Relationships among Gene Expression and Anthocyanin Composition of Malbec Grapevine Clones

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Ⓢ Supporting Information

ABSTRACT: Anthocyanin profiles are commonly used for grapevine cultivar identification because it is currently accepted that this trait is closely related to their genetic characteristics. Nevertheless, the extent of the variation for the anthocyanin profiles among clones of the same cultivar has not yet been studied in depth. The relative concentration of anthocyanins of 131 Malbec clones grown in the same vineyard was investigated by HPLC-DAD and the use of comprehensive statistic procedures. Complementarily, the expression level of structural and regulatory genes was studied via real time polymerase chain reaction. Significant variation was identified among the profiles of the clones, mainly due to variations in the amounts of malvidin derivatives. Finally, the differential expression in F3′5′H, OMT1 and AM2 genes seems to be related to the malvidin content variation. This work shows the existence of variation for the anthocyanin profiles among clones from the same grapevine cultivar and the putative involvement of genes related to hydroxylation, methylation, and transport of anthocyanins on the basis of such variation.

KEYWORDS: anthocyanins, clone, profile, grapevine, Vitis vinifera, Malbec, gene expression

■ INTRODUCTION

Anthocyanins are the main pigments responsible for the color of red grapes and the wines produced from them. The 3-O-glucosides of malvidin, petunidin, cyanidin, peonidin, and delphinidin, together with their acylated (acetyl, coumaryl) derivatives, are the anthocyanins usually detected in Vitis vinifera L. cultivars.[1−3] From véraison to maturation, anthocyanins accumulate in berry skins, but significant variations in their content have been related to several environmental and management factors, such as cultivar,[4,5] climate,[6,7] soil conditions, canopy management, and irrigation.[8,9] Although the anthocyanin concentration varies greatly, the relative content of each anthocyanin remains stable, especially in the later stages of ripening.[10] Thus, it is commonly accepted that the anthocyanin profile of a given cultivar is related to its genetic background and qualitatively independent from the environmental conditions.[11] Consequently, the anthocyanin profile generated by high-performance liquid chromatography (HPLC) has been successfully used as a fingerprint in several taxonomical studies and, with the aid of multivariate statistical analyses, it has allowed grape varieties to be distinguished.[4−6,10−12] However, the use of those kinds of profiles to differentiate clones of the same cultivar is still very limited.[10]

Grape color variation has been studied through the analysis of several genes of the anthocyanin biosynthesis pathway and some transcription factors with regulatory functions on structural genes of the pathway. Among the structural genes, UDP-glucose:flavonoid-3-O-glucosyltransferase (UFGT) has shown to be a master switch in the control of the presence of berry color, strictly regulated by MYBA1.[13−16] Castellarin et al.[17,18] have shown that the ratio of trisubstituted (blue) to dihydrohylated (red) anthocyanins is under transcriptional control of flavonoid 3′(5′)-hydroxylase genes (F3′H and F3′5′H), thus affecting the anthocyanin profile. Methylation levels could also affect the anthocyanin composition, because these pigments differ from each other in the number of methoxyl groups located on their B-ring. Delphinidin and cyanidin are unmethylated, whereas peonidin and petunidin are monomethylated and malvidin is dimethylated.[19] Indeed, genes encoding O-methyltransferases involved in anthocyanin methylation[20,21] can potentially generate differences in the anthocyanin profile.[19] On the other hand, the selective function of anthocyanin transporters could also have a role in determining the final anthocyanin profile. Whereas two grapevine multidrug and toxic extrusion (MATEs), AM1 and AM3, have been described to specifically transport acylated

Table 1. Primer Pairs Used for Real-Time Quantitative RT-PCR

gene name	gene annotation[a]	sequence for the forward and reverse primers	expected fragment size (bp)
VvF3′H	VIT_17s0000g07200	F 5′- ATTCGCCACCCTGAAATGAT-3′ R 5′-AGCCGTTGATCTCACAGCTC-3′	196
VvF3′5′H	VIT_06s0009g02810	F 5′-GAAGTTCGACTGGTTATTAACAAAGAT-3′ R 5′-AGGAGGAGTGCTTTAATGTTGGTA-3′	156
VvUFGT	VIT_16s0039g02230	F 5′-CATGTCTCAAACCACCACCAAC −3′ R 5′-TGGTGCTGAAGAAGGAGAAGAC −3′	137
VvAOMT1	VIT_01s0010g03510	F 5′-GGGACATTGGACTTTGCGATG-3′ R 5′-TAACCAACTTCAGCAGCAGC-3′	79
VvAOMT2	VIT_01s0010g03490	F 5′-CCATGTCCAGCTCAAGTCACAAGG-3′ R 5′-TTCAGCTGCTCATGCTCTCTTGGG-3′	90
VvAOMT3	VIT_01s0010g03470	F 5′-AGAGAGCATGAGCAGCTGAGAG-3′ R 5′-TCCTTCGTCAACAGGCACATTC-3′	90
VvABCC1	VIT_16s0050g02480	F 5′- AGGGTCTAGGCTCCATTGTTG-3′ R 5′-ACCAGTATCCGGCTTCTTCG-3′	106
VvAM1	VIT_16s0050g00930	F 5′-GTTCGGCTTTCAATTGCCTCG-3′ R 5′- CCTTCCCACCCATTAAAAGC-3′	146
VvAM2	VIT_16s0050g00910	F 5′- TGAATTTTAATGGGTGGGAGGC-3′ R 5′-TCATTGGAGACCCGAATGCTT-3′	73
VvAM3	VIT_16s0050g00900	F 5′- GGTTGGAGGTCGTGATGTTC-3′ R 5′-TTGGAGACCCGAATGCTCAC-3′	58
VvGST	VIT_04s0079g00690	F 5′- CCTGATTTCCTCCTTCGACAGC-3′ R 5′-GCCTGAAATCGCCATCTTCTACC-3′	64
VvMybA1	VIT_02s0033g00380	F 5′-AGGAAGCCATCATCCACTTCACC-3′ R 5′-TGCTCAGCTAACAGGCTTTCCC-3′	71
VvMyb5B	VIT_06s0004g00570	F 5′-AGTCCAGTCGTTCGGGTTC-3′ R 5′-GCAGGGTGTTGAAGCCAAAT-3′	157
VvACT1	VIT_04s0044g00580	F 5′-CTTGCATCCCTCAGCACCTT-3′ R 5′-TCCTGTGGACAATGGATGGA-3′	82

[a]According to CRIBI Grape Genome (http://genomes.cribi.unipd.it/grape/).

anthocyanins,[22] Francisco et al.[23] recently reported that an ATP binding cassette protein (ABCC1) transports anthocyanidin 3-O-glucosides, with a preferential transport for malvidin 3-O-glucoside.

In this work we demonstrated the existence of significant variation for the anthocyanin profiles among clones from the same cultivar and also identified the variation in the amount of a particular anthocyanin as the main distinguishing factor within those profiles, where genetic variation and differential gene expression of particular genes seem to be important factors to determine the clonal difference in anthocyanin profile.

■ **MATERIALS AND METHODS**

Berry Sampling. The assays were performed during the vintages 2010 and 2012, at the Bodegas Esmeralda (BESA) clone collection localized at La Pirámide vineyard (Catena Zapata Winery, Agrelo,

Mendoza; 34°55′21″ S, 69°7′4″ E). The collection included 131 clones (116 from BESA, 12 from FCA-UNCuyo, and 3 from INTA). Clones were sorted in 34 rows with four clones per row with a plantation density of 4000 plants per hectare (1.25 m × 2.00 m). The vineyard was a homogeneous field without slope, and all of the plants were under artificial irrigation. Each experimental unit (clone) was represented by approximately 45 plants. Ripening state of the samples was determined on the basis of berry density as an indicator of accumulated sugar levels. Density was estimated by berry flotation in a range of NaCl solutions, each having a decrease in salinity of 20 g NaCl L^{-1} (from 160 to 80 g NaCl L^{-1}).[24]

Varietal Identity Testing of the Clones through Microsatellite Analysis. Young leaves were sampled from every clone and kept frozen at −80 °C. DNA was extracted using a DNeasyTM Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Nine nuclear microsatellite markers were studied (VVS2, VVMD5, VVMD27, VVMD28, ssrVrZAG29, ssrVrZAG62, ssrVrZAG67, ssrVrZAG83, and ssrVrZAG112), using a multiplex PCR, according to the method of Ibáñez et al.[25]
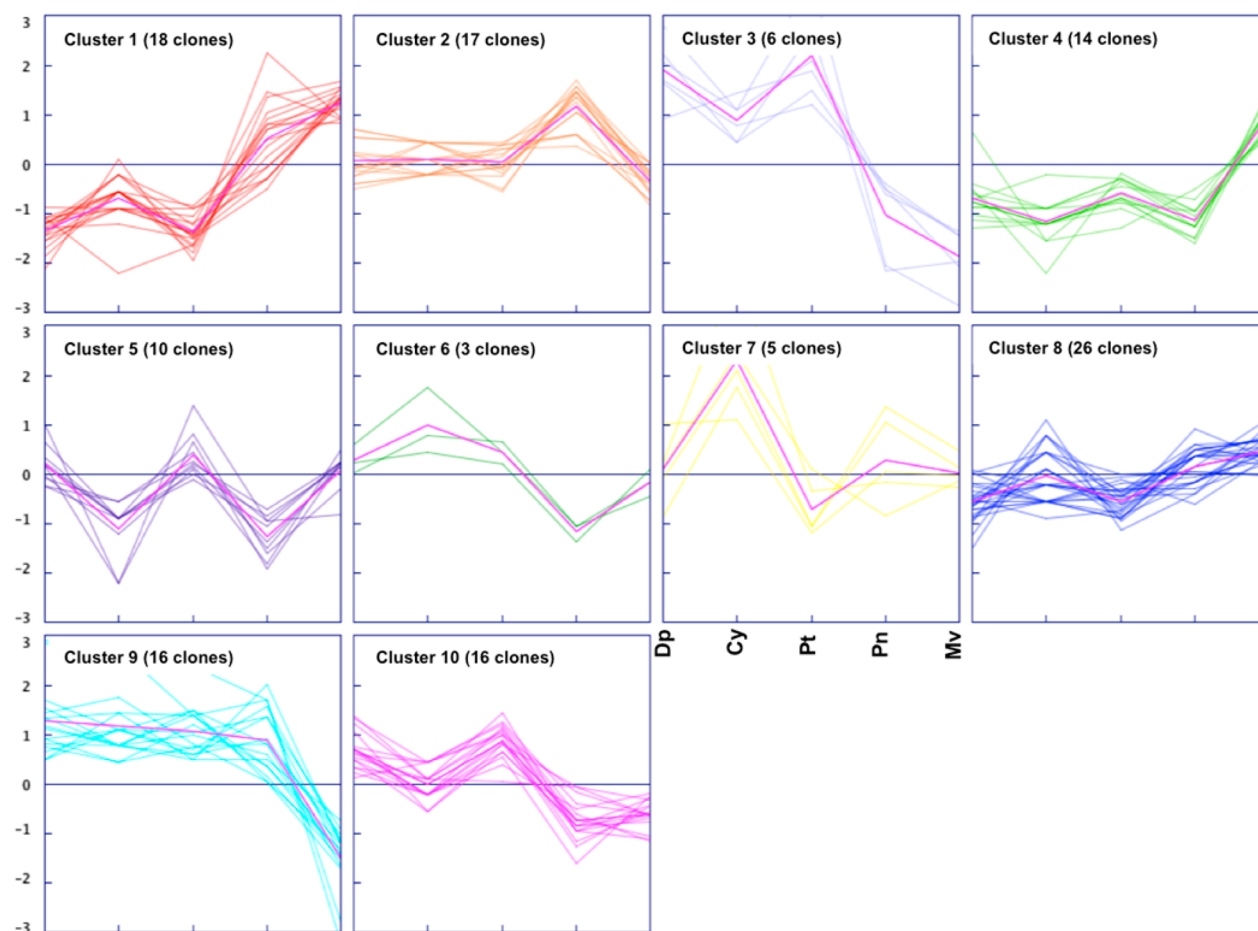
Figure 1. Clustering analysis of 131 Malbec clones according to their anthocyanin profiles using the *k*-means method. A five-point profile of each clone was obtained after adding up the glycosylated, acetylated, and coumaroylated forms for each anthocyanin: delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn), and malvidin (Mv). The number of clones per cluster is indicated in parentheses.

Extract Preparation from Skins. Three hundred berries per clone were randomly collected at a density of 140−160 g L^{-1} of NaCl (approximately 24 °Brix) in nylon bags from different positions within clusters from every plant. The samples were kept in ice to prevent dehydration and transported to the laboratory, where they were weighed, frozen, and conserved at −80 °C. Berry anthocyanins were extracted as previously described[3,26,27] with minor modifications. Briefly, skins and seeds were separated by hand from 50 berries, weighed, and ground with 30 mL of ultrapure water. Forty milliliters of hydroalcoholic solution (ethanol/water, 12:88, v/v) containing 5 g/L of tartaric acid was added to the ground material. The final weight of the suspension was adjusted to 100 g with ultrapure water. The pH of extracts was adjusted to 3.6 with NaOH or HCl. Extracts were macerated for 2 h at 25 °C using an orbital shaker at 200 rpm and then centrifuged for 15 min at 4000 rpm.

HPLC-DAD Analysis of Anthocyanins. Two milliliters of skin extracts per experimental unit were filtered through a 0.45 μm pore size nylon membrane, and then 100 μL was injected in the high-performance liquid chromatograph coupled to diode array detector (HPLC-DAD) system (PerkinElmer, Shelton, CT, USA). Separation was performed at 25 °C using a Chromolith Performance C18 column (100 mm × 4.6 mm i.d., 2 μm; Merck, Darmstadt, Germany) with a Chromolith guard cartridge (10 mm × 4.6 mm). A gradient consisting of solvent A (water/formic acid, 90:10, v/v) and solvent B (acetonitrile) was applied at a flow rate of 1.1 mL/min from 0 to 22 min and at flow rate of 1.5 mL/min from 22 to 35 min as follows: 96−85% A and 4−15% B from 0 to 12 min, 85−85% A and 15−15% B from 12 to 22 min, 85−70% A and 15−30% B from 22 to 35 min. This was followed by a final wash with 100% methanol and re-equilibration of the column. Photodiode array detection was performed from 210 to

600 nm, and the quantification was carried out by peak area measurements at 520 nm, according to the method of Fanzone et al.[28] Anthocyanin amount was expressed by using malvidin-3-glucoside chloride as standard (Extrasynthese, Lyon, France) for a calibration curve (R^2 = 0.98). Identification and confirmation of anthocyanic pigments were performed by HPLC-DAD/ESI-MS as described by Monagas et al.[29]

RNA Isolation, cDNA Synthesis, and Real-Time Quantitative PCR (qRT-PCR) Expression Analysis. For gene expression studies, post-véraison berries were harvested at a density of 100−120 g L^{-1} of NaCl, frozen, and conserved at −80 °C. Total RNA was extracted from berry skins according to the procedures described by Reid et al.[30] Final RNA purification and DNase digestion of contaminating DNA in the RNA samples were performed using the SV Total RNA Isolation System (Promega) following standard protocols. Reactions for cDNA synthesis and qRT-PCR were performed according to Lijavetzky et al.[31] using a StepOne Real-Time PCR System (Applied Biosystems, Life Technologies). Nontemplate controls were included for each primer pair, and each qRT-PCR reaction was completed in quadruplicate. Expression data were normalized against the grapevine *ACT1* gene (*VIT_04s0044g00580*). A normalization gene was chosen after the comparison of *ACT1*, *EFa1* (*VIT_06s0004g03220*) and *UBI* (*VIT_16s0098g01190*) genes using NormFinder software.[32] All three genes were previously tested for grapevine gene expression analysis.[30] Relative quantification was performed by means of the ΔΔCt method using StepOne software v2.2.2 (Applied Biosystems, Life Technologies). Gene-specific primers were designed using the QuantPrime web tool,[33] and the sequences are described in Table 1.

Statistical Methods. Clustering analysis of HPLC-DAD data was performed using the *k*-means method[34] implemented in Genesis
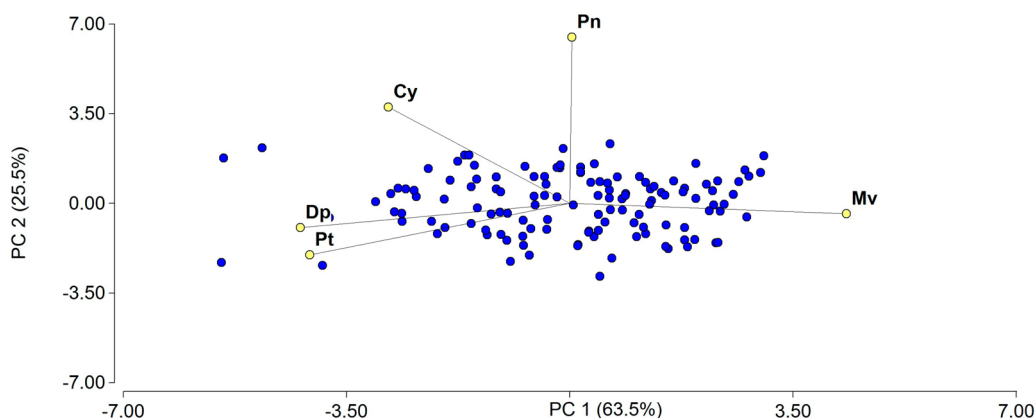
Figure 2. Principal component analysis (PCA) of the anthocyanin profile data of the 131 studied samples. The first (PC1) and second (PC2) principal components are represented. Each of the five points determining the profiles described in Figure 1 was treated as a variable (Dp, Cy, Pt, Pn, and Mv).
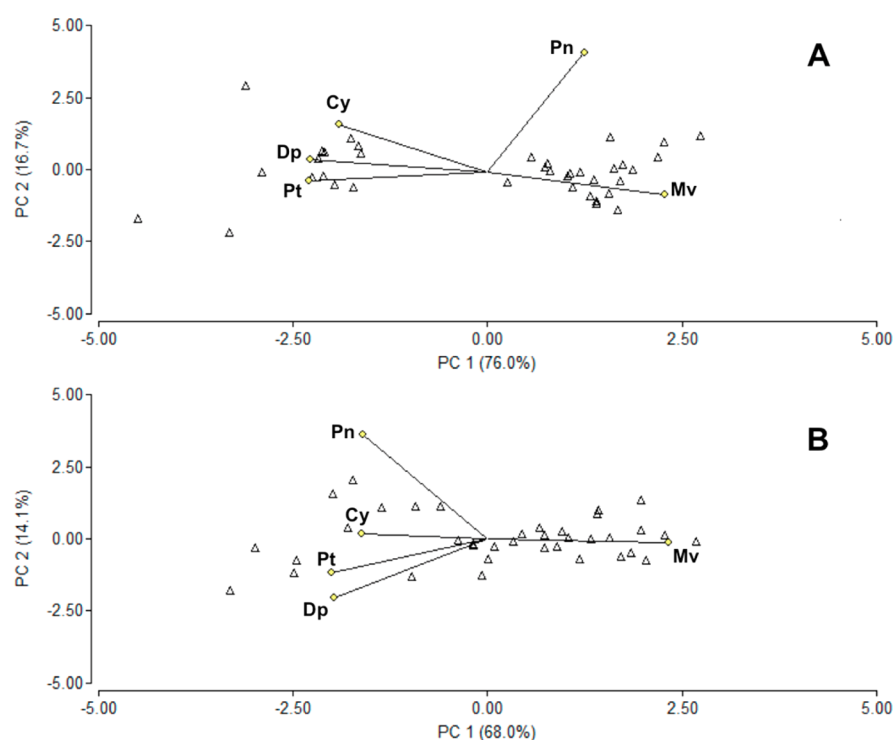

Figure 3. Principal component analysis (PCA) of the anthocyanin profile data of selected clones representing both extremes of the Mv content distribution described in Table S3 in the Supporting Information. Plots correspond to the data collected in 2010 (A) and 2012 (B). The first (PC1) and second (PC2) principal components are represented.

software v.1.7.6.[35] Principal component analysis[36] and linear regression analysis were carried out by means of InfoStat software.[37] Additional statistical calculations for the qRT-PCR analysis (group analysis: adjusted p values using Benjamini–Hochberg) were carried out with the help of DataAssist software v.3.01 (Applied Biosystems, Life Technologies).

RESULTS

All of the Clones Studied Belong to the Cultivar Malbec. Before performing any biochemical analysis, we first verified the cultivar identity of the 131 sampled clones by analyzing nine microsatellite markers. As shown in Table S1 in the Supporting Information, all of the samples presented the same genotype for the nine analyzed microsatellite markers. This genotype was compared with the ICVV database (www.

icvv.es) and the European *Vitis* database (www.eu-vitis.de) and matched with Cot (prime name for Malbec at the *Vitis* International Variety Catalogue-VIVC, www.vivc.de). The total probability of identity found for this set of nine microsatellite markers was very low (6.93 × 10^{-12}),[25] and allows concluding that all of the studied samples belong to the cultivar Malbec.

Malbec Clones Can Be Discriminated by Their Anthocyanin Profiles. To evaluate the phenotypic variation for the anthocyanin profile of the 131 Malbec accessions of the studied collection, we performed a HPLC-DAD analysis.[3,26−28] For each Malbec clone 15 anthocyanins were considered: delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn), and malvidin (Mv) in their glycosylated, acetylated, and coumaroylated derivative forms (i.e., Dp3Gl, Cy3Gl, Pt3Gl, Pn3Gl, Mv3Gl, Dp3acGl, Cy3acGl, Pt3acGl, Pn3acGl,
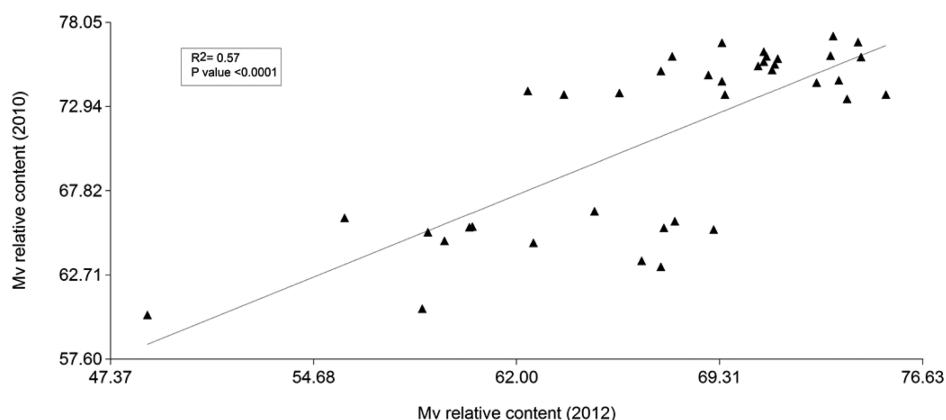
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Figure 4. Correlation between malvidin (Mv) relative content of selected extreme clones during two different seasons (2010 and 2012).

Mv3acGl, Dp3cumGl, Cy3cumGl, Pt3cumGl, Pn3cumGl, and Mv3cumGl; Supporting Information Figure S1). The absolute and relative contents of those anthocyanins in samples of the clone collection are shown in Table S2 in the Supporting Information. Surprisingly, we obtained a broad spectrum of anthocyanin profiles among the clones. To have a comprehensive outlook of those different profiles, clones were grouped on the basis of their anthocyanin profiles using a k-means clustering analysis.[34] A five-point profile of each sample was obtained after adding the glycosylated, acetylated, and coumaroylated derivatives for each anthocyanin. As observed in Figure 1, the clones presenting similar profiles were clustered in 10 different profile groups. As expected, the larger cluster (cluster 8) included clones showing little variation with respect to the median profile. However, another five clusters grouped clones displaying high or low proportion of particular anthocyanins (clusters 1, 3, 4, 7, and 9).

Malbec Clones Are Mainly Separated by Variation in Mv Content. To further evaluate the variation observed within the Malbec clone collection, a principal component analysis (PCA) over the anthocyanin profile data of the 131 studied samples was performed (Figure 2). For this analysis each of the five points determining the profiles described in Figure 1 was treated as a variable (Dp, Cy, Pt, Pn, and Mv). First and second principal components (PC1 and PC2) explained together almost 90% of the variability for the anthocyanin profile (63.5 and 25.5%, respectively). PC1 pointed out the role of Mv content variation as the main discriminating factor within the clones. Dp and Pt contents were found negatively correlated with Mv, in agreement with both Dp and Pt being Mv precursors.[38] On the other hand, Pn content seems to be independent with respect to Mv, whereas Cy, a precursor of Pn, displayed an intermediate behavior (Figure 2).

Putative Genetic Effects on Mv Content and Anthocyanin Profiles Variation. After verifying the existence of different anthocyanin profiles within the clone collection and identifying the Mv content as the main variation source, we proceeded to evaluate the extent of conservation of the observed anthocyanin profiles in different years as an indication of the existence of an underlying genetic effect. For this purpose, we selected about 30% of clones representing both extremes of the Mv content distribution (Supporting Information Table S3). Data from the selected clones displayed similar PCA results as the complete collection, with an obvious increase of the PC1 (led by Mv) and a consequent decrease in the PC2 (Figure 3A). The same selected clones were further

analyzed by HPLC-DAD in a second-year replication. The PCA performed on the selected clones during 2012 (Figure 3B) displayed again a large effect of Mv content on the evaluated phenotypic variation, as reflected in the PC1 value (68%). Besides the PCA results, this putative genetic effect could also be observed in Figure 4, where the linear regression analysis between the Mv content of the selected clones (2010 vs 2012) displayed highly statistically significant results with an $R^2 = 0.57$ (p value < 0.0001). Additionally, Dp and Pt presented also a highly significant correlation; Cy was just slightly correlated, whereas Pn showed no correlation (Table 2).

Table 2. Linear Regression Analysis between Individual Anthocyanin Contents Measured in 2010 and 2012

variable[a]	n	r^2	r^2 adj	p value
Mv	38	0.57	0.56	<0.0001
Dp	38	0.47	0.45	<0.0001
Pt	38	0.41	0.39	<0.0001
Cy	38	0.15	0.13	0.0168
Pn	38	0.04	0.01	0.2369

[a]Delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn), and malvidin (Mv) as the sum of their corresponding glycosylated, acetylated, and coumaroylated derivatives.

Variation in Mv Content Is Associated with Distinct Patterns of Gene Expression during Biosynthesis and Transport of Anthocyanins. We evaluated the putative influence of different genes related to biosynthesis and transport of anthocyanins on the variation in Mv contents (and the anthocyanin profiles) within the Malbec clone collection. To perform such analysis we designed qRT-PCR specific primers for 13 grapevine genes comprising 6 structural genes [(i) flavonoid 3′-hydroxylase (*F3′H*); (ii) flavonoid 3′,5′-hydroxylase (*F3′5′H*); (iii) UDP-glucose:flavonoid-3-*O*-glucosyltransferase (*UFGT*); (iv) *O*-methyltransferase 1 (*OMT1*); (v) *OMT2*; and (vi) *OMT3*],[17−19,39] 5 anthocyanin transporters [(i) glutathione-*S*-transferase (*GST*); (ii) ATP binding cassette C1 (*ABCC1*); (iii) anthoMATE1 (*AM1*); (iv) *AM2*; and (v) *AM3*;[18,22,23]], and 2 *MYB* transcription factors [(i) *MYBA1* and (ii) *MYB5b*].[14−16,18,40] Sequences for the forward and reverse primers as well as the PCR expected fragment size are detailed in Table 1. We measured the transcript accumulation of each gene in two groups of four clones corresponding to both Mv content extremes in the clone collection (Supporting Information Table S4). Clones selected

for each of the groups belong to (i) contrasting *k*-means clusters (Figure 1), (ii) opposite extremes at all PCAs (Figures 2 and 3), and (iii) opposite extremes of Mv content (Figure 5;
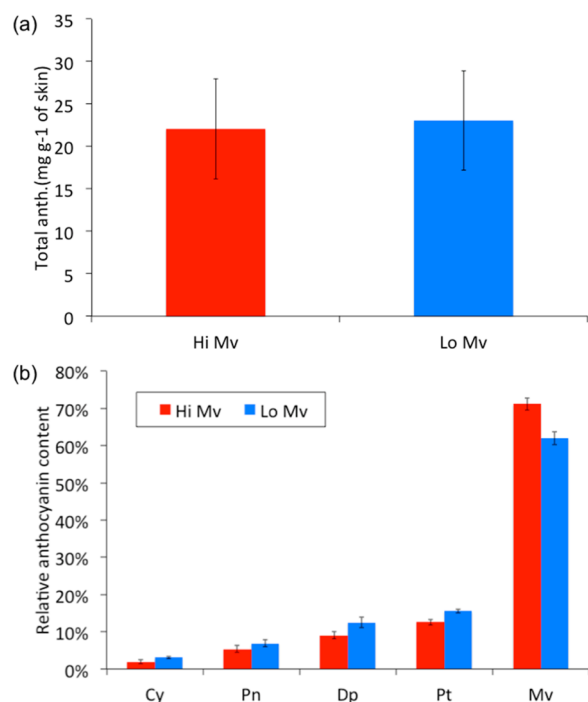

Figure 5. Anthocyanin content and metabolite profiles in berry skin of two groups of four Malbec clones corresponding to both Mv content extremes in the clone collection: (a) total anthocyanins expressed as mg g^{-1} of skin of malvidin equivalents; (b) anthocyanin profiles expressed as the relative concentration of cyanidin (Cy), peonidin (Pn), delphinidin (Dp), petunidin (Pt), and malvidin (Mv). High Mv content clones: MB25, FM5, FM4, and MB5. Low Mv content clones: MB81, MB30, MB77, and MB113.

Table S3 in the Supporting Information). Interestingly, although the anthocyanin profiles of both groups are clearly different (Figure 5b; Supporting Information Table S4), the total amount of anthocyanins is almost the same (Figure 5a). Consequently, with significant difference in Mv, the proportions of trisubstituted/disubstituted anthocyanins and methylated/unmethylated anthocyanins were higher in the high Mv content group (Supporting Information Table S4).

As previously reported by Fournier-Level et al.[19] for other colored cultivars, no expression of *OMT3* was detected in grape skins of Malbec (data not shown). Unsurprisingly, most genes displayed slight difference between the two compared groups (Figure 6). Two of the structural genes showed significant variations, one related to anthocyanin hydroxylation (*F3′5′H*) and other involved in anthocyanin methoxylation (*OMT1*). The gene showing the more extreme differences was a putative anthocyanin transporter (*AM2*) with a fold change close to 2.9× and a *p* value of 0.0088. Finally, neither of the two *MYB* transcription factors analyzed (*MYBA1* and *MYB5b*) showed gene expression differences between the high and low Mv content groups (Figure 6).

■ DISCUSSION

Anthocyanin profiles, in combination with different statistical methods, have been efficiently employed as biochemical markers to discriminate grapevine varieties, hybrids, and wild

accessions.[6,11,41,42] However, information about the use of those tools to depict intracultivar variation is very limited.[10] We tested the usefulness of anthocyanin profiles to identify variation among Malbec grapevine clones by analyzing a large collection comprising 131 accessions grown in the same vineyard. The amount of variation observed in the studied collection largely exceeded our expectations considering the close genetic relationship between the clones (Supporting Information Table S1). Accordingly, 10 different clone clusters were discriminated by means of *k*-means analysis. Even though this study shows a high proportion of clones with median-like profiles (Figure 1), about 41% of the clones are grouped in four clusters characterized by the predominance of high or low Mv relative contents (clusters 1, 3, 4, and 9). Moreover, the additional evaluation of the HPLC-DAD data by means of PCA (Figure 2) clearly indicates the key role of the Mv relative content variation for shaping the phenotypic distribution of the clones of the collection. As the value of PC1 indicates, Mv content explained 63.5% of the phenotypic variation for the anthocyanin profiles. The analysis of the anthocyanin profiles of six different Tempranillo clones was previously reported by Revilla et al.[10] The authors concluded that the analyzed clones were affected mostly by weather conditions despite slight differences in the profile of a particular clone. Conversely, the size of our collection and probably the diversity in the origin of the Malbec clones allowed in our case the detection of a broad variation spectrum (Supporting Information Table S2; Figure 1). These findings encouraged us to more deeply analyze the origins of the differences between the anthocyanin profiles among the Malbec clones.

The assessment of the genetic variation among clones of the same grapevine cultivar has been performed by means of different molecular marker techniques.[43−49] The aim of these works is looking for intravarietal variation at the DNA level of the studied cultivars. Some of the reports also describe the correlation between molecular markers and the geographical origin of the clones or with particular phenotypic traits.[43,47,49] Even though these reports clearly show the existence of intravarietal genetic variability at the DNA level, no information regarding the intravarietal analysis of genetic variation for particular quality traits has been presented so far. To contribute some information on this subject, we analyzed a large set of Malbec clones in two different seasons for their anthocyanin profiles. We particularly focused on the Mv relative content, the main source of phenotypic variation for that trait in our study (Figure 2). We selected two groups of clones from both extremes of phenotypic distribution after the 2010 experiment, and we tested them again during a subsequent season. Results from 2012 were similar to those of 2010, as observed in both the PCA and correlation analysis (Figures 3 and 4). These findings suggest a putative genetic effect on the regulation of the Mv content, because clones presenting low or high Mv content during 2010 performed correspondingly during 2012 season. Additionally, as a support of the conservation of the anthocyanin profiles, the two other trisubstituted anthocyanins (Dp and Pt) presented also a highly significant correlation. However, among the disubstituted anthocyanins, Cy was just slightly correlated, whereas Pn showed no correlation (Table 2). As expected, we also detected important environmental variation, which was presumably responsible for the more average-like behavior of some of the selected clones, that is, less extreme in 2012 than in 2010 (Figure 3). These results suggested that environmental effects seem to affect differentially
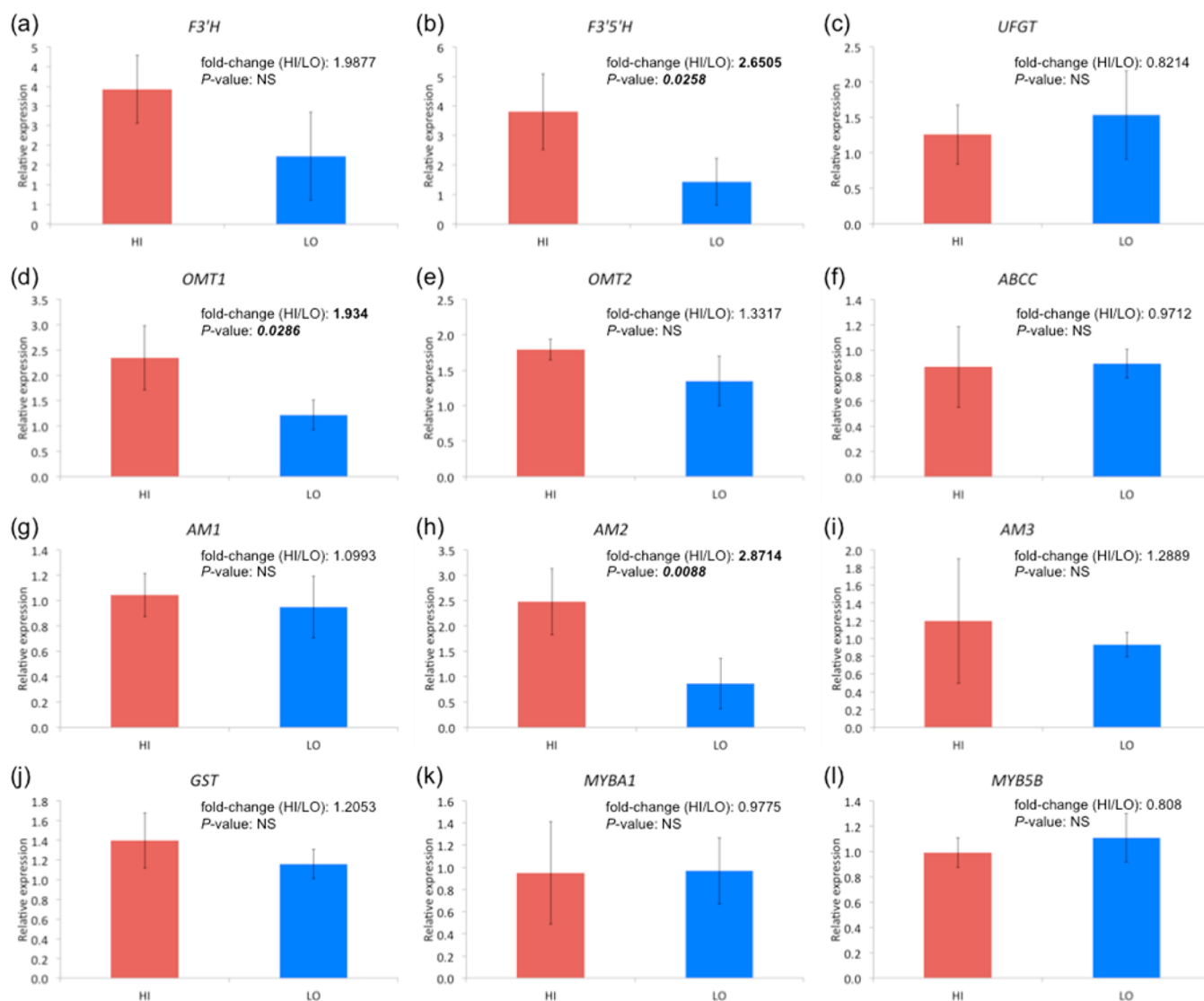
Figure 6. qRT-PCR amplification of 12 grapevine genes in berry skins of two groups of four Malbec clones corresponding to both Mv content extremes in the clone collection; comparison of the average relative expression (fold change) of high Mv content clones versus low Mv content clones: (a) F3′H; (b) F3′5′H; (c) UFGT; (d) OMT1; (e) OMT2; (f) ABCC; (g) AM1; (h) AM2; (i) AM3; (j) GST; (k) MYBA1; (l) MYB5b. NS indicates not significant Benjamini−Hochberg false discovery rate test.

the di- and trisubstituted anthocyanins, because Cy and Pn presented a higher environmental dependence than Dp, Pt, and Mv (Figure 4 and Table 2). We found little information on the analysis of genetic variation affecting anthocyanin profiles of clones of the same cultivar. In the paper on the Tempranillo cultivar mentioned above,[10] the authors concluded that the variation in the analyzed anthocyanin fingerprint depends mainly on agroclimatic factors and not on genetic differences among clones. Interestingly, the presented results showed that the only Tempranillo clone showing consistent differences was characterized by variations on the relative proportion of Mv during three different seasons. Despite the described variation indicating that the Tempranillo clones were affected mostly by weather conditions, genotypic effects seemed also to be present in that experiment.[10]

The huge natural variation for grape berry color is under the control of several genes and transcription factors that act at different points of the anthocyanin biosynthetic pathway.[50,51] Besides the master role of UFGT (and their main regulators, MYBA1 and MYBA2) in determining the presence of

anthocyanins in colored grapes,[13−16] the cultivar-specific function of other genes along the pathway is accepted to be involved in the regulation of the quantitative nature of grape skin color variation as well as of their different hues.[18,50] Whereas anthocyanin hydroxylases[18,39,52,53] participate in the modulation of anthocyanin composition upstream of UFGT, O-methyltransferases (OMTs)[19−21] and anthocyanin transporters[22,23] also play important roles downstream of UFGT. It is important to note that the molecular and biochemical studies mentioned above are mainly based on functional analyses of these significant genes by using different colored cultivars and usually by comparing white genotypes versus colored genotypes. In the present work we report for the first time the expression analysis of several of the genes involved in anthocyanin profile variation at an intravarietal level by evaluating a Malbec clone collection. We analyzed the expression of each gene in two groups of four clones corresponding to the extremes of the Mv content distribution (Supporting Information Table S4). Therefore, it is expected that differences in gene expression between the two groups

should be related to differences in the concentration of anthocyanins.

Several studies showed the correlation between the expression of *UFGT* and *GST* as well as those of the transcription factors *MYBA1* and *MYB5B* with the accumulation of total anthocyanins.[18,40,50,54] The expression of *UFGT*, *GST*, *MYBA1*, and *MYB5B* in our experiment showed no significant differences between the groups of high and low Mv (Figure 6c,j,k,l), in agreement with the total anthocyanin results displayed in Figure 5a, where both groups of clones presented similar mean values. We can infer from these results that profile differences between the groups of high and low Mv content are not due to variation in total amount of anthocyanins. As seen in Figure 5b and in Table S4 in the Supporting Information, the extreme value of Mv in the high Mv content group is compensated by a decrease in the other four anthocyanins.

Analysis of the transcriptional control of anthocyanin biosynthetic genes in different cultivars bearing extreme phenotypes for berry pigmentation showed that the predominance of trisubstituted anthocyanins (Dp, Pt, and Mv) was associated with higher ratios of *F3′5′H/F3′H* gene transcription.[18] Although our work was performed with clones of the same colored cultivar, we also observed a predominance of trisubstituted anthocyanins in the high Mv content group (Supporting Information Table S4; Figure 5b). Consistent with this result, the expression of *F3′5′H* showed a significant fold change (>2.65×; *p* value = 0.0258) between the contrasting Mv content groups (Figure 6b), whereas *F3′H* presented a lower and not significant fold change (Figure 6a). Accordingly, as mentioned above, in the comparison of 2010 and 2012 HPLC data, trisubstituted anthocyanins showed a putative higher genetic effect (Figure 4 and Table 2).

The positive correlation between the expression of different anthocyanin *OMTs* and the anthocyanin methylation level at ripening has been thoroughly studied.[18,19,51,55] Particularly, Fournier-Level et al.[19] showed that *OMT1* seems to be the fundamental gene for anthocyanin methylation, whereas *OMT2* may be responsible for fine and specific differences in the level of methylated anthocyanins. Additionally, the analysis of an OMT from Cabernet Sauvignon[20] showed that the highest specific activity of the enzyme was found for Dp, which is converted by this enzyme in two steps to Mv, the most abundant anthocyanin in most red grapes.[56] In agreement with these previous studies, we found a significant differential gene expression for *OMT1* (but not for *OMT2*) when comparing the high and low Mv content groups (Figure 6d,e). It is worth mentioning that the higher ratio of methylated/unmethylated anthocyanins reported for the high Mv content group in Table S4 in the Supporting Information is solely due to Mv, because the other methylated anthocyanins (Pn and Pt) presented lower percentages. We can infer that similarly to the observation reported by Lücker for Cabernet Sauvignon,[20] in the studied collection of Malbec clones, *OMT1* is involved in the conversion of Dp and Pt, which leads to the high contents of Mv of the extreme clones (Figures 2 and 3; Supporting Information Table S4).

Despite the increasingly deep understanding of anthocyanin biosynthesis and regulation, little is known about the molecular aspects of their transport.[22,23] In grapevine, different anthocyanin transporters have been characterized. Conn et al.[57] reported the involvement of *GST* in vacuolar accumulation of anthocyanins by exporting them to the tonoplast membrane. Two MATEs (*AM1* and *AM3*) have been described to specifically transport acylated anthocyanins, but not glucosylated ones.[22] More recently, a tonoplast-localized ABCC-type protein (ABCC1) was characterized with a preferential malvidin 3-*O*-glucoside transporter function.[23] As we mentioned above, *GST* showed no expression differences between the high and low Mv content groups (Figure 6j). Additionally, neither *AM1* and *AM3* nor *ABCC1* appeared to be involved in the variations in the profiles of Malbec clones in the collection (Figure 6f,g,i). Interestingly, the gene showing the highest expression differences between the extreme Mv content groups is a still uncharacterized anthoMATE (*AM2*).[22] As seen in Figure 6h, *AM2* presented a fold- change of 2.9× with a *p* value < 0.01, suggesting the putative involvement of this gene in the anthocyanin profile variation within the Malbec clones. As proposed by Gomez et al.,[22] the existence of other transport mechanisms has to be elucidated and characterized to have more evidence about a hypothetical relationship between the transport mechanisms and the final anthocyanin composition.

This work reveals the existence of variation for the anthocyanin profiles among clones from grapevine cultivar Malbec by using an appropriate sample size as well as suitable analytical and statistical tools. We also identified the variation in Mv derivative content as the main discriminating factor within the anthocyanin profiles, with a putative genetic variation involvement. We finally show that the increase in Mv content of the extreme clones is associated with a higher expression of different genes at distinct points of the anthocyanin biosynthesis pathway. Clones displaying relatively higher Mv content also presented elevated gene expression for anthocyanin hydroxylase (*F3′5′H*), an anthocyanin *O*-methyltransferase (*OMT1*), and a putative anthocyanin transporter (*AM2*). These results suggest focusing our attention on the gene expression variation at different steps of the anthocyanin biosynthesis pathway because modulation of the final anthocyanin profile seems to be fine-tuned by multiple genes performing different functions on the pathway.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Tables S1–S4 and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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