Table 3.1). Epigallocatechin gallate and epicatechin gallate were the most abundant in the cv. 'Moscatel Galego Branco' (2803.8 mg/ kg dried berry) and cv. 'Pinot Noir' (1521.5 mg/ kg dried berry), respectively, on green stage (Table 3.1).

The flavonols detected in the present study included derivatives of myricetin, quercetin, kaempferol, isorhamnetin and syringetin (Table 3.1). Quercetin-3-*O*-galactoside revealed to be the major flavonol in five of the nine skin color somatic variants analyzed. The cv. 'Malvasia Fina Roxo' exhibited quercetin-3-*O*-glucoside as major compound in harvest stage (463 mg/ kg dried berry) and the cv. 'Moscatel Galego Roxo', cv. 'Pique-poul Noir' and cv. 'Pinot Gris' showed isorhamnetin-3-*O*-glucoside as the major flavonol at the green stage (308.1 mg/ kg, 1042.8 mg/ kg and 1752.9 mg/ kg dried berry, respectively) (Table 3.1). Syringetin-3-*O*-glucoside was exclusive of the cv. 'Pique-poul Gris', cv. 'Pinot Blanc' and cv. 'Pinot Noir'. On the other hand, myricetin-3-*O*-glucoside was only detected in the cv. 'Pinot Gris' and cv. 'Pinot Noir'. Overall, total concentration of flavonols decreased during berry maturation, except for the cultivars 'Malvasia Fina', 'Malvasia Fina Roxo' and 'Pinot Gris' (Table 3.1).

Five anthocyanins glucosides were determined (Table 3.1). Concerning to acylated compounds, only esters with *p*-coumaric acid were found, which revealed to be exclusive of the black-skinned 'Pinot Noir' cultivar, at ripe stage (Table 3.1). As expected, anthocyanins were only found in colored cultivars. Among the black-skinned cultivars, the cv. 'Pinot Noir' had the highest concentration at ripe stage (1016.9 mg/ kg dried berry). On the other hand, the cv. 'Malvasia Fina Roxo' contained the highest amount among the red-skinned cultivars, with 76.0 mg/ kg dried berry at harvest stage. Each group of skin color somatic variants has a specific anthocyanin as major compound; malvidin-3-*O*-glucoside was the main anthocyanin in 'Pinot' family (30.4 mg/ kg in cv. 'Pinot Gris' and 689.5 mg/ kg dried in cv. 'Pinot Noir', both at ripe stage); peonidin-3-*O*-glucoside was the major compound for 'Pique-poul' (7.8 mg/ kg in cv. 'Pique-poul Noir' and 5.7 mg/ kg dried berry in 'cv. Pique-poul Gris', both at veraison) and cyanidin-3-*O*-glucoside in 'Malvasia Fina' (76.0 mg/ kg dried berry in cv. 'Malvasia Fina Roxo' at harvest stage) families (Table 3.1).

Resveratrol-3-*O*-glucoside, the only stilbene found, was noticed mainly in the last stages of the berry development. Three of the nine skin color somatic variants analyzed did not presented this compound, namely the white-skinned cv. 'Pinot Blanc', the red-skinned cv. 'Moscatel Galego Roxo' and the black-skinned cv. 'Pique-poul Noir' (Table 3.1).

Once phenols are the main category of compounds involved in shaping of berry skin color, the HPLC-DAD data were subjected to PCA analysis to highlight the differences or similarities among the grape berry skin color somatic variants studied.

A PCA was performed for each grape berry skin color somatic variant group, representing the four varieties analyzed. The skin color somatic variants of each variety were grouped according to the grape cultivar and also on the basis of developmental stage. PCA analysis for all datasets explained between 65.2 % and 81.1 % of the total variations (Figure 3.2).

As shown in Figure 3.2 A, white and red-skinned 'Malvasia Fina' cultivars were grouped close to each other by developmental stages, except for veraison. At the green stage, both cultivars grouped together, showing negative PC1 scores (group A1). Ripe stage of both white and red-skinned 'Malvasia Fina' cultivars also formed an independent group (group A2), as well as, the harvest stage, which corresponds to the third group formed (group A3).

The green stage of both white and red-skinned 'Moscatel Galego' cultivars has shown positive PC1 scores (group B1) (Figure 3.2 B). Ripe and harvest stages of the white-skinned 'Moscatel Galego Branco' cultivar clustered together (group B2), as well as veraison, ripe and harvest stages of the red-skinned 'Moscatel Galego Roxo' cultivar (group B3).

The PCA analysis on the 'Pique-poul' set of cultivars revealed that the veraison stage was the only one showing a positive PC2 score (Figure 3.2 C), being both the red and black-skinned cultivars clearly distinguished at this stage once the black-skinned cv. 'Pique-poul Noir' showed a negative PC1 score and the red-skinned cv. 'Pique-poul Gris' a positive PC1 score. The remaining stages presented negative PC2 scores, being the harvest stages of both cultivars, the green stage of the cv. 'Pique-poul Gris' and the ripe stage of the cv. 'Pique-poul Noir' clustered together (group C1).

All the developmental stages of the white and red-skinned cultivars, cv. 'Pinot Blanc' and cv. 'Pinot Gris', grouped close to each other (Figure 3.2 D, group D1). The results also showed that ripe and harvest stages of this cultivar grouped together, with a positive PC1 score (group D2).

Table 3.1 – Phenolic compounds in grape skin color somatic variants during berry development (mg/kg berry, dry basis). Within parenthesis are shown the standard deviations of three determinations

		MF				MFR				MGB				MGR			
		G	**V**	**R**	**H**	**G**	**V**	**R**	**H**	**G**	**V**	**R**	**H**	**G**	**V**	**R**	**H**
TR (min.)	**PHENOLIC ACIDS**																
	Hydroxybenzoic acids																
5.2	Gal ac	25.9 (0.2)	8.1 (1.0)	8.3 (0.1)	12.9 (1.0)	--	24.8 (0.6)	9.3 (0.8)	11.5 (1.1)	--	11.2 (0.5)	36.4 (0.9)	13.6 (0.6)	--	31.7 (1.0)	12.6 (0.2)	20.3 (1.6)
21.7	Syr ac	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	Σ	**25.9 (0.2)**	**8.1 (1.0)**	**8.3 (0.1)**	**12.9 (1.0)**	**--**	**24.8 (0.6)**	**9.3 (0.8)**	**11.5 (1.1)**	**--**	**11.2 (0.5)**	**36.4 (0.9)**	**13.6 (0.6)**	**--**	**31.7 (1.0)**	**12.6 (0.2)**	**20.3 (1.6)**
	Hydroxycinnamic acids																
8.2	Caft ac	602.6 (10.1)	127.6 (0.8)	53.5 (0.7)	192.2 (2.3)	514.8 (16.3)	106.9 (2.1)	46.4 (2.7)	164.2 (2.3)	4679.0 (114.7)	450.2 (14.5)	480.7 (8.2)	136.3 (11.9)	5028.7 (88.7)	144.5 (4.6)	163.0 (1.9)	257.4 (4.3)
11.9	Cout ac	818.5 (3.0)	136.1 (1.3)	31.1 (0.7)	123.1 (4.9)	327.6 (13.2)	105.6 (2.4)	22.3 (1.6)	106.1 (3.6)	4507.0 (107.4)	351.8 (1.7)	252.1 (5.3)	25.2 (2.1)	5228.7 (304.9)	--	96.1 (1.5)	179.4 (1.5)
	Σ	**1421.1 (13.1)**	**263.7 (2.1)**	**84.6 (1.4)**	**315.3 (7.2)**	**842.4 (29.5)**	**212.5 (4.5)**	**68.7 (4.3)**	**270.3 (5.9)**	**9186.0 (222.1)**	**802.0 (16.2)**	**732.8 (13.5)**	**161.5 (14.0)**	**10257.4 (393.6)**	**144.5 (4.6)**	**259.1 (3.4)**	**436.8 (5.8)**
	FLAVONOIDS																
	Flavan-3-ols																
14.7	Cat	120.4 (5.3)	--	--	175.2 (12.5)	892.9 (26.4)	468.1 (11.1)	--	247.4 (0.2)	434.4 (15.9)	--	93.2 (7.5)	8.0 (0.2)	--	--	--	--
22.2	Epicat	--	--	--	201.9 (25.7)	--	509.1 (0.7)	--	186.5 (4.3)	553.6 (34.1)	--	136.3 (7.3)	--	--	--	--	--
24.6	Epigallocat gall	--	--	--	--	--	52.7 (0.1)	--	--	2803.8 (157.9)	--	--	--	--	--	--	--
37.3	Epicat gall	--	--	--	168.1 (1.8)	--	476.5 (2.9)	--	140.3 (2.3)	--	--	--	--	--	--	--	--
	Σ	**120.4 (5.3)**	**--**	**--**	**545.2 (40.0)**	**892.9 (26.4)**	**1453.7 (14.8)**	**--**	**574.2 (6.8)**	**3791.8 (207.9)**	**--**	**229.5 (14.8)**	**8.0 (0.2)**	**--**	**--**	**--**	**--**
	Flavonols																
37.7	Myr-3-*O*-glu	--	nq	--	--	--	--	--	--	--	--	--	--	--	--	--	--
46.4	Quer-3-*O*-galac	--	128.3 (0.7)	43.1 (0.9)	283.9 (0.4)	--	216.2 (0.1)	126.6 (0.3)	344.2 (4.8)	2143.3 (48.7)	101.4 (1.2)	502.9 (12.0)	188.6 (10.8)	184.3 (3.0)[a]	252.7 (29.9)[a]	75.4 (1.1)	56.6 (0.9)[a]
47.3	Quer-3-*O*-rut	--	7.8 (0.1)	2.4 (0.2)	6.9 (0.0)	--	8.8 (0.2)	9.5 (0.1)	22.9 (0.3)	17.3 (0.8)	1.2 (0.1)	8.6 (0.4)	7.1 (0.5)			1.5 (0.0)	
48.5	Quer-3-*O*-glu	--	185.8 (1.5)	54.6 (2.4)	151.9 (0.3)	--	207.4 (4.4)	226.3 (1.7)	463.0 (1.2)	166.5 (3.2)	18.5 (0.5)	155.3 (2.7)	140.9 (7.8)	88.2 (1.4)	58.1 (6.2)	32.0 (0.6)	37.1 (1.3)
54.4	Kaemp-3-*O*-glu	--	40.9 (0.5)	8.2 (0.2)	19.9 (0.7)	--	31.7 (0.3)	54.1 (1.3)	53.7 (1.4)	25.5 (1.3)	2.2 (0.1)	30.0 (0.7)	28.3 (0.1)	--	30.4 (3.2)	6.2 (0.0)	7.0 (0.5)
55.9	Isor-3-*O*-glu	78.4 (4.3)	7.2 (0.0)	2.6 (0.2)	3.9 (0.3)	73.1 (3.4)	nq	13.0 (1.5)	6.4 (0.4)	17.5 (0.7)	--	8.0 (0.5)	5.3 (0.1)	308.1 (2.5)	45.8 (2.4)	2.0 (0.1)	3.1 (0.2)
56.5	**S**yr-3-*O*-glu	--	--	--	--	--	--	--	--	--	nq	--	--	--	--	--	--
	Σ	**78.4 (4.3)**	**329.1 (2.8)**	**102.7 (3.9)**	**446.6 (1.7)**	**73.1 (3.4)**	**432.4 (5.0)**	**375.4 (4.9)**	**836.5 (8.1)**	**2370.1 (54.7)**	**123.3 (1.9)**	**704.8 (16.3)**	**370.2 (19.3)**	**580.6 (6.9)**	**387.0 (41.7)**	**117.1 (1.8)**	**103.8 (2.9)**

Table 3.1
(continued)

		MF				MFR				MGB				MGR			
		G	**V**	**R**	**H**	**G**	**V**	**R**	**H**	**G**	**V**	**R**	**H**	**G**	**V**	**R**	**H**
TR (min.)	**Anthocyanins**																
12.3	Del-3-*O*-gl	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
14.6	Cya-3-*O*-glu	--	--	--	--	--	38.4 (0.6)	--	76.0 (2.1)	--	--	--	--	--	--	--	--
16.5	Pet-3-*O*-glu	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
19.1	Peo-3-*O*-glu	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
20.7	Mal-3-*O*-glu	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
32.1	Peon-3-*O*-*p*cmglu	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
32.5	Mal-3-*O*-*p*cmglu	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	Σ	--	--	--	--	--	**38.4 (0.6)**	--	**76.0 (2.1)**	--	--	--	--	--	--	--	--
	STILBENES																
38.9	Res-3-*O*-glu	--	10.8 (0.1)	3.6 (0.2)	14.8 (0.1)	--	10.5 (0.9)	8.2 (0.3)	16.0 (0.4)	--	--	2.9 (0.3)	3.4 (0.1)	--	--	--	--
	Total	**1645.8 (22.9)**	**611.7 (28.9)**	**199.2 (5.6)**	**1334.8 (50.0)**	**1808.4 (59.3)**	**2172.3 (26.4)**	**461.6 (10.3)**	**1784.5 (24.4)**	**13347.9 (484.7)**	**936.5 (18.6)**	**1706.4 (45.8)**	**556.7 (34.2)**	**10837.4 (400.5)**	**563.2 (47.3)**	**388.8 (5.4)**	**560.9 (15.7)**

Table 3.1
(continued)

		PPG				PPN				PB				PG				PN			
		G	V	R	H	G	V	R	H	G	V	R	H	G	V	R	H	G	V	R	H
TR (min.)	PHENOLIC ACIDS																				
	Hydroxybenzoic acids																				
5.2	Gal ac	18.9 (1.0)	43.5 (1.5)	30.5 (0.8)	49.5 (3.5)	--	--	29.8 (0.4)	29.7 (0.5)	--	--	--	6.6 (0.4)	nq	8.9 (0.7)	5.5 (0.1)	8.8 (0.3)	--	67.9 (1.0)	29.7 (0.1)	33.8 (2.6)
21.7	Syr ac	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	3.5 (0.1)	--	--	--	--
	Σ	**18.9 (1.0)**	**43.5 (1.5)**	**30.5 (0.8)**	**49.5 (3.5)**	--	--	**29.8 (0.4)**	**29.7 (0.5)**	--	--	--	**6.6 (0.4)**	--	**8.9 (0.7)**	**5.5 (0.1)**	**12.3 (0.4)**	--	**67.9 (1.0)**	**29.7 (0.1)**	**33.8 (2.6)**
	Hydroxycinnamic acids																				
8.2	Caft ac	328.1 (13.2)	793.1 (8.0)	322.9 (6.4)	395.8 (2.0)	1371.7 (45.8)	--	107.9 (0.4)	96.2 (3.9)	677.2 (65.9)	1042.3 (46.1)	466.4 (41.0)	143.0	50.4 (2.6)	580.6 (19.9)	138.5 (13.8)	86.8 (1.1)	4549.7 (114.4)	742.6 (1.6)	379.8 (7.9)	178.5 (15.7)
11.9	Cout ac	143.5 (4.9)	315.2 (3.4)	113.6 (2.6)	171.4 (2.4)	516.0 (0.3)	--	43.4 (1.5)	45.0 (1.9)	470.2 (26.7)	413.8 (34.0)	132.9 (9.3)	33.9 (0.9)	42.4 (4.0)	207.4 (16.3)	46.1 (3.1)	22.5 (1.1)	2259.2 (39.3)	324.5 (4.8)	215.3 (3.6)	102.8 (8.3)
	Σ	**471.6 (18.1)**	**1108.3 (11.4)**	**436.5 (9.0)**	**567.2 (4.4)**	**1887.7 (46.1)**	--	**151.3 (1.9)**	**141.2 (5.8)**	**1147.4 (92.6)**	**1456.1 (80.1)**	**599.3 (50.3)**	**176.9**	**92.8 (6.6)**	**788.0 (36.2)**	**184.6 (16.9)**	**109.3 (2.2)**	**6808.9 (153.7)**	**1067.1 (6.4)**	**595.1 (11.5)**	**281.3 (24.0)**
	FLAVONOIDS																				
	Flavan-3-ols																				
14.7	Cat	--	138.2 (2.5)	159.8 (11.5)	--	167.7 (2.0)	nq	--	--	--	--	--	--	98.7 (0.7)	90.2 (4.2)	62.0 (1.0)	--	500.3 (3.9)	969.4 (15.1)	159.7 (2.0)	111.9 (6.7)
22.2	Epicate	--	86.2 (7.1)	65.9 (2.2)	--	--	--	--	--	--	--	76.8 (4.6)	--	--	--	34.3 (0.6)	--	851.8 (19.6)	1340.5 (101.9)	87.8 (4.2)	56.4 (2.7)
24.6	Epigallocat gall	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
37.3	Epicat gall	--	639.7 (13.3)	575.4 (10.8)	--	--	--	--	--	--	--	--	--	--	--	--	--	1521.5 (97.6)	835.6 (26.2)	--	--
	Σ	--	**864.1 (22.9)**	**801.1 (24.5)**	--	**167.7 (2.0)**	--	--	--	--	--	**76.8 (4.6)**	--	**98.7 (0.7)**	**90.2 (4.2)**	**96.3 (1.6)**	--	**2873.6 (121.1)**	**3145.5 (143.2)**	**247.5 (6.2)**	**168.3 (9.4)**
	Flavonols																				
37.7	Myr-3-*O*-glu	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2.9 (0.0)	7.6 (0.2)	--	16.2 (0.4)	22.5 (0.9)	19.0 (1.7)
46.4	Quer-3-*O*-galac	--	354.7 (4.4)	185.2 (4.4)	178.4 (1.1)	787.6 (2.9)	--	127.2 (0.4)	164.5 (5.0)	65.3 (0.4)	622.1 (21.9)	131.5 (12.5)	30.6 (0.0)	109.7 (5.8)	1290.7 (7.7)	465.7 (16.3)	159.7 (0.5)	938.8 (46.3)	176.2 (1.1)	147.6 (0.6)	90.6 (7.7)
47.3	Quer-3-*O*-rut	--	--	7.2 (0.3)	7.2 (0.4)	--	--	--	4.3 (0.1)	--	4.1 (0.2)	1.5 (0.1)	1.5 (0.1)	--	--	--	7.7 (0.1)	9.5 (0.5)	2.7 (0.1)	3.6 (0.0)	4.2 (0.4)
48.5	Quer-3-*O*-glu	96.0 (2.1)	145.0 (1.8)	187.9 (1.2)	180.6 (4.3)	543.8 (2.2)	--	138.5 (0.2)	105.5 (3.1)	23.0 (0.7)	89.7 (1.4)	28.2 (0.7)	49.8 (0.3)	286.9 (7.8)	234.0 (7.6)	138.8 (3.2)	204.3 (1.1)	102.6 (6.1)	100.3 (1.6)	99.5 (0.9)	106.8 (7.6)
54.4	Kaemp-3-*O*-glu	--	20.3 (0.2)	26.5 (0.4)	23.7 (0.6)	37.8 (0.1)	--	19.6 (0.9)	12.9 (0.3)	--	4.5 (0.5)	0.3 (0.0)	6.3 (0.2)	--	21.4 (2.2)	15.7 (1.0)	25.2 (1.4)	--	7.2 (0.7)	7.7 (0.3)	8.9 (0.8)
55.9	Isor-3-*O*-glu	400.5 (8.1)	3.1 (0.3)	179.3 (0.8)	48.8 (2.2)	1042.8 (12.5)	4.8 (0.3)	25.3 (1.0)	--	170.8 (2.4)	2.7 (0.1)	1.0 (0.1)	Nq	1752.9 (24.7)	13.1 (0.1)	39.1 (1.1)	17.7 (1.0)	--	nq	5.8 (0.2)	29.7 (2.9)
56.5	Syr-3-*O*-glu	--	11.5 (0.1)	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	Σ	**496.5 (10.2)**	**534.6 (6.8)**	**578.9 (7.1)**	**431.5 (8.6)**	**2412.0 (17.7)**	**4.8 (0.3)**	**310.6 (2.5)**	**282.9 (8.5)**	**259.1 (24.1)**	**719.0 (24.1)**	**168.5 (13.4)**	**88.2 (0.6)**	**2149.5 (38.3)**	**1559.2 (17.6)**	**662.2 (21.6)**	**414.5 (4.3)**	**1041.4 (52.9)**	**302.6 (3.9)**	**295.4 (2.9)**	**269.9 (21.1)**

Table 3.1
(continued)

		PPG				PPN				PB				PG				PN			
		G	**V**	**R**	**H**	**G**	**V**	**R**	**H**	**G**	**V**	**R**	**H**	**G**	**V**	**R**	**H**	**G**	**V**	**R**	**H**
TR (min.)	**Anthocyanins**																				
12.3	Del-3-*O*-glu	--	--	--	--	--	0.4 (0.0)	--	--	--	--	--	--	--	--	2.2 (0.2)	nq	--	21.1 (0.1)	43.3 (0.8)	4.77 (0.07)
14.6	Cya-3-*O*-glu	--	5.0 (0.0)	nq	nq	--	1.5 (0.1)	nq	nq	--	--	--	--	--	--	--	nq	--	20.2 (0.1)	11.4 (0.0)	2.48 (0.05)
16.5	Pet-3-*O*-glu	--	--	--	nq	--	1.5 (0.1)	nq	nq	--	--	--	--	--	--	3.1 (0.0)	nq	--	74.3 (0.5)	144.9 (0.4)	23.37 (0.04)
19.1	Peo-3-*O*-glu	--	7.8 (0.1)	nq	1.97 (0.04)	--	5.7 (0.1)	nq	nq	--	--	--	--	--	nq	6.5 (0.5)	--	--	253.2 (8.6)	114.5 (0.6)	26.96 (0.03)
20.7	Mal-3-*O*-glu	--	0.2 (0.0)	--	--	--	4.2 (0.0)	nq	nq	--	--	--	--	--	nq	30.4 (0.6)	--	--	410.4 (7.6)	689.5 (2.0)	121.48 (0.42)
32.1	Peon-3-*O*-*p*cmglu	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	9.9 (1.1)	--
32.5	Mal-3-*O*-*p*cmglu	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	3.4 (0.7)	--
	Σ	--	**13.0 (0.1)**	--	**1.97 (0.04)**	--	**13.3 (0.2)**	--	--	--	--	--	--	--	--	**42.2 (1.3)**	--	--	**779.2 (16.9)**	**1016.9 (5.6)**	**179.1 (0.6)**
	STILBENES																				
38.9	Res-3-*O*-glu	--	33.1 (0.3)	23.2 (0.6)	--	--	--	--	--	--	--	--	--	--	--	--	1.1 (0.0)	--	18.8 (1.4)	5.1 (0.1)	4.7 (0.5)
	Total	**987.0 (29.3)**	**2596.6 (43.0)**	**1870.2 (42.0)**	**1050.2 (16.5)**	**4467.4 (65.8)**	**18.1 (0.5)**	**491.7 (4.8)**	**453.8 (14.8)**	**1406.5 (116.7)**	**2175.1 (104.2)**	**844.6 (68.3)**	**271.7 (1.0)**	**2341.0 (45.6)**	**2446.3 (58.7)**	**990.8 (41.5)**	**537.2 (6.9)**	**10723.9 (327.7)**	**5381.1 (172.8)**	**2189.7 (26.4)**	**937.1 (58.2)**

MF – 'Malvasia Fina'; MFR – 'Malvasia Fina Roxo'; MGB – 'Moscatel Galego Branco'; MGR – 'Moscatel Galego Roxo'; PPG – 'Pique-poul Gris'; PPN – 'Pique-poul Noir'; PB – 'Pinot Blanc'; PG – 'Pinot Gris'; PN – 'Pinot Noir' G – Green; V – Veraison; R – Ripe and H – Harvest; TR – retention time; Gallic acid (Gal ac); caftaric acid (Caft ac); coutaric acid (Cout ac); catechin (Cat); syringic acid (Syr ac); epicatechin (Epicat); epigallocatechin gallate (Epigallcat gall); epicatechin gallate (Epicat gall); resveratrol-3-*O*-glucoside (Res-3-*O*-glu); myricetin-3-*O*-glucoside (Myr-3-*O*-glu); quercetin-3-*O*-galactoside (Quer-3-*O*-galac); quercetin-3-*O*-rutinoside (Quer-3-*O*-rut); quercetin-3-*O*-glucoside (Quer-3-*O*-glu); kaempferol-3-*O*-glucoside (Kaemp-3-*O*-glu); isorhamnetin-3-*O*-glucoside (Isor-3-*O*-glu); syringetin-3-*O*-glucoside (Syr-3-*O*-glu); delphinidin-3-*O*-glucoside (Del-3-*O*-glu); cyanidin-3-*O*-glucoside (Cya-3-*O*-glu); petunidin-3-*O*-glucoside (Pet-3-*O*-glu); peonidin-3-*O*-glucoside (Peo-3-*O*-glu); malvidin-3-*O*-glucoside (Mal-3-*O*-glu), peonidin-3-*O*-*p*-coumaroylglucoside (Peon-3-*O*-*p*cmglu), malvidin-3-*O*-*p*-coumaroylglucoside (Mal-3-*O*-*p*cmglu).

∑: sum of the identified polyphenolic compounds; "--": not detected; [a] Quercetin-3-*O*-galactoside and quercetin-3-*O*-rutinoside were quantified together.

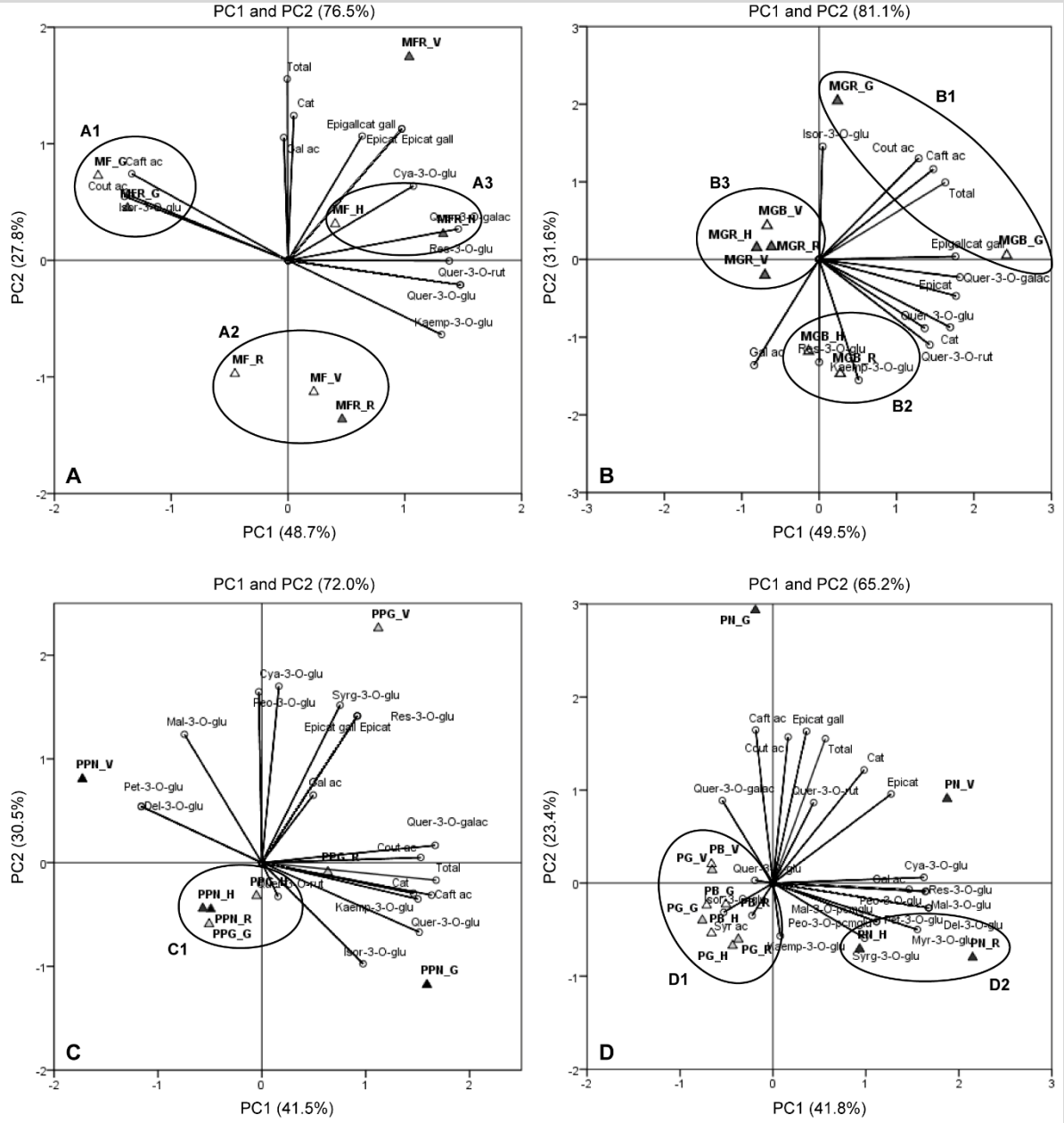

Figure 3.2 – Plot of the different grape berry skin color somatic variants in the space defined by the Principal Components 1 and 2 (PC1 and PC2) obtained by Principal Component Analysis applied to their characteristic polyphenolic profiles at four developmental stages (G – Green; V – Veraison; R – Ripe and H – Harvest) (A - D) [variables: gallic acid (Gal ac); caftaric acid (Caft ac); coutaric acid (Cout ac); catechin (Cat); syringic acid (Syr ac); epicatechin (Epicat); epigallocatechin gallate (Epigallcat gall); epicatechin gallate (Epicat gall); resveratrol-3-*O*-glucoside (Res-3-*O*-glu); myricetin-3-*O*-glucoside (Myr-3-*O*-glu); quercetin-3-*O*-galactoside (Quer-3-*O*-galac); quercetin-3-*O*-rutinoside (Quer-3-*O*-rut); quercetin-3-*O*-glucoside (Quer-3-*O*-glu); kaempferol-3-*O*-glucoside (Kaemp-3-*O*-glu); isorhamnetin-3-*O*-glucoside (Isor-3-*O*-glu); syringetin-3-*O*-glucoside (Syr-3-*O*-glu); delphinidin-3-*O*-glucoside (Del-3-*O*-glu); cyanidin-3-*O*-glucoside (Cya-3-*O*-glu); petunidin-3-*O*-glucoside (Pet-3-*O*-glu); peonidin-3-*O*-glucoside (Peo-3-*O*-glu); malvidin-3-*O*-glucoside (Mal-3-*O*-glu), peonidin-3-*O*-*p*-coumaroylglucoside (Peon-3-*O*-*p*cmglu), malvidin-*3*-*O*-*p*-coumaroylglucoside (Mal-*3*-*O*-*p*cmglu)]. Variables A: MF – 'Malvasia Fina'; MFR – 'Malvasia Fina Roxo'. Variables B: MGB – 'Moscatel Galego Branco'; MGR – 'Moscatel Galego Roxo'. Variables C: PPG – 'Pique-poul Gris'; PPN – 'Pique-poul Noir'. Variables D: PB – 'Pinot Blanc'; PG – 'Pinot Gris'; PN – 'Pinot Noir'].

3.3.3 Colorimetric measurements

During grape maturation, skin berry color changes from an initial green, typical of the unripe grapes, to a yellow/brownish and red/ blue at harvest, respectively in white and color-skinned grapes. This evolution on color parameters was evaluated and results are shown in Table 3.2.

During maturity, a decrease in b^* parameter and an increase in a^* could be generally observed, i.e. a decline of green color and a rise of yellow, red or blue, giving thus a brownish and red/blue coloring to the skin of the white and colored cultivars, respectively. This behavior is more evident regarding the colored cultivars. In what respects to redness (a^* values), values were only recorded in the red-skinned cultivars 'Moscatel Galego Roxo' and 'Pinot Gris', and in the black-skinned cultivars 'Pique-poul Noir' and 'Pinot Noir'. Especially in the red-skinned cultivars, this parameter clearly increases between veraison and ripe stages, showing an inverse trend compared to the lightness (L^*), despite without significance in the cv. 'Moscatel Galego Roxo' and cv. 'Pinot Gris' (Pearson's correlation = -0.920, P = 0.080 and -0.911, P = 0.089, respectively). Although, yellowness (b^* values) showed similar values among white and color-skinned cultivars in the green stage, the highest values were obtained in white and red-skinned cultivars during all four developmental stages analyzed. The lower values were observed in the black-skinned cultivars, showing a clear decrease between veraison and ripe stages.

Chroma values (C^*_{ab}) decreased until harvest, being more evident for red and black-skinned cultivars, particularly in the black-skinned 'Pinot Noir' cultivar from veraison until harvest, in agreement with the fall of anthocyanins. This was accompanied by changes in the values of the hue (h_{ab}), which clearly decreased not only in 'Pinot Noir' (Pearson's correlation = 0.986, $P< 0.05$) from green to harvest stages but also in all colored cultivars and in 'Moscatel Galego Branco'.

At the late developmental stage analyzed, relevant differences existed in hue, being higher in white-skinned cultivars than in red-skinned (more orange-red) and black-skinned cultivars (more blue-red), showing the black-skinned 'Pinot Noir' cultivar the highest value (359.85). Moreover, chroma (C^*_{ab}) is positively correlated with b^* in all cultivars, independently from skin color, showing correlation coefficients of 0.971 and 1.00 (all correlations were significant at $P< 0.05$). On the other hand, b^* was positively correlated with hue (h_{ab}), but only in the black-skinned cultivars, correlation coefficients

of 1.00 for the cv. 'Pinot Noir' and 0.959 for the cv. 'Pique-poul Noir' (all correlations were significant at $P < 0.05$) being observed. Usually, hue (h_{ab}) showed an inverse correlation with a^*. However, this correlation was only observed and statistically significant for the red-skinned cultivars, with correlation coefficients of -0.972 and -0.999 for the cv. 'Pinot Gris' and the cv. 'Moscatel Galego Roxo', respectively (all correlations were significant at $P < 0.05$).

Color differences were also calculated ($\Delta E^*ab = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$) between the different developmental stages analyzed. They were firstly calculated between green and harvest stages and, as expected, it was possible to distinguish the color between them in all cultivars, with values of $\Delta E^*ab \geq 4$. Color differences were also calculated between green and veraison and between ripe and harvest, the last two stages. It was observed that color differences were more pronounced between green and veraison ($17 \geq \Delta E^*ab \geq 4$) then between the last two stages ($8 \geq \Delta E^*ab \geq 2$).

Table 3.2 – Colorimetric parameters analyzed for all grape skin color variants picked at different stages of ripeness.

		MF	MFR	MGB	MGR	PPG	PPN	PB	PG	PN
Green	L^*	46.82	46.09	50.28	47.87	52.31	51.96	46.37	47.52	43.82
	a^*	-5.28	-5.18	-7.54	-5.20	-6.89	-6.46	-6.03	-5.96	-5.29
	b^*	30.17	29.89	33.35	32.05	28.62	29.22	28.50	25.67	26.07
	C^*_{ab}	30.63	30.34	34.19	32.47	29.44	29.93	29.13	26.35	26.60
	h_{ab}	99.92	99.84	102.74	99.22	103.53	102.47	101.95	103.06	101.48
Veraison	L^*	54.15	55.12	57.23	49.78	55.71	51.73	47.57	48.15	40.58
	a^*	-8.44	-2.72	-9.15	-0.38	-9.77	6.74	-6.24	-1.25	1.14
	b^*	29.41	25.29	31.06	30.19	27.58	17.42	22.95	25.67	14.72
	C^*_{ab}	30.67	25.44	32.38	30.20	29.26	18.68	23.78	25.70	14.77
	h_{ab}	106.01	96.13	106.42	90.70	109.50	68.85	105.21	92.78	85.58
Ripe	L^*	57.91	55.48	55.47	41.19	56.48	48.64	49.74	40.79	30.66
	a^*	-5.71	2.88	-5.67	11.31	-8.56	3.00	-9.00	10.30	5.30
	b^*	27.60	25.40	30.02	21.10	26.70	14.64	27.15	12.49	2.05
	C^*_{ab}	28.18	25.56	30.55	23.94	28.04	14.94	28.60	16.19	5.68
	h_{ab}	101.70	83.53	100.7	61.80	107.78	78.42	108.33	50.49	21.11
Harvest	L^*	59.14	52.87	57.54	39.26	55.92	48.07	50.02	35.26	31.24
	a^*	-7.36	0.45	-3.89	12.04	-4.77	5.12	-5.17	11.25	3.25
	b^*	27.00	27.11	33.48	21.21	23.78	16.09	25.50	5.56	-0.01
	C^*_{ab}	28.02	27.71	33.70	24.39	24.25	16.88	26.02	12.55	3.25
	h_{ab}	105.06	87.99	96.93	60.41	101.34	72.36	101.45	25.29	359.85

MF – 'Malvasia Fina'; MFR – 'Malvasia Fina Roxo'; MGB – 'Moscatel Galego Branco'; MGR – 'Moscatel Galego Roxo'; PPG – 'Pique-poul Gris'; PPN – 'Pique-poul Noir'; PB – 'Pinot Blanc'; PG – 'Pinot Gris'; PN – 'Pinot Noir'; L^* – lightness; a^* – redness; b^* – yellowness; C^*_{ab} – chroma; h_{ab} – hue angle.

3.3.4 MYBA1 and MYBA2 allelic variation

The results obtained in the two different assays performed to characterize the *MYBA1* locus are found in Table 3.3. The first assay, that allows the detection of the *Gret1* retrotransposon insertion, showed the same banding pattern for black, red and white-skinned grape somatic variants, all of them amplifying a fragment corresponding to the non-functional allele *VvmybA1a*. The second PCR reaction, performed to detect the wild-type allele and other putative functional ones, highlighted different amplification profiles. For the white-skinned cultivars and for the red-skinned cultivars 'Malvasia Fina Roxo' and 'Moscatel Galego Roxo', no amplification fragments were detected. Concerning the black-skinned cultivars and the red-skinned cultivars 'Pinot Gris' and 'Pique-poul Gris', the wild-type allele, *VvmybA1c*, was amplified.

The detection of *MYBA2* polymorphisms was performed by SNaPshot assay. The allelic profiles are listed in Table 3.3. The SNP VvMYBA2R44 or K980 showed both homozygous and heterozygous profiles for the non-functional allele (TT and TG, 44.44 and 55.56 %, respectively). As expected, all the white-skinned cultivars showed the non-functional allele (T), while the red and black-skinned cultivars showed the functional allele (G), except for the red-skinned cultivar 'Moscatel Galego Roxo', which revealed to be homozygous for the non-functional allele (TT). Moreover, VvMYBA2C22 SNP was detected in the two black-skinned cultivars, 'Pinot Noir' and 'Pique-poul Noir'.

Table 3.3 – Allelic profiles of genotypes analyzed at *MYBA1* and *MYBA2* genes.

Cultivar	Berry color	*MYBA1*		*MYBA2*	
		VvmybA1a *Gret1*	*VvmybA1c* *No Gret1*	VvMYBA2R44	VvMYBA2C22
MF	W	+	-	TT	TT
MFR	R	+	-	TG	TT
MGB	W	+	-	TT	TT
MGR	R	+	-	TT	TT
PPG	R	+	+	TG	TT
PPN	B	+	+	TG	TG
PB	W	+	-	TT	TT
PG	R	+	+	TG	TT
PN	B	+	+	TG	TG

MF – 'Malvasia Fina'; MFR – 'Malvasia Fina Roxo'; MGB – 'Moscatel Galego Branco'; MGR – 'Moscatel Galego Roxo'; PG – 'Pique-poul Gris'; PPN – 'Pique-poul Noir'; PB – 'Pinot Blanc'; PG – 'Pinot Gris'; PN –' Pinot Noir'; W – white; R – red; B – black; "+" presence of allele, "-" absence of allele.

3.4 DISCUSSION

3.4.1 Evaluation of grape berry skin color somatic variants development

A complex series of physical and biochemical changes, such as modifications in size, chemical composition, color, texture, flavor and pathogen resistance occurs during grape berry development (Ali et al., 2011). However, the knowledge about the grape metabolites of skin color somatic variants is still incomplete, since this kind of cultivars has received less attention than common cultivars with different skin colors. In this context, the present work contributes for bridging this gap in knowledge by analyzing different groups of skin color somatic variants during berry development using an integrative approach.

The common pattern of berry weight development is a considerable increase up to harvest (K. Ali et al., 2011; Degu et al., 2014; Fortes et al., 2011). Our data confirm this pattern, as shown in Figure 3.1 A–D, once almost all somatic variants doubled their berry weight from green stage until harvest.

During the last berry developmental phase, pH should increase. Along with this increase, in almost all skin color somatic variants analyzed, pH also increased between green and veraison stages, which may be related with the decline of tartaric and malic acid, resulting in an increase of overall pH (Figure 3.1 E–H).

While sucrose is described as the major sugar in the green phase, glucose and fructose accumulate in the later ripe and harvest stages (K. Ali et al., 2011; Fortes et al., 2011), as can be observed in Figure 3.1 M–P, with an exponential increase from green stage.

Zhao et al. (2015) observed that the bud sport of ‘Benitaka’ cultivar revealed a different sugar accumulation pattern when compared with its ancestor cv. ‘Italia’. In our data, the red-skinned cv. ‘Moscatel Galego Roxo’ also seems to exhibit a marked difference in sugar accumulation when compared with the white-skinned variant cv. ‘Moscatel Galego Branco’. These results should be further investigated in order to define the event that triggers and controls sugar accumulation in the berries of these skin color somatic variants.

Furthermore, the highest levels of organic acids, malic and tartaric, were detected in the green and veraison stages and decreased in ripe and harvest stages (Figure 3.1 I–L). These findings are in accordance with previous reports for both acids, since their synthesis occurs until veraison and declines in the later stages (Degu et al., 2014; Fortes

et al., 2011). Our data showed higher oscillations in malic acid concentration than in tartaric acid, even though both acids have revealed similar concentrations. Likewise, Conde et al. (2007) observed a great variation on malic acid levels during berries development and maturation, in contrast to tartaric acid. The results also showed that sugar/ acid ratio was inversely proportional in all skin color somatic variants analyzed.

Mineral soil composition has an important influence on grape quality and on the organoleptic properties of wine. Among the several minerals present in grape berries, potassium usually represents the most abundant cation (Conde et al., 2007). Our data is in accordance with this finding as shown in Figure 3.1 Q–T, once in all skin color somatic variants, potassium was the most prevalent cation, with higher amounts than calcium and magnesium.

3.4.2 Phenolic compounds

Taking into account the ancestor variety of the studied cultivars, previously confirmed as skin color somatic mutants of the varieties 'Malvasia Fina', 'Moscatel Galego', 'Pique-poul' and 'Pinot' (Ferreira et al., 2016) and the phenotypic characterization based on the colorimetric measurements, the berry color represents the main difference between the skin color somatic variants belonging to these different groups of varieties.

As color is one of the main attributes related to food acceptability in general and grapes/ wines in particular, the relationships between colorimetric parameters and the phenolic profiles were explored by means of the multiple correlations applied to all the skin color somatic variants. During color development, a decrease in b^* parameter and an increase in a^* were generally observed, which is more evident regarding the colored cultivars (Table 3.2). Independently of the variety, the colorimetric parameters were particularly correlated with anthocyanin biosynthesis/ accumulation at colored cultivars. A positive correlation was generally observed among a^* parameter and anthocyanin accumulation, i.e., the anthocyanin biosynthesis/ accumulation give rise to an increase of a^* values (Supplementary File 1). This indicate that cultivars became redness and less green during berry development. On the other hand, a negative correlation was observed among b^* parameter and anthocyanin accumulation, once this parameter represents the yellowness measure (Supplementary File 1). Thus, the anthocyanin biosynthesis/ accumulation leads to the decrease of b^* parameter, i.e., the development of blue color.

This inverse behavior between *a** and *b** parameters was particularly correlated with anthocyanins in the cv. 'Pinot Gris' and cv. 'Pinot Noir'. In the cv. 'Pinot Gris', correlation was stronger with *a** parameter, which is in agreement with a redder phenotype, regarding delphinidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside biosynthesis/ accumulation (Supplementary File 1). On the other hand, in the cv. 'Pinot Noir' *a** parameter was closely correlated with two anthocyanins (petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside). Moreover, in the cv. 'Pinot Noir', a stronger correlation was observed between *b** parameter and anthocyanins biosynthesis/ accumulation than in the cv. 'Pinot Gris', which is in accordance with its darker phenotype (Supplementary File 1).

In grapes, color differences are not only due to a low/ high pigment accumulation, phenolic biosynthetic dysfunctions should be also considered as the cause of these differences. Usually, malvidin-3-*O*-glucoside (with one hydroxyl group and two methoxyl groups on the B-ring) is the main pigment in the majority of the *V. vinifera* fruits, as observed for the black and red-skinned cultivars, 'Pinot Noir' and 'Pinot Gris', reaching its maximum value at ripe stage, although notably higher in the cv. 'Pinot Noir' (689.5 mg/kg) than in the cv. 'Pinot Gris' (30.4 mg/kg) (Table 3.1). These results indicate that both flavonoid- 3', 5'- hydroxylase and *O*-methyltransferases should be particularly active in these skin color somatic variants belonging to the same variety. Moreover, at the ripe stage, the cv. 'Pinot Noir' also revealed two acylated derivatives (peonidin-3-*O*-*p*-coumaroylglucoside and malvidin-3-*O*-*p*-coumaroylglucoside), as previously reported for the first time by Ferreira et al. (2016), which were not detected at the harvest stage, a fact that allowed the clear distinction of these two stages in these black-skinned somatic variant at the PCA analysis (Figure 3.2 D). On the other hand, the black-skinned 'Pique-poul Noir' cultivar seems to have a reduced functionality of flavonoid-3',5'- hydroxylase, despite preserving the *O*-methyltransferase activity, which results in the prevalent accumulation of peonidin-3-*O*-glucoside (5.7 mg/kg at veraison) (with one hydroxyl and one methoxyl group on the B-ring), as it was also observed for the red-skinned cv. 'Pique-poul Gris' (7.8 mg/kg) (Table 3.1). Besides peonidin-3-*O*-glucoside, the red-skinned cv. 'Pique-poul Gris' also synthesized cyanidin-3-*O*-glucoside (with two hydroxyl groups on the B-ring) at veraison stage, in a higher proportion than in the cv. 'Pique-poul Noir', which has led to the clear distinction of these two skin color somatic variants at this stage on PCA analysis (Figure 3.2 C).

The red-skinned ‘Malvasia Fina Roxo’ cultivar shows a clear prevalence of cyanidin-3-*O*-glucoside (76.0 mg/ kg at harvest stage), indicating a reduced activity of both flavonoid-3’, 5’- hydroxylase and *O*-methyltransferase (Table 3.1). This fact showed that the cv. ‘Malvasia Fina’ and cv. ‘Malvasia Fina Roxo’ are metabolically quite different, particularly from veraison stage, as observed at PCA analysis (Figure 3.2 A).

The cv. ‘Moscatel Galego Roxo’, with a red berry pigmentation (Table 3.2), did not present detectable amounts of anthocyanins. Nevertheless, low levels of anthocyanins (with a predominance of cyanidin-3-*O*-glucoside – 5.1 mg/kg) have been detected in cv. ‘Moscatel Galego Roxo’ grapes sampled on a previous year (Ferreira et al., 2016). In fact, in our study, an overall decrease in the anthocyanins content has been observed for all the cultivars analyzed comparing with the data of Ferreira et al. (2016). Nevertheless, the composition and concentration of anthocyanins in grapes is a consequence of many factors, such as climate (like sunlight exposure, UV irradiation, temperature), that changes according to the year, affecting both the expression of the structural and regulatory genes (Downey et al., 2006; He et al., 2010).

It was also observed that delphinidin and petunidin derivatives were never predominant, suggesting that, when the flavonoid-3’-5’-hydroxylase is normally functional, *O*-methyltransferases are generally strongly activated, which is in accordance with previously reports regarding the analysis of other pink berry grapes (Mattivi et al., 2006; Rustioni et al., 2016).

Other cultivars already described as showing a predominance of cyanidin-3-*O*-glucoside in its anthocyanin profile were the cv. ‘Zibbibo Nero’ (59.8 mg/kg) (De Lorenzis et al., 2015) and the cv. ‘Okuyama’ (82.97 mg/kg) (Azuma et al., 2009), considered revertant bud sports of the white-skinned cultivars ‘Muscat of Alexandria’ and ‘Italia’, respectively. Rustioni et al. (2016) also described the cv. ‘Aragatsi’ (52.21 mg/kg), cv. ‘Chardonnay Rouge’ (67.85 mg/kg), cv. ‘Faberrebe’ (95.73 mg/ kg) and cv. ‘Fernand Rose’ (97.59 mg/kg) as berry color mutants of white-skinned varieties based on their high levels of cyanidin-3-*O*-glucoside. Therefore, and considering the pedigree of other cultivars showing cyanidin-3-*O*-glucoside as the major anthocyanin, based on the present and previous results (Ferreira et al., 2016), the cv. ‘Malvasia Fina Roxo’ and the cv. ‘Moscatel Galego Roxo’, with a slightly salmon pink hue due to the prevalence of this anthocyanin, should be considered berry skin color mutant revertants of the respective white-skinned cultivars, ‘Malvasia Fina’ and ‘Moscatel Galego Branco’.

Previous works also demonstrated that flavonols, such as myricetin derivatives, were missing in white cultivars, indicating a dysfunction of the flavonoid 3', 5'-hydroxylase associated to white berried phenotypes (Mattivi et al., 2006). Our data is in agreement with these results once none of the white-skinned variants analyzed revealed myricetin derivatives in their phenolic profiles.

3.4.3 MYBA1 and MYBA2 loci characterization

It has been described that white-skinned cultivars lack the expression of the gene encoding UFGT (UDP-glucose:flavonol 3-*O*-glucosyltransferase), due to polymorphisms in the grape transcription factor genes (*MYB*-type), called *MYBA1* and *MYBA2*. In the case of *MYBA1*, the silencing is due to the insertion of *Gret1* retrotransposon in its promotor region, while two non-conservative mutations in the coding region of *MYBA2* lead to an amino acid substitution and a truncated protein (Carrasco et al., 2015; Fournier-Level et al., 2009; Kobayashi et al., 2004; Walker et al., 2007).

As expected, the *MYBA1* locus of white-skinned berry variants analyzed in this study revealed to be homozygous, determined by the *Gret1* retrotransposon insertion in the promotor region of *MYBA1* gene, while the genetic profile of colored cultivars was heterozygous at *MYBA1* locus, showing both the non-functional and the functional allele (Table 3.3).

The *MYBA1* genetic profiles were consistent with the berry color phenotype of each skin color somatic variants analyzed, unless for the cv. 'Malvasia Fina Roxo' and cv. 'Moscatel Galego Roxo'. Despite being collected as red-skinned cultivars and the colorimetric measurements reflect their pigmentation, the genetic profiles of both red-skinned cultivars were similar to that of white-skinned cultivars. Similar results were reported by Migliaro et al. (2014), where the authors were not able to discriminate among the genetic profiles of the cv.'Sauvignon Rouge' and cv. 'Sauvignon Blanc' and the cv. 'Chasselas Violet' and cv. 'Chasselas Blanc'. These cultivars were described as red/pink-skinned variants derived from a white-skinned ancestor, where the main mechanism found for color gain is the excision of *Gret1*, leaving the solo-3'LTR. However, unlike what has been referred for the cv. 'Sauvignon Rouge' and cv. 'Chasselas Violet' by Migliaro et al. (2014), the *Gret1* partial region was not detected in the cv. 'Malvasia Fina Roxo' and cv. 'Moscatel Galego Roxo', suggesting that the gain in color may be due to

the occurrence of different mutational events affecting the anthocyanin biosynthetic pathway.

Among the remaining somatic variants, where the ancestor corresponds to a colored variety, the genetic profile of the cv.'Pinot Noir' and cv. 'Pinot Gris' resulted similar to the profile obtained by Vezzulli et al. (2012) for this variety, as well as for the cv. 'Pique-poul Noir' and cv. 'Pique-poul Gris', all of them contain one copy of the functional *VvmybA1c* allele, thus being heterozygous at *MYBA1* locus.

Taking into account the haplotypes defined by Fournier-Level et al. (2010), considering both *MYBA1* and *MYBA2* loci, all white-skinned cultivars and the red-skinned cv. 'Moscatel Galego Roxo' were consistent with haplotype 'Hap B', holding the *Gret1* insertion and a mutated T allele at VvMYBA2R44 position, corresponding to a white haplotype profile. Among the colored cultivars (particularly the black-skinned cultivars) the typical haplotype 'Hap C-N', holding no *Gret1* and a functional G allele at VvMYBA2R44, described as the major colored haplotype in black berried varieties was not detected. The cv. 'Malvasia Fina Roxo' profile corresponds to a low frequency recombined "white" haplotype 'Hap Rec', holding the *Gret1* insertion and a functional G allele at VvMYBA2R44, which results in an altered color (red). The remaining colored cultivars present both *Gret1* insertion and a putative functional allele at *MYBA1* and a functional G allele at VvMYBA2R44, corresponding to the haplotype 'Hap C-Rs', the most common on cultivated grapevine.

Although the *Gret1* insertion revealed to be the main factor determining grape color, as has been previously described, the VvMYBA2R44 mutation of *MYBA2* appears to play an essential role in the *MYB* diversification, mainly contributing to the phenotypic diversity of less colored cultivars, as observed for the cv. 'Malvasia Fina Roxo', showing the same allelic composition as the remaining colored cultivars at *MYBA2* locus. However, our results also suggest that the genetic polymorphisms of the berry color locus are not able to fully explain the color variability mainly among less colored cultivars, such as the cv. 'Moscatel Galego Roxo', suggesting the influence of other genes.

Another explanation to the skin color variability observed in somatic variants could be also detected using a layer-specific approach, investigating the presence of somatic mutations occurred in the cell layers (L1 and L2) of the shoot apical meristem (Migliaro et al., 2014; Vezzulli et al., 2012). This kind of approach allowed the description of different models about the generation of somatic variants, based on cases such as the cv.

'Cabernet Sauvignon', cv. 'Pinot' and cv. 'Muscat of Alexandria' (De Lorenzis et al., 2015; Vezzulli et al., 2012; Walker et al., 2006).

3.5 CONCLUSIONS

This work represents the first attempt to perform an integrative approach combining colorimetric, metabolic and genotypic data to improve the knowledge regarding grape berry skin color diversity using skin color somatic variants. The results showed that the initial stages, green and veraison, are metabolically very different from ripe and harvest and that veraison represents a key stage from which grape berries undergo dramatic metabolic changes, namely anthocyanin biosynthesis in colored variants.

Although *Gret1* retrotransposon insertion has been identified as the major determinant for the berry skin color in all unpigmented or less pigmented variants, the genetic polymorphisms of the considered berry color locus are not able to fully explain the color variability among some red-skinned variants. Moreover, the phenotypic diversity of the skin color somatic variants analyzed is not only related to the quantitative accumulation of anthocyanins. Dysfunctions at different levels of the biosynthetic pathway are behind the color variation observed among each group of skin color somatic variants analyzed. Thus, both quantitative and qualitative differences should be taken into account to explain skin color variation.

The spontaneous emergence of skin color variation observed during grapevine domestication has provided a valuable source of diversity, considering the key importance of the berry skin color in cultivar selection and use. The data here obtained represents an important source of information regarding the biochemical characteristics of these variants that could be used for the production of novel and improved wines.

Supporting information

Supplementary data (Supplementary File 1) to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2017.03.050 and on the electronic version of this Thesis.

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

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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.

3.6 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 cv. '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 occurred 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 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* gene 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.

3.7 MATERIAL AND METHODS

3.7.1 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 Commission' (CVRVV) 'Estação Vitivinícola Amândio Galhano' in Arcos de Valdevez (41º81'N,8º41'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 ºC until being processed.

3.7.2 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 as reported by Castro et al. (2011). The remaining six microsatellites were amplified by individual PCR (VVMD28, VVMD32, VVIv37, VVIv67, VVIp31 and VMC4f3), according to the same authors.

3.7.3 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'-CCTGCAGCTTTTTCGGCATCT- 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'- TAGCTGCTGCCACTGCATAG- 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.

3.7.4 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 (QIA- GEN, 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).

3.7.5 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). Briefly, 1mL of pre-warmed (65ºC) extraction buffer [300 mM Tris HCl (pH 8), 25 mM EDTA, 2 M NaCl, 2 % CTAB, 2 % PVP, 0.05 % spermidine trihydrochloride] and 1 mL of β-mercaptoethanol were added to the ground powder tissue. Samples were incubated at 65 ºC for 10 min and then extracted twice with chloroform-isoamyl alcohol (24:1), with a centrifugation at 30.000 ×g for 20 min at 4 ºC. To the supernatant, 0.1 v 3 M NaOAc (pH 5.2) and 0.6 v isopropanol were added, mixed, and then stored at -80 °C for 30 min. Nucleic acid pellets were collected by centrifugation at 3.500 ×g for 30 min at 4 °C and then dissolved in 0.5 mL Tris-EDTA (pH 8). To selectively precipitate the RNA, 125 µL of 10 M LiCl was added and the samples were stored overnight at 4 ºC. The precipitated RNA was pelleted in the following day by centrifugation, washed with 4 ºC 70 % EtOH, air dried and dissolved in 40 µL of DEPC-treated water. Total RNA was further purified using the E.Z.N.A.® MicroElute RNA Clean Up Kit (Omega Bio-Tek, Norcross, GA, USA) following the standard protocol. RNA concentration and 260/280 nm ratios were determined before and after DNase I digestion with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA (200ng) was reverse transcribed with the qScript TM cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD, USA) according to the supplied protocol.

3.7.6 Quantitative reverse transcription PCR (qRT-PCR) expression analysis

The differential transcript level of genes involved in the biosynthesis of anthocyanins (*CHS1, CHS3, F3'H1a, F3'5'H1a, F3'5'Hj, DFR, FLS4, ANR, LAR1, LAR2, LDOX, UFGT* and *OMTc*) and transcription factors related with this pathway (*MYBA1, MYBA2, MYB4b, MYB5a* and *MYB5b*) was determined by qRT-PCR using a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Genes selection was performed based on the previously described expression levels of these genes analyzed on different grapevine cultivars during berry development (Ali et al., 2011; De Lorenzis et al., 2016; Falginella et al., 2012).

Each PCR reaction (20 µL) contained 200 mM of each primer, 3 µL of cDNA (1:30 dilution of the synthesis reaction), 1 x SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and water up to 20 µL. Thermal cycling

conditions were 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s, annealing (variable temperatures) for 30 s and 72 ºC for 30 s. A melting cycle with temperature ranging from 60 to 95 ºC was included in order to detect non-specific amplification in cDNA samples. Each one of the three biological replicates used to extract RNA and synthetize cDNA were used for qRT-PCR reaction in duplicate. Gene transcripts were quantified upon normalization to *Elongation factor 1-alpha* (*EF1α*) by comparing the threshold cycle (Ct) of each target gene with geometric mean of *EF1α* Ct. The relative quantification per each gene was calculated by the 2-ΔCt method, where ΔCt is the difference in threshold cycle between the geometric means of the target gene and the reference gene.

3.8 RESULTS AND DISCUSSION

A genotypic description of twelve SSR loci was used to ascertain the genetic identity of the highly pigmented cv. 'Vinhão' (Figure 3.3 a-b). Among the set of microsatellites analyzed, the OIV core set VVS2, VVMD5, VVMD7, VVMD27, ssrVrZAG62 and ssrVrZAG79 that correspond to OIV801 to OIV806 descriptors (OIV, 2009), established by the European Project GENRES # 81 for the identification of grapevine cultivars, was included along with six other SSR loci VVMD28, VVMD32, VVIv37, VVIv67, VVIp31 and VMC4f3 to perform a more accurate grape cultivar authentication. The genetic identity of cv. 'Vinhão' was ascertained by comparison with fingerprinting data previously reported by Castro et al. (2011) (Figure 1b). It was also concluded that cv. 'Vinhão' share a high number of alleles with the red cv. 'Amaral', another autochthonous cultivar from Vinhos Verdes DOC region that is described as an ancient cultivar, with an important role for the genetic diversity found in this wine-growing region, suggesting the likely kinship relation between these cultivars (Castro et al., 2012; Ferreira et al., 2015).

Regarding the berry skin color feature of cv. 'Vinhão', both *MYBA1* and *MYBA2* loci were molecularly characterized through their allelic variations. *MYBA1* locus was characterized by two different approaches: i) the detection of the non-functional allele (*VvmybA1a*), carried out through the presence of *Gret1* retrotransposon insertion and ii) the assessment of putative functional alleles, namely the wild-type allele without any kind of insertion, *VvmybA1c*, or other functional alleles. 'Vinhão' genotype showed to carry

only one *MYBA1* allele, being homozygous for the wild-type allele, *VvmybA1c*, which is characterized by no insertion in the promotor region (Figure 3.3 a-b). The fragment with ≈1080 bp corresponding to *VvmybA1c* (*MYBA1* promotor region and part of the coding sequence) was sequenced and compared with those sequences obtained from the reference grapevine genome Pinot noir-derived line and no deviation was found from the reference sequence. Additionally, *MYBA2* gene polymorphisms were assessed by the SNaPshot system, using VvMYBA2R44 (K980) as marker, which revealed the presence of the wild-type allele as the unique allele (G/G), called *VvmybA2r*, allowing the perfect function of *MYBA2* gene (Figure 3.3 c).

In *Vitis vinifera* cultivars, the mutational pattern for these two color genes gives rise to different genotypic combinations that consist mainly on three haplotypes, Hap C-N, Hap C-Rs and Hap-B (Fournier-Level et al., 2010). Hap C-N is considered the ancestral haplotype, consisting of the functional genes *MYBA* that carries no *Gret1* insertion at *MYBA1* and a functional G allele at *MYBA2*. Hap C-Rs haplotype contains both a functional component at *MYBA1* and a non-functional T allele on *MYBA2* and Hap-B contains two non-functional alleles for both genes. Taking into account these main haplotypes of the berry color locus, cv. 'Vinhão' carries two functional alleles on both *MYBA* genes, i.e. the ancestral haplotype Hap C-N (Figure 3.3 c). Several studies suggest that the number of functional alleles affects the capability of anthocyanin accumulation in grape berry skin (Azuma et al., 2011; Carrasco et al., 2015; De Lorenzis et al., 2015; Fournier-Level et al., 2010). This could be one of the reasons whereby cv. 'Vinhão' has the capability to accumulate higher anthocyanin amounts in berry skin, with values ranging from 7274 to 11842 mg/ kg at full maturation, as previously described by Dopico-García et al. (2008), corresponding to the cultivar with the highest content of anthocyanins among the 10 different cultivars from Vinhos Verdes DOC region analyzed. Moreover, Fournier-Level et al. (2010) and Carrasco et al. (2015) also concluded that the most ancestral haplotype C-N was found in other varieties from the Iberian Peninsula, suggesting that Portuguese and Spanish grapes have kept the ancestral haplotype, which could outcome from an isolation event affecting these varieties in a region considered a glaciation refuge zone, involving limited gene flow from the east or due to their hybridization with non-domesticated endemic *Vitis vinifera* subsp. *sylvestris*.

Since the regulation of biosynthetic anthocyanin pathway in *V. vinifera* is coordinated by transcriptional and post-transcriptional control of several structural genes, it is reasonable to speculate that the capacity of anthocyanin accumulation in cv. 'Vinhão'

is not only due to the *MYB* genes allelic composition at the berry color locus. In order to better understand the berry color trait of the cv. 'Vinhão', the expression patterns of structural genes involved in the biosynthesis of anthocyanins and transcription factors related with this pathway were examined at different developmental stages by using qRT-PCR method (Figure 3.4). Anthocyanins' biosynthetic pathway shares the first steps of the general phenylpropanoid pathway. Thus, it is important to analyze the expression levels of genes involved in different steps along the entire pathway, including earlier genes, which can affect down-stream branches, such as the anthocyanins' pathway. During the berry development, metabolism changes are typically fast, which affects flowering and berry ripening rates, even in the same plant. Therefore, in this kind of gene expression studies is more important to consider the trends of gene expression than focusing on differences in exact values at a single time point (Primetta et al., 2015).

a)

b)

Cultivar name	Vinhão							
Berry color	Black-skinned							
SSR loci	**VVS2**		**VVMD5**		**VVMD7**		**VVMD27**	
Allele size (bp)	130	131	218	222	237	261	185	185
SSR loci	**VrZAG62**		**VrZAG79**		**VMC4f3**		**VVMD28**	
Allele size (bp)	187	185	243	249	171	177	233	255
SSR loci	**VVMD32**		**VVIp31**		**VVIv37**		**VVIv67**	
Allele size (bp)	237	237	178	188	161	163	353	363
Identified SSR profile	Vinhão (Castro et al. 2011)							

c)

Figure 3.3 – Pigmentation of the cv. 'Vinhão' berries collected at ripening stage (a); varietal identity of cv. 'Vinhão' through nuclear microsatellites (b); allelic composition of *MYBA1* and *MYBA2*, with the corresponding haplotype (c).

After the first common steps of the phenylpropanoid pathway, the flavonoid biosynthesis is initiated by chalcone synthase (CHS) activity. CHS1 is described as the best characterized chalcone isoform in grapevine; however its expression is not constant during the growing cycle (Conde et al., 2016). The obtained data shows different expression patterns of *CHS1* and *CHS3* genes. *CHS1* showed a significant increase between the last two stages (ripening and full maturation), with the highest expression level observed at the full maturation stage. While, *CHS3* had the highest expression level at ripening stage (Figure 3.4), similar to the expression pattern observed in 'Corvina' by

De Lorenzis et al. (2016), which showed the highest steady-state transcript abundance also at the ripening stage. Genes involved in the hydroxylation of dihydrokaempferol via flavonoid-3', 5' hydroxylase (*F3'5'H1a* and *F3'5'Hj* transcripts) that leads to the anthocyanin precursor delphinidin, significantly increase as the berry develops from veraison to the last stage of maturation in cv. 'Vinhão'. Despite both transcripts showed the same expression trend, as it was also observed in cv. 'Alicante Bouschet' by Falginella et al. (2012), *F3'5'Hj* is more expressed than *F3'5'H1a* (Figure 3.4). The parallel biosynthetic pathway that uses the same precursor, dihydroxylkaempferol to another anthocyanin precursor (cyanidin) via the enzyme flavonoid 3' hydroxylase (*F3'H1a* transcript) only showed an increase expression at ripening stage, however with a significantly lower expression than the transcripts of the other branch of the pathway (*F3'5'H1a* and *F3'5'Hj*) (Figure 3.4). It was demonstrated that the ratio between anthocyanins trisubstituted (delphinidin, petunidin and malvidin) and disubstituted (cyanidin and peonidin) in the lateral B-ring is associated with *F3'5'H/ F3'H* transcription level, thus affecting the anthocyanin profile (Muñoz et al., 2014). Previously, Dopico-García et al. (2008) showed that delphinidin-3-*O*-glucoside and malvidin-3-*O*-glucoside were the major anthocyanins accumulated in cv. 'Vinhão', which could be correlated with the fact that *F3'5'Hs* transcripts showed a higher transcription level than the *F3'H1a* transcript in this cultivar. Flavonol synthase (FLS) is the first enzyme of the flavonol biosynthetic branch of the flavonoid pathway that is responsible for the conversion of dihydroflavonols to flavonols, which are important co-pigments that stabilize anthocyanins in wine. Gene expression analysis by qRT-PCR revealed that *FLS4* transcript levels decreased until ripening stage and then abruptly increased, being mostly expressed at the full mature stage, probably induced by the UV-radiation (Figure 3.4). The dihydroflavonol reductase enzyme (DFR) is responsible for the first committed step in the pathway leading to the synthesis of flavan-3-ols. *DFR* showed an increase transcription level between the green and ripening stages and the transcript abundance of the gene was slightly reduced at full mature stage. The same expression patter was observed for leucoanthocyanidin dioxygenase (*LDOX*), however at a much higher level than *DFR* (Figure 3.4). The expression of anthocyanidin reductase (*ANR*) and leucoanthocyanidin reductase (*LAR1* and *LAR2*), which are the key enzymes of the proanthocyanidin biosynthesis showed opposite behaviors; *ANR* expression level increased from the green stage until full maturation and the expression level of *LAR1* and *LAR2* decrease during berry maturation (Figure 3.4). The enzyme UDP- glucose: flavonol

3-*O*-glucosyltransferase (UFGT) represents the key point of anthocyanin biosynthesis pathway and catalyzes the final step of the pathway. 'Vinhão' starts to express *UFGT* at veraison, reaching the highest value at ripening stage and then decreased at the last sampling date (Figure 3.4). Comparing cv. 'Vinhão' with the teinturier cv. 'Alicante Bouschet' analyzed by Falginella et al. (2012), despite both cultivars showed a quite similar *UFGT* expression pattern, in cv. 'Alicante Bouschet' berry skin, the expression of this gene does not decrease at the end of maturation. The Portuguese cultivar 'Trincadeira' has been also reported as showing an increase transcript abundance of *UFGT* on veraison and ripening stages (Fortes et al., 2011). Regarding the transcriptional regulation of the pathway during berry development, some *MYB* factors, such as *MYB4b*, possess repressor activities and inhibit phenolic compound synthesis. This gene has been described as a direct repressor of anthocyanin synthesis once it negatively regulates the *UFGT* gene expression (Matus et al., 2009). Thus, it was expected that *MYB4b* showed an inverse expression profile to that observed for *UFGT* gene, showing the highest level of expression at green stage and then decreasing, reaching the lowest values at the last two sampling dates, corresponding to ripening and full maturation stages (Figure 3.4). Furthermore, the expression pattern of *MYBA1* and *MYBA2*, which are the two direct regulatory genes of *UFGT* expression revealed to follow the same trend, with a peak of expression at veraison, overlapping, as expected, with the expression profile of *UFGT* (Figure 3.4). Contrary to *MYBA1* and *MYBA2* genes that specifically control the last biosynthetic step of anthocyanin synthesis, *MYB5a* and *MYB5b* appear to regulate earlier steps and other sub-branches of the phenylpropanoid pathway, namely the synthesis of flavan-3-ols (Ali et al., 2011). The transcript profiles of *MYB5a* and *MYB5b* were similar during berry development on cv. 'Vinhão', being mainly expressed before veraison, reaching the highest transcription level at green stage (Figure 3.4), which showed a coordinated expression with its target genes *LAR1*/*LAR2*.

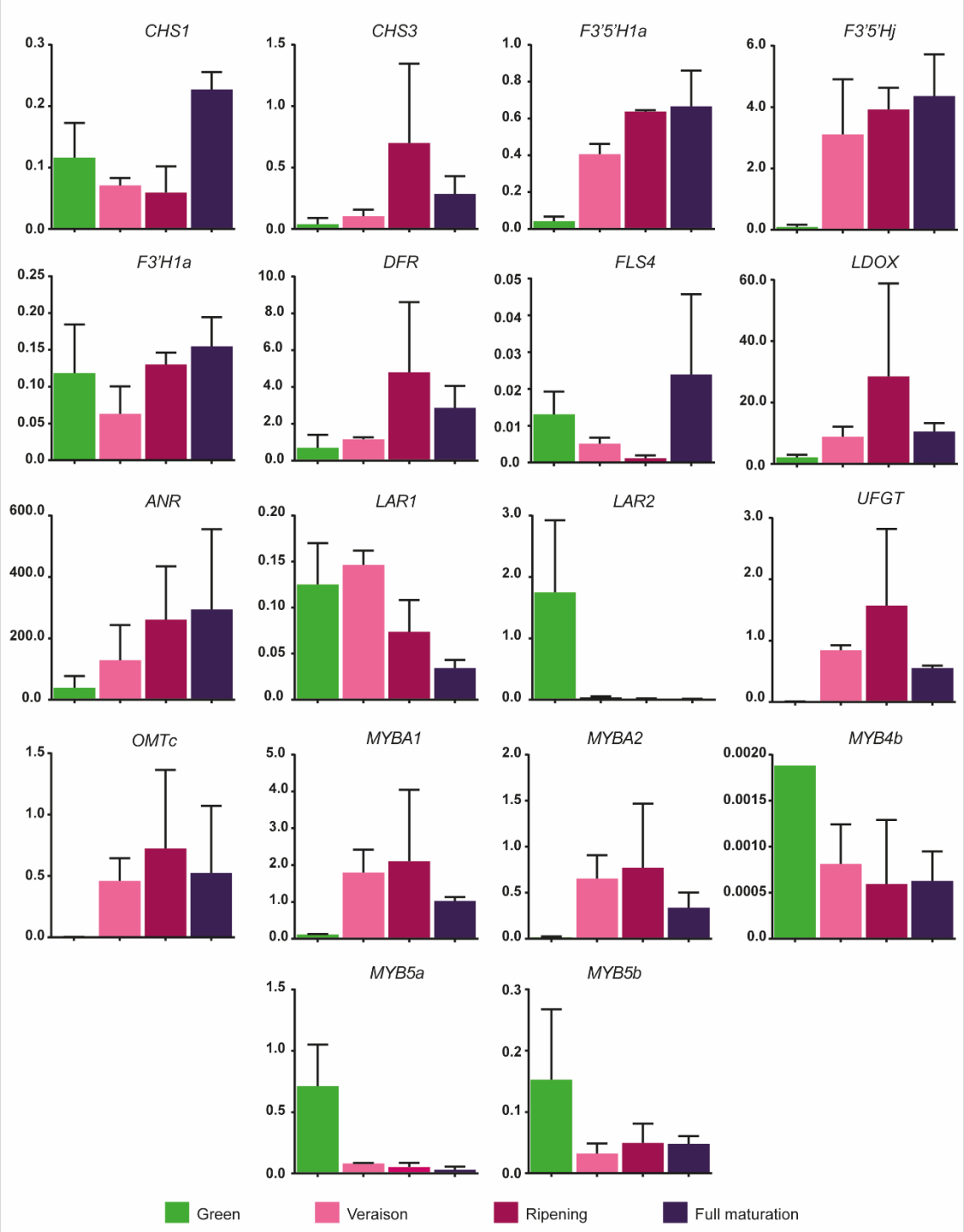

Figure 3.4 – Expression level of genes and transcription factors involved in the biosynthesis of anthocyanins (*CHS1, CHS3, F3'5'1a, F3'5'Hj, F3'H1a, DFR, FLS4, LDOX, ANR, LAR1, LAR2, UFGT, OMTc*, *MYBA1*, *MYBA2*, *MYB4b*, *MYB5a and MYB5b*) in cv. 'Vinhão' grapes at four developmental stages (x axis) during the 2015 season. The expression of each gene has been normalized using the geometric mean of expression values of one housekeeping gene (*EF1α*) at each sampling. The relative gene expression (y axis) has been determined based on the 2-ΔCt method. Bars represent the standard deviation.

O-methyltransferases (OMT) catalyzes the conversion of cyanidin-3-*O*-monoglucoside into peonidin-3-*O*-monoglucoside and delphinidin-3-*O*-monoglucoside into petunidin- and malvidin-3-*O*-monoglucoside and it has been suggested that the expression of *OMT* genes is correlated with the accumulation of methylated anthocyanins in grapevine, which may affect color stability (Ageorges et al., 2006; Hugueney et al., 2009). The results obtained for cv. 'Vinhão' showed that *OMTc* is steadily induced at veraison, which corresponds to the stage where anthocyanins start to be accumulated in pigmented cultivars, with an even higher transcript level at ripening stage, and then slightly decline towards full mature stage (Figure 3.4). This expression profile matches with the transcriptional levels of *OMT* genes on other pigmented cultivars, such as cv. 'Cabernet Sauvignon', cv. 'Norton' (Ali et al., 2011) and cv. 'Alicante Bouschet' (Falginella et al., 2012).

3.9 CONCLUSIONS

In this study, a molecular characterization focused on the genetics behind the high pigmented berries of the cv. 'Vinhão' was performed for the first time, by the analysis of *MYBA1* and *MYBA2* allelic composition and also by the analysis of the expression patterns of genes involved in the anthocyanin biosynthetic pathway during berry development.

The findings obtained helped to clarify the genetics underlying the intense berries skin pigmentation that characterizes this autochthonous Portuguese cultivar, suggesting that *MYB* genes play a major role on the berry skin color of the cv. 'Vinhão'. This highly pigmented cultivar revealed to be homozygous for functional alleles on both *MYBA1/A2* genes, corresponding to the most ancestral haplotype (Hap C-N).

This study focused on berry skin color of this cultivar is of major importance for breeding programs that could contribute to improving cv. 'Vinhão' grape quality and attract more attention to this Portuguese cultivar, being expected that it will be used to produce even more high quality red wines in the future.

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CHAPTER 4 | BERRY SKIN COLOR REVERSIONS

Molecular characterization of berry skin color reversion on grape somatic variants

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Abstract

During grapevine domestication somatic variation has been used as a source of diversity for clonal selection. This work provides additional information on the molecular mechanisms responsible for berry skin color reversion on a subset of somatic variants for berry skin color never investigated before. The berry color locus and its surrounding genomic region were genetically characterized through a layer-specific approach, which has already been proven to be a successful method to decipher the molecular mechanisms responsible for color reversions on somatic variants. Deletions of different extent and positions were detected among less-pigmented/ unpigmented variants derived from a pigmented wild-type. These deletions affected only the inner cell layer in the less pigmented variants and both cell layers in the unpigmented variants. Regarding the pigmented variants derived from an unpigmented wild-type, only one group was distinguished by the *Gret1* retrotransposon partial excision from the *MYBA1* promoter. Moreover, within this latter group, *MYBA2* showed an important role regarding the phenotypic variation, through the recovery of the functional G allele. This investigation focused on the berry color locus using somatic variants for berry skin color, promotes a better understanding of the evolutionary events behind their origin and variability, opening an opportunity for their use in the genetic improvement of varieties.

4.1 INTRODUCTION

Domestication of the wild *Vitis vinifera* ssp. *sylvestris* to *Vitis vinifera* ssp. *sativa* species was a complex and long-term evolutionary process that involved multiple loci [1]. This process dramatically changed the grape biology in order to ensure higher sugar content, greater yield, regular production and adaptation to different environments [2].

Today, most of the cultivated grapevine varieties are the result of continuous selection and vegetative propagation over centuries, spontaneously generated by sexual reproduction and via somatic mutations. These events led to grape varieties which have enough similar vegetative and reproductive features to allow them to freely interbreed [3]. Clones are usually selected for a particular character or combination of characters which, when propagated by appropriate means, retain those characters and are genetically identical, i.e., true-to-type [4].

Although vegetative propagation has been used as a conservative strategy to maintain and propagate clones, somatic mutations may occur naturally when a spontaneous morpho-physiological mutation appears on a shoot, leading to clonal polymorphism [3,5]. When a mutation appears in a single cell of a shoot apical meristem layer, the following mitotic divisions lead to the emergence of a mutated section [6], giving rise to a periclinal chimera. These structures correspond to specific types of genetic mosaicism in which one or two entire meristematic cell layers are genetically different from the others, and are developmentally independent from the adjacent layers [7]. Usually they are stable and do not threaten plant fitness, and are maintained by vegetative propagation. However, occasionally these cellular rearrangements in the periclinal chimera lead to homogenization of the genotype to the whole plant [5] in a two-step process. First, a somatic mutation emerges in one cell of the shoot apical meristem and then is propagated by cell division to an entire cell layer, generating a stable chimeric structure. Subsequently, the replacement of one cell layer by another can lead to a homogenization of the genotype of the whole plant, representing the second step of the process [8]. Therefore, divergent genotypes and, to some extent divergent phenotypes, can appear due to these molecular and cellular mechanisms, representing a valuable source of heritable variation that has been widely exploited by winemakers [5,9]. There are many examples of spontaneous variant traits in grapevines, including berry skin and flesh color or flavor, size and compactness of bunches, canopy growth, date of ripening and productivity [3,5].

At the genetic level, the most well-documented polymorphisms leading to qualitative variation within varieties are those that affect berry skin color. Currently, several pigmented varieties have certified clones with different skin color shades, such as ‘Aramon’, ‘Grenache’, ‘Pinot’ or ‘Terret’. Moreover, unpigmented skin berry varieties such as ‘Savagnin’, ‘Chardonnay’ and ‘Chasselas’ also comprise pigmented clones [5].

The first comparative study of molecular polymorphisms arising during vegetative propagation on the whole genome scale was done on spontaneously generated grape clones. Despite the small number of SNP (single nucleotide polymorphism) and indel events observed, mobile elements were identified as being involved in most of the polymorphisms detected [10].

Overall, the grapevine somatic variants for berry skin color can be divided in two main groups: A) unpigmented and less-pigmented variants from a pigmented ancestor, namely white and grey/pink-skinned variants derived from a black-skinned ancestor; B) pigmented variants from an unpigmented wild-type, namely red/pink-skinned variants derived from a white-skinned wild-type [11–13].

Research on grape berry color shows that the color locus is a cluster of four *MYB* and *MYB*-like genes, spread along a 200 kb-region located on chromosome 2, which comprises *MYBA1* and *MYBA2*, two adjacent transcription factors that regulate the transcription of *UFGT* (UDP-glucose:flavonoid 3-*O*-glucosyltransferase), a key point in the anthocyanin pathway. In white-skinned cultivars, the absence of anthocyanins has been related with the insertion of the *Gret1*, a 10,422 bp long retrotransposon, in the promoter region of *MYBA1* gene combined with two non-conservative mutations in the coding sequence of *MYBA2*, a non-synonymous point mutation that 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]) and a 2-bp deletion (CA) altering the reading frame, that results in a smaller protein [14–17]. An additional point mutation of *MYBA2* gene (C22) related to berry pigmentation was described by Carrasco et al. [18] in wild grapevine accessions with low anthocyanin content. These mutations in both genes lead to the loss of transcription factors expression, which consequently prevents anthocyanin biosynthesis.

Researchers have recently focused on the somatic variation affecting berry skin color in different varieties, using a layer-specific approach to identify the molecular mechanisms responsible for the polymorphisms occurring at berry color locus and to understand the evolutionary events behind their origin [5,13,19]. Vezzulli et al. [19]

observed a homozygous-like region in ‘Pinot Gris’ and ‘Pinot Blanc’ clones, suggesting that the mutation impairing the color locus was directly related with the presence of deletions, extending for at least 4.2 Mb for the L2 cell layer of the cv. ‘Pinot Gris’ and ranging from 100 to 179 kb for both cell layers of the cv. ‘Pinot Blanc’. Based on these results, they concluded that the cv. ‘Pinot Noir’ gave rise to the cv. ‘Pinot Blanc’ and cv. ‘Pinot Gris’ independently, suggesting a parallel evolutionary model. Considering the same family of somatic variants, Pelsy et al. [5] investigated a collection of ‘Pinot Noir’, ‘Pinot Gris’ and ‘Pinot Blanc’ clones and proposed a model integrating both mutation and cell layer rearrangements to explain the mechanism of clone diversification. On the other hand, Migliaro et al. [13] identified polymorphisms that enable the distinction of less pigmented/unpigmented and pigmented mutants from the corresponding wild-type genotype, concluding that phenotypic variation is due to deletion events that can result in either gain- or loss-of-gene function. Deletions of different extent and position were detected among less pigmented/unpigmented variants derived from a pigmented wild-type. Regarding the pigmented variants derived from an unpigmented wild-type, the same authors observed that the main mechanism for color gain was the partial *Gret1* retrotransposon excision from the *MYBA1* promoter, leaving only the solo-3’LTR region.

The present study was focused on the genetic characterization of the berry color locus and its surrounding genomic region, aiming to uncover the molecular mechanisms responsible for the skin color phenotype reversion on a set of somatic variants for berry skin color, analyzed here for the first time. The results obtained represent an important addition to the current knowledge about the structural dynamics along the distal arm of chromosome 2 and the evolutionary events behind the origin of somatic variants for berry skin color.

4.2 MATERIAL AND METHODS

4.2.1 Plant material

The molecular mechanisms of color variation in grapevine somatic variants for berry skin color were analyzed in 26 accessions belonging to 10 *V. vinifera* spp. *sativa* varieties, including the 'Pinot 'family (cv. 'Pinot Noir', cv. 'Pinot Gris' and cv. 'Pinot Blanc') which was used as a reference (Table 4.1). The different accessions were provided by the Institut National de la Recherche Agronomique – INRA, Centre of Grapevine Genetic Resources (Montpellier, France); Universidade de Trás-os-Montes e Alto Douro – UTAD (Vila Real, Portugal) and the Instituto Nacional de Investigação Agrária e Veterinária – INIAV, Coleção Ampelográfica Nacional (Dois Portos, Portugal). All accessions were divided in two groups (A and B) according to their botanical origin (Table 4.1).

4.2.2 Genomic DNA extraction

To implement the layer-specific approach, two genomic DNA samples for each accession were isolated: one from 100 to 200 mg of young leaf (L1+L2 derived tissue), corresponding to stage E (2 to 3 leaves unfolded) of the Baggiolini scale, and the other from 200 to 300 mg of woody shoot pith (L2 derived tissue) at stage A (winter bud) of the same scale, for a total of 52 tissue-specific DNA samples. The leaf and woody shoot pith materials were ground in 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. DNA concentration was determined by UV spectrometer (Nanodrop™ ND-1000, ThermoFisher Scientific, USA). DNA quality was also checked with a 1 % (w/v) agarose gel electrophoresis using 1x TBE buffer followed by ethidium bromide staining. Necessary dilutions were performed (approximately 10 ng/µL) and kept at 4 º C for further use. Subsequent molecular analyses were performed independently on both DNA samples from each accession.

Table 4.1 – List of the studied somatic variants for berry skin color divided in two groups (A and B), respective berry skin color, repository and accession number.

Cultivar	Berry skin color [1]	Repository[2]	Accession number
Group A			
Aramon	B	INRA/CGGR	22Mtp1
Aramon Gris	G	INRA/CGGR	23Mtp2
Aramon Blanc	W	INRA/CGGR	24Mtp1
Aspiran Noir	B	INRA/CGGR	1Mtp3
Aspiran Gris	G	INRA/CGGR	3Mtp2
Aspiran Blanc	W	INRA/CGGR	2Mtp1
Grolleau	B	INRA/CGGR	297Mtp11
Grolleau Gris	G	INRA/CGGR	305Mtp2
Grolleau Blanc	W	INRA/CGGR	356Mtp2
Pinot Noir	B	UTAD	F2.2.3
Pinot Gris	G	UTAD	F2.13.2
Pinot Blanc	W	UTAD	F2.13.1
Pique-poul Noir	B	INRA/CGGR	11Mtp8
Pique-poul Gris	G	INRA/CGGR	12Mtp5
Pique-poul Blanc	W	INRA/CGGR	13Mtp7
Terret Noir	B	INRA/CGGR	14Mtp3
Terret Gris	G	INRA/CGGR	15Mtp2
Terret Blanc	W	INRA/CGGR	16Mtp3
Group B			
Folgasão Roxo	R	INIAV/CAN	52709
Folgasão	W	INIAV/CAN	52708
Malvasia Cândida Roxo	R	INIAV/CAN	50911
Malvasia Cândida	W	INIAV/CAN	50810
Malvasia Fina Roxo	R	UTAD	F2.3.1
Malvasia Fina	W	UTAD	F2.13.5
Mourisco	B	INIAV/CAN	52002
Mourisco Roxo	R	INIAV/CAN	52001

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

[2] INRA - Institut National de la Recherche Agronomique, CGGR - Centre of Grapevine Genetic Resources (Montpellier, France); 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).

4.2.3 Simple sequence repeat (SSR) analyses

To determine whether the berry color variants used in the study were true-to-type, i.e. if all berry skin color variants were actually somatic variants of the original variety (wild type) genotype, a set of 10 markers (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79, VMC4f3, VVMD28, VVMD32 and VVIv67), which included the Organisation Internationale de la Vigne et du Vin (OIV) core set established by the European Project GENRES#81 for grapevine cultivars identification, was used. Two multiplex PCRs were carried out with the OIV SSR core set as reported by Castro et al. [20], the first one involving VVS2, VVMD5 and VVMD7 (set A), and the second VVMD27, ssrVrZAG62 and ssrVrZAG79 (set B). Individual reactions were performed with the remaining four primer pairs (VMC4f3, VVMD28, VVMD32 and VVIv67) according to the same authors. The SSR allelic profile of each accession was compared with its published reference profile (Table 4.2).

Another set of 10 markers (SC8_0146_010, SC8_0146_026, VVNTM1, VVNTM2, VVNTM3, VVNTM4, VVNMT5, VVNTM6, VVIU20, VMC7G3) surrounding the berry color locus and distributed along the distal arm of chromosome 2 was used to investigate polymorphisms in this region (Table 4.2). PCR reactions were performed as described by Vezzulli et al. [19]. Capillary electrophoresis was carried out in an ABI 3130xl Genetic Analyzer (Life Technologies, Foster City, CA, USA) and the fragments were sized with Peak Scanner V1.0 software (PE Applied Biosystems, CA, USA) using the GeneScan 500 LIZ size standard (Life Technologies) as an internal ladder.

4.2.4 MYBA1 and MYBA2 gene structure

The *MYBA1* and *MYBA2* gene polymorphisms (the detection of functional and non-functional alleles) were also investigated.

Regarding characterization of the *MYBA1* locus, the presence or absence of *Gret1* retroelement in the *MYBA1* promoter region was detected using the primers a (5'-AAAAAGGGGGGCAATGTAGGGACCC-3') and d3 (5'-CCTGCAGCTTTTTCG GCATCT-3') as described by Lijavetzky et al. [14]. PCR amplifications and detection of amplified fragments were performed as reported in Lijavetzky et al. [14]. In order to detect putative functional alleles, the primers used were F2 (5'-GGACGTTAAAAAATGGTTGCACGTG-3') described by Azuma et al. [21] and R1 (5'-GAACCTCCTTTTTGAAGTGGTGACT-3') by Lijavetzky et al. [14]. PCR

amplifications and detection were performed as reported in Azuma et al. [21] and Carrasco et al. [18], respectively.

For the *MYBA2* gene, the single nucleotide polymorphism (SNP) related to berry color VvMYBA2R44 [11] was investigated by a SNaPshot assay. *MYBA2* amplification, SNP genotyping by ABI PRISM SNaPshot Multiplex kit (Life Technologies Corporation, Carlsbad, California) and SNP detection on ABI PRISM 310 Genetic Analyzer Sequencer were performed, as reported in Carrasco et al. [18].

4.3 RESULTS

4.3.1 Varietal identification

Ten SSR loci were used to ascertain the genetic identity of the somatic variants and their relative ancestor to confirm their trueness-to-type. This fingerprinting system revealed that the pigmented and unpigmented variants of each variety are closely related, showing the same genetic profile, which confirms that all the berry skin color variants analyzed were somatic variants of their respective wild-type genotype. Cultivar identification was achieved by comparing each SSR profile with the European *Vitis* database (http://www.eu-vitis.de/) or public profile (Supplementary File 2).

4.3.2 MYBA1 and MYBA2 allelic composition

To characterize the structural dynamics of the berry color locus two *MYB*-related genes (*MYBA1* and *MYBA2*) were analyzed, since they are known to be functionally involved in berry pigmentation.

Regarding these two *MYB*-related genes, their allelic composition allowed inferring some of the color differences observed among the studied variants. For all the wild-type pigmented ancestors of the group A, the *MYBA1* promoter was heterozygous, containing both the *Gret1* (non-functional *VvmybA1*^{ITA}) and non-*Gret1* (functional *VvmybA1*^{AFL}) alleles in the two cell layers, as well as in the L1 + L2 cell layer of the red/ grey-skinned derivate variants (Table 4.3). In turn, only the *Gret1* (non-functional *VvmybA1*^{ITA}) allele, present in homozygosity, was detected in the L2 of red/ grey-skinned variants and in both cell layers of unpigmented variants (Table 4.3).

Table 4.2 – Primer information of the microsatellite markers used to all the studied loci.

SSR Locus	LG	Use	5'-3' Forward primer sequence	5'-3' Reverse primer sequence	Reference
VVS2[1, 2]	11	True-to-type confirmation	CAGCCCGTAAATGTATCCATC	AAATTCAAAATTCTAATTCAACTGG	Thomas and Scott (1993); This et al. (2004)
VVMD5[1, 2]	16	True-to-type confirmation	CTAGAGCTACGCCAATCCAA	TATACCAAAAATCATATTCCTAAA	Bowers et al. (1996, 1999); This et al. (2004)
VVMD7[1, 2]	7	True-to-type confirmation	AGAGTTGCGGAGAACAGGAT	CGAACCTTCACACGCTTGAT	Bowers et al. (1996, 1999); This et al. (2004)
VVMD27[1, 2]	5	True-to-type confirmation	GTACCAGATCTGAATACATCCGTAAGT	ACGGGTATAGAGCAAACGGTGT	Bowers et al. (1999); This et al. (2004)
VrZAG62[1, 2]	7	True-to-type confirmation	GGTGAAATGGGCACCGAACACACGC	CCATGTCTCTCCTCAGCTTCTCAGC	Sefc (1999); This et al. (2004)
VrZag79[1, 2]	5	True-to-type confirmation	AGATTGTGGAGGAGGGAACAAACCG	TGCCCCCATTTTCAAACTCCCTTCC	Sefc (1999); This et al. (2004)
VMC4f3	12	True-to-type confirmation	AAAGCACTATGGTGGGTGTAAA	TAACCAATACATGCATCAAGGA	Di Gaspero et al. (2000)
VVMD282	3	True-to-type confirmation	AACAATTCAATGAAAAGAGAGAGAGAGA	TCATCAATTTCGTATCTCTATTTGCTG	Bowers et al. (1999)
VVMD32[2]	4	True-to-type confirmation	TATGATTTTTTAGGGGGGTGAGG	GGAAAGATGGGATGACTCGC	Bowers et al. (1999)
VVIv67	15	True-to-type confirmation	TATAACTTCTCATAGGGTTTCC	TTGGAGTCCATCAAATTCATCT	Merdinoglu et al. (2005)
SC8_0146_010	2	Deletion delimitation	-	-	Adam-Blondon A.F., personal communication
SC8_0146_026	2	Deletion delimitation	-	-	Adam-Blondon A.F., personal communication
VVNTM1	2	Deletion delimitation	CCACGCCACTATTGCTAAAC	TGCACCGTATCAAGATCATGTC	Fournier-Level et al. (2009)
VVNTM2	2	Deletion delimitation	TACCTGCTAACAATGCATTATG	TATTTGGTTTTTTCTAAATAGA	Fournier-Level et al. (2009)
VVNTM3	2	Deletion delimitation	TGCTGACCTGAATCATTTTCTACT	GATGTTCTGGAGAGATGCTTATC	Fournier-Level et al. (2009)
VVNTM4	2	Deletion delimitation	TTTGCATGACTGCTTGGTGTAT	CCCATTGCTAAACCCTACTCCT	Fournier-Level et al. (2009)
VVNTM5	2	Deletion delimitation	AGGAGGAATCCACATCAAAAGA	TGATTCAAAGGAATAAATAACCATCA	Fournier-Level et al. (2009)
VVNTM6	2	Deletion delimitation	CCTTCTTGGACACCCATACAAA	TTCCCTATCAACAAACTTGAGG	Fournier-Level et al. (2009)
VVIU20	2	Deletion delimitation	ACAACCTTAATGCTTCTACCAA	TCACCATGGAGATTTTCTGTAG	Merdinoglu et al. (2005)
VMC7G3	2	Deletion delimitation	TTACTAGTGCTGTCCTGCTCCA	TGCTTCCTCTCTTTCAACTTTCA	Pellerone et al. (2001)

[1] OIV core set, [2] VIVC database

The *MYBA1* gene did not allow discrimination of most variants of group B where the wild-type ancestor corresponds to a white-skinned variant, since they share the same allelic composition for this gene (Table 4.4). The partial excision of the *Gret1* retrotransposon was only observed in the 'Mourisco' family of group B (Table 4.4). The presence of the functional G allele at VvMYBA2R44 position was detected in some less-pigmented light red variants (cv. 'Malvasia Fina Roxo', cv. 'Folgasão Roxo' and cv. 'Malvasia Cândida Roxo') derived from a white-skinned ancestor (Table 4.4).

4.3.3 Genetics behind black-to-grey and/ or white skin color reversions

As previously mentioned, all the pigmented ancestor accessions of group A were heterozygous, containing the *Gret1* (*VvmybA1*^{ITA}) and non-*Gret1* (*VvmybA1*^{AFL}) alleles of *MYBA1* in both cell layers (Table 4.3). Moreover, the clonal differences between the pigmented and the derived unpigmented variants was shown to be caused by deletions of different extents, resulting in the removal of the functional colored allele of both *MYBA* genes, leading to the white phenotype. In cultivars derived from asexual reproduction, such as the ones analyzed here, the emergence of the homozygous-like pattern compared with the ancestor (usually heterozygous) can only be assigned to a deletion (grey-shaded region in Table 4.3). The homozygous-like term corresponds to the genetic hemizygous term (which refers to a null allele, called *VvmybA1*^{PNB}), which will be used henceforth.

The black-skinned cv. 'Pinot Noir' was homozygous for two microsatellite markers (VVNTM6 and VVNTM4) and heterozygous for eight, as well as for the *Gret1* retrotransposon insertion and *MYBA2* SNP, in either L1 + L2- or L2- cell layers. The cv. 'Pinot Gris' differed from the cv. 'Pinot Noir' in having only one allele in the region between the VVNTM1 and VMC7G3 SSR markers, as well as only the *Gret1* allele in L2-derived tissues. In turn, the cv. 'Pinot Blanc' was hemizygous in a smaller region, between SC08_010 and VVNTM5, both on L1 + L2-, and L2-derived tissues (Table 4.3). A similar situation was observed for 'Pique-poul' and 'Aramon' families, where both less-pigmented and unpigmented variants probably derived from the wild-type pigmented ancestor in an independent way due to the different deletion extent and position. The cv. 'Pique-poul Blanc' differ from the cv. 'Pique-poul Noir' since it has only one allele in the region between the SC8_010 and VVNTM6 SSR markers, as well as only the *Gret1* allele (*VvmybA1*^{ITA}) in both L1 + L2- and L2-derived tissues. On the other hand, the hemizygous profile of the cv. 'Aramon Blanc' was between the VVNTM2 and VVNTM6 SSR

markers. Regarding the less-pigmented variants of both varieties, they only showed a difference from their wild-type pigmented ancestor in the L2 cell layer. The cv. 'Picpoul Gris' revealed the presence of only one allele in the region encompassed by the VVNTM1 and VVNTM6 markers, also affecting the non-*Gret1* allele. The cv. 'Aramon Gris', similar to the less pigmented variants already described, can only be distinguished from its wild-type cultivar in L2-derived tissues, between the VVNTM2 and VVNTM3 SSR markers (Table 4.3).

For the cv. 'Aspiran Gris' and cv. 'Aspiran Blanc', one allele was observed from VVNTM1 to VVNTM6 SSR markers in the L2 cell layer of the cv. 'Aspiran Gris' and in the L1 + L2-derived tissues and the L2 cell layer of the cv. 'Aspiran Blanc'. The same deletion pattern was also detected for the cv. 'Terret Gris' and cv. 'Terret Blanc' (Table 4.3). Finally, in the cv. 'Grolleau Gris', as in the two previous cases ('Aspiran' and 'Terret'), the homozygous status was restricted to the L2 cell layer, this being the main difference when compared with its wild-type. This deletion extended, at least, from the VvMYBA2 marker region to VVNTM6 SSR marker. Due to the homozygosity of the black-skinned ancestor cv. 'Grolleau' upstream of the VvMYBA2 marker it was not possible to have an accurate resolution of the deletion on the 5' border. The same deletion pattern observed for the cv. 'Grolleau Gris' was found in the cv. 'Grolleau Blanc', but in this color variant it was also present in L1 + L2-derived tissues. Besides that, the homozygosity of the black-skinned ancestor for the cv. 'Grolleau' and cv. 'Terret' in VVNTM4 SSR marker also prevents a more precise delimitation of the deletion on the 3' border (Table 4.3).

4.3.4 Genetics behind white-to-red skin color reversion

The molecular characterization of group B (pigmented variants derived from an unpigmented wild-type) followed the same layer-specific approach described above for group A. No genetic differences between layers were found within each family of cultivars, comparing the wild-type ancestral and its corresponding variants based on the markers used in this study (Table 4.4).

Table 4.3 – Genetic profile of berry color locus and its surrounding genomic region on black-to-grey and/ or white revertant somatic variants and its ancestors. The grey background indicates the deleted region. he – heterozygous; ho – homozygous; *Gret1* – non-functional allele; Non-*Gret1* – functional allele.

			Molecular marker and genomic coordinate on chromosome 2 (in Mb)											
Cultivar	**Layer**	**Berry skin color[1]**	**SC8_010**	**SC8_026**	**VVNTM1**	**VVNTM2**	**VvMYBA2R44**	**VvMYBA1**	**VVNTM3**	**VVNTM5**	**VVNTM6**	**VVNTM4**	**VVIU20**	**VMC7G3**
			12674	**12970**	**14149**	**14151**	**14181**	**14248**	**14288**	**14325**	**14330**	**14384**	**16539**	**18270**
Aramon	L1+L2	B	ho	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	he	he	he
	L2		ho	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	he	he	he
Aramon Gris	L1+L2	G	ho	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	he	he	he
	L2		ho	he	he	ho	T	*Gret1*	ho	he	he	he	he	he
Aramon Blanc	L1+L2	W	he	he	he	ho	T	*Gret1*	ho	ho	ho	he	he	he
	L2		he	he	he	ho	T	*Gret1*	ho	ho	ho	he	he	he
Aspiran Noir	L1+L2	B	ho	he	he	ho	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
	L2		ho	he	he	ho	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
Aspiran Gris	L1+L2	G	ho	he	he	ho	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
	L2		ho	he	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
Aspiran Blanc	L1+L2	W	ho	he	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
	L2		ho	he	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
Grolleau	L1+L2	B	ho	ho	ho	ho	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
	L2		ho	ho	ho	ho	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
Grolleau Gris	L1+L2	G	ho	ho	ho	ho	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
	L2		ho	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
Grolleau Blanc	L1+L2	W	ho	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
	L2		ho	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
Pinot Noir	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
Pinot Gris	L1+L2	G	he	ho	he	he	T/G	*Gret1*/Non-*Gret1*	he	he	ho	ho	he	he
	L2		he	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	ho	ho
Pinot Blanc	L1+L2	W	he	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
	L2		he	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he

Table 4.3
(continued)

Cultivar	Layer	Berry skin color[1]	Molecular marker and genomic coordinate on chromosome 2 (in Mb)											
			SC8_010	**SC8_026**	**VVNTM1**	**VVNTM2**	**VvMYBA2R44**	**VvMYBA1**	**VVNTM3**	**VVNTM5**	**VVNTM6**	**VVNTM4**	**VVIU20**	**VMC7G3**
			12674	**12970**	**14149**	**14151**	**14181**	**14248**	**14288**	**14325**	**14330**	**14384**	**16539**	**18270**
Pique-poul Noir	L1+L2	B	he	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
	L2		he	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
Pique-poul Gris	L1+L2	G	he	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
	L2		he	he	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
Pique-poul Blanc	L1+L2	W	ho	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
	L2		ho	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
Terret Noir	L1+L2	B	he	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
	L2		he	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
Terret Gris	L1+L2	G	he	he	he	he	T/G	*Gret1*/Non-*Gret1*	ho	he	he	ho	he	he
	L2		he	he	ho	ho	T	*Gret1*	ho	ho	he	ho	he	he
Terret Blanc	L1+L2	W	he	he	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he
	L2		he	he	ho	ho	T	*Gret1*	ho	ho	ho	ho	he	he

[1] B – Black, G – Grey, W – White

Table 4.4 – Genetic profile of berry color locus and its surrounding genomic region on white-to-red revertant somatic variants and its ancestors. The pink background indicates the putatively homozygous regions. he – heterozygous; ho – homozygous; *Gret1* – non-functional allele; ? – undetected functional allele; Non-*Gret1* – functional allele.

			Molecular marker and genomic coordinate on chromosome 2 (in Mb)											
Cultivar	**Layer**	**Berry skin color[1]**	**SC8_010**	**SC8_026**	**VVNTM1**	**VVNTM2**	**VvMYBA2R44**	**VvMYBA1**	**VVNTM3**	**VVNTM5**	**VVNTM6**	**VVNTM4**	**VVIU20**	**VMC7G3**
			12674	**12970**	**14149**	**14151**	**14181**	**14248**	**14288**	**14325**	**14330**	**14384**	**16539**	**18270**
Folgasão Roxo	L1+L2	R	he	he	ho	ho	T/G	*Gret1*/?	ho	ho	ho	ho	ho	ho
	L2		he	he	ho	ho	T/G	*Gret1*/?	ho	ho	ho	ho	ho	ho
Folgasão	L1+L2	W	he	he	ho	ho	T	*Gret1*	ho	ho	ho	ho	ho	ho
	L2		he	he	ho	ho	T	*Gret1*	ho	ho	ho	ho	ho	ho
Malvasia Cândida Roxo	L1+L2	R	he	he	ho	ho	T/G	*Gret1*/?	ho	he	ho	ho	ho	ho
	L2		he	he	ho	ho	T/G	*Gret1*/?	ho	he	ho	ho	ho	ho
Malvasia Cândida	L1+L2	W	he	he	ho	ho	T	*Gret1*	ho	he	ho	ho	ho	ho
	L2		he	he	ho	ho	T	*Gret1*	ho	he	ho	ho	ho	ho
Malvasia Fina Roxo	L1+L2	R	ho	ho	ho	ho	T/G	*Gret1*/?	ho	ho	ho	ho	ho	ho
	L2		ho	ho	ho	ho	T/G	*Gret1*/?	ho	ho	ho	ho	ho	ho
Malvasia Fina	L1+L2	W	ho	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	ho	ho
	L2		ho	ho	ho	ho	T	*Gret1*	ho	ho	ho	ho	ho	ho
Mourisco Tinto	L1+L2	B	he	he	ho	ho	T/G	Non-*Gret1*	he	he	ho	ho	he	he
	L2		he	he	ho	ho	T/G	Non-*Gret1*	ho	he	ho	ho	he	he
Mourisco Roxo	L1+L2	R	he	he	ho	ho	T/G	Non-*Gret1*	he	he	ho	ho	he	he
	L2		he	he	ho	ho	T/G	Non-*Gret1*	ho	he	ho	ho	he	he

[1] B – Black; R – Red; W – White

Although showing the same genetic pattern in L1+ L2- and L2- derived tissues, the red and black-skinned 'Mourisco Roxo and 'Mourisco Tinto' cultivars were both homozygous for a putative functional allele, described as a double insertion of 111- and 44-bp within the promoter region of *MYBA1*, which should correspond to *VvmybA1*^{SUB} allele. The remaining variants of group B were putatively homozygous for the *Gret1* insertion (*VvmybA1*^{ITA} allele) in the entire shoot apical meristem (Table 4.4).

Regarding the results obtained for *MYBA2*, all cultivars showed to be homozygous for the non-functional T allele, except cv. 'Folgasão Roxo', cv. 'Malvasia Cândida Roxo' and cv. 'Malvasia Fina Roxo', which carried the functional G allele (Table 4.4).

With the exception of 'Mourisco' family, in the remaining families of group B, the markers used were homozygous and monomorphic along an extensive genomic region on the distal arm of chromosome 2. While in 'Malvasia Fina' family this putatively homozygous and monomorphic region is present along the entire distal arm of chromosome 2, in 'Folgasão' and 'Malvasia Cândida' families, this homozygosity is of a smaller length, restricted to the region between VVNTM1 and VMC4f3 and between VVNTM1 and VVNTM3 SSR markers, respectively (Table 4.4).

4.4 DISCUSSION

4.4.1 True-to-type confirmation

One of the most challenging and unresolved problems for viticulture and germplasm management worldwide concerns the fact that grapevine is a species with alternative nomenclatures, with several cases of synonymies and homonymies. For this reason, it is particularly relevant to confirm that somatic variants correspond to true-to-type of the wild-type ancestor variety at the molecular level. Based on the results obtained by SSR genotyping, possible homonymy cases, as previously observed by Ferreira et al. [22] for cultivars of the families 'Alvarelhão', 'Bastardo', 'Carrega' and 'Touriga' were discarded.

4.4.2 Berry skin color reversions associated to somatic variation

The genetic analysis of group A varieties plainly showed that the deletion length encompassing the non-*Gret1* allele (*VvmybA1*^{AFL}) of *MYBA1* is highly variable among different varieties and also within the same variety, resulting in different color

phenotypes. Thus, this mutational event on the distal arm of chromosome 2 is likely to happen in a repeated and independent way. All white-skinned variants of group A can be differentiated from the respective wild-type counterpart. In the white-skinned variants, both layers are affected by the deletion and, consequently, the hemizygous state is detectable in the entire shoot apical meristem. For the less-pigmented variants from group A, a layer-specific molecular approach is required to discriminate them from the pigmented ancestor, because the same hemizygous state described for the white-skinned variants was only detected in the L2 cell layer. Thus, all less pigmented variants showed a chimerical status, while in all unpigmented ones the deletion was conserved, involving the entire shoot apical meristem.

Although no differences in the deletion extension of grey- and white-skinned variants could be observed in ‘Aramon’, ‘Aspiran’, ‘Grolleau’ and ‘Terret’ families with the layer-specific molecular approach adopted, these data do not exclude the hypothesis that the white-skinned variant evolved from the grey-skinned one, or that these variants evolved independently from their pigmented wild-type ancestor. Thus, it could be helpful to use an integrative analysis considering the available historical information. Migliaro et al. [13] concluded that the cv. ‘Tempranillo Gris’ probably evolved from the cv. ‘Tempranillo’ due to an independent mutational event, considering the historical documentation about the direct selection of the cv. ‘Tempranillo Blanco’ from the cv. ‘Tempranillo’. The authors inferred that the loss of the functional allele associated with the berry skin color was followed by L1 colonization and displacement to the L2 cell layer by the mutant cell, avoiding the grey berry chimerical status. Therefore, the same process could have occurred for ‘Aramon’, ‘Aspiran’, ‘Grolleau’ and ‘Terret’ families where no differences in the deletion extension of grey- and white-skinned mutants could be detected. However, the sequential evolutionary model, described by Walker et al. [15] for the cv. ‘Cabernet Sauvignon’ cannot be excluded since the grey-skinned berries could represent a periclinal chimera of the black-skinned wild-type ancestor. In turn, the white-skinned berries could be the bud sport of the grey-skinned through a process that includes a deletion in the berry color locus in the L2 cells, while the white berry phenotype results from a cellular rearrangement (displacement) in the grey-skinned berries where the L2 cell layer (unpigmented) replaces the L1 cells (pigmented). Furthermore, although considered a rare event due to the stability of the anticlinal cell divisions the opposite phenomenon, L1 cell invasion of the inner layer (a process called replacement), can also be considered [9].

The main mechanism found for color gain in cultivars that belong to group B is the partial excision of *Gret1*, called *VvmybA1*^{RUO} allele, which leaves the last part of the retrotransposon (solo-3'LTR region) at *MYBA1* promotor as described by Migliaro et al. [13]. However, a similar mechanism was only observed in the 'Mourisco' family. Considering the results obtained for the 'Mourisco' family, both cultivars (cv. 'Mourisco Roxo' and cv. 'Mourisco Tinto') should have derived from an unpigmented wild-type ancestor, homozygous for the *Gret1* insertion (*VvmybA1*^{ITA}). At least the L2 layer must have been affected by the subsequent partial excision of the *Gret1*, although it is not possible to verify if the mutation resulting in the partial excision of *Gret1* affected the L1 layer as well, since this layer-specific approach was not applied on tissues containing only L1 cells. Thus, the skin color differences observed among these two somatic variants are probably due to specific interferences on the anthocyanin biosynthetic pathway regulated by different regulatory transcription factors, since they both showed the same allelic composition for *MYBA1* and *MYBA2* genes.

The same wild-type genotype could be affected by different mutations resulting in color gain, as previously reported for two variants of the cv. 'Italia'. The recovery of *MYBA1* expression on the less-pigmented 'Ruby Okuyama' cultivar was caused by intra-LTR recombination within *Gret1* retrotransposon, while for the more intense pigmented 'Benitaka' cultivar, color gain was caused by homologous recombination between *MYBA1* and *MYBA3*, called *VvmybA1*^{BEN} allele, which was identified as a novel functional allele that restored *MybA1* transcripts [16].

Although the *Gret1* insertion at the promoter region of *MYBA1* has been considered as the main factor determining grape skin color variation and responsible for the recent and extremely rapid diffusion of the white phenotype regarding the grapevine domestication history, the *MYBA2* polymorphisms also seem to play a relevant role in the *MYB* diversification process [23], as observed for some less-pigmented variants of group B, namely cv, 'Folgasão Roxo', cv. 'Malvasia Cândida Roxo' and cv. 'Malvasia Fina Roxo'.

As previously mentioned, except for the 'Mourisco' family, in the remaining varieties of group B, the analyzed molecular markers were homozygous and monomorphic along an extensive genomic region of chromosome 2. These results are in accordance with the homozygosity found by Migliaro et al. [13]. Thus, all these findings agree with the history of grape evolution and domestication. Although domestication and breeding are usually associated with a decrease of grape diversity, its influence, on a

genome-wide scale, seems to be weak due to the different changes in morphology observed since grape domestication, including a wide range of berry colors. Myles et al. [23] identified a 5-Mb region on chromosome 2 encompassing the *MYB* transcription factor genes having observed a positive selection for white grapes around this locus, which is in agreement with the intense breeding for lighter berry color and the rapid dissemination of the *MYB* mutations responsible for reduced pigmentation previously referred to by Fournier-Level et al. [17].

In summary, this layer-specific approach was informative regarding the discrimination of skin color somatic variants with a pigmented ancestor, particularly the VVNTM1 and VVNTM5 SSR markers. Moreover, VVNTM3 and VVNTM4 SSR markers were the less informative markers since they were shown to be homozygous for almost all the somatic variants for berry skin color analyzed. Additionally, for somatic variants derived from an unpigmented ancestor this approach was shown to be not very informative regarding the discrimination of the variants, due to the extensive homozygosity observed either between variants or between layers of each variant.

4.4.3 Evolutionary mechanisms behind the origin of skin color somatic variants

Based on layer localization and the differences in the size of the deletion on the distal arm of chromosome 2, it is suggested that the less-pigmented and unpigmented somatic variants analyzed in this study can be ascribed from a pigmented ancestor (group A) in accordance with two theoretical models: 1) the Sequential model, which is the first evolutionary model proposed to explain the somatic color mutants development within a variety, also reported as ‘Cabernet Sauvignon-model’ by Walker et al. [11]; according to this model, the black-skinned wild-type ancestor gives rise to a less-pigmented variant which in turn gives rise to the unpigmented variant (Figure 4.1 A); and 2) the Parallel model, first proposed for ‘Pinot’ family by Vezzulli et al. [19], where the black-skinned berry ancestor gives rise to the less-pigmented (cv. ‘Pinot Gris’) and the unpigmented (cv. ‘Pinot Blanc’) variants independently (Figure 4.1 B). Based on the results obtained, three varieties seem to follow the Sequential model, namely; ‘Aspiran’, ‘Grolleau’ and ‘Terret’. The remaining families (‘Aramon’, ‘Pinot’ and ‘Pique-poul’) seem to be in agreement with the Parallel model.

Regarding the experimental data obtained for group B, where the wild-type ancestor is a white-skinned cultivar giving rise to a less-pigmented variant, a Revertant

model (Figure 4.1 C) is proposed. As mentioned above, the main mechanism described as being responsible for color gain involves the partial excision of *Gret1* retrotransposon from the *MYBA1* promoter. However, this mechanism can only be applied to the 'Mourisco' variety, in both pigmented variants. Our data suggests a novel mechanism for the genetic make-up of less-pigmented variants evolving from an unpigmented ancestor. For the cases of the less-pigmented variants (cv. 'Folgasão Roxo', cv. 'Malvasia Cândida Roxo' and cv. 'Malvasia Fina Roxo') color gain seems to result from the recovery of the functional G allele on VvMYBA2R44 position. This event might be playing a fundamental role, probably by restoring *MYBA2* transcripts (Figure 4.1 C).

4.5 CONCLUSIONS

These findings represent a significant breakthrough regarding the mechanisms behind the formation of somatic variants for berry skin color, namely unpigmented or pigmented-related grape cultivars with different color shades, since such molecular information has not been previously available for somatic variants derived from the pigmented ancestors 'Aramon', 'Aspiran', 'Grolleau', 'Pique-poul' and 'Terret', nor for the unpigmented ancestors, 'Folgasão', 'Malvasia Cândida', 'Malvasia Fina' and 'Mourisco'.

These data support the conclusion that, besides *MYBA1* and *MYBA2* playing an important role regarding the phenotypic variation observed among the berry skin color somatic variants due to black-to-grey and/ or white reversions, the molecular mechanism leading to skin color variation results from different deletion patterns encompassing the berry color locus. Moreover, the experimental data regarding the white-*to*-red reversion suggest that *MYBA2* gene might play a fundamental role for color gain through the recovery of functional G allele on VvMYBA2R44 position.

This approach was also shown to be particularly informative regarding the discrimination of somatic variants for berry skin color with a pigmented ancestor, therefore it could be used to solve problems of cultivar identification and newly appearing somatic variants for berry skin color.

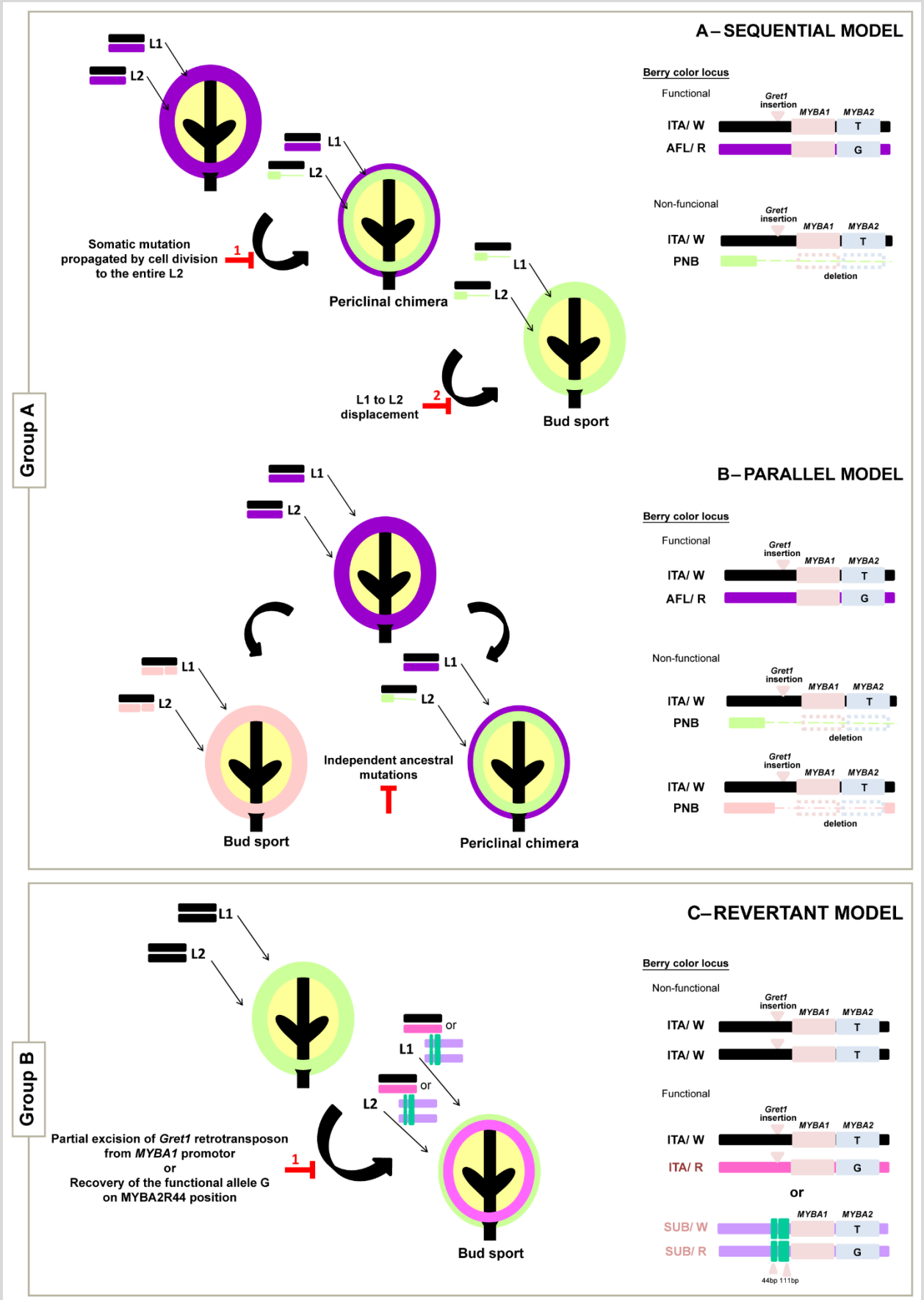

Figure 4.1 – Schematic representation of the evolutionary models describing the structural dynamics at the berry color locus in the L1 and L2 cell layers. Variants of the Group A can originate either through the Sequential model (A) or the Parallel model (B). The origin of variants from the Group B can be explained by the Revertant model (C), from one out of two different mechanisms, the partial excision of the Gret1 retrotransposon from the *MYBA1* promotor or the recovery of the G functional allele of *MYBA2*. On the right side are represented the structures of the different alleles and genotypes found in the somatic variants for berry skin color studied.

Supporting information

Supplementary data (Supplementary File 2) to this article can be found online at http://dx.doi.org/10.3233/JBR-170289 and on the electronic version of this Thesis.

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Molecular characterization of berry color locus on the Portuguese cv. 'Fernão Pires' and cv. 'Verdelho' and their red-berried somatic variant cultivars

Vanessa Ferreira, Isaura Castro, David Carrasco, Olinda Pinto-Carnide, Rosa Arroyo-García. Molecular characterization of berry color locus on the Portuguese cv. 'Fernão Pires' and cv. 'Verdelho' and their red-berried variants. *Ciência e Técnica Vitivinícola*. 2018; 33 (2): 184 – 190
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Abstract

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 red-berried variants cv. 'Fernão Pires Rosado' and cv. 'Verdelho Roxo', respectively. The analysis of *Gret1* insertion within the *MYBA1* gene revealed no polymorphism responsible for white-*to*-red shift of the red-skinned variants cv. 'Fernão Pires Rosado' and cv. 'Verdelho Roxo'. Moreover, *MYBA2* 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 *MYBA1* and non-functional T allele on *MYBA2* in homozygosity for both cell layers of shoot apical meristem, suggesting the occurrence of other mutational events responsible for the color gain.

4.6 INTRODUCTION

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 cv. ‘Pinot Noir’ and cv. ‘Cabernet Sauvignon’ (Walker et al., 2006; Yakushiji et al., 2006) and more recently in others such as cv. ‘Alfrocheiro Preto’ (Zanol et al., 2011), cv. ‘Muscat of Alexandria’ (De Lorenzis et al., 2015) and cv. ‘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, *MYBA1* and *MYBA2*, that induce the transcription of *UFGT* 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).

MYBA1 gene silencing results from a *Gret1* retrotransposon insertion in its promotor region. The presence of *Gret1* retrotransposon in the promoter of the *MYBA1* gene was firstly described and associated with a loss-of-function in cv. 'Italia' and cv. 'Muscat of Alexandria' cultivars, being called *VvmybA1a* non-functional allele (Kobayashi et al., 2004). The most likely original sequence of *MYBA1*, before the *Gret1* retrotransposon insertion, corresponds to the allele *VvmybA1c*, described as wild-type allele, that lacks *Gret1* completely. Regarding *MYBA2*, a non-synonymous single nucleotide polymorphism in the *MYBA2* 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 *MYBA1* and *MYBA2* 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 *MYBA1* and *MYBA3* 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). 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 *MYBA1* 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 (cv. 'Malvasia Fina', cv. 'Moscatel Galego' and cv. 'Pinot') through the analysis of *MYBA1* 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 *MYBA2* 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 18th 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 cv. '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).

4.7 MATERIAL AND METHODS

4.7.1 Plant material

The molecular mechanisms of color variation in grapevine somatic variants for berry skin color was 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 4.5. 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 4.5).

Table 4.5 –List of plant material divided in two groups (A and B) based on the berry skin color of the ancestor cultivar.

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, Coleção Ampelográfica Nacional (Dois Portos, Portugal).

4.7.2 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 (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 mechanism behind color reversions. For that, two, genomic DNA samples were isolated from each cultivar, namely 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.

4.7.3 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, *MYBA1* and *MYBA2* gene polymorphisms were investigated to understand the effect of the berry color locus genotype on the color variation in 'Fernão Pires', 'Verdelho' and 'Pinot' (as reference) families. The detection of functional and non-functional alleles for *MYBA1* (*Gret1* insertion and other length polymorphisms) and gene polymorphism for *MYBA2* (R44 SNP) was performed according to Carrasco et al. (2015).

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 (*MYBA1* and *MYBA2* genes) 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 Madrid Science

Park (fpcm.es/en/servicios-cientificos) 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).

4.8 RESULTS AND DISCUSSION

4.8.1 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 variants cv. 'Fernão Pires Rosado', cv. 'Verdelho Roxo', cv. 'Pinot Gris' and cv. 'Pinot Blanc', in order to 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 between cv. 'Pinot Blanc', cv. 'Pinot Gris' and cv. 'Pinot Noir', confirming that the somatic variant cultivars and their relative ancestor cultivar shared the same genetic profile (Supplementary File 3). 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.

4.8.2 Layer-specific genotyping at the berry color locus

Based on the analysis of *MYBA1* gene for the detection of the non-functional allele (with *Gret1* insertion, called *VvmybA1a*) and putative functional alleles, both white-skinned Portuguese cultivars, cv. 'Fernão Pires' and cv. 'Verdelho', and the white-skinned reference cv. 'Pinot Blanc', contained only the non-functional allele (*VvmybA1a*). The pigmented-skinned reference cultivars, cv. 'Pinot Gris' and cv. 'Pinot Noir' revealed both non-functional (*VvmybA1a*) and functional (*VvmybA1c*) alleles, while the color-skinned cv. 'Fernão Pires Rosado' and cv. 'Verdelho Roxo' showed only the non-functional allele (*VvmybA1a*) (Table 4.6).

To further understand the genetic basis of cv. 'Fernão Pires Rosado' and cv. 'Verdelho Roxo' berry color, *MYBA2* SNP polymorphisms were also examined. Like what happened for *MYBA1* 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 *MYBA2* 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 cv. 'Fernão Pires Rosado', allowing to explain the color gain of this red-berried somatic variant cultivar derived from its white-skinned ancestor cv. 'Fernão Pires' (Table 4.6).

Considering both genes (*MYBA1* and *MYBA2*) Fournier-Level et al. (2010) define three different haplotypes, considering the presence or absence of the *Gret1* insertion in the promotor region of *MYBA1*, and the presence of a functional G allele or a mutated/ non-functional T allele in VvMYBA2R44 position: haplotype 'colored' (Hap C-N), 'altered color' (Hap C-Rs) or 'white' (Hap-B). The control colored variants, cv. 'Pinot Noir' and cv. 'Pinot Gris', both hold the *Gret1* insertion and a putative functional allele at *MYBA1* (*VvmybA1c*) and a functional G allele at VvMYBA2R44, corresponding to the haplotype Rs.

Table 4.6 – *MYBA1*and *MYBA2* allelic polymorphisms.

	Cultivar code	Berry color[1]	*MYBA1*		*MYBA2*
			VvmybA1a Gret1	*VvmybA1c No Gret1*	VvMYBA2R44
Group A	**PN**	B	x	x	T/G
	PG	G	x	x	T/G
	PB	W	x	-	T/T
Group 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

The profile of the cv. 'Fernão Pires Rosado' profile revealed a low frequency haplotype, recombined "white" Hap-Rec, holding the *Gret1* insertion and a functional G allele at VvMYBA2R44, resulting in an altered color (red), as previously described for cv. 'Malvasia Fina Roxo' by Ferreira et al. (2017). The white-skinned Portuguese cultivars, cv. 'Fernão Pires' and cv. 'Verdelho', as well as the white-skinned reference, cv. 'Pinot Blanc', were consistent with the haplotype Hap-B. Surprisingly, also the red-berried cv. 'Verdelho Roxo', revealed this 'white' haplotype B.

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 variant derived somatic variant cv. 'Pinot Blanc' (Table 4.7), 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, cv. 'Chasselas Blanc' and cv. '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 4.7). This homozygosity has been previously described for other skin color somatic variants derived from an unpigmented ancestor cutlivars, which was associated with a selective sweep of this genomic region (Migliaro et al., 2017).

The experimental data obtained suggest that *MYBA2* 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, *MYBA1* and *MYBA2* 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.

Table 4.7 – 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.

				Molecular marker and genomic coordinate on chromosome 2 (in Mb)													
	Cultivar code	**Layer**	**Berry color[1]**	**SC8_010**	**SC8_026**	**VVNTM1**	**VVNTM2**	**VvMYBA2**		**VvMYBA1**		**VVNTM3**	**VVNTM5**	**VVNTM6**	**VVNTM4**	**VVIU20**	**VMC7G3**
				12674	**12970**	**14149**	**14151**	**14181**		**14248**		**14288**	**14325**	**14330**	**14384**	**16539**	**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

Supporting information

Supplementary data (Supplementary File 3) to this article can be found on the electronic version of this Thesis.

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Genetic analysis of a white-*to*-red berry skin color reversion and its transcriptomic and metabolic consequences in grapevine (*Vitis vinifera* cv. 'Moscatel Galego')

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Abstract

Somatic mutations occurring within meristems of vegetative propagation material have had a major role in increasing the genetic diversity of the domesticated grapevine (*Vitis vinifera* L.). The most well studied somatic variation in this species is the one affecting fruit pigmentation, leading to a plethora of different berry skin color features. Color depletion and reversion are often observed in the field. However, there are no genome-wide studies to date that account for the consequences of this somatic variation. In this study we analyzed the origin of a white-*to*-red skin color reversion and its transcriptomic and metabolic consequences on the cv. 'Moscatel Galego', a member of the large family of Muscats. The red-skinned variant, characterized by a preferential accumulation of dihydroxylated anthocyanins, showed in heterozygosis a partially-excised *Gret1* retrotransposon from the promoter region of the *MYBA1* anthocyanin regulator, while *MYBA2* was still in homozygosis for a non-functional allele. Through metabolic and transcriptomic analyses, we show that within a near-isogenic background, the transcriptomic consequences of color reversion are largely associated to diminished light/UV-B responses probably as a consequence of the augment of metabolic sunscreens (i.e. anthocyanins). We also proposed that the reduced activity of the flavonoid trihydroxylated sub-branch and decreased anthocyanin methylation/acylation are the potential causes for the mild red-skinned coloration in cv. 'Moscatel Galego Roxo'. The observed positive relation between anthocyanins and stilbenes could be attributable to an increased influx of phenylpropanoid intermediaries due to the replenished activity of *MYBA1*.

4.9 INTRODUCTION

The grapevine is one of the oldest perennial domesticated fruit crops in the world and it has been widely cultivated and valued either for its fruit or wine. Cultivation of domesticated grape (*Vitis vinifera* subsp. *vinifera*) started 6,000-8,000 years ago from its wild ancestor *V. vinifera* subsp. *sylvestris* in the Near East [1]. The large number of grape varieties known nowadays is certainly the result of many different processes, including multiple domestication centers from local *Vitis sylvestris* vines [2], subsequent crosses, and to a lesser extension, the conventional breeding practiced during the last century.

Vegetative propagation has been widely used as a strategy within breeding programs for multiplication of plants with desired features, creating clones that are genetically identical to the original donor. However, somatic mutations, naturally occurring during plant growth, can accumulate over time and generate divergent genotypes and occasionally lead to morphological and agronomical differences. These new interesting phenotypes can stabilize in grapevine plants as periclinal chimeras or extend to all cell layers, giving rise to new cultivars, in a process referred as clonal variation [3]. Consequently, somatic mutations combined with vegetative propagation have had a major role in increasing the genetic diversity in grapevine accessions. The use of these mutants in genomic studies is continuously helping to assign functions and roles to specific genes [3–6].

There are many examples of spontaneous variant traits, including berry color or flavor, ripening date, size and compactness of bunches, canopy growth or yield [5]. Vine growers have been exploring them as a source of diversity for both wine and table grapes. Genetic alterations responsible for these emergent phenotypes result from single nucleotide variation (SNV), insertion-deletions (INDELs) and from chromosomal rearrangements due to complex genome structural variation (SV) [4,7]. The most well studied polymorphisms leading to somatic variations within grapevine varieties are those that affect berry skin pigmentation. Diversity in fruit color has led to a substantial classification of grape cultivars and wine classes in the market, a process that gained cultural significance and extends thousands of years into human history [8]. Grape skin color shows a great diversity of colors ranging from white or green to grey, pink, red and black. This color palette is determined by the differential accumulation of anthocyanins, a group of flavonoids, in epidermal and sub-epidermal cell layers of the berry skin.

The regulation of anthocyanin synthesis is directly related with the activity of several R2R3-MYB transcription factors [9], some of which are located in two well-described grape color loci. The recently identified 'vegetative color locus' [10] harbors *MYBA5/6* and *MYBA7* genes, while the 'berry color locus' comprises *MYBA1* and *MYBA2* genes [11], two essential genes that determine berry skin color variation (Fournier-Level et al., 2009). Both loci share the regulation of late biosynthetic and modification/ transport-related genes, such as UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) and anthocyanin 3-*O*-glucoside-6''-*O*-acyltransferase (3AT) [10–12]. However, they differ in regulating the expression of the flavonoid-3'5'-hydroxylase (F3'5'H) family, directly influencing the proportion of tri- and disubstituted anthocyanins [10], ultimately affecting color characteristics in terms of hues, values and saturations.

Mutations in *MYBA1* and *MYBA2* genes can cause a loss of transcription factor activity on anthocyanin biosynthetic genes, leading to a 'white' phenotype. The loss of berry skin pigmentation has been mostly associated with the insertion of *Gret1* retrotransposon in the 5' regulatory region of the *MYBA1* gene [13]. Additionally, two mutations in the coding sequence of *MYBA2* (a point mutation and a 2 bp CA deletion that alters its reading frame) can also contribute to the loss of berry skin pigmentation [11]. These altered gene structures are commonly designated as non-functional alleles (*mybA1a* and *mybA2w*, respectively), being frequently present in homozygosis in white-skinned cultivars [8,14].

Several types of mutations have been identified at the berry color locus being responsible for color reversions in grapevine. Occasionally, black-skinned cultivars that are heterozygous for the non-functional and functional alleles give rise to color somatic variants, characterized by red, grey or white-skinned berries depending on whether the mutations at the berry color locus occurred only in the L1 or both L1 and L2 cell layers [15–18]. A large deletion removing both functional *MYBA1* and *MYBA2* alleles has also been associated with color reversions from the black-skinned cultivars 'Cabernet Sauvignon' and 'Pinot Noir' to their white-skinned bud sports, cv. 'Shalistin' and cv. 'Pinot Blanc', respectively [18,19]. Moreover, in cv. 'Koshu', a weakly colored grape cultivar, a 33 bp insertion in the second intron of the *MYBA1* red allele affects mRNA stability [20]. More recently, Carbonell-Bejerano et al. [7] demonstrated that the loss of color in cv. 'Tempranillo Blanco', occurs in response to an unbalanced chromoanagenesis, a process in which large numbers of complex rearrangements occur in a single catastrophic event, as often observed in cancer cells.

On rare occasions, reversions from mutated-to-functional allelic versions may occur in white-skinned cultivars giving rise to red-skinned variants. The main mechanism described for color gain is the partial *Gret1* retrotransposon excision from the *MYBA1* promoter, leaving behind its solo-3'LTR region (*VvmybA1b* allele). This mechanism has been firstly described in cv. 'Ruby Okuyama' and cv. 'Flame Muscat' by Kobayashi et al. [13] but has been associated with several other red-skinned somatic variants derived from white-skinned cultivars [16]. In addition, the pink-skinned somatic variant cv. 'Benitaka' derived from the white-skinned cv 'Italia' was reported as a result of homologous recombination between the non-functional allele of *MYBA1* and the truncated *MYBA3* gene at their promoter region, resulting in the recovery of *MYBA1* genomic integrity (and therefore its transcription) on cv. 'Benitaka' [21].

Skin color reversion is a rather common event in grapevine and currently, several pigmented and unpigmented varieties have certified clones with different skin color shades. For cv. 'Muscat à Petits Grains Blancs' (synonym cv. 'Moscato Bianco', cv. 'Moscatel de Grano Menudo' or cv. 'Moscatel Galego Branco' as it is known in Portugal), pink and red berry color variants are commonly known (http:// plantgrape.plantnet-project.org/). In this study we analyzed the genetic origin of a white-*to*-red skin color reversion on color somatic variants of the cv. 'Moscatel Galego', belonging to the large family of Muscats. In addition, through metabolic and transcriptomic analyses we studied the possible consequences of pigment depletion and reversion.

4.10 MATERIALS AND METHODS

4.10.1 Plant material and geographical description of sampling sites

Young leaves, woody shoot and berries of cv. 'Moscatel Galego Branco' and cv. 'Moscatel Galego Roxo' were sampled from experimental vineyards planted in rows, located at Quinta de Santa Bárbara (near Pinhão) in Douro Region (41º 10' N, 7º 33' W, 130 m elevation). Only healthy plants were sampled, presenting no visible signs of fungal attack or other microbiological alteration. In general, soils from this region have a high content of fine sand and silt and a low content of clay in the fine earth. They are acid and poor in organic matter. The climatic regime is Mediterranean, with rains concentrated in winter and autumn, and a typical severe soil water deficit in summer. The average annual precipitation over 30 years is 650 mm. The maximum average precipitation generally

occurs in winter (December, 100 mm approximately) while the minimum is in summer (August, 10 mm approximately). The mean annual air temperature is 16 ºC and ranges from 8 ºC in January, to 25 ºC in July [22].

Fruit samples were collected around 10 a.m. in 2015 at two different developmental stages: 1) veraison, being characterized by the beginning of anthocyanin accumulation in cv. 'Moscatel Galego Roxo' (time point when 50 % of berries have changed their color; cv. 'Moscatel Galego Branco' was sampled at the same time); and 2) early ripening, corresponding to 2 weeks after veraison (WAV). On each sampling date, three independent biological replicates, each including 15-20 berries, were randomly picked from a single row of plants (both from the sunny and shady sides of the plants) and pooled. Berries were immediately frozen in liquid nitrogen and kept at -80 ºC.

4.10.2 Analysis of anthocyanin composition and content of berry skins

Anthocyanins were extracted from berry skins and liquid chromatography coupled to mass spectrometry (HPLC–MS) was used to perform the identity of standards as recommended by García-Beneytez et al. (2003). All analyses were carried out using an HP 1100 system (Hewlett-Packard) with a PDA UV-vis coupled to a mass spectrometer equipped with an ESI interface. MS parameters were: capillary voltage, 4000 V; fragmenter ramped from 90 to 120 V; drying gas temperature, 325 °C; and gas flow (N_2), 12 mL/min. The instrument was operated in positive ion mode scanning from *m/z* 50 to 2000 at a scan rate of 1.47 sec/cycle.

A liquid chromatograph composed by a 600 quaternary pump, a 717 automatic injector, a TC2 controller for a column oven, a 996 photodiode array detector and a Millennium 32 workstation (Waters) was used to perform the HPLC analysis of anthocyanins. The separation was carried out using a Waters Nova-Pak C18 steel cartridge (3.9 × 250 mm), filled with 5 μm particles, and furnished with a Waters Sentry Nova-Pack C18 guard cartridge (20 × 3.9 mm), both thermostated at 55 °C. The two mobile solvent phases consisted of water/acetonitrile (95:5) adjusted to pH 1.3 with trifluoracetic acid (solvent A), and water/acetonitrile (50:50) adjusted to pH 1.3 with trifluoracetic acid (solvent B), using a gradient elution program as follows: linear gradient from 15 % B to 35 % B in 20 min, from 35 % B to 50 % B in 10 min, 50 % B for 6 min, from 50 % B to 100 % B in 5 min, 100 % B for 5 min, 100 % B to 15 % B in 1 min. A flow rate of 0.8 mL/min was applied. Samples (20 μL) were analyzed in triplicate. Spectra detection was

registered every second between 250 and 600 nm, with a bandwidth of 1.2 nm. Samples, standard solutions, and mobile phases were filtered before analysis through a 0.45 μm pore size membrane.

4.10.3 Genomic and transcriptomic analyses of cv. 'Moscatel Galego' white- and red-skinned variants

4.10.3.1 Simple sequence repeat (SSR) analysis

Four genomic DNA samples were extracted for cv. 'Moscatel Galego Branco' and cv. 'Moscatel Galego Roxo' from 100 to 200 mg of young leaf and skin berry (L1+L2 derived tissues) and from 200 to 300 mg of woody shoot pith and roots from greenhouse canes (L2 derived tissues) as previously described by Ferreira et al. [23].

Genomic DNA samples extracted from young leaves were analyzed for 12 SSR loci (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79, VVMD28, VVMD32, VVIv67, VVIv37, VVIp31 and VMC4f3) in order to determine the genetic identity of both cv. 'Moscatel Galego' skin color variants. PCR conditions and further analysis were performed as described in Castro et al. [24]. Primer sequences are found in Supplementary File 4.

A set of 10 SSR markers (SC8_0146_010, SC8_0146_026, VVNTM1, VVNTM2, VVNTM3, VVNTM4, VVNMT5, VVNTM6, VVIU20, VMC7G3) flanking the berry color locus and distributed along the distal arm of chromosome 2 was used to investigate polymorphisms in this region, using genomic DNA samples, extracted from young leaf and woody shoot, taking advantage of a well-established layer-specific approach described by Vezzulli et al. [15]. For each marker, the genetic difference between the L1+L2 (leaf) and L2 (woody shoot pith) should correspond to the make-up of the L1 cell layer. Primer sequences are found in Supplementary File 4. PCR reactions were performed as recommended in Vezzulli et al. [15]. Capillary electrophoresis was carried out in an ABI PRISM^{R} 310 Genetic Analyzer sequencer (Life Technologies) and the fragments were sized with Peak Scanner V1.0 software (Applied Biosystems) using as internal ladder the GeneScan 500 LIZ size standard (Life Technologies).

4.10.3.2 *MYBA1* and *MYBA2* gene structure analysis

Polymorphisms in *MYBA1* and *MYBA2* genes were determined as a proxy for the determination of functional and non-functional alleles. Genomic DNAs of young leaf, skin berry, woody shoot pith and roots were used to perform the *MYBA1* locus characterization. Primer pairs used for PCR analyses of *MYBA1* promoter region were VvMYBA1(1) for *VvmybA1a* allele (*Gret1* insertion) detection, and VvMYBA1(2) for the detection of *VvmybA1b* allele. Primer sequences are found in Supplementary File 4. The PCR reaction mixture (25 µL final volume) contained 50 ng total DNA, 0.3 mM dNTPs, 1 mM MgSO4, 0.3 µmol of each primer and 1 U Platinum™ *Pfx* DNA Polymerase (Life Technologies). The touch-down PCR was performed in a Veriti 96 well thermal cycler (Applied Biosystems) with the following steps: 5 min at 94 ºC; one cycle at 94 ºC for 15 s, 60 ºC for 30 s, 68 ºC for 2 min and 30 s; annealing temperature (Ta) decreased of 1 ºC at each cycle up to 50 ºC which was the Ta of the following 25 cycles; 68 ºC for 5 min and a final step of at least 10 min at 4 ºC to stop the reaction. Presence of PCR products was assessed by electrophoresis with a 1.5 % agarose gel and quantified by comparison with NZYDNA Ladder III (NZYTech).

For *MYBA2* gene, the single nucleotide polymorphism (SNP) related to berry color VvMYBA2R44 (K980) was investigated by a SNaPshot assay. *MYBA2* amplification, SNP genotyping by ABI PRISM SNaPshot Multiplex kit (Life Technologies) and SNP detection on ABI PRISM 310 Genetic Analyzer Sequencer were performed as reported in Carrasco et al. [25].

4.10.3.3 RNA isolation

For gene expression studies (qPCR and RNA deep sequencing), total RNA was extracted from berry skins according to the procedures described by Reid et al. [26]. To selectively precipitate the RNA, 125 µL of 10 M LiCl was added and the samples were stored overnight at 4ºC. RNA was pelleted next day, washed with cold 70 % EtOH, air dried and dissolved in 40 µL DEPC-treated water. Total RNA was further purified using the E.Z.N.A.® MicroElute RNA Clean Up Kit following the standard protocol. RNA concentration and 260/280 nm ratios were determined before and after DNase I digestion with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

4.10.3.4 Transcriptome analyses

RNA-Seq analysis was performed on 12 samples corresponding to veraison (V) and early ripening (R, 2WAV) berries of cv. 'Moscatel Galego Roxo' (RV and RR) and cv. 'Moscatel Galego Branco' (WV and WR), with three biological replicates each. Pair-ended sequencing was performed on Illumina HiSeq2000 with a sequencing lane of 2 x 50 bp and a depth of 40 million of sequences approximately.

RNA-Seq data analysis was performed on the AIR platform (www.transcriptomics.cloud). More specifically, the quality of the raw reads was checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then a trimming step was performed to remove low quality bases and sequencing adaptors with the tool BBDuk (https://sourceforge.net/projects/bbmap/). A minimum Phred-like score of 25 was set and a minimum length of the reads of 25 nucleotides. High quality reads were then mapped on the reference genome (IGPP 12Xv1) of *Vitis vinifera* cv. 'Pinot Noir' PN40024 with STAR (https://github.com/alexdobin/STAR) considering Pinot and Moscatel as different genotypes. Read summarization was then performed with featureCounts (http://bioinf.wehi.edu.au/featureCounts/) using only the reads with a mapping quality higher than 30. The statistical analysis was performed with R. Specifically, lowly expressed genes were removed with the package HTSFilter (http://www.bioconductor.org/packages/release/bioc/html/HTSFilter.html), selecting "TMM" as the normalization method. Then, the filtered genes were used to perform a differential analysis with DESeq2. Genes were considered statistically differentially expressed if the corrected p-value (false discovery rate, FDR) was lower than 0.05 (Supplementary File 5). Gene Ontology Enrichment Analysis (GOEA) was performed with in house scripts based on a hypergeometric test on the proportion of GO categories between the differentially expressed genes (DEGs) and the whole genome, GO categories were considered enriched if the FDR of the test was less than 0.05.

The original sequence data were submitted to the Sequence Read Archive (SRA) database of the NCBI under the accession SRP156198 (BioProject ID PRJNA484195). Expression heatmaps were constructed for different list of genes of interest (corresponding to different branches of the phenylpropanoid pathway) using the web-based tool Heatmapper (http://www3.heatmapper.ca/expression/), transforming FPKM (fragments per kilobase of transcript per million mapped fragments) values using a Z-score scaling whenever needed.

4.10.3.5 Quantitative reverse transcription PCR (qRT-PCR) expression analysis

Total RNA (200 ng) was reverse transcribed with the qScript ™ cDNA SuperMix (Quanta Biosciences) according to the manufacturer's guideline. The differential transcript level of *MYBA1/2*, *UFGT*, *F3'5'H1a* and *F3'H1a* was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using a 7300 Real Time PCR System (Applied Biosystems) (Supplementary File 4). Each reaction (20 µL) contained 200 nM of each primer, 3 µL of cDNA (1:30 dilution of the synthesis reaction), 1x SYBR Green Real-Time PCR Master Mix (Thermo Fisher) and water up to 20 µL. Thermal Cycling conditions were 95ºC for 10 min, followed by 95ºC for 15 s, annealing (variable temperatures) for 30 s and 72ºC for 30 s for 40 cycles. A melting cycle with temperature ranging from 60 to 95ºC was included in order to detect non-specific amplification in cDNA samples. Each one of the three biological replicates used to extract RNA and synthetize cDNA were used for qRT-PCR reaction in duplicate. Gene transcripts were quantified upon normalization to Elongation factor 1-alpha (*EF1α*, VIT_06s0004g03220) by comparing the threshold cycle (Ct) of each target gene with geometric mean of *EF1α* Ct. The relative quantification per each gene was calculated by the $2^{-\Delta Ct}$ method, where ΔCt is the difference in threshold cycle between the geometric means of the target gene and the reference gene.

4.11 RESULTS AND DISCUSSION

4.11.1 Berry color phenotypes of cv. 'Moscatel Galego' variants

Skin color reversion is a rather common event in grapevine and currently, several pigmented and unpigmented cultivars have certified clones with different skin color shades. For cv. 'Muscat à Petits Grains Blancs' (synonym cv. 'Moscato Bianco', cv. 'Moscatel de Grano Menudo' or cv. 'Moscatel Galego Branco' as it is known in Portugal), pink and red berry color variants are commonly known (http:// plantgrape.plantnet-project.org/). The 'Muscat à Petits Grains Blancs' cultivar is considered one of the main progenitors of the large family of Muscats that is extensively spread all over the world and appreciated since ancient times, mainly due to its highly terpenic flavor [27]. Historically, the appearance of the ampelographic reference to cv. 'Moscato Rosso' perfectly resembling the already known cv. 'Moscato Bianco' suggested that the red-skinned variant derived from the white-skinned cultivar, probably as the result of a

selection episode in a cv. 'Moscato Bianco' vine. Although different color variants with red shades are known, a previous study analyzing three accessions of cv. 'Moscatel Galego' with different color shades (white, red and black) revealed that only the white and red-skinned accessions have the same SSR profile, suggesting that the black-skinned accession was a different variety [28].

The different color phenotypes of 'Moscatel Galego' cultivars used in this study are shown in Figure 4.2, in which cv. 'Moscatel Galego Branco' is a typical white-skinned cultivar, whereas its color-reverted variant cv. 'Moscatel Galego Roxo' shows a red blush coloration. In a previous study we measured different colorimetric parameters (*a**, *b**, *L**, hue angle and chromaticity) during berry development, in order to investigate the differences between the two skin color phenotypes of cv. 'Moscatel Galego' [29]. Here, we observed an inverse correlation between *a** and *b** values with anthocyanin accumulation (and therefore ripening) in cv. 'Moscatel Galego Roxo'; the strong correlation with *a** agreeing with a redder (and less blueish) phenotype.

4.11.2 Dihydroxylated anthocyanin derivatives are the most abundant in cv. 'Moscatel Galego Roxo'

We performed a temporal profiling of anthocyanin derivative compounds in both Muscat variants at two developmental stages: veraison and early ripening (2 WAV). As expected, anthocyanins were only detected in cv. 'Moscatel Galego Roxo'. Only four anthocyanins were identified (all being monoglucoside derivatives): delphinidin, cyanidin, peonidin and malvidin. At veraison and early ripening stages, cyanidin-3-*O*-glucoside accounted for 83.46 % and 88.80 % of the total amount of anthocyanins, respectively (Figure 4.2). The next most abundant anthocyanin was peonidin-3-*O*-glucoside, accounting for 12.24 % at veraison and 8.57 % at ripening. The trihydroxylated anthocyanin derivatives represented the less abundant anthocyanins, where delphinidin-3-*O*-glucoside showed 4.2 and 1.7 % at veraison and ripening stage (1.7 %), respectively, and malvidin-3-*O*-glucoside was only detected at the ripening stage (0.86 %) (Figure 4.2). These results are in agreement with Ferreira et al. [28], where in fully mature berries (around 6-8 WAV) the dihydroxylated cyanidin-3-*O*-glucoside was the most abundant. Also, at this period, a fifth minor anthocyanin corresponding to petunidin-3-*O*-glucoside was found suggesting its accumulation at ripening stages later than 2WAV. Altogether, the results obtained for the red variant of cv. 'Moscatel Galego' are in contrast to the fact that

trihydroxylated anthocyanins are generally found more abundant in black-skinned cultivars, clarifying the reason behind its red coloration.

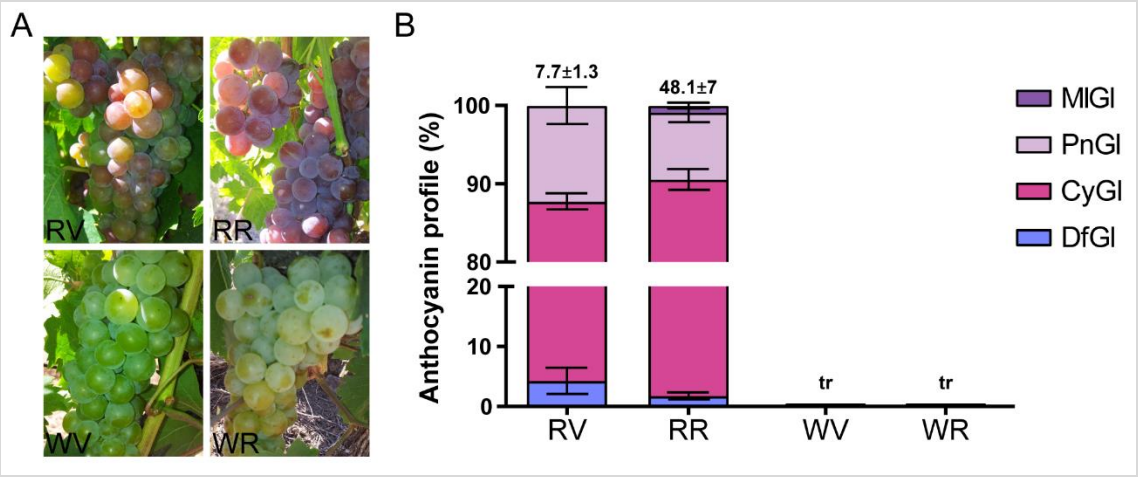

Figure 4.2 – Color reversion in cv. 'Moscatel Galego Roxo' is mainly attributable to the recovery of dihydroxylated anthocyanin accumulation. A) Representative plant phenotypes in the field at veraison and early ripening of cv. 'Moscatel Galego Roxo' and cv. 'Moscatel Galego Branco'. B) Anthocyanin quantifications. RV and RR: red-skinned variant at veraison and early ripening, respectively. WV and WR: white-skinned variant at veraison and early ripening, respectively.

4.11.3 The color reversion in cv. 'Moscatel Galego Roxo' is due to Gret1 retrotransposon partial excision from MYBA1 promoter

Twelve SSR markers, including the six SSR markers adopted by the *Organisation Internationale de la Vigne et du Vin* [30] for varietal identification, were used to genotype the white-skinned cv. 'Moscatel Galego Branco' and its color reverted variant cv. 'Moscatel Galego Roxo' in order to ascertain the genetic identity between them. In fact, this fingerprinting system showed an exact match between cv. 'Moscatel Galego Branco' and cv. 'Moscatel Galego Roxo' allelic profiles for all the 12 SSR loci analyzed, confirming that both cultivars are very closely related and probably they were originated recently from each other (Table 4.8). This genetic identity was further confirmed by comparison with the fingerprinting reported in previous works [24,28].

To further investigate the genetic structure of the berry color locus and its surrounding genomic region, twelve molecular markers were screened, 10 SSRs spread throughout this region of chromosome 2 and two R2R3-MYB genes, *MYBA1* and *MYBA2*, which were analyzed regarding their functional and non-functional allelic configuration (Table 4.8). This investigation was performed by using a well-established layer-specific approach, which has already been proven to be a successful method to

decipher the molecular mechanisms responsible for color reversions on other grape somatic variants [15,16,23].

Different assays were performed to characterize the *MYBA1* locus; the first one [VvMYBA1(1)] aimed to investigate the insertion of the *Gret1* retrotransposon at the gene promoter region (*VvmybA1a* allele), which was detected in both white and red-skinned cultivars of 'Moscatel Galego' in both L1 + L2- and L2-derived tissues.

The second assay [VvMYBA1(2)] was carried out to specifically detect the *Gret1* partial excision [solo-3' long terminal repeat (LTR) allele, also known as *VvmybA1b* allele], which was detected for cv. 'Moscatel Galego Roxo', also in both L1 + L2- and L2-derived tissues.

Our analysis (Figure 4.3) shows that cv. 'Moscatel Galego Branco' is putatively homozygous for the presence of the *Gret1* allele (non-functional) in both L1 and L2 cell layers, whereas cv. 'Moscatel Galego Roxo' is heterozygous for the presence of the *Gret1* and the solo-3' LTR alleles in both the L1 + L2 (skin and leaves) and L2 (wood and roots) layer-derived tissues, as it has been described for cv. 'Moscato Bianco' and cv. 'Moscato Rosso' by Migliaro et al. [16] and also for other red-skinned cultivars derived from a white-skinned ancestor, such as cv. 'Chasselas Rouge', cv. 'Italia Rubi', cv. 'Malvasia Rosa' and cv. 'Sultanina Rosa'. Consequently, it can be hypothesized that the partial excision of the *Gret1* retrotransposon, leaving the solo-3' LTR region, must have affected at least the L2 cells of the homozygous ancestor cv. 'Moscatel Galego Branco' for the presence of the *Gret1* allele, giving rise to the red-skinned somatic variant. This hypothesis agrees with the historical background of cv. 'Moscatel Galego' and has also been described as the main mechanism for color recovery on white-skinned cultivars.

Similarly to what has been previously observed in other studies for white-skinned ancestor cultivars [16,23], an extensive putatively homozygous and monomorphic region was found along the distal arm of chromosome 2, including the presence of the non-functional T allele of *MYBA2* in homozygosis both in cv. 'Moscatel Galego Branco' and cv. 'Moscatel Galego Roxo' (Table 4.8). Altogether we suggest that the non-black, but red skin coloration was recovered from the white phenotype by a partial *MYB* activation (i.e. excluding *MYBA2* gain of function) occurring probably and exclusively in the L2 cell layer.

Table 4.8 – Genetic profiles of cv. 'Moscatel Galego Branco' and its red-skinned revertant variant cv. 'Moscatel Galego Roxo' based on a set of microsatellite markers used for true-to-type confirmation (12 SSR loci) and for characterization of the berry color locus and its surrounding genomic region (10 SSR loci). The pink background indicates the putatively homozygous regions. ho – homozygous; *Gret1* – non-functional allele; Solo3'LTR – functional allele.

		LG[1]	**11**	**16**	**7**	**5**	**7**	**5**	**12**	**3**	**4**	**19**	**10**	**15**
			VVS2	**VVMD5**	**VVMD7**	**VVMD27**	**VrZAG62**	**vrZAG79**	**VMC4f3**	**VVMD28**	**VVMD32**	**VVIp31**	**VVIv37**	**VVIv67**
Cultivar	**Layer**	**Berry skin Color[2]**												
Moscatel Galego Roxo	L1+L2	R	130-130	224-232	231-247	175-191	185-195	249-253	165-204	243-265	261-269	182-186	159-161	360-371
Moscatel Galego Branco	L1+L2	W	130-130	224-232	231-247	175-191	185-195	249-253	165-204	243-265	261-269	182-186	159-161	360-371
		LG[1]						**2**						
			SC8_010 12674	**SC8_026 12970**	**VVNTM1 14149**	**VVNTM2 14151**	**VvMYBA2R44 14181**	**VvMYBA1 14248**	**VVNTM3 14288**	**VVNTM5 14325**	**VVNTM6 14330**	**VVNTM4 14384**	**VVIU20 16539**	**VMC7G3 18270**
Cultivar	**Layer**	**Berry skin Color[2]**												
Moscatel Galego Roxo	L1+L2	R	ho	ho	ho	ho	T/T	*Gret1*/Solo 3'LTR	ho	ho	ho	ho	ho	ho
	L2		ho	ho	ho	ho	T/T	*Gret1*/Solo 3'LTR	ho	ho	ho	ho	ho	ho
Moscatel Galego Branco	L1+L2	W	ho	ho	ho	ho	T/T	*Gret1*	ho	ho	ho	ho	ho	ho
	L2		ho	ho	ho	ho	T/T	*Gret1*	ho	ho	ho	ho	ho	ho

[1] LG – Linkage group; [2] R – Red; W - White

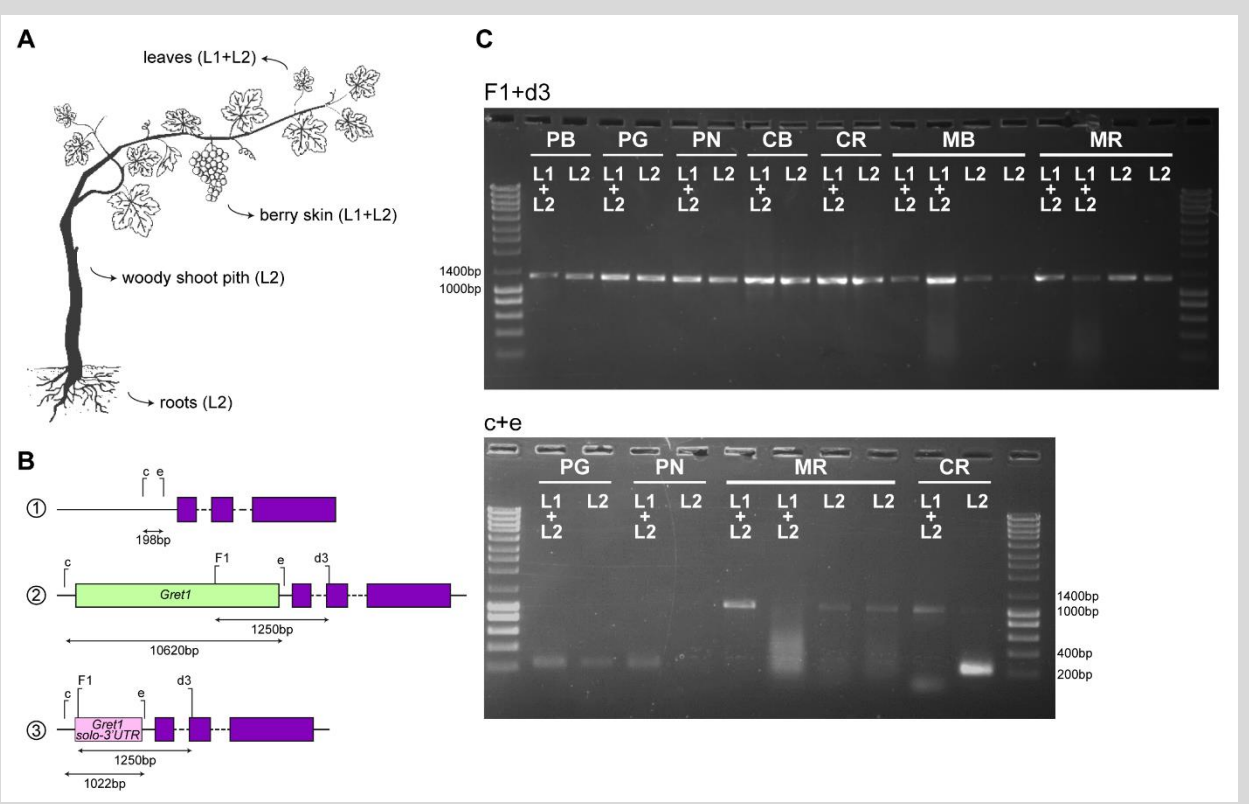

Figure 4.3 – Molecular marker analysis reveals the genetic nature of color reversion in cv. 'Moscatel Galego' variants. A) Cartoon depicting the different plant tissues and respective cell layers used for molecular analyses; B) Schematic representation of *MYBA1* alleles (1- *VvmybA1c*; 2 – *VvmybA1a*; 3 – *VvmybA1b*) and genomic location of the primer combinations used in the PCR assays [F1+d3 – VvMYBA1(1) and c+e – VvMYBA1(2)]; C) PCR assays performed on cv. 'Pinot Blanc' (PB), cv. 'Pinot Gris' (PG), cv. 'Pinot Noir' (PN), cv. 'Chasselas Blanc' (CB), cv. 'Chasselas Roxo' (CR), cv. Moscatel Galego Branco' (MB) and cv. 'Moscatel Galego Roxo' (MR) using VvMYBA1(1) (1250 bp – *VvmybA1a*) and VvMYBA1(2) (198 bp – *VvmybA1c* and 1022 bp – *VvmybA1b*) primer combinations.

4.11.4 Transcriptomic comparison of color variants reveals a specific modulation of light responsive genes and secondary metabolism

Since both cv. 'Moscatel Galego' variants represent almost near-isogenic lines one from the other, we decided to explore the transcriptomic differences caused by their color variation. mRNA libraries were constructed for the four previously tested samples: red and white, at veraison and ripening (RV, RR, WV and WR) and pair-ended sequenced by Illumina (three biological replicates per condition). After adaptor and low-quality base trimming, 952 357 192 clean reads (39.68 million reads in average per condition) remained. An average of 88.7 % of reads/condition mapped uniquely to the reference genome, while 4.5 % mapped to multiple loci and were discarded. Principal component analysis (PCA) showed that a majority of the variation in abundances of mRNAs between libraries is associated with developmental stage (PC1 of 69.2 %; Supplementary File 6),

while PC2 was inferred to capture predominantly somatic variant variation (24.5 %). The differential expression analysis, run through DESeq2, showed 2551 and 2785 genes to be up- and down-regulated (FDR <0.05) by color reversion at veraison, compared with 4275 and 4223, occurring at early ripening, respectively (Supplementary File 6). This indicates that the biggest differences between cv. 'Moscatel Galego Roxo' and cv. 'Moscatel Galego Branco', in terms of the number of DEGs, are found after the onset of ripening.

We analyzed the proportion of enriched gene ontology (GO) categories in the color reverting variant and found that different environmental, metabolic and stress responses were enriched (Supplementary File 7, 8 and 9). As expected, flavonoid metabolism was enriched in the up-regulated genes, as reflected by many different categories such as 'chalcone isomerase activity', 'phenylalanine ammonia-lyase activity' and 'phenylpropanoid biosynthetic process'. Interestingly, among up-regulated genes found both at veraison and ripening, there is an enrichment of 'anaerobic respiration' (GO:0009061), 'cutin biosynthetic process' (GO:0010143), 'trihydroxystilbene synthase activity' (GO:0050350) and 'response to heat' (GO:0009408) terms. Veraison-specific highly enriched biological processes included 'production of siRNA involved in RNA interference' (GO:0030422, FDR=0.008) and 'histone acetyltransferase activity' (GO:0004402, FDR=0.02), while at early ripening the terms 'regulation of auxin mediated signaling pathway' (GO:0010928, FDR=0), 'trehalose metabolism in response to stress' (GO:0070413, FDR= 0.01), 'xyloglucan biosynthetic process' (GO:0009969, FDR=0.02) and 'cell wall biogenesis' (GO:0042546, FDR=0.02) were enriched.

Enriched GO categories of down-regulated genes in response to color reversion showed three major processes occurring with higher rank in the white-skinned variant: photosynthesis, light responses and isoprenoid metabolism. Within the former, at least 20 related terms were enriched at both stages, harboring photosystem components, chloroplast structures and chlorophyll synthesis. Several light-signaling categories were enriched at both developmental stages including 'response to high light intensity' (GO:0009644) and 'response to low fluence blue light stimulus by blue low-fluence system' (GO:0010244). Metabolic responses to be down-regulated with color reversion were mainly subscribed to the metabolism of lipids (e.g. GO:0030148, GO:0006633, GO:0008610), steroids (GO:0006694 and GO:0006696), farnesyl-diphosphate (GO:0004310), squalene (GO:0051996), xanthophylls (GO:0016123) and carotenoids (GO:0016117). These results suggest that color reversion arrests photosynthesis and the accumulation of accessory pigments as a response to sunlight filtering, mainly exerted by

anthocyanins. In contrast to the 'heat response' term identified among genes induced by color reversion, we found the term 'response to cold' (GO:0009409) enriched in down-regulated genes at both developmental stages, suggesting that white and red skinned berries may have different daytime temperatures possibly due to the physico-chemical properties of pigments in sunlight reflection and absorption. Additionally, phosphate ion transport-related terms (e.g. GO:0035435 and GO:0006817) were also enriched among down-regulated genes at both developmental stages.

4.11.5 Inactivation of the flavonoid trihydroxylated sub-branch and decreased anthocyanin methylation/acylation as potential causes for the red-skinned coloration in cv. 'Moscatel Galego Roxo'

We further inspected the expression of early-phenylpropanoid and anthocyanin-related genes between both color variants to corroborate the metabolic data and gene ontology analysis. Phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL), being key enzymes catalyzing the first three steps of the phenylpropanoid pathway (PP), had many transcripts being up-regulated on cv. 'Moscatel Galego Roxo', particularly at the berry ripening stage (Figure 4.4). This higher expression levels suggest an increased influx of the PP pathway, ultimately affecting down-stream pathways.

From all the genes of the PP shown in Figure 4.4, the most affected in the color reverting condition were those related to anthocyanin synthesis (defined by purple dots). This increase on anthocyanin-related genes (e.g. *UFGT*-VIT_16s0039g02230, the last committed step for anthocyanin synthesis) in cv. 'Moscatel Galego Roxo' coincides with the accumulation of anthocyanins in the red-skinned variant. The transcript expression pattern of the major anthocyanin regulators *MYBA1* (VIT_02s0033g00410), *MYBA2* (VIT_02s0033g00390) and *MYB5B* (VIT_06s0004g00570) completely matches with the expression of their target genes (such as *UFGT* [30], *3AT* [12], *GST4* and *AOMT1*) on cv. 'Moscatel Galego Roxo'. *MYBA1* expression also agrees with our genetic data (merely no detection on white berries, <0.5 FPKM) corroborating with the allelic composition of the *MYBA1* gene in both variants. Despite *MYBA2* transcripts were highly detected in white-skinned berries (70-130 FPKM) although the highest levels were found in the red variant. Either way, these expression patterns should not be relevant once our genetic data showed that both color variants are homozygous for the non-functional T alleles. We

further validated the molecular marker data by inspecting all reads mapping at the *MYBA2* locus. Both the G-*to*-T single nucleotide polymorphism at position 131 of the CDS (that leads to a $R^{44}\rightarrow L^{44}$ amino acid substitution), and the CA dinucleotide deletion in Exon 3 (disrupting the C-terminal) were found in all reads belonging to veraison and ripening samples of both somatic variants. These two mutations are responsible, according to Walker et al. [18], for *MYBA2's* inactivity in the white allele.

Flavonoid 3′-hydroxylases (F3'H) and 3′5′-hydroxylases (F3'5'H) are the enzymes that catalyze the hydroxylation of the B-ring of flavonoids, producing the corresponding dihydroxylated and trihydroxylated derivatives, respectively (i.e. found in both flavonol and anthocyanin compounds). In grapevine, the variation in anthocyanin composition is strongly influenced by the expression of genes coding for flavonoid hydroxylases [31–33]. Usually F3'5'H activity prevails over F3'H, and the products of flavonoid hydroxylases are predominately channeled into the branch of the pathway involved in the biosynthesis of delphinidin (which is latter transformed into malvidin, all with blue-purplish coloration) at the expense of those involved in the synthesis of cyanidin (reddish derivatives). Jeong et al. [31] suggested that the levels of *F3'Hs* and *F3'5'H* expression agreed well with the ratios of cyanidin- and delphinidin-based anthocyanins, which is in accordance with Castellarin et al. [32,33], who found a strong relationship between the expression of *F3'H* and *F3'5'H* genes and the kinetics of accumulation of dihydroxylated and trihydroxylated anthocyanins in the dark blue-skinned cv. 'Merlot'. In the current study, transcripts coding for both *F3'H* and *F3'5'H* were relatively lowly abundant and expressed without differences between both color variants (Figure 4.4). In addition, a higher expression of a few *F3'H* transcripts was observed, but with no differences between red and white-skinned berries. These data suggest that flavonoids in both variants are majorly channeled into the dihydroxylated branch due to the activity of *F3'Ha* (VIT_17s0000g07200), *F3'H1* (VIT_09s0002g01090) and *F3'H5* (VIT_05s0094g01190). In the case of the red-skinned variant, these flavonoids are then transformed into anthocyanin compounds. The idea of a deficient activation of the trihydroxylated branch would correlate with the higher accumulation of cyanidin-3-*O*-glucoside (83.46 % at veraison and 88.80 % on ripe berries) in the downstream steps of this pathway in the red-skinned cv. 'Moscatel Galego Roxo' whereas trihydroxylated anthocyanins were only slightly accumulated (Figure 4.3).

Matus et al. [10] observed that after the overexpression of *MYBA1* in grapevine hairy roots, most of the *F3'5'H* genes were activated. On the contrary, the closely related *MYBA6* and *MYBA7* were not able to activate the family, in correlation with higher di/ trihydroxylated anthocyanin ratios. The differences between *MYBA1/A2* and *A6/A7* mostly relies on the presence of a C-terminal motif conferring high induction capacity of target genes, in addition to other residues with non-proven functionality yet. Despite the sequence of *MYBA1* in cv. 'Moscatel Galego Roxo' is identical to that of other red and black-skinned cultivars, our data suggests that the inability of activating the trihydroxylated branch could be due to a lower expression of *MYBA1*, together with the less number of cells expressing this gene (i.e. those possible located exclusively in the L2 cell layer) in comparison to black-skinned berries (expressing high amounts of *MYBA1* in both L1 and L2 cell layers). Other possibility could represent the epigenetic regulation of the F3'5'H family, an idea that trusts in the tandem localization of all these genes in the grapevine genome [34].

The methylation of phenolic compounds, as catalyzed by *O*-methyltransferases (OMTs), is an important step in flavonoid metabolism [32]. In grapevine, increasing evidence suggests that the expression of *OMT* genes is correlated with the accumulation of methylated anthocyanins [35,36], For instance, cyanidin and delphinidin are respectively converted to peonidin and malvidin by the action of 3'-*O*-methyltransferase. We determined that peonidin-3-*O*-glucoside was the second major anthocyanin in cv. 'Moscatel Galego Roxo' in correlation with the expression profile observed for *AOMT1* despite it was found in low levels. Additionally, the acylation step of anthocyanin biosynthesis is mediated by anthocyanin acyltransferases, which promotes color stability and intensity of anthocyanins [37]. In the present study acylated forms of anthocyanins were not detected, which agrees with the fact that the unique transcript detected *3AT* (VIT_03s0017g00870) is expressed at very low levels in cv. 'Moscatel Galego Roxo'.

Altogether, we hypothesize that the particular red coloration in cv. 'Moscatel Galego Roxo' berries results from the poor inactivation of the flavonoid trihydroxylated sub-branch and decreased anthocyanin-methylation/acylation activities due to low expressions of *3AT* and *AOMT* genes.

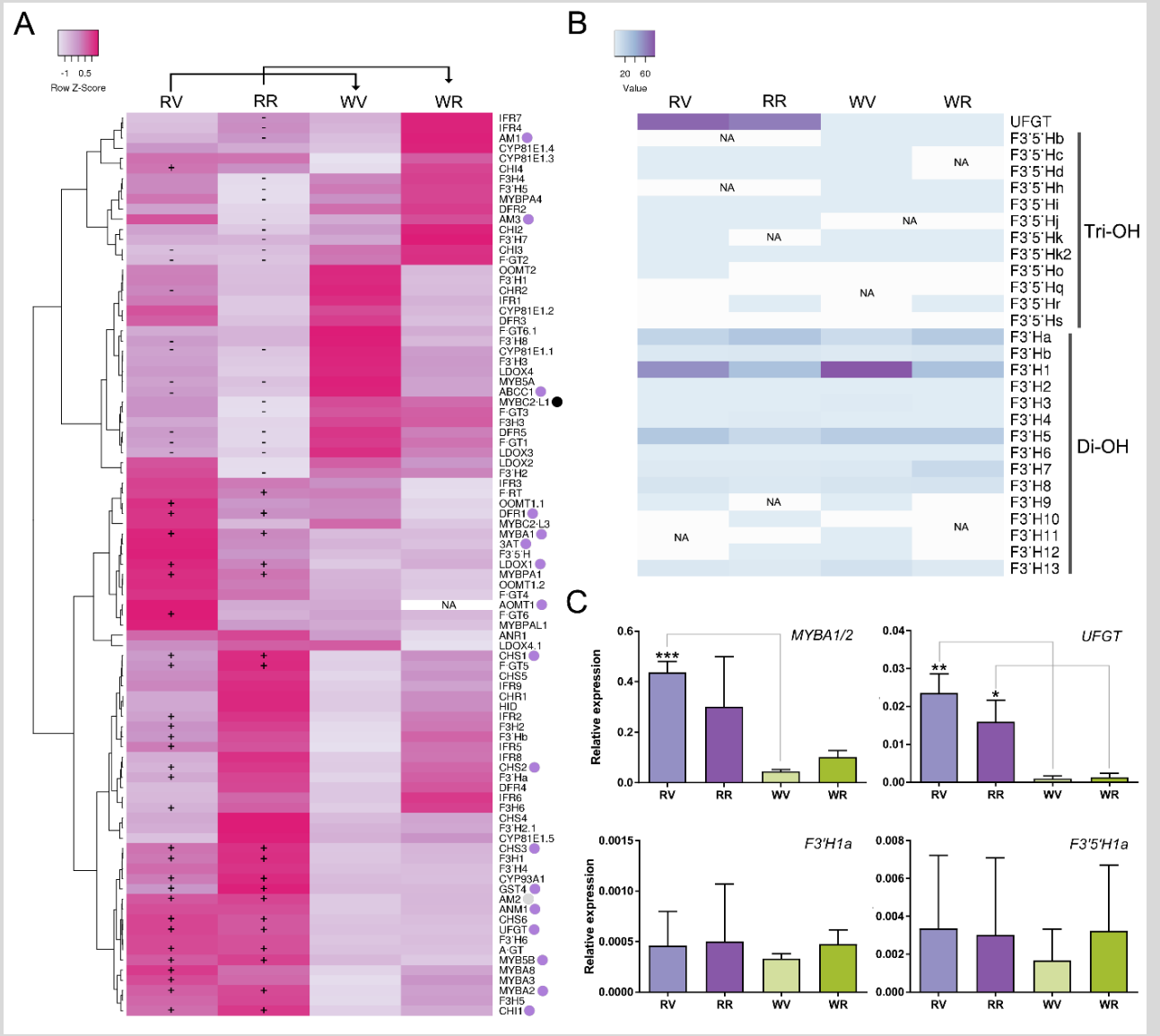

Figure 4.4 – Expression behavior of phenylpropanoid and anthocyanin related genes in color reverted somatic variants. A) Expression heatmap for early and late phenylpropanoid pathway genes. Genes with significant expression differences between the color reverting and the white-skinned cultivar (as reference) are shown with '+' and '-' symbols representing up and down-regulation, respectively (FDR <0.05). Purple dots next to gene names represent those with proven roles in anthocyanin synthesis. The anthocyanin-specific repressor MYBC2-L1 (subgroup 4) is highlighted with a black dot. Negative and positive Z-scored FPKM values represent low and highly expressed genes, respectively, while values around zero correspond to mild expression levels. Average linkage clustering and Pearson distance measurement methods were used for clusterization. NA= not assigned value due to very low expression. Gene IDs, together with their FPKM can be found in Supplementary File 10. B) FPKM values of flavonoid (3' and 3',5') hydroxylase family genes in cv. 'Moscatel Galego' color variants. *UFGT* expression was used as a reference. C) qRT-PCR expression analysis of selected genes: *MYBA1*, its target *UFGT* and the flavonoid hydroxylases, *F3'H-1a/F3'Hb* (VIT_17s0000g07210) and *F3'5'H-1a/F3'5'Hd* (VIT_06s0009g02840).

4.11.6 Stilbenes differentially accumulate in red-skinned somatic variants through the increased expression of their regulators

Stilbenes constitute an important group of phenylpropanoids that share the first steps of the general phenylpropanoid pathway with flavonoids and have been extensively studied due to their nutraceutical properties and also because of their plant protective roles against pests, pathogens and abiotic stresses [38]. Since we observed enriched stilbene-related ontology terms in the red skinned somatic variant, we further evaluated the expression of the complete stilbene pathway, including their characterized regulators: the R2R3-MYB transcription factors (TFs) *MYB14* and *MYB15* [39], as well as the recently reported candidate WRKY regulators *WRKY3*, *WRKY24* and *WRKY43* [38].

The cv. 'Moscatel Galego Roxo' showed an increased expression of stilbene related genes compared to its white variant. At the ripening stage it presented a significant up-regulation of many *STS* genes (22 transcripts among 37) that were not induced in the white variant at the same developmental stage (Figure 4.5). Despite being less expressed when compared to ripening, 14 *STS* transcripts were also up-regulated in the red-skinned variant at veraison. The transcript profiles are in accordance with the resveratrol and piceid contents, accumulating 5.3 times higher in the red variant at ripening (Figure 4.5). These results suggest, that despite the fact that the flavonoid and stilbene branches are competing with each other for *p*-coumaroyl-CoA and cinnamoyl-CoA substrates [40] [STS and chalcone synthases (CHS) belong in fact to overlapping homologous enzyme superfamilies; Interpro IPR012328], an increased flux into the phenylpropanoid pathway may have an effect in the availability of stilbene precursors which in turn may activate the transcription of *STS* genes by positive feedback.

Since several transcriptional regulators of the stilbene biosynthetic pathway have been identified in grapevine (all of them showing a strong co-expression with *STS* genes) we hypothesized that the activation of *STS* genes in cv. 'Moscatel Galego Roxo' could be due to an increase in the expression of their regulators. The R2R3-MYB gene *MYB14* (VIT_07s0005g03340) showed increased expression in the red-skinned variant at both developmental stages (Figure 4.5) although its expression was much higher at ripening, correlating with the significant higher red-*to*-white (R/W) expression ratio of its proven targets *STS29* (VIT_16s0100g01010) and *STS41* (VIT_16s0100g01130) [41] at this later stage. *MYB15* (VIT_05s0049g01020) had a significantly increased R/W expression ratio only at veraison, suggesting that this *MYB* may have a more initial role in *STS* activation.

Wong et al. [39] showed that *MYB13* , sharing a close similarity with *MYB14* and *MYB15* (all being part of Subgroup 2), was highly co-expressed with several *STS* transcripts. Despite our results in cv. 'Moscatel Galego Roxo' do not show such correlation this could be due to the fact that *MYB13* (VIT_05s0049g01010) is more expressed in other tissues than fruits, such as roots and leaves.

Very recently, Vannozzi et al. [38] identified and characterized TFs from the WRKY TF family (*WRKY03*, *WRKY24*, *WRKY43* and *WRKY53*) as potential *STS* regulators. These authors observed that *WRKY24* seems to act as a direct effector in the activation of the *STS29* promoter, independently of *MYB14* and *MYB15*. We observed that the expression levels of the *WRKY24* (VIT_08s0058g00690) and *STS29* were coordinated in cv. 'Moscatel Galego Roxo'. Vannozzi et al. [38] showed that *WRKY03* did not have an effect on *STS29* promoter activity on its own, but acted in combination with *MYB14*. These two WRKY TFs were differently increased in cv. 'Moscatel Galego Roxo' compared to the white-skinned variant, at both developmental stages. For the case of *WRKY43* (VIT_14s0068g01770), despite an increased tendency in the red-skinned variant was observed, its differences were not statistically significant.

Our findings reveal a differential regulation of stilbene metabolism in color reverting berries. In grapevine, the overexpression of *MYBA* genes activate the initial steps of the phenylpropanoid pathway (activating genes such as *PAL* or *4CL*, Matus et al. [10]) despite they are not able to activate *MYB14* nor *MYB15* expression (note that these overexpression studies were conducted on grapevine hairy roots). Therefore, the stilbene pathway may be activated as a secondary response to the higher phenylpropanoid precursor availability. In addition, it may be also determined as a counterbalance mechanism to avoid the excessive accumulation of flavonoid compounds.

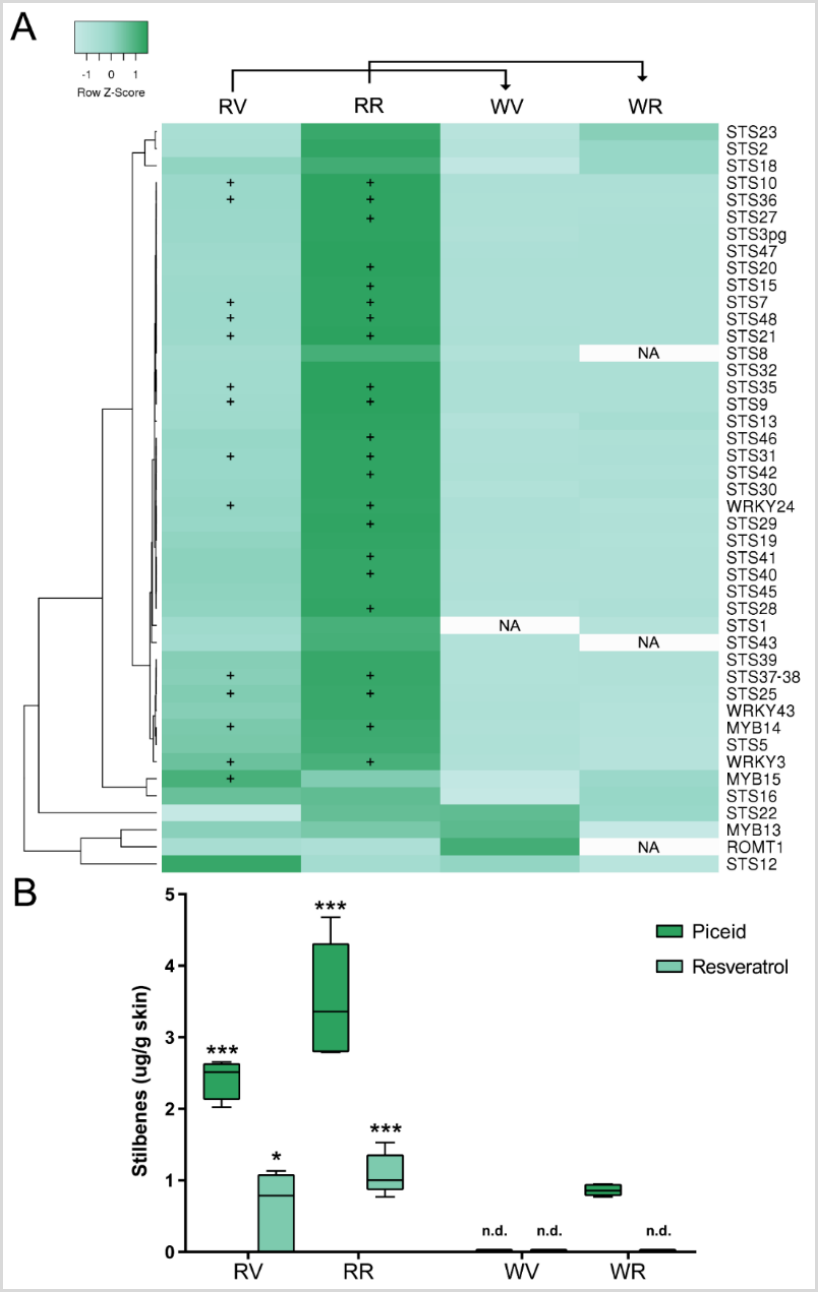

Figure 4.5 – Activation of the stilbene pathway in the berry skin of the color reverting variant.
A) Expression changes of structural and regulatory genes of the stilbene pathway. Genes with significant expression changes between the color reverting and the white-skinned cultivar (as reference) are shown with the '+' symbol representing up-regulation (FDR <0.05). Average linkage clustering and Pearson distance measurement methods were used for clusterization. NA= not assigned value due to very low expression. Gene IDs, together with their FPKM can be found in Supplementary File 10. B) Resveratrol and piceid quantifications in berry skins of cv. 'Moscatel Galego' variants. One, two and three asterisks represent significant differences of $p<0.1$, $p<0.05$ and $p<0.01$, respectively in the red to white variant comparisons independently on each developmental stage (unpaired t test, two-tailed). nd: not detected.

4.12 CONCLUSIONS

In this study, we characterized the genetic, metabolic and transcriptomic differences caused by a white-*to*-red skin color reversion in a near-isogenic background by comparing a red-skinned somatic variant and its white-skinned ancestor in the 'Moscatel Galego' cultivar.

Several authors reported cyanidin derivatives as the minor group of anthocyanins in colored cultivars [42,43]. Taking into account that cyanidin is the precursor of dihydroxylated anthocyanins, it is usual to find low concentrations of its derivatives in dark colored grapes [43]. Despite in black-skinned cultivars cyanidin derivatives constitute the minor group of anthocyanins, the data here obtained suggest that in both variants of cv. 'Moscatel Galego' the phenylpropanoid pathway is being majorly channeled into the dihydroxylated flavonoid sub-branch (probably through the preferential activity of *F3'Ha, F3'H1, F3'H5*), resulting in a higher accumulation of cyanidin-3-*O*-glucoside in the red-skinned variant cv. 'Moscatel Galego Roxo' in contrast to the slight accumulation of trihydroxylated anthocyanins. Thus, we hypothesized that the main reason behind the pigmentation of the red-skinned variant is probably through a deficient activation of the trihydroxylated flavonoid sub-branch along with the decreased activity of anthocyanin-methylation/acylation enzymes.

The genetic data showed that the coloration of the red-skinned variant was recovered from the white-skinned phenotype of cv. 'Moscatel Galego Branco' by the partial activation of the *MYBA1* gene within the berry color locus. This conclusion was based on the fact that the red-skinned variants showed an heterozygotic partial excision of the *Gret1* retrotransposon from the promotor region of the anthocyanin regulator *MYBA1*, probably occurring exclusively in the L2 cell layer, while *MYBA2* was still in homozygosis for a non-functional allele. Altogether, these results allow to hypothesize a predictive model for the white-*to*-red color reversion in grapevine, specifically in cv. 'Moscatel Galego', where the partial *Gret1* excision from the promoter region of *MYBA1* [only in one allele and probably in only one cell layer (L2)] plus the lack of *MYBA2's* function, lead to a decreased berry color locus activity and consequently to a decreased target activation that results in a red-skinned instead of a blue/black-skinned coloration.

Finally, and probably as a consequence of the reactivation of the anthocyanin pathway in the red-skinned somatic variant, other related phenylpropanoid sub-branches may result affected. Such is the case of the stilbene pathway, a branch that is thought to

compete against the flavonoid pathway despite we found it positively correlated to flavonoids in our analysis. Further studies are needed to demonstrate a direct effect of the anthocyanin pathway over the synthesis of stilbenes.

Supporting information

Supplementary data (Supplementary File 4-10) to this article can be found on the electronic version of this Thesis.

Acknowledgments

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CHAPTER 5 | CONCLUDING REMARKS AND OUTLOOK

Conclusions and outlook

The studies described in this Thesis aimed to deepen the current knowledge about the metabolomics and genetics behind grapes berry skin color trait, in order to uncover the molecular mechanisms that contribute for the existing natural variation in the cultivated grapevine (*Vitis vinifera* L.).

Although vegetative propagation has been used as a strategy for multiplication of plants with desired features, natural crossings between the newly introduced cultivars and the local germplasm can also occur, alongside with the emergence of somatic mutation events. Consequently, grapes' phenotype has become greatly diversified, in particularly regarding grape berry skin coloration, ranging from yellow-green to pink, red and black. Despite the basic mechanisms of fruit coloration are now established for several plants, the combined application of metabolomic, genomic and transcriptomic methodologies can make a valuable contribution towards a better characterization of the still poorly understood fine tuning of berry color trait in the cultivated grapevine.

Somatic variants could potentially have a commercial interest if they improve an important agronomical trait or if they allow product innovation. Nowadays, several pigmented varieties, such as 'Aramon', 'Grenache', 'Pinot' or 'Terret', have certified clones with distinct color shades. On the other hand, unpigmented skin berry varieties such as 'Savagnin', 'Chardonnay' and 'Chasselas' also comprise pigmented clones (Pelsy et al., 2015). For example, berry color somatic variants of cv. 'Cabernet Sauvignon' (cv. 'Malian' and cv. 'Shalistin') gave the opportunity to produce some unique wines in South Australia (Torregrosa et al., 2011). Moreover, in other countries, some grapevine somatic mutants are more widespread than their corresponding wild type, which is the case for cv. 'Pinot Gris' and cv. 'Traminer Rot', in Italy. 'Pinot Gris' cultivar is a grey-skinned mutant of the black-skinned cv. 'Pinot Noir' and is grown in a vineyard area in Italy three times larger than the cultivated area of its wild-type progenitor. In its turn, the cv. 'Traminer Rot' cultivar derives from the white-skinned cv. 'Savagnin Blanc' and shows two mutations, one affecting the berry skin color and the other the terpene content and, in this particular case, only the mutant variety is registered in the Italian Grapevine Variety Catalogue (Migliaro et al., 2014).

The first decisive progress on the genetic basis of berry color somatic variation was reported by Kobayashi et al. (2004) based on the characterization of two somatic variants, cv. 'Ruby Okuyama' and cv. 'Flame Muscat', derived from cv. 'Italia' and cv. 'Muscat

of Alexandria', respectively. On this study it was described that a retrotransposon-induced mutation in *MYBA1*, a transcription factor that regulates anthocyanin biosynthesis, is associated with the loss of pigmentation in white cultivars of *V. vinifera*. Since then, using somatic polymorphisms, several studies investigated the specific function of structural genes (Ageorges et al., 2006; Castellarin and Di Gaspero, 2007), the genetic and molecular mechanisms of the polyphenolic pathway regulation (Lijavetzky et al., 2006; This et al., 2007; Walker et al., 2007) and the role of chimerism in the phenotype determination (Walker et al., 2006).

In the present work, different approaches were applied to study uncharacterized somatic variants for berry skin color, derived both from pigmented or unpigmented ancestors, as well a major skin-pigmented Portuguese cultivar, 'Vinhão'.

The identification of grape berry skin color mutants, reported on *Chapter 2*, was performed by genotyping a germplasm set of twenty-five grapevine accessions with twelve microsatellite loci. Among the eleven groups of putative berry skin color mutants genotyped, nine accessions were identified as true color mutants, including related black, grey or red and white-skinned variants derived from a single variety, which were grouped in four different families: 'Malvasia Fina', 'Moscatel Galego', 'Pinot' and 'Pique-poul'. The phenolic profile of the confirmed berry skin color mutants revealed that they could be distinguished according to their non-colored compounds (phenolic acids, flavan-3-ols, flavonols and stilbenes) and anthocyanins composition. As expected, anthocyanins were the main group of compounds that allowed a clear division among colored and non-colored related mutant variants and it was also observed that, in different skin color mutants, their discrimination was due to the contribution of different anthocyanins. This work benefits from the complementary use of molecular and chemical approaches in the correct identification of the berry skin color mutants analyzed.

Grapevine berries are complex organs formed by diverse tissues that follow a double sigmoid growth pattern of development, divided into three distinct phases, as described in *Chapter 1*. A complex series of physical and biochemical changes, such as modifications in size, chemical composition, color, texture, flavor and pathogen resistance occurs during grape berry development (Ali et al., 2011). Thus, understanding grape berry development and the metabolism of different classes of compounds responsible for traits like berry color is imperative to control and improve quality aspects of grapes. In *Chapter 3*, a first attempt to perform an integrative approach combining colorimetric, metabolic and genotypic data was presented, focusing on the changes that

occur during berry development, to improve the knowledge regarding grape berry skin color diversity using somatic variants for berry skin color. The results showed that the initial stages, green and veraison, are metabolically very different from ripe and harvest. Additionally, veraison revealed to be a key stage from which grape berries undergo dramatic metabolic changes, namely through anthocyanin biosynthesis in colored variants. The process of anthocyanin biosynthesis/ accumulation also showed a correlation with the colorimetric parameters analyzed, particularly in cv. 'Pinot Gris' and cv. 'Pinot Noir'. The skin color phenotype of these two somatic variants could be associated with the correlation of *a** and *b** parameters and their anthocyanin profile. The berry skin color variability observed among the somatic variants analyzed was not fully explained by the berry color locus genotype, specifically among some of the red-skinned variants. However, the phenolic profiles allowed to infer about specific interferences, namely some possible dysfunctions at different levels of the biosynthetic pathway, which could be behind the color variation observed among each group of the skin color somatic variants analyzed. For example, data were consistent concerning that grapes showing cyanidin-3-*O*-glucoside as the major anthocyanin, as well as flavonols with two substituent groups in the lateral B-ring, such as cv. 'Malvasia Fina Roxo' and cv. 'Moscatel Galego Roxo', are generally originated by a white-skinned ancestor. Thus, it was hypothesized that after retro-mutation, i.e. color recovery, these grapes seem to keep the dysfunction on flavonoid hydroxylases enzymes, which negatively affect the synthesis of flavonols and anthocyanins with three substituent groups in the lateral B-ring. This hypothesis was further discussed at *Chapter 4*, in the case study of a white-*to*-red berry skin color reversion and its transcriptomic and metabolic consequences in grapevine (*Vitis vinifera* cv. 'Moscatel Galego').

Briefly, the results obtained indicate that the color differences observed between somatic variants are not solely the result of the total amount of the compounds synthesized, but rather reflect a different dynamics of the phenolic pathway, that should be taken into account to explain skin color variation. In this context, and considering the fact that the knowledge about the grape metabolites of somatic variants for berry skin color is still incomplete, since some of these cultivars have received less attention, this study presented on *Chapter 3* contributes for bridging this gap in knowledge by analyzing different groups of somatic variants for berry skin color during berry development using an integrative approach.

An interesting case study, also presented in *Chapter 3* was about the autochthonous Portuguese cultivar 'Vinhão', which have an intense black-bluish skin color that dyes like no other Portuguese cultivar. 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 quantitative reverse transcription 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 (Hap C-N), which is consistent with the high colored phenotype that characterizes this cultivar. The expression patterns in four berry developmental stages of genes playing key functional roles in anthocyanin biosynthesis, showed the dynamics occurring throughout grape berry development, revealing their involvement in the progression of key developmental 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, which could contribute to improve the grape quality of this Portuguese cultivar, which could be used to produce even higher quality red wines. In the future, it will be interesting to deepen into the controversial question behind this major pigmented Portuguese cultivar: Is 'Vinhão' a *teinturier* cultivar or not? A *teinturier* cultivar, by definition, synthesizes and accumulates anthocyanins both in berry skins and in its flesh. Despite cv. 'Vinhão' be described as dying like no other Portuguese cultivar, our phenotypic observations do not demonstrate an anthocyanin accumulation in its flesh. Thus, berries of the cv. 'Vinhão' should be used as a natural model for studying the histological anthocyanin bodies, HPLC anthocyanins measurements and gene expression analysis of flavonoid/ anthocyanin genes in skin and flesh, particularly during ripening and even pos-harvest developmental stages.

Research on grape berry color showed that mutations in *MYBA1* and *MYBA2* genes (berry color locus) are the major cause for the loss of transcription factor activity on anthocyanin biosynthetic genes, leading to a 'white' phenotype. As previously detailed on *Chapter 1*, the loss of berry skin pigmentation has been mostly associated with the insertion of *Gret1* retrotransposon in the 5' regulatory region of the *MYBA1* gene (Kobayashi et al., 2004). Additionally, two mutations in the coding sequence of *MYBA2* (a point mutation and a 2 bp CA deletion that alters its reading frame) can also contribute

to the loss of berry skin pigmentation (Walker et al., 2007). Several types of mutations have been identified at the berry color locus as being responsible for color reversions in grapevine. Occasionally, black-skinned cultivars that are heterozygous for the non-functional and functional alleles give rise to color bud sports, characterized by red, grey or white-skinned berries depending on whether the mutations at the berry color locus occurred only in the L1 or in both L1 and L2 cell layers (Migliaro et al., 2014; Pelsy et al., 2015; Vezzulli et al., 2012; Walker et al., 2006).

The studies presented on *Chapter 4* provide additional information on the molecular mechanisms responsible for berry skin color reversion on a subset of somatic variants for berry skin color that have never been investigated before, including the red-skinned somatic variants of the Portuguese white-skinned cultivars 'Fernão Pires' and 'Verdelho'. These studies took advantage of a layer-specific approach to genetically characterize the berry color locus and its surrounding genomic region, which has already been proven to be a successful method to decipher the molecular mechanisms responsible for color reversions on somatic variants (Migliaro et al., 2014; Vezzulli et al., 2012).

Three evolutionary models have been reported to explain the origin of color reversions on grapevine somatic variants: the 'Sequential Model', the 'Parallel Model' and the 'Revertant Model'. The obtained data support the conclusion that deletions of different extent and positions are behind the origin of less-pigmented/ unpigmented variants derived from a pigmented ancestor. These deletions affected only the inner cell layer in the less pigmented variants and both cell layers in the unpigmented variants. Considering the extent and positions of the deletions, both the 'Sequential' ('Aspiran', 'Grolleau' and 'Terret') and the 'Parallel' ('Aramon', 'Pinot' and 'Pique-poul') models were observed (*Chapter 4*, Figure 4.1 A-B). Regarding less-pigmented variants derived from an unpigmented ancestor, which were characterized by an extensive homozygous and monomorphic genomic region along the distal arm of chromosome 2, the main mechanism described as being responsible for color gain involves the partial excision of *Gret1* retrotransposon from the *MYBA1* promotor. This mechanism was only determined on the cv. 'Mourisco Tinto' and cv. 'Mourisco Roxo' and also on the white-*to*-red berry skin color reversion case study in cv. 'Moscatel Galego'. Moreover, the data obtained and described on *Chapter 4* suggest a novel mechanism for the genetic make-up of less-pigmented variants evolving from an unpigmented ancestor since, namely on the cv. 'Folgasão Roxo', cv. 'Malvasia Cândida Roxo', cv. 'Malvasia Fina Roxo' and on the cv. 'Fernão Pires Rosado', color gain seems to result from the recovery of the functional G

allele on *MYBA2*, which might be playing a fundamental role, probably by restoring *MYBA2* transcripts.

Color depletion and reversion are often observed in the field and the studies conducted for this Thesis showed that the mutational events responsible for color gain/ recovery are less understood and different from those described for color loss. Thus, a case study of a white-*to*-red berry skin color reversion and its transcriptomic and metabolic consequences in grapevine (*Vitis vinifera* cv. 'Moscatel Galego') was performed. The main results obtained can be summarized as follow:

i. Reddish skin color phenotype;
ii. Preferential accumulation of dihydroxylated anthocyanins (cyanidin-3-*O*-glucoside);
iii. Extensive homozygosity along the distal arm of chromosome 2;
iv. Partial activation of *MYBA1* gene within the color locus.

Based on these findings it can be hypothesized that the main reason behind the pigmentation of the red-skinned variant cv. 'Moscatel Galego Roxo' is probably through a deficient activation of the trihydroxylated flavonoid sub-branch, along with the decreased activity of anthocyanin-methylation/acylation enzymes. Additionally, a predictive model was proposed for the white-*to*-red color reversion in grapevine, specifically in cv. 'Moscatel Galego', where the partial *Gret1* excision from the promoter region of *MYBA1* [only in one allele and probably in only one cell layer (L2)] combined with the lack of *MYBA2's* function lead to a decreased activity of the berry color locus and consequently to a decreased target activation, which results in a red-skinned instead of a blue/black-skinned coloration.

As a consequence of the reactivation of the anthocyanin pathway in the red-skinned somatic variant, other related phenylpropanoid sub-branches may probably result affected. Such is the case of the stilbene pathway, a branch that is thought to compete against the flavonoid pathway despite we find it positively correlated to flavonoids in our analysis. Further studies are needed to demonstrate a direct effect of the anthocyanin pathway over the synthesis of stilbenes.

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SUPPORTING INFORMATION

CHAPTER 3

Supplementary File 1 – Pearson's correlation analysis applied to colorimetric measurements and phenolic profiles from grape skin color somatic variants during berry development. Color gradient represents the strength of Pearson's correlation, from dark blue (strong negative correlation) to white (no correlation) and dark red (strong positive correlation).

CHAPTER 4

Supplementary File 2 – Genetic profiles of the studied accessions based on a set of 10 microsatellite markers used for true-to-type confirmation.

Supplementary File 3 – Genetic profiles of the studied accessions based on a set of nine microsatellite markers used for true-to-type confirmation.

Supplementary File 4 – List of primers used for molecular analyses.

Supplementary File 5 – Volcano plots of gene expression changes in the four comparisons tested.

Supplementary File 6 – A) Principal component analysis (PCA) of red and white-skinned somatic variant transcriptomic data sets. B) Number of differentially expressed genes in each comparison.

Supplementary File 7 – Common and unique gene ontology terms in up- and down-regulated genes at both veraison and ripening stages.

Supplementary File 8 – Gene Ontology Enrichment Analysis in up- and down-regulated genes (DESeq2) within RV vs WV comparison (FDR=<0,05; Query Item>=3).

Supplementary File 9 – Gene Ontology Enrichment Analysis in up-regulated genes (DESeq2) within RR vs WR comparison (FDR=<0,05; Query Item>=3).

Supplementary File 10 – Phenylpropanoid pathway genes with normalized expression values (FPKM) obtained from this study. R: red-skinned variant, W: white-skinned variant, V: veraison, R: early ripening (2WAV).