A group of grapevine MYBA transcription factors located in chromosome 14 control anthocyanin synthesis in vegetative organs with different specificities compared with the berry color locus

The R2R3 MYBA5, MYBA6 and MYBA7 Transcription Factors Within the Grapevine Vegetative Color Locus Control Anthocyanin Synthesis with Different Specificities Compared to the Berry Color Locus

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Abstract

Grapevine organs accumulate anthocyanins in a cultivar-specific and environmentally induced manner. The *MYBA1-A2* genes within the berry color locus represent the major genetic determinants of fruit color. The simultaneous occurrence of transposon insertions and point mutations in these genes is responsible for most white-skinned phenotypes. However, red pigmentation found in vegetative organs suggests the presence of additional regulators. This work describes a genomic region of chromosome 14 containing three closely related R2R3-MYB genes, named *MYBA5*, *MYBA6* and *MYBA7*. Ectopic expression of the latter two in grapevine hairy roots (HR) promoted anthocyanin accumulation without affecting other phenylpropanoids. Transcriptomic profiling of HR expressing *MYBA1*, *MYBA6.1* and *MYBA7* showed that these regulators share the activation of late biosynthetic and modification/transport-related genes but differ in the activation of the *FLAVONOID-3´5´-HYDROXYLASE* (*F3'5'H)* family. However, the alternatively spliced *MYBA6.2* is incapable of activating anthocyanin synthesis due to the lack of a MYC1-interaction domain. MYBA1, MYBA6.1 and MYBA7 activated the promoters of *UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE* (*UFGT*) and *ANTHOCYANIN 3-O-GLUCOSIDE-6''-O-ACYLTRANSFERASE* (*3AT*) but only MYBA1 induced *F3'5'H* in concordance with the low proportion of tri-hydroxylated anthocyanins found in *MYBA6-A7* HR. These loci are related to the red/cyanidic pigmentation of vegetative organs in black and white-skinned cultivars and form part of the UV-B radiation response pathway orchestrated by ELONGATED HYPOCOTYL 5 (VviHY5). These results demonstrate the involvement of additional anthocyanin regulators in grapevine and suggest an evolutionary divergence between the two grape color loci for controlling additional targets of the flavonoid pathway.

Introduction

Colors are omnipresent in living organisms and rely on the presence of iridescent structures or the accumulation of pigments. In plants, pigments accumulate in different organs providing a wide range of signals to animals. Among these, anthocyanins provide a variety of colors to flowers, fruits and vegetative organs, ranging from red and orange to blue and violet. In addition to their role as co-evolutionary traits, anthocyanins have many other functions in the plant kingdom, such as filtering and attenuating ultraviolet radiation, scavenging free radicals (antioxidant activity) and modulating reactive oxygen signaling cascades involved in growth, development and stress adaptation (reviewed by Hatier and Gould, 2009).

Anthocyanins are synthesized as part of the flavonoid branch within the phenylpropanoid pathway. The transcriptional network regulating flavonoid accumulation is conserved in eudicots (Albert et al., 2014; Albert, 2015). Grapevine (*Vitis vinifera* L.) is an interesting model for examining this regulation due to the expansion and diversification of genetic factors controlling the synthesis of these compounds (Hichri et al., 2011; Wong et al., 2016). In this species, anthocyanins can accumulate in both reproductive and vegetative organs in a cultivar and environmental-specific manner. Despite this, all of the attention has been directed to their presence in the berries, given that they are crucial in determining the quality of fruit and wine, and due to their health benefits on consumption.

The genes coding for the enzymes of the flavonoid pathway are transcriptionally regulated by the combinatorial interaction of MYB, basic helix-loop-helix (bHLH) and WD40 transcription factors (TF). As in fleshy fruit species, both MYB and bHLH factors denote gene target specificity (Heppel et al., 2013; Montefiori et al., 2015). In grape, distinctive R2R3-MYB TFs have been associated with the regulation of anthocyanin synthesis, from which at least four have been directly demonstrated to control this branch *in planta* (*MYBA1*, Kobayashi et al., 2004; *MYBA2*, Walker et al., 2007; *MYB5B*, Cavallini et al., 2014; *MYBC2-L1*, Cavallini et al., 2015). These factors activate or repress different steps of the pathway, restricting anthocyanin accumulation to specific developmental stages and in response to environmental factors and agricultural practices (reviewed by Matus, 2016).

The color of the grape berry skin (ranging from green or white to grey, pink, red and black) relies on the allelic condition of a major locus on chromosome 2 (chr2,

reviewed by Pelsy, 2010). This berry color locus harbors at least two functional R2R3-MYB genes; *MYBA1* and *MYBA2* (Walker et al., 2007), and represents the major genetic determinant of skin color variation (Fournier-Level et al., 2009). The simultaneous occurrence of different types of mutations in these genes is responsible for the un-pigmented phenotype of most white-skinned cultivars. These mutations have been largely assessed in different genotypes and somatic variants (Lijavetzky et al., 2006; Migliaro et al., 2014) and can be grouped in: i) inactivation of *MYBA1* through the insertion of the *Gret1* retrotransposon in its promoter/5'UTR region (Kobayashi et al., 2004; 2005), ii) non-synonymous single-nucleotide polymorphisms 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). Other less frequent genetic alterations may result in loss of pigmentation (pink-skinned phenotypes), such as short intron insertions that impede a correct splicing of the *MYBA1* mRNA (Shimazaki et al., 2011). Reversions from mutated-to-functional allelic versions may occur by excision of *Gret1* (This et al., 2007) or by illegitimate homologous recombination between *MYBA1* and *MYBA3* (Azuma et al., 2009). All these previous studies confer sufficient evidence for the genetic variability of anthocyanin composition in fruit tissues but do not explain pigmentation in other organs. Indeed, red pigmentation is typically observed in the field in vegetative organs of white cultivars at very early developmental stages (e.g. leaves just after bud burst) or in response to environmental stresses (e.g. excessive radiation). Recently, Blanco-Ulate et al. (2015) demonstrated that the anthocyanin pathway was transcriptionally modulated by *Botrytis cinerea* infection in white-skinned berries of cv. 'Semillon', but this response was independent of *MYBA1* or *MYBA2* expression. All these data suggest the presence of additional regulators of anthocyanin synthesis.

Grape *R2R3-MYB* genes related to flavonoid synthesis have greatly expanded by segmental and tandem duplications (Matus et al., 2008; Wong et al., 2016). However, these events are exclusive to the anthocyanin, proanthocyanidin (PA) and C2- repressor motif clades (Subgroups 6, 5 and 4, respectively), excluding other closely related groups such as the flavonol (Subgroup 7) or trichome (Subgroup 15) clades. These studies have pioneered the isolation and characterization of several regulators of PA and anthocyanin synthesis (Terrier et al., 2009; Huang et al., 2014; Koyama et al., 2014; Cavallini et al., 2015), demonstrating that these expansion events had originated functional, and thus sub-specialized members. Finding novel anthocyanin-related MYB genes in the

grapevine genome suggests that pigmentation is a complex trait possibly involving distinct loci in its regulation.

The following work describes a genomic region of chr14 containing the closely related genes *MYBA5*, *MYBA6* and *MYBA7*. To demonstrate that these genes control anthocyanin synthesis, we ectopically expressed them in grapevine hairy roots. Further transcriptomic and metabolomic analyses, together with transient gene reporter assays, demonstrated their ability to activate the anthocyanin branch. These genes shared many targets with *MYBA1*, but were insufficient in promoting the accumulation of tri-hydroxylated anthocyanins. We searched for regulatory components of these new *MYBA* genes and show that they form part of the UV-B response signaling, and that this regulation is in part achieved by the photomorphogenic factor ELONGATED HYPOCOTYL 5.

Results and Discussion

Sequence comparison of *R2R3-MYB* genes within the anthocyanin-related clade

In Arabidopsis, anthocyanin synthesis is regulated by four R2R3-MYB proteins belonging to Subgroup 6 (Dubos et al., 2010), all of which act at different stages of plant development and in response to diverse stimuli (Gonzalez et al., 2008). In *V. vinifera*, MYBA1 and MYBA2 directly regulate and switch on the expression of anthocyanin-related genes, including *UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE* (*UFGT, VIT_16s0039g02230*) and *ANTHOCYANIN 3-O-GLUCOSIDE-6''-O-ACYLTRANSFERASE* (*3AT*, *VIT_03s0017g00870*) by the onset of berry ripening (Walker et al., 2007; Rinaldo et al., 2015). Recently, large-scale transcriptomic data and gene co-expression networks have supported the existence of several additional targets in the late anthocyanin biosynthetic branch that are activated at veraison (Fasoli et al., 2012; Palumbo et al., 2014; Wong et al., 2016).

The sequencing of the highly homozygous grapevine genome of Pinot Noir PN40024 (8.4X assembly, Jaillon et al., 2007) initially allowed the prediction of 108 R2R3-MYB genes (Matus et al., 2008). To date, this number has increased to 134 (Wong et al., 2016). Several genes belonging to Subgroup 6 (anthocyanin-related) were identified within this updated collection: a group of nine R2R3-MYB genes was found in a 150 kb cluster on chromosome 2 (chr2, Figure 1A), including the anthocyanin

regulators *MYBA1* and *MYBA2*. The remaining seven consist of *MYBA3* and *MYBA4*, shown to be truncated proteins and non-functional (Walker et al., 2007), and five uncharacterized *MYBA-like* genes that with the exception of *MYBA8* (*VIT_02s0033g00380*, truncated) all codify for proteins with similar sizes to MYBA1. Part of this group may represent pseudogenes, a subject yet to be demonstrated. In addition to the cluster in chr2, we found five other anthocyanin-related *MYB* genes distributed on chr1 (*VIT_01s0011g01190* and *VIT_01s0011g01200*) and chr14 (*VIT_14s0006g01340*, *VIT_14s0006g01290* and *VIT_14s0006g01280*). A deeper analysis of the 440 kb cluster of chr14 showed that the three protein sequences derived from these models possessed the characteristic PRPR[S/T]F motif of Subgroup 6 (Stracke et al., 2001), suggesting their involvement in the control of the anthocyanin pathway. We therefore named them *MYBA5*, *MYBA6* and *MYBA7*, respectively.

Inspection of previously published RNA-Seq data from cv. 'Corvina' (Venturini et al., 2013) confirmed the integrity (sequence) and expression of *MYBA5*, *MYBA7* and two spliced versions of *MYBA6*. We designed primers in the *MYBA6* coding and untranslated regions to amplify these transcript variants (Supplemental Figure 1) and cloned three mRNAs corresponding to *MYBA6.1* (Genebank accession no. FJ556912), *MYBA6.2* (Genebank accession no. FJ556913) and *MYBA6.3* (Genebank accession no. FJ556914). The sequence of *MYBA7* was also cloned for further characterizations (Genebank accession no. FJ265729). *MYBA6.2* results from a non-canonical exon-intron junction while *MYBA6.3* and the second variant identified in the RNA-Seq data (*MYBA6.4*) consist of intron-retaining versions of *MYBA6* (Figure 1B). In the case of *MYBA6.2*, a premature splicing event in exon 2 and a delayed start of exon 3 produce a protein of identical sequence to MYBA6.1, except for the absence of the R3 repeat of the DNA-binding domain. As single-R proteins can still bind to DNA and control the expression of target genes (Wang et al., 2008), we hypothesized that the MYBA6.2 protein may still regulate transcription in grapevine. *MYBA6.3* and *MYBA6.4* mRNAs potentially code for truncated proteins, due to a premature stop codon in their retained intronic sequences.

Sequence comparison of grape MYBA proteins with members of Subgroup 6 from other species allowed the identification of the more frequently diverging residues within the highly conserved DNA binding domain (Supplemental Figure 2), in addition to several other features from anthocyanin regulators that are also present in these

proteins (Supplemental Figure 3). As described by Heppel et al. (2013) an Arginine at position 39 in the R2 domain combined with a four amino acid Ala–Asn–(Asp/Ala)–Val signature between positions 90 to 93 in the R3 domain favor specificity towards the anthocyanin pathway. As seen in Supplemental Figure 2, MYBA5/A6/A7 share the same residues except for an Asp-to-Ser change in the first position of the R3 domain signature. According to Lin-Wang et al. (2010) this residue appears in over 5% of cases of other anthocyanin regulators of the Rosaceae lineage, suggesting that it is a functional residue. These residues are adjacent to those that bind DNA directly (Ogata et al., 1994; Solano et al., 1997), raising the importance of their conservation in promoter binding specificity.

As shown by their phylogenetic analysis (Supplemental Figure 3A), one interesting feature concerning these new MYBA proteins is that they are positioned in the anthocyanin clade at a similar distance between grape MYBA1-2 homologues and anthocyanin MYB regulators belonging to other plant species. In fact, a deeper analysis of their sequences shows that MYBA proteins from chr2 exclusively possess a C-terminal domain consisting of 11 residues, termed the A1-related motif (A1M, Supplemental Figure 3B-C, Supplemental Figure 4).

Ectopic expression of MYBA6 and MYBA7 in grapevine hairy roots promotes the accumulation of di-hydroxylated anthocyanins

Due to the extremely high sequence similarity between MYBA5 and MYBA6, we decided to investigate the function of MYBA6.1, its single-R spliced version MYBA6.2 and MYBA7. We ectopically expressed them in hairy roots (HR) of the white berry-skinned cultivar (cv.) 'Maccabeu'. HR devoid of any transgene and *MYBA1* overexpressing HR were included as negative and positive controls for anthocyanin accumulation, respectively. From all the transgenic clones obtained, *MYBA6.1* (4 clones) and *MYBA7* (2 clones) exhibited pink/reddish pigmentation (Figure 2A), whereas *MYBA1* hairy roots presented a stronger dark-red appearance (Supplemental Figure 5A, Cutanda-Perez et al., 2009). None of the four positive clones for *MYBA6.2* showed a red coloration and appeared identical to the untransformed HR.

In correlation with the observed phenotypes, metabolite-profiling analyses of MYB overexpressing (OE) HR revealed that MYBA6.1 and MYBA7 were able to induce an increase of total anthocyanins, though to a lesser extent compared to MYBA1

(Figure 2B). *MYBA6.1*-OE lines showed higher percentages of non-acylated anthocyanins compared to MYBA1, suggesting differences at the expression level or enzymatic activity of anthocyanin acyltransferases. The relative amount of acylated forms in *MYBA7*-OE lines was between those obtained for MYBA1 and MYBA6. Interestingly, *MYBA6.1* and *MYBA7*-OE HR exhibited a lower proportion of tri-hydroxylated anthocyanins (Figure 2B, Supplemental Figure 5B). The content of malvidin derivatives was in fact reduced in *MYBA6.*1 and completely absent in *MYBA7* overexpressing hairy roots. Similarly, delphinidin derivatives were only present in the *MYBA1*-OE HR. The overexpression of all four MYB genes did not affect the accumulation of PAs whose total amounts remained unchanged (Supplemental Figure 5C). The accumulation of stilbenoid compounds decreased depending on the type of stilbenoid (Supplemental Figure 5D). *Cis*-piceids showed the most dramatic decrease in *MYBA6.1, MYBA7* and *MYBA1-OE* lines compared to HR devoid of transgene.

Modulation of anthocyanin biosynthetic genes by MYBA6 and MYBA7

To gain a deeper insight into the quantitative differences found between these potential anthocyanin regulators, we further selected one *MYB-OE* HR line for each gene based on their similar transgene expression levels (Supplemental Figure 6), and evaluated their transcriptomes by microarray analysis. Three biological replicates of each transgenic HR were considered for the NimbleGen microarray Vitis exp HX12 (Roche, NimbleGen Inc.) that allows the expression analysis of 29,549 unigenes. To identify the gene expression profiles with the greatest contribution to the differences between each transgenic line and the untransformed-HR transcriptome, a multiclass comparison analysis was carried out using Significance Analysis of Microarray (SAM) with a false discovery rate (FDR) of 0.3% (TMev 4.3). Considering a fold change > 1.9, 2294 oligonucleotides presented an increase in their hybridization signal for *MYBA6.1-OE*, 1908 for *MYBA6.2-OE*, 2129 for *MYBA7-OE* and 841 for *MYBA1-OE* (Supplemental Table 1). All transcripts were annotated against the 12Xv1 release of the grapevine genome (http://genomes.cribi.unipd.it/DATA/).

The ectopic expression of the four *MYB* genes in the cv. 'Maccabeu' HRs resulted in the modulation of genes belonging to 19 functional categories, as evidenced by Gene Ontology (GO) analysis (Supplemental Figure 7). By discerning between up-

and downregulated genes, we found that those related to 'secondary metabolic process' and 'cellular process' were among the most upregulated for *MYBA1-OE* and *MYBA6.1-OE*, whereas in *MYBA7-OE*, genes related to secondary metabolism were less representated. In the case of *MYBA6.2-OE*, all functional categories were represented by a similar percentage of both up- and downregulated genes. The 'signal transduction' category was among the most represented terms in the downregulated genes in all overexpressors (Supplemental Figure 7).

As anthocyanins are synthesized via the shikimate and phenylpropanoid pathways, we considered the complete set of the corresponding modulated genes. We found that a larger numbers of genes were activated in *MYBA6.1*-OE, followed by *MYBA1-OE* and *MYBA7-OE* (Figure 2C, Supplemental Table 2). However, a larger activation (reflected as fold change values) was observed when *MYBA1* was overexpressed. Many of the activated phenylpropanoid-related genes were shared by *MYBA1-*, *MYBA6.1-* and *MYBA7-OE* datasets (Supplemental Figure 8A, Table 1). In the case of downregulated genes, a shared set corresponded to *STILBENE SYNTHASES* and uncharacterized genes that possibly belong to the early steps of the pathway (i.e. necessary for the synthesis of small weight phenolic compounds, Supplemental Figure 8B). In the case of MYBA6.2, the number of phenylpropanoid-downregulated genes was larger compared to the rest (Figure 2C).

A comparison between the lists of upregulated phenylpropanoid-related genes obtained by all microarray analyses revealed that MYBA6.1, MYBA7 and MYBA1 all induce genes specifically related to anthocyanin synthesis (Table 1). This common gene set showed the highest gene expression modulation in the three transgenic lines *MYBA6.1-*, *MYBA7-* and *MYBA1-OE* (Table 1, Supplemental Table 1). In the case of MYBA6.2, despite the large number of DEGs encountered, none of the upregulated genes were clearly related to the anthocyanin pathway.

Among anthocyanin late biosynthetic genes induced by all three R2R3-MYBs, we found *UFGT* (*VIT_16s0039g02230), ANTHOCYANIN-O-METHYLTRANSFERASE 1, 2* and *3* (*AOMT1-3*, *VIT_01s0010g03510, VIT_01s0010g03490* and *VIT_01s0010g03470*), *3AT* (*VIT_03s0017g00870*) and the vacuolar transport-related *GLUTATHIONE-S-TRANSFERASE 4* (*GST4*, *VIT_04s0079g00690*). A putative *UDP GLUCOSE:FLAVONOID 7-O- GLUCOSYLTRANSFERASE* (*VIT_09s0002g06590*) and

a still uncharacterized gene annotated as "*ANTHOCYANIN MEMBRANE PROTEIN 1*" (*ANM1*, *VIT_08s0007g03560*) defined as a SOLUTE CARRIER 35 (SLC35), were also induced. In addition to *UFGT* and *3AT*, all the remaining genes represent high confidence targets based on the high expression observed here and in previous transcriptomic/co-expression studies (Cutanda-Perez et al., 2009; Fasoli et al., 2012; Palumbo et al., 2014). This metabolite and transcriptomic profiling of HRs provides the first evidence that MYBA6 and MYBA7 are regulators of the anthocyanin branch. The control of their putative downstream targets (*UFGT*, *GST4*, *AOMT,*[1] *3AT, ANTHOMATE 3, ANM1*) was further extrapolated and confirmed by quantitative PCR (qPCR) for all the transformed HR lines obtained (Supplemental Figure 6B).

Some early flavonoid structural genes (those common for the anthocyanin, PA and flavonol branches) were differentially regulated by the R2R3-MYBs. Four *CHALCONE SYNTHASE (CHS)* and three *FLAVONOID-3'-5'-HYDROXYLASE (F3'5'H)* genes were strongly induced by MYBA1 and mildly by MYBA6.1 (Table 1). Several other members of the *F3'5'H* family were exclusively induced by MYBA1. As F3'5'H enzymes are responsible for the tri-hydroxylation of flavonoid backbones, our transcriptomic data suggests that only MYBA1 is significantly capable of inducing these modifications. This hypothesis is consistent with our metabolic data, where *MYBA1-OE* shows a higher proportion of the tri-hydroxylated delphinidin, petunidin and malvidin derivatives. Accordingly, only in *MYBA6.1-OE*, three *FLAVONOID 3'-HYDROXYLASE (F3'H)* genes (related to the synthesis of the di-substituted cyanidin and peonidin, Forkmann, 1991; Winkel-Shirley, 2001) were found slightly upregulated (Supplemental Table 1-2). These results highlight the activation of different sub-branches for anthocyanin synthesis as a possible functional difference between the R2R3-MYBs from chr2 and chr14.

As previously documented in grapevine, we also show that the ectopic activation of *R2R3-MYBA* genes modified the expression of several other *R2R3-MYB* family members with either activation or repression activities (Supplemental Table 1; Cutanda-Perez et al., 2009; Terrier et al., 2009; Cavallini et al., 2015). The still uncharacterized *MYB196* gene (*VIT_16s0039g01920*) was highly induced in *MYBA6.1*, *MYBA6.2* and *MYBA7-OE* HR. This gene was recently shown to be associated to cytochromes P450 and biotic stress responses (Wong et al., 2016). A strong upregulation of *MYBA6* expression was detected in *MYBA7-OE*, suggesting the existence of a transcriptional

control between the two MYBs. Our data also shows that a homolog of Arabidopsis *CAPRICE* (*VviCPC*, *VIT_12s0059g02360*) was induced in *MYBA1*, *MYBA6.1* and *MYBA7-OE* lines. In Arabidopsis, CPC acts as a positive regulator of root hair development and a repressor of anthocyanin synthesis (Zhu et al., 2009). Additionally, four members of the MYB C2-motif repressor clade were induced in MYBA1 HR. These data suggest the existence of a conserved regulatory mechanism that fine-tunes the synthesis of flavonoids.

MYBA6 and MYBA7 interact with components of the MBW complex and activate the promoter of anthocyanin related genes

The involvement of a MYB-bHLH-WD40 (MBW) protein complex in the regulation of the anthocyanin pathway has been largely studied in model herbaceous and crop species. R2R3-MYB proteins are known to play a central role in this regulatory mechanism by determining the specificity of the complex for the different target genes (Koes et al., 2005). Grapevine MYBA1-A2 proteins are not able to induce their target gene promoters in the absence of bHLH- and WD40-interacting cofactors (Hichri et al., 2010). The presence of the conserved amino acid signature [D/E]Lx2[R/K]x3Lx6Lx3R in the R3 domain of MYBA5, A6 and A7 suggested that these proteins also interact with bHLH proteins belonging to the III-f sub-group. We performed a yeast two-hybrid assay to test the ability of MYBA proteins to physically interact with the bHLH grapevine flavonoid regulator MYC1 (Hichri et al., 2010). The TF MYBA1 was used as a positive control in the assay. MYB proteins were fused with the GAL4 activation domain (GAL4-AD) and the bHLH factor with the GAL4 binding domain (GAL4-BD). The reciprocal combination could not be performed because of the inherent ability of MYB to activate the transcription of all the yeast reporter genes when fused to GAL4-BD (*data not shown*). Our results showed the ability of MYBA6.1 and MYBA7 to interact with MYC1 (Figure 3A) suggesting their participation in the MBW regulatory complex controlling the flavonoid pathway. On the other hand, the single-R MYBA6.2, which lacks the R3 repeat (and thus the bHLH-interacting signature) was unable to interact with MYC1.

To determine the capacity of MYBA6 and MYBA7 to regulate the transcription of anthocyanin structural genes, a transient expression assay was conducted using cv.

'Chardonnay' grapevine suspension cell cultures and a dual luciferase reporter system (Horstmann et al., 2004). The response of the previously isolated promoter fragments of *UFGT* and *F3´5´H* to the transient expression of different grape MYBs (Bogs et al., 2007) was tested. We also isolated a 1828 bp promoter sequence of *3AT* and fused it to the *LUCIFERASE* gene for this purpose. The induction of the *UFGT* promoter activity by MYBA6.1 and MYBA7 was dependent on the presence of the grapevine bHLH factor MYC1 in the same manner as for MYBA1 (Figure 3B); for this reason, all subsequent transfections were performed in the presence of this bHLH factor. Cell suspensions transiently expressing these promoter:luciferase reporter constructs showed increases in the promoter activity of 50-to-100-fold for *UFGT* and ~10-fold for *3AT* when co-transformed with MYBA6 or MYBA7, in comparison to the control bombardments in the absence of these TFs. These values were doubled or tripled when co-transformed with MYBA1, suggesting an extended ability of MYBA proteins from chr2 to regulate common MYBA targets (similar results were obtained with MYBA2, *data not shown*). The exclusive activation of *F3´5´H* promoter by MYBA1 (and to a greater extent by MYBPA1) reinforces the idea that MYBA6 and MYBA7 lack the capacity to activate the tri-hydroxylated branch of the anthocyanin pathway, although this should be further demonstrated in the rest of the *F3'5'H* gene family. MYBA6.2 was unable to activate any of the three promoters tested, a fact probably related to its failure to interact with MYC1. Taken together, the results of these transient promoter and yeast two-hybrid experiments strongly support the hypothesis that MYBA6 and MYBA7 have a role in the transcriptional regulation of anthocyanin biosynthesis in grapevine.

The expression of newly identified MYBA genes correlates with anthocyanin accumulation in vegetative organs of red and white-skinned cultivars

We used the global gene expression atlas of cv. 'Corvina' (Fasoli et al., 2012) for establishing the expression domains of *MYBA5*, *MYBA6* and *MYBA7*. The data was compared to the expression of *MYBA1, MYBA2* and *MYBA3* (Walker et al., 2007) in order to gain information about their *in planta* roles as anthocyanin regulators and to evaluate any sub specialization and diversification among all *MYBA* genes.

The heatmap reported in Figure 4A shows that several organs possess similar *MYBA5, MYBA6* and *MYBA7* expression patterns that greatly differ from those of *MYBA1, MYBA2* and *MYBA3*. The highest transcript levels of *MYBA5, MYBA6* and *MYBA7* were detected in vegetative organs and green berry stages while *MYBA* genes from chr2 were specific to reproductive organs at ripening stages (i.e. after veraison). *MYBA5* and *MYBA6* are preferentially expressed in young and well-developed tendrils, in young leaves, green stems, buds at bud break and after bud break and finally, in seedlings. Despite the high similarity between *MYBA5* and *MYBA6,* their corresponding Nimblegen probes were designed in the most divergent regions of their sequences, and the atlas shows differential expression in senescent leaves, seedlings and stems. *MYBA7* expression is instead high in mature leaves, seedlings and most of the bud developmental stages. In berry tissues, seeds and rachis, *MYBA5, MYBA6* and *MYBA7* are highly expressed in early development (fruit set and post fruit set) and start decreasing after the onset of ripening (veraison).

We performed a second heatmap that included the expression data of several characterized flavonoid structural genes and we removed from the set of organs/developmental stages those characterized by high *MYBA1* gene expression. As a result, we confirmed a strong transcriptomic relationship between *MYBA5*, *MYBA6* and *MYBA7* and the anthocyanin-related genes *AOMT1, GST4, UFGT, LDOX* and *3AT* (Supplemental Figure 9A). Moreover, hierarchical clustering revealed that these genes are co-expressed specifically in vegetative organs (i.e. stems, leaves, inflorescence, tendrils, buds and seedlings; Supplemental Figure 9B).

Our data allowed us to hypothesize that MYBA5, MYBA6 and MYBA7 are anthocyanin regulators specifically acting in vegetative organs. In order to validate this hypothesis we attempted to correlate their expression with the presence of anthocyanins in organs such as young leaves and tendrils of cv. 'Pinot Noir' and buds of cv. 'Corvina'. These results were then combined with the anthocyanin profiles obtained by HPLC-MS (Figure 4B, Supplemental Figure 9C). In all red vegetative organs, *MYBA1* expression was not related to anthocyanin accumulation, thus excluding MYBA1 as the regulator responsible for this pathway in young leaves, buds and tendrils. A higher *MYBA6.1* expression was specifically detected in leaves and tendrils with anthocyanin pigmentation, while *MYBA7* transcript levels were higher in red buds and, to a lesser extent, in red tendrils, when compared to the corresponding green organs. Therefore, the

accumulation of anthocyanins in these organs correlates with an increase of *MYBA6.1* and *MYBA7* expression and not with *MYBA1*.

The metabolic profile of leaves, tendrils and buds determined by HPLC-MS revealed a preferential accumulation of peonidin 3-glucoside and cyanidin 3-glucoside, which were more abundant in the pigmented organs (Supplemental Figure 9C). The higher content of these di-hydroxylated anthocyanins, whose synthesis is mediated by F3'H, resembles the metabolic profiles of the HR overexpressing *MYBA6.1* and *MYBA7* (Figure 2B). This strengthens the idea that these TFs are mostly responsible for the synthesis of di-hydroxylated anthocyanins, by passing through F3'H enzymes. On the contrary, the low or null expression of *MYBA1* may explain why red vegetative organs accumulate fewer tri-substituted delphinidin-, malvidin- and petunidin-derivatives (product of F3'5'H action). MYBA6.1 did not activate the promoter of *F3'5'H VIT_06s0009g02830* (Figure 3B) and only faintly induced the expression of *VIT_06s0009g02970*, *VIT_06s0009g02840* and *VIT_06s0009g02810* in transgenic HR (Table 1). As seen in the transcriptomic or dual luciferase assays, these *F3'5'Hs* were activated by MYBA1, corroborating a limited ability of MYBA6 to affect the synthesis of tri-substituted anthocyanins.

To rule out the possibility that MYBA1 or MYBA2 could be somehow responsible for the pigmentation observed in vegetative organs, we analyzed leaves from white berry-skinned cultivars. These are characterized by both the presence of the *Gret1* retrotransposon in their *MYBA1* promoter regions and a non-functional *MYBA2* allele as previously reported by Walker et al. (2007). Reddish pigmentation in vegetative organs of white berry-skinned cultivars is generally observed in plants grown in the field, and has been previously reported in cv. 'Semillon' grapes infected with the noble rot agent *B. cinerea* (Blanco-Olate et al., 2015). In order to associate a potential occurrence of anthocyanins with the expression of *MYBA* genes in leaves of white cultivars, we sampled fully expanded leaves of cv. 'Sauvignon blanc' and 'Chardonnay' with different degrees of coloration (either light green or dark purplish green, Supplemental Figure 10).

After individually collecting and quantifying the leaf samples for the presence of anthocyanins, we found a relation between dark colored leaves and a higher presence of anthocyanins (ranging from 30 to 50 μg/g fresh weight, Figure 5A). In correlation with the anthocyanin quantification, we found higher expression of *MYBA6* and *MYBA7* in dark colored leaves (Figure 5B). In both cultivars, neither *MYBA1* nor *MYBA2* were

expressed. Instead, the higher amount of anthocyanins detected in pigmented leaves was consistent with the higher expression levels of *MYBA6* and *MYBA7,* confirming that MYBA6 and MYBA7 are the specific regulators of anthocyanin synthesis in vegetative organs. These results suggest that the regulatory functions of the different MYBA TFs may not be redundant, as already shown by the microarray data of transgenic HR (Supplemental Table 1), and indicate that this new anthocyanin-related locus on chr14 controls pigmentation in organs and developmental stages where MYBA1 and MYBA2 are not active. Despite this, we cannot rule out that in particular cases the pigmentation of vegetative organs of red cultivars could also be promoted by MYBA1. This is for example the case of anthocyanin accumulation in GLRaV virus-infected leaves (Gutha et al., 2010), senescing leaves (as seen in the expression heatmap of Figure 4) or in leaves from '*teinturier*' (red-fleshed) genotypes (Jeong et al., 2006).

Environmental control of *MYBA6* and *MYBA7* expression

In Arabidopsis, *AtPAP1* is induced in response to photomorphogenic UV-B exposure and activates anthocyanin synthesis. This response is directly controlled by the bZIP transcription factor ELONGATED HYPOCOTYL 5 (AtHY5) as it binds to ACGT-containing elements in the *AtPAP1* promoter region after the radiation signaling pathway is activated (Shin et al., 2013). We recently characterized the grapevine HY5 ortholog (VviHY5), which directly regulates flavonol synthesis by activating *MYBF1* and its targets *FLS4* and *GT5* (Loyola et al., 2016). Here, *HY5* expression was correlated to that of *MYBA* genes in vegetative and reproductive organs exposed to irradiating UV-B (Figure 6). The induced expression of *VviHY5* upon the UV-B stimulus correlated with that of *MYBA6* and *MYBA7* in both plantlets and fruits (Figure 6A-B). *MYBA1*, on the contrary, was not induced in plantlets exposed to UV-B, whereas in fruits it was positively regulated in response to UV-B radiation but only in the latest sampled ripening stage and in an opposite pattern compared to *VviHY5*. The activation of the anthocyanin pathway following UV-B treatment is corroborated by the activation of the structural anthocyanin genes *AOMT1*, *AOMT2* and *GST4* (Figure 6A), which despite also being targets of MYBA1, were not correlated to its expression.

The general tendency of *MYBA6* and *MYBA7* of being downregulated towards berry ripening (also seen in Figure 4A) is delayed in response to UV-B, following the exact same expression pattern as *VviHY5*. This observation suggests that under severe environmental conditions such as increased radiation, the vegetative color locus may

also influence fruit pigmentation at least at the beginning of ripening. In fact, this was supported at the metabolite level, as the amounts of total di-hydroxylated anthocyanin derivatives found at harvest (9 weeks after veraison, WAV) was increased in response to UV-B, in correlation to the increase in the expression of *MYBA6* and *MYBA7* (Supplemental Figure 11, Supplemental Table 3). However, the total amount of malvidin, delphinidin and petunidin derivatives was not statistically different between irradiated and non-irradiated conditions, probably due to the fact that the increase in *MYBA1* expression in the last week was insufficient to increase tri-hydroxylated anthocyanins levels in response to UV-B.

Flavonol structural and regulatory genes are controlled by bZIP TFs in grapevine and present several MYB and bZIP binding sites in their promoters (Czemmel et al., 2009; Malacarne et al., 2016; Loyola et al., 2016). Thus, we searched *MYBA* promoter sequences and found that all three *MYBA* genes from chr14 had ACE elements in a similar distribution and proximity to the transcriptional start site (TSS) of those found in the *AtPAP1* promoter (Figure 6C; two close bZIP binding sites are bound by AtHY5 as shown by Shin et al., 2013). A similar distribution was observed for MYB recognition elements (MRE) that had been previously associated to the autoregulatory activation of anthocyanin MYB factors (Espley et al., 2009). Despite the fact that the *MYBA1* promoter presents more distant elements to the TSS, this observation is insufficient to prove the hypothesis that HY5 does not regulate *MYBA1*. Next, we tested the activation of *MYBA* genes by transiently expressing a VP16x4 (VP64)-VviHY5 fusion protein in agroinfiltrated cv. ‘Sultana’ plantlets as described in Loyola et al. (2016). After keeping plants in low light conditions (avoiding high basal levels of the endogenous *HY5* caused by light induction), plants were tested. Those with the highest expression of the transgene also had increased levels of *MYBA6* and *MYBA7*, but not *MYBA1* (Figure 6D). Our results suggest that MYBA6 and MYBA7 are part of the photomorphogenic response to UV (specifically involved in the production of UV-absorbing compounds) and that VviHY5 orchestrates this response.

Insights into the evolution of grape anthocyanin-related MYBs from subgroup 6

The size and diversity of the R2R3-MYB family in the plant kingdom has been largely influenced by segmental duplications in large chromosome regions (Du et al., 2015). However, and especially regarding the anthocyanin clade (Subgroup 6), tandem duplications have also largely contributed to MYB expansion and divergence (Cannon et al., 2004). In grapevine, all *MYBA* genes from chr2, 1 and 14 arose from tandem and proximal duplication events but only the latter two share synteny (Wong et al., 2016). *MYBA5*, *A6* and *A7* are located at the beginning of a region that is packed with many other *R2R3-MYB*s that arose from segmental and dispersed duplications. On the other hand, *MYBA* genes from chr2 are inside a region (paralogue to chr15 and chr16) almost devoid of *MYB* genes (Wong et al., 2016). Additional differences between MYBs from chr2 and 14 were identified in the phylogenetic analysis, showing a clear separation from each other and different proximities to anthocyanin-promoting MYBs from other species (Supplemental Figure 3). Thus, it is possible that *MYBA* genes from either chromosome had diverged at some point in evolution, acquiring the capacity to regulate anthocyanin synthesis with different efficiencies and even gaining different expression domains and regulation.

The idea of MYBA sub-functionalization in grapevine is reinforced by the presence of a terminal motif found in MYBA1-related proteins (Supplemental Figure 4). In order to test this hypothesis, we generated different MYBA1 mutant variants by removing the C-terminal A1M and M3 motifs (Figure 7A, Supplemental Figure 12) and testing them for the activation of the *UFGT*, *3AT* and *F3'5H* gene promoters. When the A1M motif was absent (like in MYBA6) the activation of *UFGT* and *3AT* by MYBA1 dropped to a similar level as when MYBA6 was transfected (Figure 3B). Removing the M3 motif decreased this activation even further. Thus, both A1M and M3 may be responsible for the intensity of the activation of late anthocyanin biosynthetic genes. However, these motifs do not appear to be responsible for the activation of the early flavonoid *F'3'5H* gene promoter, and probably this is controlled by other divergent residues between MYBA1 and MYBA6-A7. In fact, different residues can be found in the M1 or M2 motifs, or in the dicots anthocyanin-promoting motif 2 (DAPM2, Heppel et al., 2013), located within the R3 repeat. As described earlier, MYBA5, A6 and A7 share an Asp-to-Ser change at the beginning of this element, and this change is also present in other regulators found in the Rosaceae lineage.

Based on these results it is plausible to suggest that the increased activity of MYBA1 versus MYBA6-A7 is in part due to the presence of the A1M motif and that further testing would be needed to check if this motif defines specificity in regulating other *F3'5'H* promoters or if other residues are responsible for *F3'5'H*-exclusive activation by MYBA1.

Reconciling all our results, we propose that R2R3-MYB regulators from chromosome 14, found in a region we refer to as the new vegetative color locus, regulate anthocyanin pigmentation in the vegetative organs of the grapevine. Our results also suggest that like MYBA1, the regulatory mechanism of MYBA6 and MYBA7 is mediated by their participation in the MBW complex. However, we showed that the vegetative color locus possesses quantifiable differences compared to the berry color locus, especially regarding gene target and metabolic sub-branch activation. When overexpressed in the grapevine hairy root system, MYBA6 and MYBA7 almost exclusively promoted the accumulation of cyanidin and peonidin, in contrast to the well-characterized MYBA1, which promotes the synthesis of larger amounts of tri-hydroxylated anthocyanins. The selective induction of the di- or tri-hydroxylated branches within the anthocyanin pathway was maintained when testing the capacity of MYBA proteins to activate different anthocyanin-related promoters and also when comparing organs exposed to higher ultraviolet radiation conditions. These new genes are largely regulated at the transcriptional level by development and light, constituting a new mechanism for integrating ultraviolet radiation to control anthocyanin biosynthesis in grapevine. *MYBA* genes show differential responses to UV-B in a tissue-specific and time-dependent manner and it seems that these differences are closely related to the transcriptional regulation exerted by the photomorphogenic factor HY5.

Methods

Gene Cloning

Grapevine organs (leaves, inflorescences, tendrils and berry skins from different growth stages) were collected from the cv. 'Cabernet Sauvignon' in a commercial vineyard in the Maipo Valley, Chile (latitude 33°36'S, longitude 70°39'W). Reddish roots that

developed from canes grown in a hydroponic system in a greenhouse were also sampled. Organs were frozen in liquid nitrogen and stored at -80°C.

Based on the updated identification of the grape *R2R3-MYB* family (Wong et al., 2016), primers were designed (Supplemental Table 4) for amplifying the coding sequences and untranslated regions of *MYBA6* (*VIT_14s0006g01290*) and *MYBA7* (*VIT_14s0006g01280*). In the case of *MYBA6*, different bands were observed depending on the cDNA samples used. *MYBA6.1* was amplified from a mixture of red root, tendril and veraison berry skin cDNA, *MYBA6.2* was amplified from veraison berry skin cDNA, *MYBA6.3* was amplified from root cDNA and *MYBA7* was amplified from red root cDNA. The resulting PCR products were purified and directionally cloned into the pENTR/ D-TOPO vector (Invitrogen) and verified by sequencing.

Bioinformatics

Grapevine MYB proteins were aligned against the full-predicted amino acid sequences of anthocyanin-related proteins belonging to *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa*, *Petunia x hybrida*, *Antirrhinum majus*, *Fragaria x ananassa*, *Malus x domestica*, *Prunus avium*, *Capsicum annuum*, *Solanum lycopersicum* and *Ipomoea batatas*. Sequence alignments were assembled using the MUSCLE algorithm-based AlignX module from Mega5 software (Tamura et al., 2007). Phylogenetic trees were constructed using the maximum likelihood tree method and computed using the WAG model, with g-distributed (G + I) rates among sites and partial deletion gap treatment. Tree nodes were evaluated by bootstrap analysis for 1,000 replicates. The trees obtained were generated in MEGA5 and visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree).

The online MEME Suite (http://meme.nbcr.net/; Bailey et al., 2009) was used for discovering motifs within C-terminal protein sequences (excluding the R2R3 domain). The search parameters used were: six to nine residues, any number of motif repetitions and search of five motifs maximum. ZmC1, OsC1, VviMYBA2, VviMYBA3, VbeMYBA1, VlMYBA1-2, VlMYBA2, VpsMYBA2 and VamMYBA1 were excluded from the search.

Gene expression profiles were assessed in the global *V. vinifera* cv. 'Corvina' (clone 48) gene expression ATLAS of different organs at various developmental stages (Fasoli et al., 2012). The expression data were analyzed using T-MeV v4.81 (Saeed et

al., 2006). The fluorescence intensity values of each transcript in the tissues/organs were calculated as log2 in order to generate clustered heat maps.

Plant transformation and hairy root culture

pENTR/D-TOPO containing *MYBA6.1*, *MYBA6.2* and *MYBA7* were recombined into the binary vector pK2GW7 (Karimi et al., 2002) with the use of LR clonase II (Invitrogen). The resulting constructs (expressing each *MYB* gene under the control of the 35S promoter) were then transformed into *Agrobacterium rhizogenes* strain A4 by electroporation and used for grapevine plantlet transformation of ten-week old plants from the white-skinned cv. 'Maccabeu'. Induction and culture of transgenic hairy roots (HR) was performed as described by Torregrosa and Bouquet (1997), with modifications reported by Gomez et al. (2009). HRs produced from culturing with non-transformed *A. rhizogenes* were used as a negative control (HR devoid of any transgene). Samples from *MYBA1*-expressing HR in the cv. 'Maccabeu' background (Cutanda-Perez et al. 2009) were also taken for comparison.

Metabolite profiling of grapevine hairy roots

Determination of anthocyanin composition (100 mg of ground frozen tissue) was performed by high-performance liquid chromatography (HPLC) as described by Ageorges et al. (2006). Stilbenes were analyzed in the injections without phloroglucinolysis and detected at 320 nm. Proanthocyanidin (PA) extraction and composition analysis was assessed by HPLC as described by Verriès et al. (2008). Total PAs were analyzed after phoroglucinolysis using an Agilent 1100LC HPLC-DAD-fluorimeter system. Total PAs were obtained by subtracting the concentrations of free monomers determined after direct HPLC analysis from the sum of concentrations of all flavan 3-ol units released after phloroglucinolysis (as flavan-3-ol monomers for terminal units and as phloroglucinol adducts for extension units). Concentrations were determined from standard curves calculated from pure monomers and their phloroglucinol derivatives (Souquet et al., 2004). Free flavan 3-ol monomers (catechins, epicatechins) were analyzed with a 5 µl injection on a Waters Millennium HPLC system equipped with a diode-array detector [PDA 996 (190-700nm)] and were quantified using a W2475 fluorimeter detector (λem=275nm and λex=322nm). Concentrations were determined from standard curves calculated from pure monomers.

RNA extraction and expression analysis by real-time quantitative PCR (qPCR)

Total RNA from transgenic and control HRs was extracted using the RNeasy Plant Mini kit (Qiagen) with modifications reported in Gomez et al. (2009). RNA quality and quantity were determined using a Nanodrop 2000 instrument (Thermo Scientific). All analyses were conducted on three biological replicates corresponding to subculture clones from each selected transgenic and control lines. Reverse transcription and qPCR were performed as described in Huang et al. (2013).

For qPCR analyses of grapevine vegetative organs and of agroinfiltrated cv. 'Sultana' plantlets, total RNA was isolated using Spectrum Plant Total RNA Kit (Sigma-Aldrich) and following the manufacturer's instructions. One µg aliquots were treated with DNase I (Promega) and reverse transcribed using Improm-II Reverse Transcriptase (Promega) according to the manufacturer's instructions. The qPCR analyses were performed using the SYBR Green PCR Master Mix (Applied Biosystems) and an Mx3000P Real-Time PCR System (Stratagene).

For all qPCR analyses performed, gene expression data (Ct values) were employed to quantify relative gene expression by using the efficiency corrected method described by Pfaffl (2001). *ELONGATION FACTOR 1* (*EF1, VIT_06s0004g03220*) and *UBIQUITIN1* (*UBI1*, *VIT_16s0098g01190)* were used as the reference genes for normalization. All primers used are listed in Supplemental Table 4.

Microarray construction, hybridization and data analysis

For microarray analysis of transgenic HR lines, three biological replicates (sub-cultured clones) of *MYBA6.1-2*, *MYBA6.2-4*, *MYBA7-1* and *MYBA1-1* were selected. The previously extracted RNA was checked on a Bioanalyzer Chip RNA 7500 series II (Agilent). cDNA synthesis, labeling, hybridization and washing were performed according to the NimbleGen Arrays User's Guide (V 3.2). Each hybridization was carried out on a NimbleGen microarray 090818 Vitis exp HX12 (Roche, NimbleGen Inc.), representing 29,549 predicted genes on the basis of the 12X grapevine V1 gene prediction version (https://urgi.versailles.inra.fr/Species/Vitis/Annotations). The microarray was scanned using a ScanArray 4000XL (Perkin-Elmer) at 532 nm (Cy-3 absorption peak) and GenePix Pro7 software (Molecular Devices) according to the manufacturer's instructions. Images were analyzed using NimbleScan v2.5 software

(Roche), which produces Pair Files containing the raw signal intensity data for each probe and Calls Files with normalized expression data derived from the average of the intensities of the four probes for each gene. The normalized gene expression data were finally converted to log2 values to process the data. A Pearson Correlation analysis was carried out to evaluate the robustness of the three biological replicates in each sample. To identify the genes significantly modulated between each transgenic line and the control HRs, a two-class unpaired comparison analysis was carried out using Significance Analysis of Microarray (SAM) with a false discovery rate (FDR) of 0.3% for each analysis (TMev 4.3).

Yeast two-hybrid assay

The coding sequences of MYB (*MYBA6.1, MYBA6.2, MYBA7, MYBA1*) and bHLH (*MYC1*) genes were transferred by site-specific recombination from the pENTR/D-TOPO vectors into pDEST22 (downstream of the *GAL4 AD* sequence) and pDEST32 (downstream of *GAL4 BD* sequence) (Laboratory of Plant Systems Biology, PSB; Ghent University, Belgium), respectively. The yeast two-hybrid assay was performed as described in Cavallini et al. (2015).

Transient promoter assays

A cell suspension of *V. vinifera* cv. 'Chardonnay' petiole callus culture was used as previously described (Bogs et al., 2007; Walker et al., 2007). The 35S:MYB vectors were used as effector constructs whereas different grapevine promoters fused to the *LUCIFERASE* gene were used as reporter constructs. *MYBA1* (Walker et al., 2007) and *MYBPA1* (Bogs et al., 2007) effector constructs were used as controls depending on each assay. MYBA1 mutant versions were generated by deleting one or two C-terminal motifs. These were amplified from a MYBA1 'wild type' sequence using primers listed in Supplemental Table 4. The cloning of the promoter fragments of *VviUFGT* (AY955269, *VIT_16s0039g02230*) and *VviF3'5'H* (AJ880356, *VIT_06s0009g02830*) into pLuc (Horstmann et al., 2004) was described by Bogs et al. (2007). For *Vvi3AT* (*VIT_03s0017g00870*), a 1828 bp promoter fragment was amplified with *AAT2proF* and *AAT2proR* primers from cv. 'Shiraz' and subsequently cloned into pLUC. The dual luciferase assay protocol was modified based on Czemmel et al. (2009). Cells were bombarded with 1.6 µm gold particles coated with 0.5 µg of each plasmid with a total amount of 2 µg of plasmid per transformation for reporter/effectors bombardments. In

addition, the *Renilla luciferase* plasmid pRluc was used as an internal control in each transfection experiment. Transient assays were performed with and without the bHLH and WD40 co-factors VviMYC1 (EU447172) and AtTTG1 (NM_180738). Measurements of the luciferase activities were taken 48h after bombardment and incubation in the dark at 22 °C on agar-containing plates with GC medium. Relative activities were calculated as ratios between Firefly and Renilla (control) LUCIFERASE. The non-bombarded, grounded cell luminescence was used as background and was subtracted from all measured values. All transfection experiments (3 biological replicates) were independently repeated twice from different cell culture preparations.

Anthocyanin analysis of grapevine vegetative organs of white and black-skinned cultivars

Pigmented and non-pigmented buds were collected at bud burst from *V. vinifera* cv. 'Corvina', while pigmented and non-pigmented young leaves and tendrils were collected from cv. 'Pinot Noir' (three biological replicates). Samples were divided in two groups for gene expression analysis (qPCR, as previously described) and anthocyanin quantification.

For total anthocyanin quantification, samples were powdered and extracted in 2 volumes (w/v) of methanol, acidified with 1% (v/v) hydrochloric acid in an ultrasonic bath at room temperature at 40 kHz for 15 min. The total amount of anthocyanins was determined by spectrophotometry at λ = 540 nm using malvidin 3-glucoside as a standard. For the determination of anthocyanin profiles, HPLC-electrospray ionization-mass spectromety (ESI-MS) analysis was carried out as described in Cavallini et al. (2014), with the following modifications: the chromatographic gradient started from 0% to 10% of solvent B in 2 min, from 10% to 20% in 10 min, from 20% to 25% in 2 min, from 25% to 70% in 7 min, from 70% to 90% in 1 min and finally from 90% to 0% in 1 min. Then, the column was equilibrated for 20 min in 100% of solvent A. Metabolites were identified by comparing the *m/z* values, fragmentation patterns (MS/MS and MS^3) and retention times of each signal with those of available commercial standards and by comparison with values reported in the literature. To further confirm anthocyanin identity, the methanolic extracts were analyzed by LC-DAD (diode array detector), maintaining the same elution conditions. Specifically, molecules identified as anthocyanins in LC-MS were confirmed by measuring their absorbance at 520 nm.

Young grapevine leaves from white skinned cv. 'Sauvignon blanc' and cv. 'Chardonnay' were screened for visible differences in pigmentation and collected during the 2014-2015 growing season from an experimental field in the Curacaví Valley, Chile (33°36' S, 70°39' W). Three red and three green leaves were collected from three different plants of each cultivar (n=3). A total of 18 leaves per cultivar were sampled, immediately frozen in liquid nitrogen, and stored at -80ºC until required for anthocyanin quantification (following the protocol described by Jeong et al., 2010) and RNA extraction. For RT-PCR analyses on grapevine white-skinned cultivars, total RNA was extracted from leaves according to Reid et al. (2006), using a CTAB-Spermidine extraction buffer. One μg aliquotes were treated with RQ1 RNase-free DNase (Promega) and reverse transcribed using GoScript™ Reverse Transcription System (Promega).

UV-B treated *in vitro* plantlets and fruits from potted vines

Grapevine (cv. 'Cabernet Sauvignon') plantlets were exposed to 6h of low UV-B radiation (0.15 W m^{-2} irradiance), as described in Cavallini et al. (2015). After the treatment ended, plants were immediately frozen in liquid nitrogen and then stored at -80ºC until required for RNA extraction.

Fruit clusters from nine year-old potted cv. 'Cabernet Sauvignon' plants, growing in a UV-free greenhouse, were exposed to high UV-B irradiance (~0.3 W m^{-2}) applied daily for 5 h (daily biologically effective UV-B exposure, UV-B_{BE} 5.4 kJ m^{-2} d^{-1}), as described in Loyola et al. (2016). Samples were collected at -3, 0, 3, 6 and 9 weeks after veraison (WAV) for gene expression analyses and at 9 WAV for anthocyanin profile quantification.

Vacuum-induced agroinfiltration of *VP64-VviHY5* in grapevine plantlets

Six *in vitro* grapevine plantlets of cv. 'Sultana' were agroinfiltrated with a *Agrobacterium tumefaciens* C58C1 strain, either transformed with a *2xPro35S*:*VP64-HY5* construct or an empty vector, as described by Loyola et al. (2016). After agroinfiltration, plantlets were rinsed with sterile water and allowed to recover (*in vitro* conditions) for five days before collecting leaves and proceeding with RNA extraction. Plantlets characterized by i) highest and comparable expression levels of the *HY5* transgene and ii) showing low and comparable *HY5* expression were selected as

transformed and control samples, respectively. qPCRs were performed as described previously using the three selected lines as biological replicates.

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Author Contributions

Conceived and designed the experiments: J.T.M., G.B.T., A.A, P.A.J. Performed the experiments: J.T.M., E.C., R.L., L.F., J.H., S.V., F.~~G~~R[2]. Analyzed the data: J.T.M., A.A., E.C. and G.B.T. Contributed reagents/materials/analysis tools: J.A.A., J.B., A.S. Wrote the paper: J.T.M., E.C., G.B.T.

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Accession Numbers

Protein sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: ACL97979 (VviMYBA6.1), ACL97980 (VviMYBA6.2), ACL97981 (VviMYBA6.3), ACI96116 (VviMYBA7), BAE96751 (VviMYBA1), ABL14065 (VviMYBA2), ABL14067 (VviMYBA3), JG294244 (VamMYBA1, translated), AGH68551 (VpsMYBA2), BAG55462 (VlMYBA1-2), BAC07540 (VlMYBA2), AGH68552 (VbeMYBA1), AAG42001 (AtMYB75/PAP1), NP_176813 (AtMYB90/PAP2), NP_176811 (AtMYB113/PAP3), NP_176812/ AY008379 (AtMYB114/PAP4), AAQ55181 (SlANT1), ABO21074

(PhAN2), ADQ00392 (PhAN4), ADQ00391 (PhPHZ), ADW94950 (PhDPL), CAE75745 (CaA), BAF45115 (IbMYB1), ABB83826 (AmROSEA1), ABB83827 (AmROSEA2), ABB83828 (AmVENOSA), ACQ45201 (MdMYB10), ABX71493 (PavMYB10), ABX79947 (FaMYB10), NP_001106010 (ZmC1) and CAA75509 (OsC1). The VviMYBA5 sequence was retrieved from CRIBI, translated from VIT_14s0006g01340 and corrected based on RNA-Seq data from cv. 'Corvina' and on comparisons with *MYBA1*, *MYBA2*, *MYBA6* and *MYBA7*.

Gene promoter sequences from grapevine and apple MYB genes were taken from the GenBank/EMBL data libraries under the following accession numbers: FJ418598 (VviMYBA1 cv. 'Autumn Royal'), AM444376.2 (VviMYBA7, Contig VV78X104294.6), AM444005 (VviMYBA6, Contig VV78X240536.5), AM483462 (VviMYBA5, Contig VV78X053225.13), EU518249.2 (MdMYB10 cv. 'Royal Gala' R1), EU518250 (MdMYB10 cv. 'Niedzwetzkyana' R6). The promoter sequence of AtMYB75/PAP1 was retrieved from TAIR v10 (AT1G56650).

Supplemental Information

Supplemental Figure 1. RT-PCR detection of *MYBA6* splicing variants (with and without UTRs) in different grapevine organs. C-: Negative control (water), 6.1+ and 6.2+: Positive controls of *MYBA6.1* and *MYBA6.2* (cloned in pENTR/TOPO-D), gCS: genomic DNA of cv. 'Cabernet Sauvignon'. cDNA samples of tendrils (T), inflorescences at pre (F1) and post (F5) capfall stages and berry skins at pre (B1, B2) and post (B5, B6) veraison stages were used. St: 1kb plus ladder standard. Transcripts corresponding to *MYBA6.1*, *MYBA6.2* and *MYBA6.3* are indicated. Primer sequences are listed in Supplemental Table 4.

Supplemental Figure 2. Protein alignment of the DNA binding domains of anthocyanin-related MYBAs from Subgroup 6. Asterisks denote conserved residues. Subgroup variations are highlighted underneath the alignment.

Supplemental Figure 3. Relationship between MYB anthocyanin regulators from Subgroup 6 belonging to grapevine and other plant species. A) Unrooted phylogenetic tree showing selected plant MYB transcription factors. Node bootstrap values are shown with numbers and colors. B-C) Protein domain/motif organization represented by colored boxes. Specific amino acid features are highlighted with vertical bars inside each box. M1-*to*-M5 motifs identified by MEME Suite are reported in (C).

Supplemental Figure 4. C-terminal protein alignment of MYBs from Subgroup 6. Motifs 3 and A1M are highlighted.

Supplemental Figure 5. A) Differences in pigmentation between MYBA6 and MYBA1 overexpressing HR. B-D) Phenylpropanoid and Flavonoid composition of each independent HR line of the *MYBA* overexpressors. B) Relative abundances of anthocyanin derivatives. Proanthocyanidin (C) and stilbene (D) total amounts. NT: control HR devoid of transgene.

Supplemental Figure 6. qPCR expression analysis in *MYBA*-overexpressing HR. Transgene (A) and anthocyanin-related gene (B) expression quantifications of each independent transgenic line. nd: not detected.

Supplemental Figure 7. Gene Ontology (GO) analysis of differentially expressed genes in each *MYBA*-overexpressing HR.

Supplemental Figure 8. A) Venn Diagram with general overview of phenylpropanoid DEGs resulting from the overexpression of *MYBA* genes in HR (fold change >1.9 and FDR =0.3). B) Categorization of phenylpropanoid-structural genes according to their type of expression in each *MYBA*-overexpressing HR. SWP: small weight phenolics. Number and type of genes can be found in Supplemental Table 2.

Supplemental Figure 9. A-B) Expression analysis of the *MYBA* genes in relation to flavonoid (A) and anthocyanin-exclusive (B) structural genes in the cv. 'Corvina' atlas using a restricted set of organs and developmental stages with low *MYBA1* expression. Genes related to the general flavonoid pathway are *CHS1* (*VIT_14s0068g00920*), *CHS2* (*VIT_14s0068g00920*), *CHS3* (*VIT_05s0136g00260*), *F3'5'H* (*VIT_06s0009g02830*) and *LDOX (VIT_00s0521g00010)*. Proanthocyanidin (PA) structural genes are *ANR* (*VIT_00s0361g00040), LAR1 (VIT_01s0011g0296)* and *LAR2 (VIT_17s0000g04150*). Anthocyanin-related genes are *UFGT (VIT_16s0039g02230), AOMT1 (VIT_01s0010g03510)*, *3AT* (*VIT_03s0017g00870)* and *GST4 (VIT_04s0079g00690*). The gene expression data were calculated as $\log_2$. In the case of heatmap A, these were normalized on the median of each gene. Genes (in heatmap A) and samples (in heatmap B) were hierarchically clustered based on average Pearson's distance metric. Abbreviations after organ names correspond to: FS, fruit set; PFS, post fruit set; Bud - L, latent bud; Bud - S, bud swell; Bud - B, bud burst; Bud - AB, bud after burst; Inflorescence - Y, young; Inflorescence - WD, well developed; Flower - FB, flowering begins; Flower - F, flowering; Tendril - Y, young; Tendril - WD, well developed; Tendril - FS, mature; Leaf - Y, young; Leaf - FS, mature; Stem - G, green; Stem - W,

woody. Red and green boxes indicate high and low expression levels, respectively, for each gene. C) Anthocyanin profiles determined by HPLC-MS in green and red tendrils (cv. 'Pinot Noir'), leaves (cv. 'Pinot Noir') and buds (cv. 'Corvina'). White segments correspond to glycosylated anthocyanins, while those in grey indicate coumaroylated hexoside anthocyanins. Numbers refer to 1: delphinidin 3-O-glucoside, 2: cyanindin 3-O-glucoside, 3: petunidin 3-O-glucoside, 4: peonidin 3-O-glucoside, 5: malvidin 3-O-glucoside, 6: peonidin (*cis*-coumaroyl) hexoside, 7: peonidin (*trans*-coumaroyl) hexoside.

Supplemental Figure 10. A) Photographs of green and dark green purpluish colored leaves of cv. 'Sauvignon blanc'. B) Schematic representation of white and red alleles in the *MYBA1* locus (Adapted from Shimazaki et al., 2011). Primers a+c and b+c amplify the white and red alleles, respectively, while the b+c combination is not able to generate a PCR product due to the large size of the *Gret1* retrotransposon (Kobayashi et al., 2004). C) PCR for defining *MYBA1* allelic composition of cv. 'Sauvignon blanc' and cv. 'Chardonnay' replicates used in Figure 5. Genomic DNA of white-skinned (cv. 'Muscat' and cv. 'Superior') and red/black-skinned (cv. 'Flame' and cv. 'Cabernet Sauvignon') cultivars was used for comparison.

Supplemental Figure 11. Quantification of total di- and tri-hydroxylated anthocyanins at harvest (9 weeks after veraison) in berry skins of fruits irradiated with high UV-B. Asterisks represent statistical significance ($p<0.05$).

Supplemental Figure 12. Protein alignment of MYBA1, MYBA6 and MYBA1 mutant versions. Domains and motifs are highlighted. M1 motif corresponds to G-6 box in Heppel et al. (2013). The dicots-specific anthocyanin promoting motifs DAPM1 and DAPM2 correspond to box 1 and box 3, respectively (Heppel et al., 2013).

Supplemental Table 1. List of differentially expressed genes for all MYBA overexpressing hairy roots. FDR: false discovery rate.

Supplemental Table 2. Expression of phenylpropanoid-structural genes in *MYBA* HRs.

Supplemental Table 3. Quantification of anthocyanin derivatives at harvest (9 weeks after veraison) in berry skins of fruits irradiated with high UV-B. Statistical significance (showing *p* values) was calculated by one-way ANOVA followed by a posteriori comparison (Tukey test).

Supplemental Table 4. List of primers used in this work.

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FIGURES

Figure 1. Cluster of *MYBA* homologue genes found in the berry color locus 1 (chromosome 2) and a genomic region of chromosome 14. A) Schematic representation of the anthocyanin-related *MYB* genes in the grapevine genome of cv. Pinot Noir PN40024. Gene names correspond to those defined by Wong et al. (2016). Gene sizes (bp, square brackets) consider intron lengths (intergenic regions are not to scale). Gene sequence similarities and protein identities (round parenthesis) are shown with grey arrows. All *MYBA* genes are disposed in the same orientation within each chromosome, mostly positioned in tandem, with the exception of *MYBA1*/*MYBA10* and *MYBA5*/*MYBA6* pairs that share the presence of non-*MYB* genes between them. The grapevine retrotransposon 1 (*Gret1*) is shown in the *MYBA1* promoter-5'UTR region. SV: splicing variants found in cv. 'Corvina' RNASeq data. B) Genomic organization and splicing variants of *MYBA6*. Non-canonical splicing sites and the extent of the DNA binding domain (R2 and R3 repeats) are shown with red and black arrows, respectively. Asterisks: Stop codons. IR: intron retention.

Figure 2. The ectopic expression of *MYBA6.1* and *MYBA7* induces a major accumulation of di-hydroxylated anthocyanins in grapevine hairy roots (HR). A) Phenotypes of *MYBA*-overexpressing HR (cv. 'Maccabeu'). NT: HR devoid of any transgene. *MYBA6.2* overexpression did not promote anthocyanin synthesis (not shown). B) Anthocyanin profiles obtained for different independent transgenic lines (see also Supplemental Figure 5 for total amounts of other phenylpropanoids). C) General overview of differentially expressed genes (DEG) resulting from the overexpression of *MYBA* genes in HR. DEGs that are structural genes of the shikimate and phenylpropanoid pathways, with fold change >1.9 and FDR =0.3, are shown.

Figure 3. MYBA proteins interact with the flavonoid-related MYC1 for promoting the expression of anthocyanin-related genes. A) Yeast-two-hybrid analysis of MYBA-MYC1 interactions. B) Dual luciferase assays in *Vitis vinifera* cv. 'Chardonnay' grape cell cultures. The Firefly *LUCIFERASE* gene was transcriptionally fused to *UFGT* (*VIT_16s0039g02230*), *3AT* (*VIT_03s0017g00870*) and *F3´5´H* (*VIT_06s0009g02830*) promoters, and co-cultured with different MYB factors, as indicated. Fold induction

represents the activity of the respective promoter comparing the cells transfected with and without the MYB factor. In each experiment (unless described otherwise) the bHLH factor MYC1 and the WD40 protein TTG1 were used for co-transfection. The normalized LUCIFERASE activity was calculated as the ratio between the firefly and the Renilla LUCIFERASE activity. Each column represents the mean value of three independent experiments with error bars indicating standard error. *UFGT1* is not activated by MYBPA1 as previously shown by Bogs et al. (2007), so it was not included in the analysis.

Figure 4. *MYBA*5, *MYBA*6 and *MYBA7* are predominantly expressed in vegetative organs of black-skinned berry cultivars and correlate with the presence of anthocyanins in these organs. A) *MYBA* expression analysis in the grapevine cv. 'Corvina' atlas (Fasoli et al., 2012). The gene expression data were calculated as log2 and normalized on the median value for each gene. Genes were hierarchically clustered based on average Pearson's distance metric. Red and green boxes indicate high and low expression levels, respectively, for each gene. Bud-AB, Bud after burst; Bud-B, bud burst; Bud-L, latent bud; Bud-S, bud swell; Bud-W, winter bud; Flower-F, flowering; Flower-FB, flowering begins; FS, fruit set; Inflorescence-WD, well developed; Inflorescence-Y, young; Leaf-FS, mature; Leaf-S, senescing leaf; Leaf-Y, young; MR, midripening; PFS, postfruit set; PHWI, postharvest withering (1st month); PHWII, postharvest withering (2nd month); PHWIII, postharvest withering (3rd month); R, ripening; Stem-G, green; Stem-W, woody; Tendril-FS, mature; Tendril-WD, well developed; Tendril-Y, young; V, veraison. B-D) qPCR expression data of *MYBA* genes in pigmented and un-pigmented vegetative organs of cv. 'Corvina' (CV) and 'Pinot Noir' (PN). Bursted buds (B), young fully expanded leaves (C), and tendrils (D) were collected from plants grown in the same field conditions. Average anthocyanin values are indicated for each organ and are shown under each representative photograph. Expression data correspond to the means of three biological replicates.

Figure 5. Accumulation of anthocyanins in leaves of the white-skinned cultivars 'Chardonnay' and 'Sauvignon blanc' is associated with the expression of *MYBA*6 and *MYBA7*. A) Total anthocyanin quantification for each biological replicate (R). B) RT-PCR of selected genes (saturated, 35 cycles). *UBIQUITIN1* (*UBI1*, *VIT_16s0098g01190*) was used as a housekeeping gene. C+1 and C+2: berry pre- and

post-veraison cDNA samples, respectively (cv. 'Cabernet Sauvignon'). C-: negative control (water). Determination of the *MYBA1* allelic condition for each replicate used in this experiment is shown in Supplemental Figure 10.

Figure 6. UV-B responsiveness of the vegetative color locus is mediated by VviHY5. Gene expression of *VviHY5*, *MYBA* genes and anthocyanin-related genes in plantlets (A) and berry skins (B) of cv. 'Cabernet Sauvignon' in response to UV-B exposure. Irradiation treatments (irradiances of 0.15 W m^{-2} for plantlets and ~0.3 W $m^{-2}$for fruits) were conducted as in Cavallini et al. (2015) and Loyola et al. (2016). C) Distribution of bZIP and MYB transcription factor binding sites in the promoters of grape *MYBA* genes and their homologues in Arabidopsis and apple. MRE1 ([A][C/A][C][T][A][C][C]) is the functional MYB recognition element found in *AtCHS* and *AtFLS* (Hartmann et al., 2005) while, MRE2 ([G][G][T][A][G][T/C][T], in reverse of MRE1) is the core of the regulatory element found in the promoter of *MdoMYB10* (blue lines, Espley et al., 2009). ACGT-containing elements (ACE, [A][C][G][T]) and G-boxes ([C][A][C][G][T][G]) correspond to light responsive units bound by bZIP TFs. The dashed line represents the fragment of AtPAP1 where AtHY5 is bound (Shin et al., 2013). Black arrows indicate start codons. Dashed lined boxes represent variant ACE elements. D) Transient expression of *VviHY5* in cv. 'Sultana' grapevine plantlets induces *MYBA6* and *MYBA7* but not *MYBA1*. Normalized gene expression values and induction fold changes in response to *VviHY5* ectopic expression. Grapevine *in vitro* plants were agroinfiltrated with either a 35S:VP64-HY5 construct or an empty vector, and kept in low light conditions for 5 days before gene expression quantification as in Loyola et al. (2016). Values below the asterisks indicate significant differences when compared to the control.

Figure 7. The C-terminal A1M motif of MYBA1 plays a role in the high activation capacity of the anthocyanin-branch genes. A) Schematic representation of N- and C-terminal domains/motifs found in MYBA1, MYBA6 and MYBA1 mutant versions. Consensus sequence LOGOs derived from MEME can be found in Supplemental Figure 3. B) Dual luciferase assays in cv. 'Chardonnay' grape cell cultures. Each transfection was conducted as in Figure 3B, together with VviMYC1 and AtTTG1. Each column represents the mean value of three independent experiments with error bars indicating standard error.

TABLES

Table 1. Set of phenylpropanoid-related genes (structural and regulatory) that are commonly or exclusively upregulated by R2R3-MYBA transcription factors. n.s.: non-significant expression (false discovery rate = 0.3). Light grey shows induced genes above fold change threshold (1.9). Dark grey represents downregulation.

Gene Identification	Description	MYBA1	MYBA7	MYBA6.1	MYBA6.2
Genes commonly regulated by MYBA1, MYBA6.1 and MYBA7					
VIT_04s0079g00690	Anthocyanin glutathione S-transferase (GST4)	315,41	161,98	206,34	-2,59
VIT_01s0010g03510	Anthocyanin O-methyltransferase (AOMT1)	251,09	72,9	137,53	*n.s.*
VIT_16s0039g02230	UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT)	161,39	44,72	76,93	-1,53
VIT_03s0017g00870	Anthocyanin acyltransferase (3AT)	143,89	64,73	64,76	*n.s.*
VIT_09s0002g06590	Putative UDP-glucose:flavonoid 7-O-glucosyltransferase	115,68	33,08	33,43	-2,53
VIT_01s0010g03490	Anthocyanin O-methyltransferase (AOMT2)	115,17	61,46	86,27	*n.s.*
VIT_01s0010g03470	Anthocyanin O-methyltransferase (AOMT3)	71,5	56,97	80,75	*n.s.*
VIT_08s0007g03560	Anthocyanin membrane protein 1 (ANM1)	17,98	2,91	6,5	*n.s.*
VIT_16s0050g00910	AnthoMATE 2 efflux family protein (AM2)	12,3	3,9	5,67	1,65
VIT_12s0059g02360	MYB CAPRICE-like (CPC)	10,13	2,92	4,25	1,53
Genes regulated by MYBA1 and MYBA6.1					
VIT_16s0022g01020	Chalcone synthase	134,41	*n.s.*	6,58	*n.s.*
VIT_16s0022g01140	Chalcone synthase	76,11	*n.s.*	3,08	-4,15
VIT_16s0022g01190	Chalcone synthase	66,16	*n.s.*	2,39	*n.s.*
VIT_16s0022g01000	Chalcone synthase	28,55	*n.s.*	3,14	-2,71
VIT_06s0009g02970	Flavonoid 3',5'-hydroxylase (F3'5'Hj)	13,13	-1,4	1,94	-1,34
VIT_06s0009g02840	Flavonoid 3',5'-hydroxylase (F3'5'H)	11,81	*n.s.*	1,93	*n.s.*
VIT_06s0009g02810	Flavonoid 3',5'-hydroxylase (F3'5'H)	11,28	*n.s.*	1,97	-1,29
VIT_04s0008g01810	Proanthocyanidin related MYBPA8 (TT2-like)	5,47	1,72	2,61	1,83
VIT_15s0048g02430	Naringenin,2-oxoglutarate 3-dioxygenase	3,41	*n.s.*	6,48	n.s.

VIT_02s0087g00370	Anthranilate N-hydroxycinnamoyl/benzoyltransferase	3,07	*n.s.*	4,04	-1,46
VIT_08s0007g07720	2-Hydroxyisoflavanone synthase (CYP93A1)	2,89	-16,29	2,71	-5,97
VIT_12s0028g02870	Isoflavone / Orcinol O- methyltransferase	2,65	-2,02	4,58	-5,2
VIT_07s0031g03070	Isoflavone reductase	2,3	-1,26	2,2	1,57
VIT_18s0001g12690	Isoflavone reductase	2,13	*n.s.*	2,07	*n.s.*
VIT_03s0038g03080	Catechol O-methyltransferase	2,44	*n.s.*	2,32	-1,55
VIT_03s0038g03090	Catechol O-methyltransferase	2,1	*n.s.*	2,55	-1,42
Genes regulated by MYBA6.1 and MYBA7					
VIT_03s0110g00330	Cinnamyl alcohol dehydrogenase	*n.s.*	1,97	1,9	1,68
Genes exclusively regulated by MYBA6.1					
VIT_14s0006g01340	MYBA5	*n.s.*	*n.s.*	134,19	1,36
VIT_11s0016g01850	Anthocyanidin-3-glucoside rhamnosyltransferase	*n.s.*	1,58	2,29	*n.s.*
VIT_00s0218g00140	Anthocyanidin rhamnosyl-transferase	*n.s.*	1,61	2,56	*n.s.*
VIT_15s0046g01950	Anthocyanidin rhamnosyl-transferase	*n.s.*	1,62	1,97	1,71
VIT_16s0115g00360	Anthocyanidin 3-O-glucosyltransferase	*n.s.*	*n.s.*	2,07	*n.s.*
VIT_09s0002g01090	Flavonoid 3'-monooxygenase (F3'H)	*n.s.*	*n.s.*	2,41	*n.s.*
VIT_05s0062g00520	Flavonoid-glucosyltransferase	*n.s.*	*n.s.*	2,75	1,62
VIT_16s0022g01540	Flavonoid 3',5'-hydroxylase	*n.s.*	*n.s.*	2,07	*n.s.*
VIT_05s0062g00460	UDP-glucose:flavonoid 7-O-glucosyltransferase	*n.s.*	*n.s.*	2,29	*n.s.*
VIT_05s0062g00570	UDP-glucose:flavonoid 7-O-glucosyltransferase	*n.s.*	*n.s.*	2,69	1,54
VIT_05s0062g00430	UDP-glucose:flavonoid 7-O-glucosyltransferase	*n.s.*	*n.s.*	2,8	*n.s.*
VIT_16s0022g01510	Flavonoid hydroxylase-like	*n.s.*	*n.s.*	2,2	*n.s.*
VIT_18s0001g11520	Flavonoid 3-monooxygenase	*n.s.*	*n.s.*	2,13	1,43
VIT_03s0132g00260	Flavonol 3-O-methyltransferase	*n.s.*	1,81	2,09	1,7
VIT_13s0019g03430	Anthocyanin 5-aromatic acyltransferase	*n.s.*	-1,72	1,99	-2,65
VIT_12s0028g02900	Isoflavone / Orcinol O-methyltransferase	*n.s.*	-1,83	3,44	-3,82
VIT_12s0028g02860	Isoflavone / Orcinol O-methyltransferase	1,74	-2,89	2,31	-4,22
VIT_12s0028g02950	Isoflavone / Orcinol O-methyltransferase	*n.s.*	*n.s.*	1,98	*n.s.*
VIT_03s0038g04630	Isoflavone reductase related protein	*n.s.*	1,73	1,99	1,7

VIT_11s0016g01640	Phenylalanine ammonia-lyase	*n.s.*	*n.s.*	3,12	*n.s.*
VIT_11s0016g01520	Phenylalanine ammonia-lyase	*n.s.*	*n.s.*	3,41	1,34
VIT_11s0016g01660	Phenylalanine ammonia-lyase	*n.s.*	*n.s.*	2,44	1,44
VIT_03s0110g00340	Cinnamyl alcohol dehydrogenase	*n.s.*	1,50	2,03	1,7
VIT_18s0072g00920	Caffeate 3-O-methyltransferase 1	*n.s.*	*n.s.*	3,94	*n.s.*
VIT_12s0028g03110	Caffeoyl-CoA 3-O-methyltransferase	*n.s.*	*n.s.*	2,27	*n.s.*
VIT_07s0031g00280	Anthranilate N-hydroxycinnamoyl/benzoyltransferase	*n.s.*	*n.s.*	2,06	1,87
VIT_11s0037g00580	Anthranilate N-hydroxycinnamoyl/benzoyltransferase	*n.s.*	*n.s.*	2,24	1,69
VIT_12s0134g00580	Hydroxycinnamoyl-CoA quinate hydroxycinnamoyltransferase	*n.s.*	-1,58	2,11	-2,26

Genes exclusively regulated by MYBA1

VIT_02s0033g00380	Anthocyanin related MYBA8	240,28	*n.s.*	*n.s.*	*n.s.*
VIT_02s0033g00450	MYBA3	134,34	*n.s.*	*n.s.*	*n.s.*
VIT_02s0033g00440	Anthocyanin related MYBA11	107,85	*n.s.*	*n.s.*	*n.s.*
VIT_02s0033g00390	MYBA2	102,42	*n.s.*	*n.s.*	*n.s.*
VIT_02s0033g00410	MYBA1	61,38	*n.s.*	*n.s.*	-1,59
VIT_06s0009g02920	Flavonoid 3',5'-hydroxylase (F3'5'H)	13,86	-1,42	1,83	-1,4
VIT_06s0009g03000	Flavonoid 3',5'-hydroxylase (F3'5'H)	12,73	*n.s.*	1,63	*n.s.*
VIT_06s0009g02830	Flavonoid 3',5'-hydroxylase (F3'5'H)	10,73	*n.s.*	*n.s.*	-1,42
VIT_06s0009g02880	Flavonoid 3',5'-hydroxylase i (F3'5'Hi)	10,38	-1,36	1,84	-1,24
VIT_06s0009g03010	Flavonoid 3',5'-hydroxylase (F3'5'H)	10,06	*n.s.*	1,76	*n.s.*
VIT_06s0009g03050	Flavonoid 3',5'-hydroxylase m (F3'5'Hm)	8,13	-1,53	1,28	*n.s.*
VIT_02s0033g00430	Anthocyanin related MYBA10	8,11	*n.s.*	*n.s.*	*n.s.*
VIT_06s0009g02860	flavonoid 3',5'-hydroxylase f (F3'5'Hf)	8,0	-1,79	1,29	*n.s.*
VIT_06s0009g03040	flavonoid 3',5'-hydroxylase m (F3'5'Hm)	7,45	-1,23	1,47	*n.s.*
VIT_06s0009g03110	Flavonoid 3',5'-hydroxylase (F3'5'H)	7,45	-1,61	*n.s.*	-1,34
VIT_00s0521g00010	Leucoanthocyanidin dioxygenase	4,29	-3,47	1,36	-1,88
VIT_00s0687g00010	Leucoanthocyanidin dioxygenase	4,23	-8,7	*n.s.*	-2,02
VIT_12s0134g00630	Quercetin 3-O-glucoside-6"-O-malonyltransferase	4,03	*n.s.*	*n.s.*	1,49

VIT_12s0134g00590	Anthocyanidin 3-O-glucoside-6"-O-malonyltransferase	3,9	*n.s.*	*n.s.*	*n.s.*
VIT_14s0006g01620	MYBC2-L3, R2R3-MYB Flavonoid Repressor	3,29	*n.s.*	1,44	1,37
VIT_01s0011g04760	MYBC2-L1, R2R3-MYB Flavonoid Repressor	2,99	*n.s.*	1,73	1,46
VIT_17s0000g02650	MYBC2-L4, R2R3-MYB Repressor type	2,89	*n.s.*	1,57	1,29
VIT_10s0003g04910	Sinapyl alcohol dehydrogenase	2,79	*n.s.*	*n.s.*	*n.s.*
VIT_13s0019g04460	Phenylalanine ammonia-lyase 2 (PAL2)	2,74	1,26	1,64	-1,3
VIT_11s0016g01300	MYBPAR (TRANSPARENT TESTA 2-like)	2,7	*n.s.*	1,77	*n.s.*
VIT_08s0105g00380	Leucoanthocyanidin dioxygenase	2,64	*n.s.*	*n.s.*	-2,89
VIT_07s0151g01060	Chalcone isomerase	2,63	*n.s.*	*n.s.*	1,46
VIT_04s0008g01800	Proanthocyanidin related MYBPA7 (TT2-like)	2,5	*n.s.*	1,76	1,3
VIT_14s0083g00320	Cinnamoyl-CoA reductase	2,34	*n.s.*	1,33	1,38
VIT_06s0009g01990	Anthocyanin 3-O-galactosyltransferase	2,23	*n.s.*	1,79	*n.s.*
VIT_16s0039g02040	4-coumarate-CoA ligase 3	2,13	*n.s.*	*n.s.*	*n.s.*
VIT_17s0000g02660	MYBC2-L2, R2R3-MYB Repressor type	2,09	*n.s.*	1,74	1,4
VIT_16s0050g01680	UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase	2,08	-1,62	1,43	1,81
VIT_16s0050g00900	AnthoMATE 3 (AM3)	2,06	1,79	1,64	1,32
VIT_12s0059g01750	Caffeic acid O-methyltransferase	1,95	*n.s.*	1,64	1,43
VIT_18s0001g12790	Dihydroflavonol reductase	1,9	1,12	*n.s.*	*n.s.*