

# The Phenylpropanoid Pathway Is Controlled at Different Branches by a Set of R2R3-MYB C2 Repressors in Grapevine<sup>1</sup>

Erika Cavallini<sup>2</sup>, José Tomás Matus<sup>2</sup>, Laura Finezzo, Sara Zenoni, Rodrigo Loyola, Flavia Guzzo, Rudolf Schlechter, Agnès Ageorges, Patricio Arce-Johnson, and Giovanni Battista Tornielli\*

Department of Biotechnology, University of Verona, 15–37134 Verona, Italy (E.C., L.F., S.Z., F.G., G.B.T.); Center for Research in Agricultural Genomics-Consejo Superior de Investigaciones Científicas-Institut de Recerca i Tecnologia Agroalimentàries-Universitat Autònoma de Barcelona-Universitat de Barcelona, 08193 Bellaterra, Barcelona, Spain (J.T.M.); Departamento de Fruticultura y Enología, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, 6904411 Santiago, Chile (R.L.); Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológica, Pontificia Universidad Católica de Chile, 8331150 Santiago, Chile (R.L., R.S., P.A.-J.); and Institut National de la Recherche Agronomique, Unité Mixte de Recherche 1083 Sciences pour l’Oenologie, F–34060 Montpellier, France (A.A.)

ORCID ID: 0000-0001-5027-0269 (G.B.T.).

Because of the vast range of functions that phenylpropanoids possess, their synthesis requires precise spatiotemporal coordination throughout plant development and in response to the environment. The accumulation of these secondary metabolites is transcriptionally controlled by positive and negative regulators from the MYB and basic helix-loop-helix protein families. We characterized four grapevine (*Vitis vinifera*) R2R3-MYB proteins from the C2 repressor motif clade, all of which harbor the ethylene response factor-associated amphiphilic repression domain but differ in the presence of an additional TLLLFR repression motif found in the strong flavonoid repressor *Arabidopsis* (*Arabidopsis thaliana*) AtMYBL2. Constitutive expression of *VvMYB4a* and *VvMYB4b* in petunia (*Petunia hybrida*) repressed general phenylpropanoid biosynthetic genes and selectively reduced the amount of small-weight phenolic compounds. Conversely, transgenic petunia lines expressing *VvMYBC2-L1* and *VvMYBC2-L3* showed a severe reduction in petal anthocyanins and seed proanthocyanidins together with a higher pH of crude petal extracts. The distinct function of these regulators was further confirmed by transient expression in tobacco (*Nicotiana benthamiana*) leaves and grapevine plantlets. Finally, *VvMYBC2-L3* was ectopically expressed in grapevine hairy roots, showing a reduction in proanthocyanidin content together with the down-regulation of structural and regulatory genes of the flavonoid pathway as revealed by a transcriptomic analysis. The physiological role of these repressors was inferred by combining the results of the functional analyses and their expression patterns in grapevine during development and in response to ultraviolet B radiation. Our results indicate that *VvMYB4a* and *VvMYB4b* may play a key role in negatively regulating the synthesis of small-weight phenolic compounds, whereas *VvMYBC2-L1* and *VvMYBC2-L3* may additionally fine tune flavonoid levels, balancing the inductive effects of transcriptional activators.

The phenylpropanoid pathway is regulated at the level of transcription by developmental, environmental, and stress-related cues (for review, see Jaakola, 2013).

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<sup>2</sup> These authors contributed equally to the article.

\* Address correspondence to giovannibattista.tornielli@univr.it.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors ([www.plantphysiol.org](http://www.plantphysiol.org)) is: Giovanni Battista Tornielli ([giovannibattista.tornielli@univr.it](mailto:giovannibattista.tornielli@univr.it)).

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Phenylpropanoids act as free radical-scavenging or UV-absorbing compounds, pigments of coevolutionary traits, or modulators of developmental signaling cascades. Their accumulation in plant organs is based on the ability of the cell to activate or repress different metabolic modules of the pathway, giving rise to a variety of compounds, such as stilbenes, flavonoids, small-weight phenolics (SWPs), and lignins among many others.

The phenylpropanoid pathway is finely tuned and tightly controlled by transcription factors (Albert et al., 2011; Xu et al., 2014). In this control, several protein families participate and mutually interact, giving rise to regulatory specificity (Grotewold et al., 2000). This is the case of the combinatorial interaction of MYB, basic helix-loop-helix (bHLH), and WD40 proteins, the main components of a regulatory complex (MBW complex) that determines the set of anthocyanin or proanthocyanidin (PA) genes to be expressed (Winkel-Shirley, 2001; Koes et al., 2005; Ramsay and Glover,

2005). Depending on the plant lineage, these transcriptional regulatory proteins may affect all biosynthetic genes in a single module (as in maize [*Zea mays*]) or separately, regulating the so-called late structural genes, like in petunia (*Petunia hybrida*), snapdragon (*Antirrhinum majus*), and Arabidopsis (*Arabidopsis thaliana*; Mol et al., 1998). In these species, the expression of the early biosynthetic genes is controlled by other regulators (sometimes redundantly with the MBW complex; Tornielli et al., 2009; Xu et al., 2014). In some cases (e.g. flavonol or phlobaphene synthesis), a simpler mechanism just involving MYB factors has been described (Grotewold et al., 1994, 2000; Mehrtens et al., 2005; Stracke et al., 2007).

The conserved cooperation between MYB, bHLH, and WD40 proteins activates transcription, although the specificity for a given target is generally set by the participation of the appropriate R2R3-MYB factor. Several negative regulators of flavonoid synthesis have also been isolated, such as truncated bHLH or single-domain R3-MYB proteins (Burr et al., 1996; Kroon, 2004; Dubos et al., 2008; Albert et al., 2014). Some R2R3-MYB factors also operate as repressors. Members of subgroup 4 (which form part of the C2 repressor motif clade) share the presence of an ethylene response factor-associated amphiphilic repression (EAR) motif (Kranz et al., 1998) involved in the repression of transcription (Jin et al., 2000).

A hierarchical and feedback gene regulatory network for flavonoid synthesis has been suggested in eudicots, where active and passive MYB repressors are able to interfere with the proper assembly of the MBW activation complex (Albert et al., 2014). However, the differences observed in MYB repressors regarding their capacity to affect distinctive points of the phenylpropanoid pathway are still far from being clarified. Many R2R3-MYB repressors have been previously characterized in monocots and eudicots. The MYB4 homologs from Arabidopsis and petunia directly repress phenylpropanoid biosynthetic genes, such as *CINNAMATE-4-HYDROXYLASE* (*C4H*) or *4-COUMARATE CoA LIGASE* (*4CL*), responsible for the synthesis of UV-protecting sinapate esters or the floral volatiles phenylacetaldehyde and methyl benzoate (Jin et al., 2000; Colquhoun et al., 2011). In maize, ZmMYB42 and ZmMYB31 repress lignin genes affecting cell wall structure, composition, and degradability (Sonbol et al., 2009; Fornalé et al., 2010). MYB repressors specifically regulating flavonoid synthesis have also been found. Such is the case of AtMYB7, a flavonol branch repressor (Fornalé et al., 2014), or the anthocyanin-related strawberry (*Fragaria* sp.) FaMYB1 and PhMYB27, which are either restricted to reproductive organs (FaMYB1; Aharoni et al., 2001; Schaart et al., 2013) or ubiquitous but differentially regulated by light or developmental signals through the MBW complex (PhMYB27; Albert et al., 2011, 2014).

The scenario for the regulation of flavonoid synthesis depends on the plant species and the origin of its genome structure, which is influenced by natural variation and crop domestication episodes. In the case

of grapevine (*Vitis vinifera*), the sequencing of its genome has provided examples of divergence within the R2R3-MYB transcription factor family (Matus et al., 2008). Although the main amplification event of this family may have occurred before the separation of monocots and eudicots (Rabinowicz et al., 1999), maize and grapevine families may have experienced later duplication events associated with their allotetraploid or hexaploid genome origins, respectively (Rabinowicz et al., 1999; Vision et al., 2000; Jaillon et al., 2007). In addition, phylogenetic comparisons reveal that specific duplications of some subgroups or clades occurred, such as in the case of the flavonoid-related clades in maize (Dias et al., 2003) and grapevine (Matus et al., 2008). In the case of grapevine, most of these groups correspond to positive regulators, among which we found the well-characterized anthocyanin-related MYBA1-A2 (Walker et al., 2007) and the PA-related MYBPA1-PA2 (Bogs et al., 2007; Terrier et al., 2009). However, the C2 repressor motif clade is also expanded (Matus et al., 2008). Nine gene models are found in grapevine, which is in contrast to the five and six genes found in rice (*Oryza sativa*) and Arabidopsis, respectively. From these, MYBC2-L1 was recently studied in its repressive role for PA accumulation (Huang et al., 2014). A function in anthocyanin synthesis was not tested, although a repressive role may also be inferred.

The aim of this work was to test the role of different members of the grapevine C2 repressor motif clade in the regulation of the phenylpropanoid pathway. Functional information was gathered by expression of these genes in a colored genotype of petunia and also, transient expression in grapevine plantlets. Furthermore, we overexpressed the uncharacterized MYBC2-L3 gene in a homologous system of grapevine hairy roots. Our results suggest that C2 repressors fine tune different branches of the phenylpropanoid pathway for achieving different metabolic outputs according to a specific organ or in response to environmental stress.

## RESULTS

### Isolation and Sequence Analysis of C2 Repressor R2R3-MYB Transcription Factors

Nine gene models form part of the C2 repressor motif clade in grapevine (Supplemental Table S1). These are all independent genes, because they are positioned in different chromosome regions. Eight of these models were originally found by Matus et al. (2008) in the PN40024 grapevine 8.4X genomic sequence, where two were isolated from cv Cabernet Sauvignon and named MYB4a (*VIT\_03s0038g02310*; EF113078) and MYBC2-L1 (*VIT\_01s0011g04760*; EU181425). MYBC2-L1 was also isolated from the nonpigmented cv Maccabeu by Huang et al. (2014). With the idea of isolating the closest homologs of MYB4a and MYBC2-L1, we further designed primers for amplifying the open reading frames of *VIT\_04s0023g03710* and *VIT\_17s0000g02660* from cv

Cabernet Sauvignon prebloom inflorescence complementary DNA (cDNA) and *VIT\_14s0006g01620* from a pool of cDNAs originating from different organs and developmental stages of cv Corvina. These were cloned and named *MYB4b*, *MYBC2-L2*, and *MYBC2-L3*, respectively, and they were submitted to GenBank under the accession numbers FJ792820, GQ903730, and KM046932, respectively. Analysis of the deduced amino acid sequences revealed that all of these proteins possess the conserved N-terminal R2R3 repeat, which corresponds to the DNA-binding domain of MYB-type proteins (Fig. 1A; Supplemental Fig. S1). The C-terminal C1 (I<sub>sr</sub>GIDP<sub>x</sub><sup>T</sup>/<sub>N</sub>HR) and C2 (p<sub>d</sub>LNL<sup>D</sup>/<sub>E</sub>L) motifs, characteristic of subgroup 4 (Kranz et al., 1998), were also present in all of these grapevine sequences and located subsequently after the R2R3 repeats. *MYB4a* is a 251-amino-acid-long protein identical to the predicted sequence from the PN40024 12X V1 genome, whereas *MYB4b*, which is 242 amino acids in length, shares 99% identity between the cv Cabernet Sauvignon and the reference cv Pinot Noir sequences. This single-amino acid substitution is located in the C1 motif. *MYB4a* and *MYB4b* share 75% identity. *MYBC2-L1*, *MYBC2-L2*, and *MYBC2-L3* proteins are 225, 226, and 228 amino acids long, respectively; all are identical to their corresponding predicted gene models in PN40024.

Phylogenetic analyses together with protein motif discovery using the Multiple EM for Motif Elicitation (MEME) bioinformatic tool (Fig. 1) allowed us to divide the C2 repressor motif clade into additional subclades and redefine the motifs previously classified by Kranz et al. (1998) and Aharoni et al. (2001). *MYB4a* and *MYB4b* were closely related to *AtMYB4* and *PhMYB4*, whereas *MYBC2-L1*, *MYBC2-L2*, and *MYBC2-L3* were more related to the strawberry *FaMYB1* and petunia *PhMYB27* anthocyanin repressors (Fig. 1A). In addition to the C1 and C2 motifs, *MYB4a* and *MYB4b* possess a putative Zinc Finger (ZnF) domain (C3) that is also present in other *Arabidopsis* and maize homologs but absent in *AtMYB6*, *AtMYB8*, and the grapevine *PhMYB27*-like homologs. The inspection of all of these sequences in MEME confirmed a fourth motif identified by Shen et al. (2012), termed C4, with the conserved dFLGL and LD<sup>F</sup>/<sub>v</sub>RxLEMK amino acid signatures (Fig. 1B). By including in the motif search analysis the single-repeat MYB and the flavonoid repressor *AtMYB12* (Dubos et al., 2008; Matsui et al., 2008), a putative TLLLFR-type repressor motif (named C5) was found in *MYBC2-L1*, *MYBC2-L2*, and *MYBC2-L3* at the very end of their carboxyl terminus (Fig. 1B; Supplemental Fig. S1). Based on these sequences and motif analyses, we were able to categorize C2 repressors into four subclades (A–D) according to the presence of C1, C2, C3, and C4 motifs. Subclade D was further subgrouped based on the presence of the TLLLFR (C5) motif. Despite the fact that *FaMYB1* does not possess a proper C1 motif, all members of subclade D are more closely related than the other subclades because of the occurrence of similar amino acid changes in the conserved R2R3 domain (depicted with vertical bars in

the protein diagrams in Fig. 1B and red crosses in the alignment in Supplemental Fig. S1).

MYB repressors and activators used in this phylogenetic analysis showed the highest similarity within their R2R3 domain. This domain is responsible for DNA binding and mediating response specificity, but it is also in charge of the interaction with other cofactors. C2 repressors showed differences in one of two elements that, in MYB activators, mediate the specificity for either the anthocyanin or PA pathway. This conserved element, named A2 box (Stracke et al., 2001) or element 3 (Heppel et al., 2013), possesses the signature ANDV (or SNDV in fewer cases) within anthocyanin regulators belonging to more than 15 different plant species (Lin-Wang et al., 2010). Instead, if the sequence for this element is exchanged to DNEI, the specificity is redirected to the regulation of PA synthesis (Heppel et al., 2013). The importance of this element in C2 MYB repressors has not been assessed before. However, while in subclades A, B, and C, the sequence of this element is DNEI; in subclade D, the sequence is DNEV (Supplemental Fig. S1).

We assessed the presence of six bHLH interaction residues described by Grotewold et al. (2000) for the maize C1 activator, which is located in the R3 repeat of every MYB activator of anthocyanin and PA biosynthesis. These residues form part of a signature ([DE]Lx2[RK]x3Lx6Lx3R) described by Zimmermann et al. (2004) for *Arabidopsis* bHLH-interacting MYBs. Grape C2 repressor proteins share most of the amino acids described for C1 (Supplemental Fig. S2) and have a perfect match with the signature described in Zimmermann et al., 2004 (Supplemental Fig. S1), suggesting a putative interaction with bHLH cofactors.

### Spatiotemporal Expression of the C2 MYB Repressors

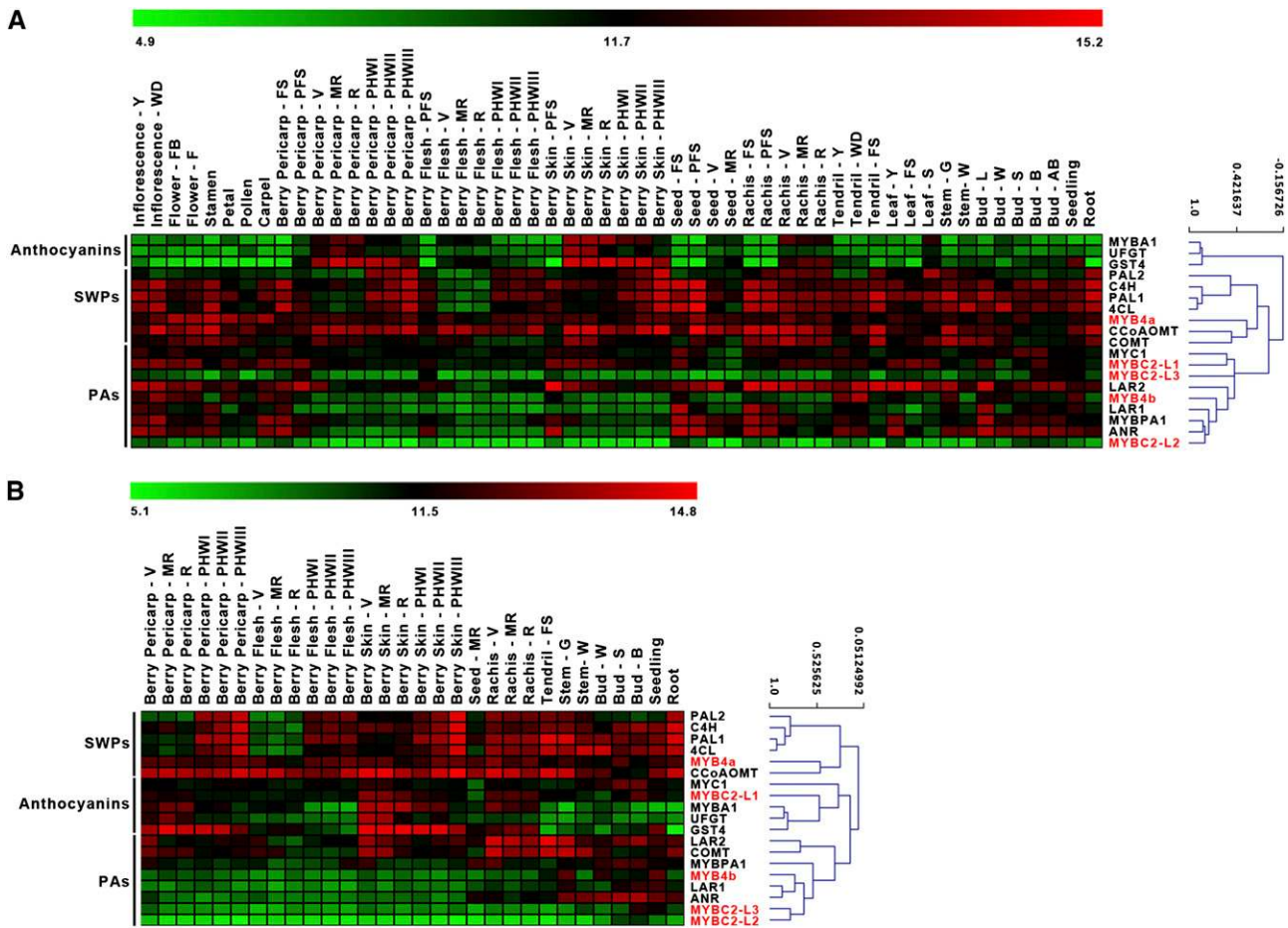
In an attempt to analyze the spatiotemporal expression of the isolated MYB repressors in grapevine, we made use of the previously published grapevine ‘Corvina’ global gene expression atlas (Fasoli et al., 2012). To assess putative transcriptional relationships between MYB repressors and phenylpropanoid-related genes, we included the expression data from key enzymatic players of different branches of the pathway. In particular, genes coding for *PHE AMMONIA LYASE (PAL)*, *C4H*, *4CL*, *CAFFEYOYL-CoA O-METHYLTRANSFERASE (CCoAOMT)*, and *CAFFEIC ACID 3-O-METHYLTRANSFERASE (COMT)* were selected as SWP-related genes. Additionally, the anthocyanin-related genes *MYBA1*, *UDP GLC: FLAVONOID-3-O-GLUCOSYLTRANSFERASE (UFGT)*, and *GLUTATHIONE S-TRANSFERASE4 (GST4)*; Walker et al., 2007; Conn et al., 2008) and the PA-related genes *MYBPA1*, *LEUCOANTHOCYANIDIN REDUCTASE1 (LAR1)*, *LAR2*, and *ANTHOCYANIDIN REDUCTASE (ANR)*; Bogs et al., 2005, 2007) were included together with the bHLH regulator of the flavonoid pathway *MYC1* (Hichri et al., 2010).



The constructed heat map allows visualization of the general gene expression tendencies of the repressors isolated in this work (Fig. 2A). *MYB4a* is ubiquitously expressed with the highest levels in floral organs and seeds after fruit set, whereas *MYB4b* shows a high expression throughout early inflorescence stages and in all mature floral organs. Also, high transcript levels were detected for *MYB4b* in vegetative organs, such as tendrils, green stems, and seedlings. *MYBC2-L1* showed a distinctive expression profile in berry skin and rachis, where high mRNA levels were found until ripening. Additionally, it was expressed in early- and

late-stage flowers (petals and carpels), all leaf developmental stages, latent buds, and buds at bud break. *MYBC2-L2* showed very low expression levels in almost all organs, with a slight expression detected in berry tissues and seeds at early stages of development, young leaves, and buds close to the bud-break period. Similarly, *MYBC2-L3* was expressed weakly in berry tissues at postfruit set and also, buds at or after bud break.

Different relationships between the expression of MYB repressors and phenylpropanoid-related genes were established based on the use of two different sets



**Figure 2.** Expression analysis in the grapevine 'Corvina' atlas in 54 organs/tissues during development (A) and in a restricted set of organs selected for low expression of PA-related genes (B). The genes are the five C2 MYB repressors (red); the regulators *MYBA1* (VIT\_02s0033g00410), *MYBPA1* (VIT\_15s0046g00170), and *MYC1* (VIT\_07s0104g00090); the SWP-related genes *PAL1* (VIT\_08s0040g01710), *PAL2* (VIT\_16s0039g01170), *C4H* (VIT\_06s0004g08150), *4CL* (VIT\_11s0052g01090), *CCoAOMT* (VIT\_07s0031g00350), and *COMT* (VIT\_16s0098g00850); the PA structural genes *ANR* (VIT\_00s0361g00040), *LAR1* (VIT\_01s0011g02960), and *LAR2* (VIT\_17s0000g04150); and the anthocyanin-related genes *UFGT* (VIT\_16s0039g02230) and *GST4* (VIT\_04s0079g00690). The gene expression data were calculated as  $\log_2$ , and 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, véraison.



of organs (Fig. 2). A first combination using the whole set of organs from the atlas showed that *MYB4a* clustered with SWP-related *COMT* and *CCoAOMT* genes, all of which show ubiquitous expression (Fig. 2A). However, *MYB4b*, *MYBC2-L1*, *MYBC2-L2*, and *MYBC2-L3* clustered together with PA-related genes, which have expression that is remarkably increased at green berry stages. Using this first set of organs, none of the repressors clustered with genes specifically related to the anthocyanin branch (i.e. *MYBA1*, *UFGT*, and *GST4*). Considering that the spatiotemporal accumulation of anthocyanins and PAs is highly divergent in grapevine organs, we performed a hierarchical cluster analysis in a selection of organs, excluding those with the highest expression of PA-related genes. This allowed establishment of a relationship between *MYBC2-L1* and anthocyanin genes, whereas *MYB4b*, *MYBC2-L2*, and *MYBC2-L3* still maintained the high correlation with genes of the PA branch. This additional analysis suggests that *MYBC2-L1* may not only regulate PA synthesis but also, control multiple branches of the flavonoid pathway.

Given the high level of identity among subclade D2 members (Fig. 1A), the general low expression of *MYBC2-L2* (Fig. 2A), and the highly similar expression profile between *MYBC2-L2* and *MYBC2-L3* (Fig. 2B), we did not further investigate the function of *MYBC2-L2*.

Quantitative PCR (qPCR) was conducted to test the developmental expression of MYB repressors on berry skins, inflorescences, and seeds of field-grown cv Cabernet Sauvignon plants. The results confirmed the expression profiles retrieved from the cv Corvina microarray expression atlas (Fig. 3A; Supplemental Fig. S3). In particular, *MYB4a* and *MYBC2-L1* showed similar expression profiles in berry skin development, with a drop point at véraison (onset of ripening) and an increase thereafter, whereas *MYB4b* and *MYBC2-L3* were mainly expressed during early berry development (Fig. 3A).

To correlate these expressions with the phenylpropanoid composition found throughout berry skin development, we performed HPLC analysis of berry skin extracts from the same field plants. Anthocyanins increased from véraison onward, whereas SWP compounds all tended to decrease toward ripening (Fig. 3B), which was largely proven in several previous works (Matus et al., 2009; Toffali et al., 2011). The main hydroxycinnamic acids found in grape skins correspond to caffeic and ferulic acid and also, derivatives of the former, such as caftaric and chlorogenic acid (Lee and Jaworski, 1987), which result from the esterification of caffeate with tartaric and quinic acid, respectively (Supplemental Fig. S4). PAs are largely known to accumulate at early stages during berry development (Bogs et al., 2007; Toffali et al., 2011) and were not quantified in this work.

Taken together, the transcript profile analyses indicate that the expression pattern of the C2 MYB repressors is generally consistent with their possible involvement in the regulation of specific branches of

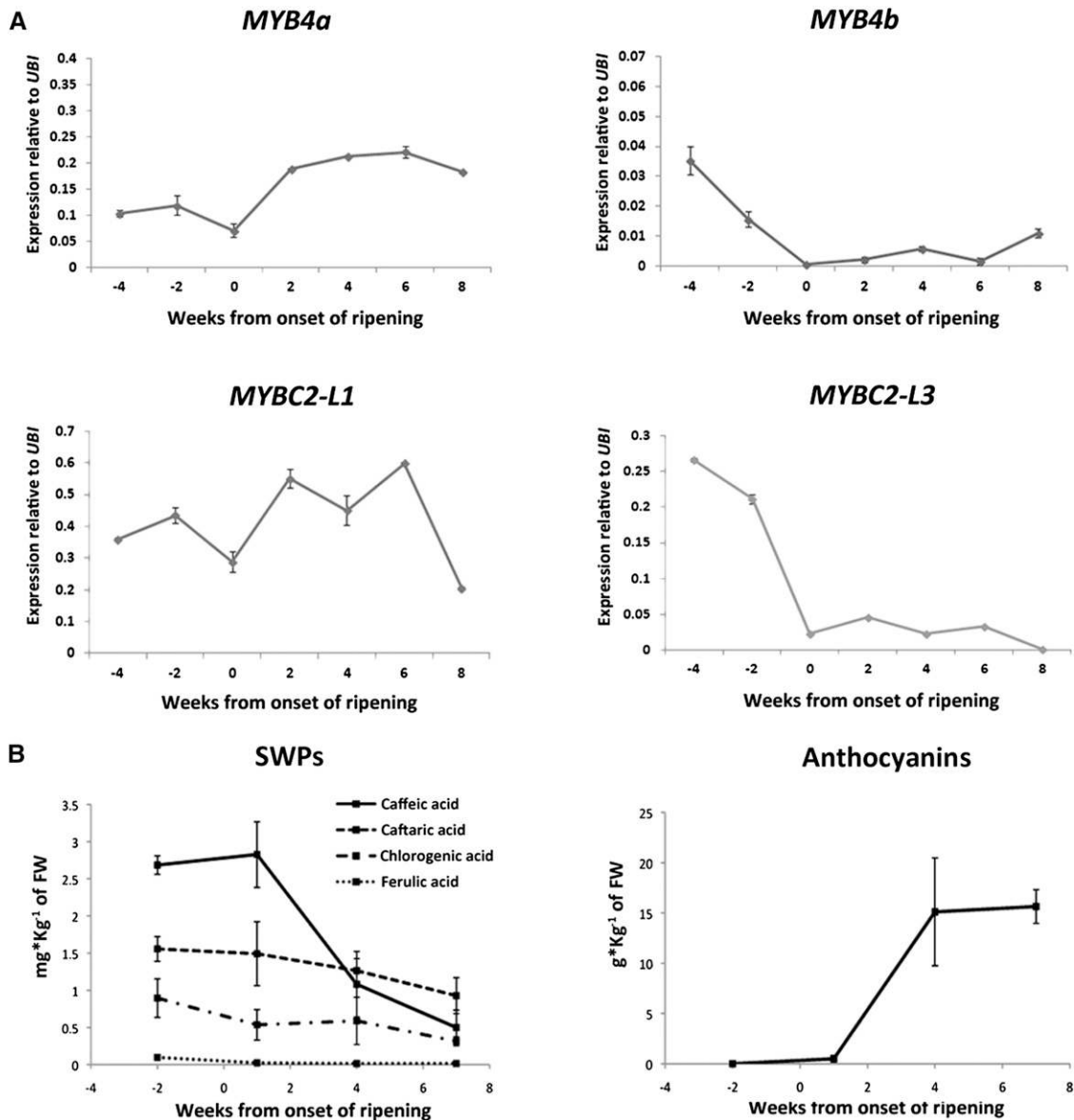
the phenylpropanoid metabolism. Specifically, *MYB4a* expression is consistent with a developmental role in the regulation of SWPs, whereas *MYB4b* and *MYBC2-L3* expressions coincide with PA accumulation, and the expression of *MYBC2-L1* suggests its involvement in both anthocyanin and PA biosynthesis.

#### Heterologous Expression of C2 MYB Repressors in *Petunia* Modifies the Phenylpropanoid Composition of Petals

To explore the function of *MYB4a*, *MYB4b*, *MYBC2-L1*, and *MYBC2-L3*, we exploited the model plant *petunia* for heterologous expression experiments. The coding sequences of each *MYB* gene were cloned under the control of the 35S promoter and used to transform the wild-type V30xM1 line, which is characterized by purple petal pigmentation. We obtained eight independent lines for each transgene. All plants were analyzed by qPCR for determining the individual transgene expression level. To correlate the expression levels of each transgene with phenotypic traits of the plants, we selected three independent lines for each transgene characterized by different transgene transcription levels (Supplemental Fig. S5). However, all *MYB4a* transformants presented similar expression levels. For each transgene, the line with the highest expression level was selected for additional phenotypic analyses. Apart from the pigmentation phenotypes described below, the transgenic plants did not show any additional alterations, consistent across independent lines.

The expression of *MYB4a* and *MYB4b* did not cause any apparent change in petal pigmentation compared with the wild-type line (Fig. 4A). Occasionally, *MYB4b*-expressing plants showed erratic whitening in the distal part of corollas (Supplemental Fig. S6A). In contrast, the expression of *MYBC2-L1* and *MYBC2-L3* led to a clear reduction in petal pigmentation, albeit with different intensity and distribution patterns. *MYBC2-L1*-transformed plants exhibited residual pink pigmentation predominately localized around the main veins and a paler color in the petal margins (Fig. 4A). A range of other phenotypes and color patterning was observed in flowers of the same transgenic line, in which distinct regions of the corolla were distinguished with different intensity and/or irregular distribution of pigmentation (Supplemental Fig. S6B). *MYBC2-L3*-expressing lines showed a more severe petal color phenotype, resulting in an almost complete loss of pigmentation, with white flowers and only a few sporadic pigmented sectors (Fig. 4A). For 35S:*MYBC2-L1* and 35S:*MYBC2-L3* plants, the strongest phenotypes correlated with the highest expression levels of the respective transgenes (Supplemental Fig. S5). Quantification analysis by spectrophotometer (pink bars in Fig. 4A) showed no significant differences in anthocyanin accumulation in 35S:*MYB4a* and 35S:*MYB4b* petals compared with the wild type, whereas a strong reduction was observed in *MYBC2-L1*- and *MYBC2-L3*-expressing petals.

Because in *petunia*, the anthocyanin and the vacuolar acidification pathways share a common regulatory



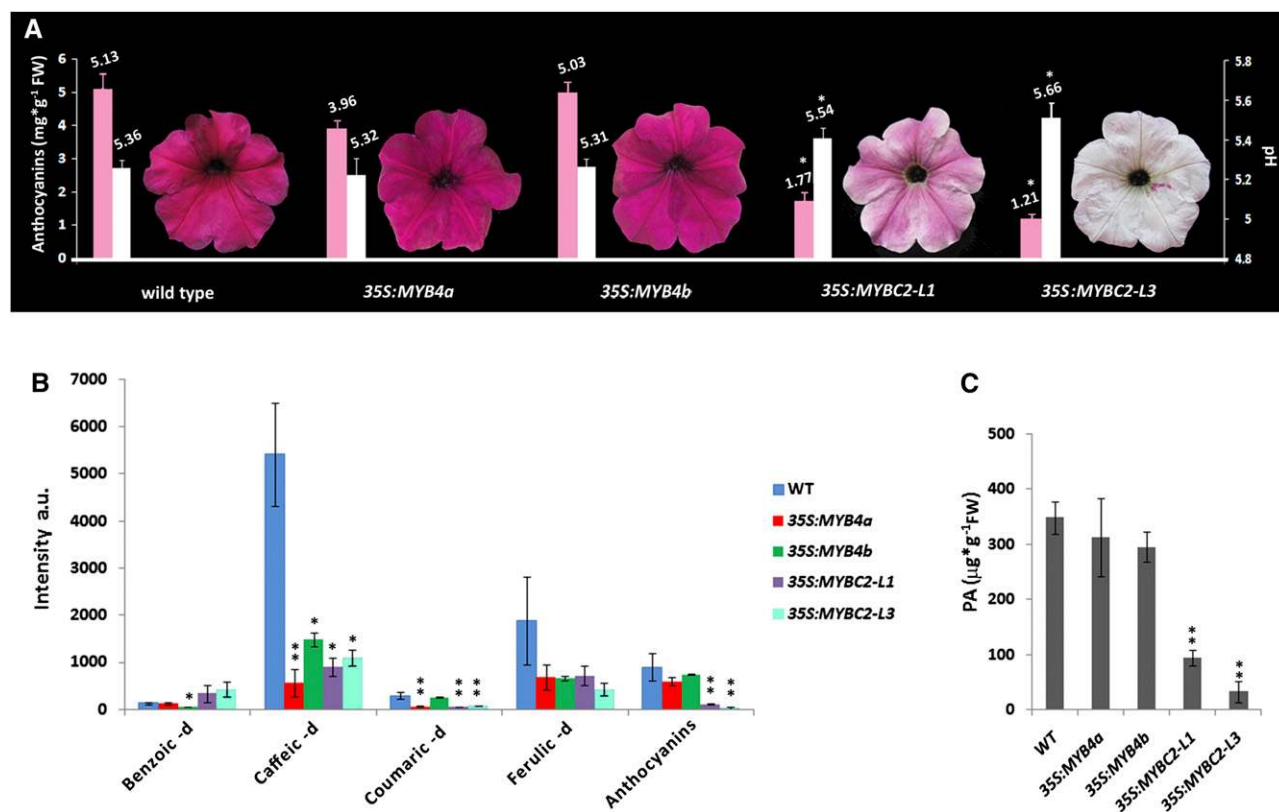
**Figure 3.** A, Expression analysis of C2 MYB repressors by qPCR in berry skin of cv Cabernet Sauvignon at different times from the onset of ripening (véraison). The data correspond to the means  $\pm$  SE of three biological replicates normalized against the expression of *UBIQUITIN1 (UBI1)*. B, Metabolomic analysis by HPLC of berry skin of cv Cabernet Sauvignon sampled at different times from the onset of ripening (véraison). The data correspond to the means  $\pm$  SD of four biological replicates. FW, Fresh weight.

mechanism mediated by the MBW complex (Spelt et al., 2002; Quattrocchio et al., 2006), we measured the pH of crude petal extracts of all transgenic and wild-type plants (white bars in Fig. 4A). The analysis revealed negligible changes in the pH of *35S:MYB4a* and *35S:MYB4b* petals compared with the control, whereas significantly increased values were detected in *MYBC2-L1* and more substantially, *MYBC2-L3* lines.

The metabolic profiles of transgenic petals were investigated by liquid chromatography (LC)-mass spectrometry (MS; Fig. 4B; Supplemental Data S1). To be able to detect in depth the biochemical phenotypes induced by

the constitutive expression of the MYB genes, an untargeted approach was used. The expression of *MYB4a* and *MYB4b* led to a decrease in the amount of hydroxycinnamic acid derivatives, whereas only in *MYB4b* plants, benzoic acid derivatives were reduced (Fig. 4B). In *MYBC2-L1* and *MYBC2-L3* transgenic lines, we detected a general decrease of hydroxycinnamic acid compounds and a strong reduction of anthocyanins, with a stronger effect observed in *35S:MYBC2-L3* petals (Fig. 4B; Supplemental Data S1).

To evaluate the effect of each repressor on the PA branch of the flavonoid pathway, we used the seeds of



**Figure 4.** Phenotypic analysis of transgenic petunia plants (line V30xM1) expressing *MYB4a*, *MYB4b*, *MYBC2-L1*, and *MYBC2-L3*. A, Flower phenotype of untransformed plant compared with *35S:MYB4a*, *35S:MYB4b*, *35S:MYBC2-L1*, and *35S:MYBC2-L3* lines. The relative anthocyanin content determined spectrophotometrically at 540 nm represents the means  $\pm$  SD of three biological replicates (pink bars). The pH of crude petal extracts represents the means  $\pm$  SD of 10 biological replicates (white bars). \*, Significant differences compared with the wild type (WT;  $P < 0.05$ ). B, Comparison of benzoic acid, hydroxycinnamic acid, and anthocyanin levels in petunia petals of the wild type and *35S:MYB4a*, *35S:MYB4b*, *35S:MYBC2-L1*, and *35S:MYBC2-L3* lines. The total amount of each group of molecules was obtained by adding together the content of the respective compound derivatives (-d) from each line. Each value is the mean  $\pm$  SD of three biological replicates. Asterisks indicate significant differences of the total amounts compared with the wild type. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . C, PA content in seeds of the wild type and *35S:MYB4a*, *35S:MYB4b*, *35S:MYBC2-L1*, and *35S:MYBC2-L3* lines. Each value is the mean  $\pm$  SD of three biological replicates. FW, Fresh weight; \*\*, Significant differences compared with the wild type ( $P < 0.01$ ).

self-fertilized transgenic and wild-type lines. The seed coat pigmentation appeared similar to the wild type for the *35S:MYB4b* line, slightly less colored for the *35S:MYB4a* line, and paler for *35S:MYBC2-L1* and *35S:MYBC2-L3* seeds (Supplemental Fig. S6C). However, the analysis of PA content showed that only *35S:MYBC2-L1* and *35S:MYBC2-L3* seeds were characterized by a strong reduction of these flavonoids (Fig. 4C).

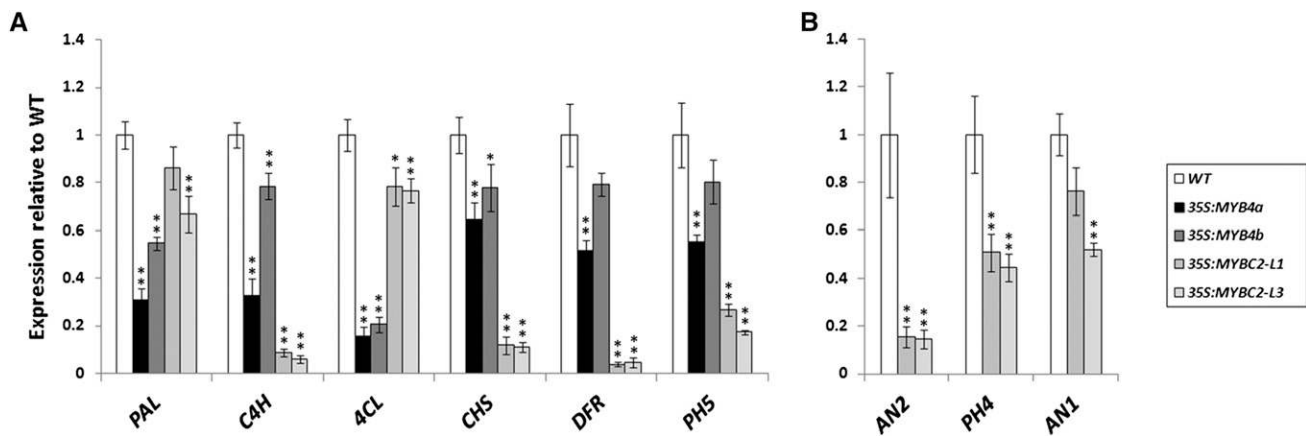
These results suggest that all C2 MYB repressors may interfere with the general phenylpropanoid pathway and that, additionally, only *MYBC2-L1* and *MYBC2-L3* are able to affect anthocyanin and PA accumulation and the vacuolar acidification pathway in petunia.

#### Reduction of Specific Phenylpropanoids in Transgenic Petunias Correlates with Down-Regulation of Different Genes of the Biosynthetic Pathway

To investigate the phenotypic and metabolic changes related to SWP/flavonoid biosynthesis and vacuolar

acidification of petals in the generated transgenic plants, we followed the expression of structural genes belonging to different branches of the pathway: the general phenylpropanoid genes *PAL*, *C4H*, and *4CL*, the early flavonoid gene *CHALCONE SYNTHASE (CHS)*, the late flavonoid gene *DIHYDROFLAVONOL REDUCTASE (DFR)*, and the proton P-type  $H^+$ -ATPase-encoding gene *PH5*. The expression of *MYB4a* and to a lesser extent, *MYB4b* led to a general decrease in the expression of these genes (Fig. 5A). However, the strongest down-regulation was detected in both transgenic lines for *PAL*, *C4H*, and *4CL*, which is in agreement with the decreased amount of SWPs revealed in the metabolomic analysis (Fig. 4B). The slight reduction of *CHS*, *DFR*, and *PH5* expression by *MYB4a* and *MYB4b* is consistent with the nonsignificant changes in anthocyanin accumulation and vacuolar acidification in these plants (Fig. 4, A and B), indicating that the impact of these MYBs on these pathways is very limited. Regarding *MYBC2-L1*- and *MYBC2-L3*-expressing lines,





**Figure 5.** Expression analysis of phenylpropanoid- and pH-related genes in petunia petals by qPCR. A, Expression of structural genes *PAL*, *C4H*, *ACL*, *CHS*, *DFR*, and *PH5* in the wild type (WT) and all transgenic lines. B, Expression of regulatory genes *AN2*, *PH4*, and *AN1* in the wild type and *35S:MYBC2-L1* and *35S:MYBC2-L3* lines. The data correspond to the means  $\pm$  SE of three biological replicates relative to an *ACTIN* housekeeping control and normalized against the wild-type value. Asterisks indicate significant differences compared with the wild type. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

we observed a strong down-regulation of *C4H*, which represents a crucial point in the metabolism of hydroxycinnamic acids, and it may, therefore, explain the reduced content of many phenylpropanoid compounds in these petals (Fig. 4B). A strong down-regulation was detected for the structural genes *CHS*, *DFR*, and *PH5*, which correlate with the anthocyanin reduction and the pH increase of the *MYBC2-L1*- and *MYBC2-L3*-expressing petals (Fig. 4, A and B). To ascertain if the expression of anthocyanin and vacuolar pH regulatory genes could also be impaired in these two transgenic lines, we analyzed the mRNA levels of the anthocyanin MYB regulator *ANTHOCYANIN2* (*AN2*), the vacuolar pH MYB regulator *PH4*, and the bHLH regulator of both pathways *AN1* (Quattrocchio et al., 1999, 2006; Spelt et al., 2000; Fig. 5B). In both *35S:MYBC2-L1* and *35S:MYBC2-L3*, a strong reduction of *AN2* and *PH4* gene expression was observed, whereas *AN1* down-regulation was less pronounced.

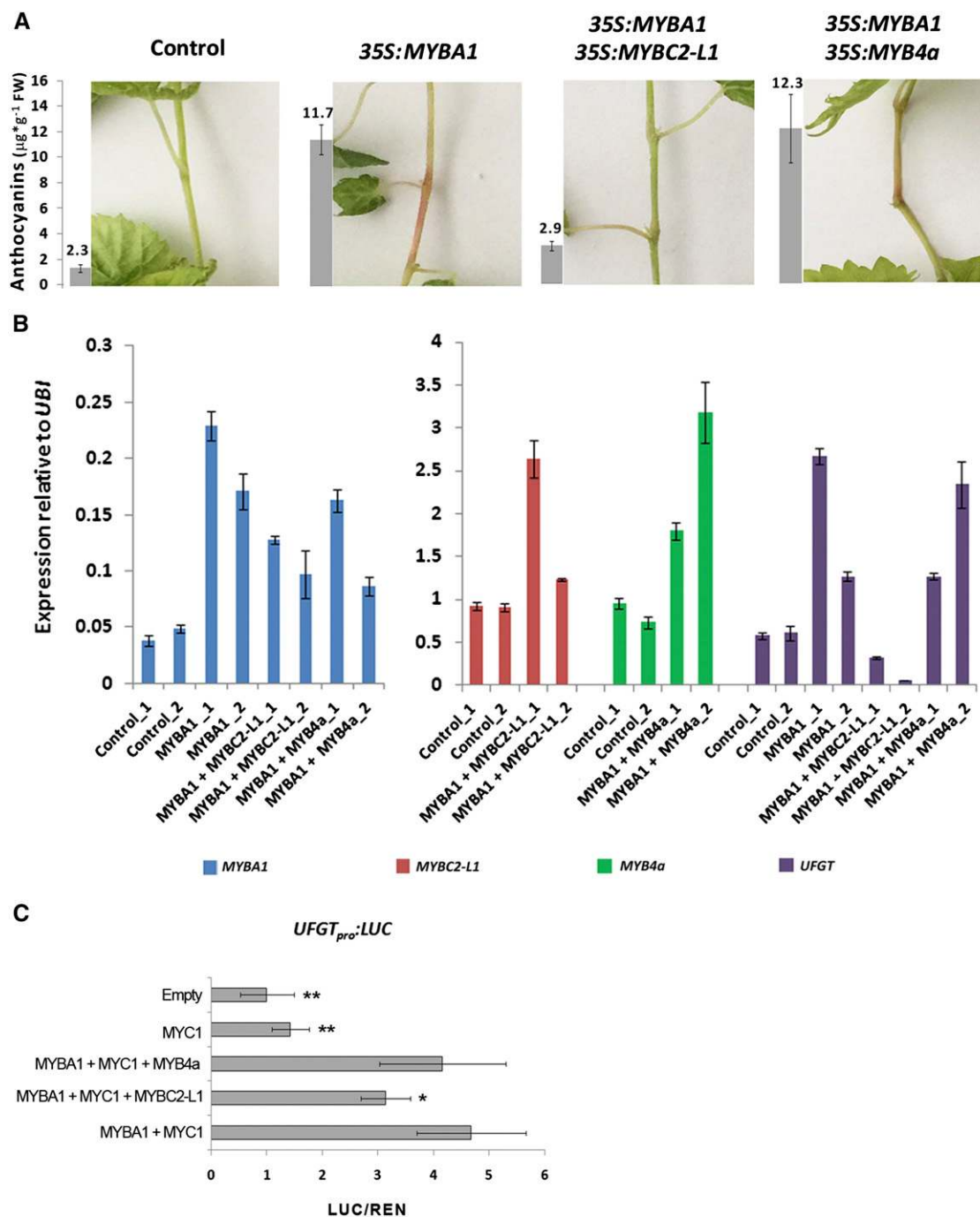
These results indicate that, in the petunia heterologous system, subclades A and D grapevine repressors participate in the control of the general phenylpropanoid pathway through the negative regulation of different enzymatic steps. Only *MYBC2-L1* and *MYBC2-L3* seem able to affect anthocyanin synthesis, possibly by direct repression of the structural genes or indirectly through the down-regulation of the *MYB* and *bHLH* activator genes.

#### Transient Expression of *MYBC2-L1* in Grapevine Represses Anthocyanin Accumulation Orchestrated by *MYBA1*

Based on the co-occurrence of high *MYB4a* and *MYBC2-L1* expression with anthocyanin accumulation (Figs. 2 and 3), we tested their ability to repress this

flavonoid branch by approaching a transient transformation assay in grapevine. Plantlets of cv Sultana were vacuum infiltrated with *Agrobacterium tumefaciens* to overexpress the anthocyanin activator *MYBA1* (1) independently, (2) combined with *MYBC2-L1*, or (3) combined with *MYB4a*. Among five plantlets agroinfiltrated for each combination, we selected two independent lines overexpressing the respective *MYB* genes to a similar extent (Fig. 6, A and B). The overexpression of *MYBA1* alone resulted in an increased pigmentation that was mainly observed in stems (Fig. 6A). A similar effect was observed in *MYBA1/MYB4a* lines, whereas *MYBA1/MYBC2-L1* plantlets were more similar to the control lines in terms of stem pigmentation (Fig. 6A). Anthocyanin content was increased for *MYBA1* and *MYBA1/MYB4a* stems with respect to *MYBA1/MYBC2-L1* and control lines, consistent with the observed pigmentation phenotypes (Fig. 6A). The expression analysis of the anthocyanin structural gene *UFGT*, which is directly regulated by *MYBA1* (Walker et al., 2007), showed that it was clearly down-regulated in *MYBA1/MYBC2-L1* lines compared with *MYBA1* and *MYBA1/MYB4a* (Fig. 6B). These data support the ability of *MYBC2-L1* to inhibit the anthocyanin pathway in grapevine, counterbalancing the action of *MYBA1*. On the contrary, *MYB4a* has no impact on this flavonoid branch. Finally, we also analyzed these plantlets for the expression of the SWP-related gene *CCoAOMT*, which had an expression profile that was closely correlated to *MYB4a* in the gene hierarchical clustering from the microarray expression atlas (Fig. 2). The results reveal that *CCoAOMT* is repressed in *MYBA1/MYB4a* and *MYBA1/MYBC2-L1* plantlets compared with control lines (Supplemental Fig. S7).

The functional diversification of *MYB4a* and *MYBC2-L1* in repressing the accumulation of anthocyanins was further evaluated by analyzing their ability to interfere



**Figure 6.** *Agrobacterium* sp.-mediated transient expression of *MYBA1*, *MYBA1/MYBC2-L1*, and *MYBA1/MYB4a* in grapevine 'Sultana.' A, Pigmentation phenotypes of stems in control and *MYBA1*, *MYBA1/MYBC2-L1*, and *MYBA1/MYB4a* lines. The relative anthocyanin content determined spectrophotometrically at 540 nm is represented by a gray bar at the left of each image. Values correspond to the means  $\pm$  se of two biological replicates. B, Expression analysis of *MYBA1*, *MYBC2-L1*, *MYB4a*, and *UFGT* on two independent lines of each combination by qPCR. The data correspond to the means  $\pm$  se of three technical replicates. C, Dual-luciferase assay of the *UFGT* promoter activation in *Agrobacterium* sp.-infiltrated tobacco (*Nicotiana benthamiana*) leaves. Firefly luciferase (LUC) values are reported relative to the REN control and normalized to the negative control (empty). Each value represents the mean  $\pm$  sd of three biological replicates. FW, Fresh weight; *UBI1*, *UBIQUITIN1*. Asterisks indicate significant differences of the total amounts compared with the wild type. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

in the activation of the *UFGT* promoter by performing a dual-luciferase assay in agroinfiltrated leaves of tobacco (Fig. 6C). The MYBA1/MYC1-directed activation of the *UFGT* promoter was significantly lower when *MYBC2-L1* was cotransfected, and no changes were observed in the *MYBA1/MYC1/MYB4a* combination. These results give additional evidence that, in contrast to *MYB4a*, *MYBC2-L1* is a negative regulator of anthocyanin biosynthesis in grapevine.

#### Yeast Two-Hybrid Assays Reveal Different Affinities of C2 MYB Repressors with Flavonoid-Related bHLH Proteins

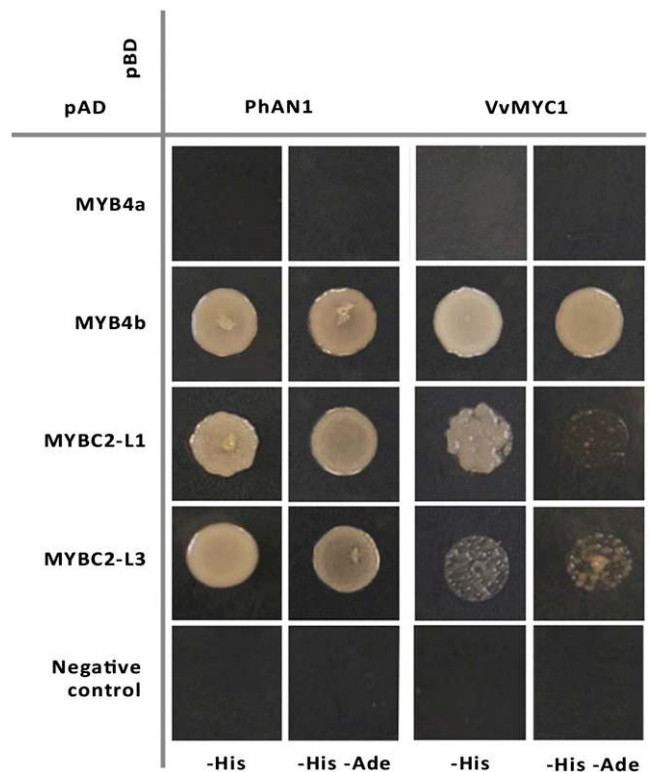
In many plant model systems, it has been established that the combinatorial interactions between MYB and bHLH transcription factors within the MBW complex are crucial for the regulation of the specific branches of the flavonoid pathway (Zimmermann et al., 2004; Hichri et al., 2010). All MYB repressors in this study possess the bHLH-interacting signature [DE]Lx2[RK]x3Lx6Lx3R in the R3 repeat (Supplemental Figs. S1 and S2). Their ability to interact with bHLH factors was, therefore, investigated in yeast (*Saccharomyces cerevisiae*) two-hybrid assays using two TRANSPARENT TESTA8 (TT8)-like bHLH proteins, PhAN1 and its close homolog VvMYC1, which had been previously characterized as regulators of the flavonoid pathway in petunia and grapevine, respectively (Spelt et al., 2000; Hichri et al., 2010). The bHLHs were fused to the GAL4 Binding Domain (GAL4 BD), and the different MYB proteins were fused to the GAL4 Activation Domain (GAL4 AD). A construct containing a short noncoding sequence fused to the AD was used as negative control. Yeasts cotransformed with bHLH-BD and MYB-AD constructs were tested for the expression of the reporter genes *ADE2* and *HIS3*, and the strength of the interactions was evaluated by growing the transformants on media lacking Trp, Leu, and His (weak) or Trp, Leu, His, and adenine (strong; Fig. 7).

As shown by growth on  $-His -Ade$ , *MYB4b* strongly interacted with both PhAN1 and VvMYC1, whereas no interaction with these bHLH factors was observed for *MYB4a* (Fig. 7). *MYBC2-L1* and *MYBC2-L3* strongly bound to petunia AN1, suggesting that the negative regulation of the flavonoid and acidification pathways observed in *MYBC2-L1*- and *MYBC2-L3*-expressing plants (Fig. 4) occurs through the participation of the MBW complex. They also bound the grapevine MYC1, but the strength of these interactions seemed very weak.

Albeit that these results need to be confirmed in planta, the different interactions with the bHLHs assayed in yeast indicate that the grapevine C2 MYB repressors may have distinct regulatory roles exerted by distinct molecular mechanisms.

#### UV-B Radiation Modifies the Expression of Subclade A C2 Repressors

Several R2R3-MYB proteins from subgroup 4 have been previously shown to be involved in UV radiation-



**Figure 7.** Interaction of the MYB repressors *MYB4a*, *MYB4b*, *MYBC2-L1*, and *MYBC2-L3* with the bHLH MYC1 of grapevine and AN1 of Petunia by yeast two-hybrid analysis. Yeasts were cotransformed with different combinations of plasmids expressing the MYB proteins fused to the AD and bHLHs fused to BD and selected for activation of the *HIS* and *ADE* reporter genes. The negative control contains a short noncoding sequence fused to the AD.

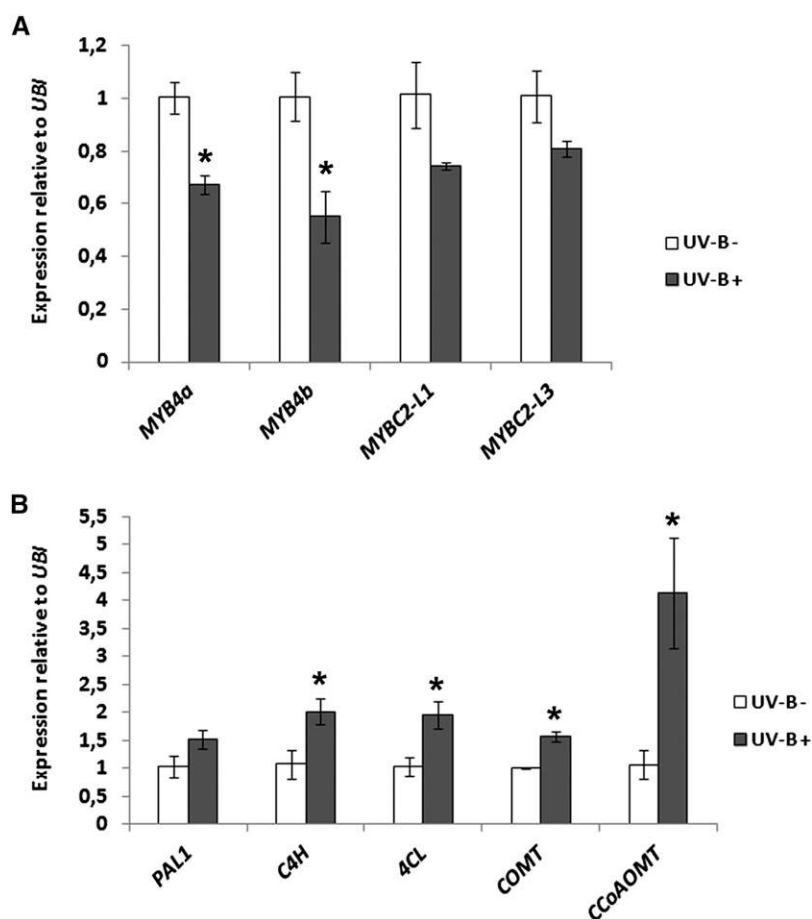
protective responses (Jin et al., 2000; Fornalé et al., 2010). The Arabidopsis *myb4* mutants were more UV-B tolerant than wild-type plants, whereas *AtMYB4* overexpression increased sensitivity to radiation, enhancing seedling death (Jin et al., 2000). *AtMYB4* repressed *C4H* expression and negatively regulated the synthesis of UV-B-absorbing sinapate esters. In relation to this function, the expression of *AtMYB4* was increased in response to blue light (early response) and x-rays (late response) but diminished after 6 h of UV-B radiation (Jin et al., 2000). To test the possible involvement of grapevine C2 repressors in UV-B responses, we irradiated young grape plantlets with low fluence UV-B (approximately  $15 \mu W cm^{-2}$  of irradiance with a dose of  $1.6 J cm^{-2} d^{-1}$ ) and followed gene expression changes after 6 h of radiation. *MYB4a* and *MYB4b* were significantly down-regulated upon radiation (Fig. 8A). *MYBC2-L1* and *MYBC2-L3* had a slight tendency toward repression, but at least in our experimental setup (6 h at low fluence UV-B), this was not significant. In correlation to these findings, we observed a significant up-regulation of *C4H*, *4CL*, *COMT*, and *CCoAOMT* genes, all related to the synthesis of SWP compounds (Supplemental Fig. S4A).

However, the *PAL* gene that we tested was not significantly affected. The increase in the expression of these genes supports the UV-induced SWP accumulation observed in previous experiments (Del-Castillo-Alonso et al., 2015). These results propose a conserved behavior and role of MYB4 homologs in response to UV radiation and suggest a set of putative targets.

#### Ectopic Expression of *MYBC2-L3* in Grapevine Hairy Roots Reduces PA Accumulation and Represses a Narrow Set of Flavonoid-Related Genes

Recently, it was shown that *MYBC2-L1* was able to inhibit PA biosynthesis when expressed in grapevine hairy roots (Huang et al., 2014). To analyze the involvement of *MYBC2-L3* in this pathway and further investigate its role in grapevine, we ectopically expressed its coding sequence in hairy roots of grapevine 'Maccabeu.' Of 10 independent transgenic hairy root clones obtained, we selected two lines with the highest expression of *MYBC2-L3* (185- and 181-fold compared with the control; Supplemental Fig. S8A). The PA amount was reduced in both transgenic lines (0.70 and 0.77 mg g<sup>-1</sup> of fresh weight) compared with the control (2.53 mg g<sup>-1</sup> of fresh weight; Supplemental Fig. S8B), suggesting that, similar to the overexpression of *MYBC2-L1* (Huang et al., 2014),

*MYBC2-L3* is able to impair the synthesis of PAs in grapevine. To test this hypothesis and also, determine the whole set of downstream genes modulated by *MYBC2-L3*, a microarray analysis was carried out on transgenic and wild-type hairy roots using the Nimblegen platform (Microarray Gene Expression Omnibus database accession no. GSE58742). Three biological replicates corresponding to three clones of the highest overexpressor and the control line were used. The transcriptomes of 35S:*MYBC2-L3* and wild-type lines were analyzed using both a coexpression-based approach (Usadel et al., 2009) to identify genes showing negatively correlated profiles with respect to *MYBC2-L3* and a two-class unpaired comparison using significance analysis of microarray (SAM; Table I; Supplemental Data S2). All genes selected by these approaches were automatically annotated against the V1 gene prediction version of the grapevine genome (Grimplet et al., 2012) and manually improved where possible. Among the genes most negatively correlated to *MYBC2-L3* expression, we found many structural genes related to phenylpropanoid, general flavonoid, or PA synthesis and transport (Table I; Supplemental Data S2). These included a *PAL*, a cinnamoyl-CoA reductase, *CHS1*, *CHS2*, *CHS3* (Goto-Yamamoto et al., 2002; Harris et al., 2013), *FLAVANONE3-HYDROXYLASE1* (*F3H1*), *LAR*,



**Figure 8.** Expression analysis by qPCR in young grape plantlets after UV-B radiation exposure of the C2 repressors *MYB4a*, *MYB4b*, *MYBC2-L1*, and *MYBC2-L3* (A) and the phenylpropanoid structural genes *PAL1*, *C4H*, *4CL*, *COMT*, and *CCoAOMT* (B). *UBI1*, *UBIQUITIN1*. \*, Significant differences against the control ( $P < 0.05$ ).

*ANR*, the putative PA transporters *MULTIDRUG AND TOXIC COMPOUND EXTRUSION1 (MATE1)* and *MATE2* (Pérez-Díaz et al., 2014), and the three glucosyltransferases (*GT1*, *GT2*, and *GT3*) recently proposed to be involved in PA galloylation (Khater et al., 2012). Interestingly, several positive and negative regulators of the flavonoid pathway were found among the genes that were most negatively correlated to *MYBC2-L3*. These were the regulator of PA biosynthesis *MYBPA1* (Bogs et al., 2007), the regulator of the general flavonoid pathway *MYB5a* (Deluc et al., 2006, 2008), the bHLH regulator of flavonoid/anthocyanin biosynthesis *MYC1* (Hichri et al., 2010), the two C2 MYB repressors *MYBC2-L1* and *MYB4a* described in this research work, and an homolog of the Arabidopsis R3 MYB repressor *CAPRICE* (Schellmann et al., 2002; Song et al., 2011).

The comparison between the 35S:*MYBC2-L3* and the wild-type transcriptomes by SAM analysis led to the identification of 124 genes modulated by *MYBC2-L3*. These included 107 down-regulated and 17 up-regulated genes (Supplemental Data S2). Among the most down-regulated genes, we found many phenylpropanoid-/general flavonoid-/PA-related genes that occupied the top of the list of genes with expressions that were negatively correlated with *MYBC2-L3* expression (Table I; Supplemental Data S2). This reinforces the possibility that these genes are targets of *MYBC2-L3* in grapevine. The down-regulation of some flavonoid-related genes (i.e. *MYC1*, *MYBC2-L1*, *MYBPA1*, *ANR*, *LDOX*, and *MATE2*) was confirmed by qPCR (Supplemental Fig. S9).

In addition to the genes encoding enzymes of the flavonoid pathway, this experiment revealed the down-

**Table I.** The most negatively correlated genes with *MYBC2-L3* expression (with Pearson's correlation coefficient < -0.98) associated with their fold change in 35S:*MYBC2-L3* grapevine hairy roots

For each gene, the gene identification, description, Pearson's correlation coefficient, and fold change are reported. FC, Fold change; PPC, Pearson's correlation coefficient.

Gene Identification	Description	PCC	FC
VIT_12S0028G01150	Transparent testa 12-like (VvMATE2)	-0.999	-3.69 <sup>a</sup>
VIT_01S0127G00800	Polyamine oxidase precursor	-0.999	-1.84 <sup>a</sup>
VIT_14S0083G00320	Cinnamoyl-CoA reductase	-0.996	-3.91 <sup>a</sup>
VIT_12S0028G01160	Transparent testa 12-like (VvMATE1)	-0.996	-2.56
VIT_14S0068G00920	Chalcone synthase 2 (VvCHS2)	-0.995	-4.32 <sup>a</sup>
VIT_05S0136G00260	Chalcone synthase 3 (VvCHS3)	-0.995	-3.28 <sup>a</sup>
VIT_04S0023G03370	Flavanone 3-hydroxylase 1 (VvF3H1)	-0.994	-2.64
VIT_14S0068G00930	Chalcone synthase 1 (VvCHS1)	-0.993	-4.01 <sup>a</sup>
VIT_14S0030G00290	No hit	-0.993	-3.44 <sup>a</sup>
VIT_04S0079G00680	Phytoene synthase (VvPSY1)	-0.993	-1.95 <sup>a</sup>
VIT_14S0219G00040	ZnF (C3HC4-type RING finger)	-0.993	-1.87 <sup>a</sup>
VIT_18S0001G09530	ZnF (C3HC4-type RING finger)	-0.992	-2.49
VIT_06S0004G02620	Phe ammonia-lyase (VvPAL)	-0.992	-3.82 <sup>a</sup>
VIT_01S0011G02960	Leucoanthocyanidin reductase 1 (VvLAR1)	-0.991	-4.51 <sup>a</sup>
VIT_07S0104G00090	Basic helix-loop-helix (VvMYC1)	-0.991	-2.98 <sup>a</sup>
VIT_04S0023G00210	GRAM domain-containing protein/ABA-responsive	-0.99	-1.86
VIT_14S0030G00260	Sugar transporter ERD6-like 3	-0.989	-2.87 <sup>a</sup>
VIT_13S0067G02870	Chalcone isomerase 2 (VvCHI2)	-0.989	-2.56
VIT_14S0030G00280	Sugar transporter ERD6	-0.989	-2.82
VIT_03S0180G00200	Hydroxybenzoate/hydroxycinnamate UDP-glucosyltransferase (VvGT2)	-0.988	-2.9 <sup>a</sup>
VIT_10S0071G00560	GRAM domain-containing protein/ABA-responsive	-0.988	-2.23 <sup>a</sup>
VIT_03S0063G00810	Carboxyesterase CXE12	-0.988	-2.22
VIT_06S0009G01110	Cation exchanger CAX7	-0.987	-3.23 <sup>a</sup>
VIT_02S0025G04720	Leucoanthocyanidin dioxygenase (VvLDOX)	-0.987	-2.29
VIT_02S0025G02390	Oligopeptide transporter OPT7	-0.986	-2.75 <sup>a</sup>
VIT_00S0361G00040	Anthocyanidin reductase (VvANR)	-0.986	-2.48
VIT_01S0011G04760	MYB domain protein 4 (VvMYBC2-L1)	-0.985	-4.64 <sup>a</sup>
VIT_03S0091G00040	Hydroxybenzoate/hydroxycinnamate UDP-glucosyltransferase (VvGT1)	-0.983	-3.09 <sup>a</sup>
VIT_09S0002G04340	Glabra2-like	-0.983	-2.59 <sup>a</sup>
VIT_14S0006G02310	No hit	-0.983	-2.56 <sup>a</sup>
VIT_06S0080G00330	Esterase/lipase/thioesterase family protein	-0.983	-2.37
VIT_06S0080G00570	Unknown protein	-0.983	-1.74
VIT_08S0007G03800	Unknown protein	-0.982	-5.6 <sup>a</sup>
VIT_01S0010G02070	Ethylene-responsive protein	-0.982	-1.89 <sup>a</sup>
VIT_17S0000G02650	MYB domain protein 7 (VvMYBC2-L4)	-0.981	-4.17 <sup>a</sup>
VIT_11S0052G01700	CBL-interacting protein kinase CIPK21	-0.981	-2.36 <sup>a</sup>
VIT_03S0063G01790	Transducin protein	-0.981	-2.66 <sup>a</sup>
VIT_00S0125G00280	PSI reaction center subunit III (PSAF)	-0.98	-2.88 <sup>a</sup>

<sup>a</sup>Genes significantly modulated by SAM analysis.



regulation of (1) several transcripts related to ion and sugar transport, (2) several members of the family of patatin-like phospholipases, and (3) transcription factors of still unknown function. These genes may represent additional targets of MYBC2-L3.

Overall, these data indicate that the overexpression of *MYBC2-L3* led to the reduction of PA content in the transgenic grapevine hairy roots through the down-regulation of many structural and regulatory genes related to the biosynthesis of PAs. MYBC2-L3 can, therefore, act like its close homolog MYBC2-L1 in the control of PA synthesis. Moreover, the repression of *MYBC2-L1* as well as other flavonoid regulators supports the hypothesis that the synthesis of these compounds is controlled by a complex hierarchical regulatory network.

## DISCUSSION

### Divergence and Evolutionary Features of the Grapevine C2 Repressor Motif Clade

The C2 repressor motif clade was named by Matus et al. (2008) while inspecting the R2R3-MYB family in the 8.4X genome assembly of the near-homozygous genotype of cv Pinot Noir (Jaillon et al., 2007). These gene annotations are highly similar to the Arabidopsis homologs belonging to subgroup 4, which were originally described by Kranz et al. (1998). Subgroup 4 was originally defined by the presence of C1, C2, and ZnF motifs. AtMYB3, AtMYB7, AtMYB4, and AtMYB32 form part of this subgroup, but AtMYB6 and AtMYB8, which do not have a ZnF motif, were excluded (Stracke et al., 2001; Dubos et al., 2010). Our phylogenetic analyses show that R2R3-MYB repressors from subgroup 4 are always associated with other C2 repressors that may lack the C4 or ZnF motifs. Therefore, we believe that a more accurate classification of this R2R3-MYB subgroup should include all proteins holding a C2 repressor motif (subclades A–D as in Fig. 1).

The C2 motif, also known as the EAR repression domain, is present in a wide range of repressors from different transcription factor families (Kagale et al., 2010). The ability of such a domain to convert a transcriptional activator into a repressor has been widely shown by chimeric protein fusion experiments (Hiratsu et al., 2003; Matsui and Ohme-Takagi, 2010). Arabidopsis EAR domain-containing proteins show the DLNxxP or LxLxL sequence conservation pattern within the core sites (Kagale et al., 2010). Most grape C2 MYB repressors studied here together with the Arabidopsis repressors AtMYB4, AtMYB7, and AtMYB32 have the exact LxLxL pattern, whereas PhMYB27 has a DLNSPP signature. MYBC2-L1 is only different from this pattern in the last residue of the core sequence (Val replacing Leu). However, both are nonpolar residues, suggesting that the function of this motif is conserved in MYBC2-L1.

The C4 motif was described by Shen et al. (2012) and confirmed with our study to be only present in subclade A repressors. This group seems to be generalized

for both monocot and dicotyledoneous lineages. The C4 motif is present in the petunia MYB4 homolog, principally involved in the negative regulation of floral volatile benzoid/phenylpropanoid compounds (Colquhoun et al., 2011), present in switchgrass (*Panicum virgatum*) PvMYB4, and present in maize ZmMYB31 and ZmMYB42 negative regulators of the early phenylpropanoid and lignin pathways (Sonbol et al., 2009; Fornalé et al., 2010; Shen et al., 2012). The C4 motif is not present in the snapdragon AmMYB308 (Tamagnone et al., 1998), despite the fact that this flavonoid repressor is much closer to subclade A members (data not shown).

Subclades C and D may have evolved differently in each species. For instance, D1 may be exclusive to strawberry and closely related species. The role of C1 motif has not been characterized yet, but it may not be related to any repression role, because FaMYB1, which lacks this motif, strongly inhibits flavonoid synthesis (Aharoni et al., 2001; Paolucci et al., 2011). The D3 subclade members share the TLLLFR repression motif that is also found in AtMYBL2, a single-repeat MYB with a strong repression activity over flavonoid synthesis (Dubos et al., 2008; Matsui et al., 2008). Arabidopsis has no subclade D members, although AtMYBL2 may have evolved from this group (e.g. by lineage-specific deletion of the R2 repeat). AtMYBL2 shares most of the subclade D-specific residues, leaving the possibility that it was originally an R2R3 MYB (Fig. 1B; Supplemental Fig. S1). Matsui et al. (2008) determined that the last six amino acids (TLLLFR) constituted a minimal repression domain different from the EAR motif. The presence of this motif is at the very end for MYBC2-L1, MYBC2-L2, and MYBC2-L3. To our knowledge, these are the first described plant R2R3-MYB transcription factors to have a TLLLFR motif, giving support to their strong repression activity found in our work for anthocyanin and PA synthesis.

The regulatory specificity of MYB proteins is given by the DNA-binding domain (Ogata et al., 1995). In addition, the R3 domain is involved in bHLH interactions. The conserved amino acid signature ([DE]Lx2 [RK]x3Lx6Lx3R) positioned between the R3 $\alpha$ 1 and R3 $\alpha$ 2 helices forms a characteristic surface-exposed pattern of charged residues, which constitute the structural basis for the MYB-bHLH interaction (Zimmermann et al., 2004). Although the maize C1 MYB factor, responsible for anthocyanin production, depends on the bHLH R factor for its regulatory function, the closely related P MYB does not (for review, see Mol et al., 1998). Grotewold et al. (2000) showed that four residues from C1 in the R3 $\alpha$ 1 helix were enough to confer P the ability to interact with R, and two additional residues in R3 $\alpha$ 2 were able to make P dependent on R for transcriptional activation. Grape genes involved in flavonoid synthesis show high identities in these residues with the maize ZmC1 protein (Supplemental Fig. S2). FaMYB1 and PhMYB27, both of which can interact with petunia bHLH AN1 and JAF13 (Aharoni et al., 2001; Albert et al., 2014), share similar residues as those found in the grapevine homologs. Thus, the

C2-like MYB repressors of grapevine were potential candidates for interacting with flavonoid bHLH factors.

#### MYB4a and MYB4b Are Negative Regulators of the General Phenylpropanoid Pathway

We explored the function of MYB4a and MYB4b to assess their potential role in the repression of the phenylpropanoid pathway. Our results show that these proteins behave similarly to other subgroup 4 homologs, such as AtMYB4 and PhMYB4, by retaining a conserved repression capacity toward a similar group of genes. Among these genes, we could find those involved in the synthesis of hydroxycinnamic acids, which constitute a starting point for the production of lignins, flavonoids, stilbenes, and many other compounds. The expression of *MYB4a* and *MYB4b* in petunia reduced the amount of specific SWP compounds in petals, with little or no impact on anthocyanin content. This is likely the result of the repressive action primarily exerted on early phenylpropanoid structural genes, such as *PAL*, *C4H*, and *4CL*, as previously shown for AtMYB4 and PhMYB4 (Jin et al., 2000; Colquhoun et al., 2011). Gene expression analysis also revealed a weak down-regulation of flavonoid- and acidification-related genes (more evident in petals of *MYB4a*-expressing plants), although this down-regulation might not have been sufficient to cause an apparent effect on petal pigmentation and pH. We suggest that MYB4a and MYB4b may repress genes acting downstream in the general phenylpropanoid pathway, albeit with a lower efficiency. However, the possibility of a reduced selectivity toward target genes related to the high levels of the 35S-driven transgene cannot be ruled out.

Consistent with the hypothesis of a preferential role in the general phenylpropanoid pathway, transient expression of *MYB4a* in grapevine revealed that MYB4a down-regulates the expression of *CCoAOMT*, the gene related to the metabolism of SWP (Supplemental Fig. S7). Also, it did not have any impact on the synthesis of anthocyanins. Recently, Salazar (2013) showed that maize *COMT* and *CCoAOMT* genes were directly bound and transcriptionally regulated by ZmMYB31, ZmMYB42, and ZmMYB11 repressors.

We could also highlight some functional differences between MYB4a and MYB4b. LC-MS metabolite profiling of transgenic petunia petals revealed some differences between *MYB4a* and *MYB4b* transformants. Other than a common impact on caffeic acid derivatives, *MYB4a* expression caused the reduction of coumaric acid derivatives, whereas in *MYB4b* lines, benzoic acid derivatives were affected. This suggests different abilities of these MYB4 homologs to regulate the genes of the SWP pathway in addition to the shared repressive action on *PAL* and *4CL*. MYB4b is able to reduce intermediates of the benzenoid pathway, thus behaving more similarly to the endogenous PhMYB4 than MYB4a.

The interaction between MYB and bHLH transcription factors is crucial for the establishment of a regulatory complex controlling the late enzymatic steps of the anthocyanin and PA pathways (Xu et al., 2014). The inability of MYB4a and MYB4b to affect the anthocyanin and acidification pathways and the very weak repressive action on the MBW-regulated *DFR* and *PH5* genes suggest that these genes do not participate in the MBW complex and do not control MBW-dependent pathways. Only in some rare cases, we did observe a slight pigmentation reduction in petals of *MYB4b*-expressing petunias, suggesting an interference with the MBW-dependent anthocyanin pathway. Yeast two-hybrid assays revealed that MYB4b interacted with petunia PhAN1 and grapevine MYC1. Considering also that *MYB4b* clusters with *MYC1* in hierarchical analysis of expression profiles (Fig. 2A), these results lead to intriguing questions about the possibility that MYB4b may affect the transcription of MBW targets through a competitive interaction with a bHLH partner. Conversely, MYB4a was unable to bind both bHLH proteins, suggesting that the mechanism of repression of MYB4a in grapevine may not involve at least the MYC1 protein.

#### MYBC2-L1 and MYBC2-L3 Are Repressors of the Anthocyanin and PA Pathways

Our phylogenetic analysis showed that MYBC2-L1 and MYBC2-L3 clustered separately from subclade A and B members and were grouped together with strawberry FaMYB1 and petunia PhMYB27 (Fig. 1). Here, we provide evidence for such phylogenetic separation by showing different regulative functions of subclade D genes, because MYBC2-L1 and MYBC2-L3 act as repressors of both anthocyanin and PA synthesis. Petunia plants expressing *MYBC2-L1* and *MYBC2-L3* showed a strong reduction in petal and seed pigmentation caused by a decrease in anthocyanin and PA content and a down-regulation of flavonoid structural genes. A role as an anthocyanin-negative regulator was confirmed for MYBC2-L1 by its transient expression in grapevine and tobacco, showing that, differently from MYB4a, MYBC2-L1 had a specific ability to control the expression of the MYBA1-regulated *UFGT* gene.

The alteration of the anthocyanin and vacuolar acidification pathways, controlled in petunia by the MBW complex (Spelt et al., 2002; Quattrocchio et al., 2006), suggests that MYBC2-L1 and MYBC2-L3 exert their repressive functions by participating with this complex, which has already proposed for FaMYB1 and PhMYB27 (Aharoni et al., 2001; Albert et al., 2014). This is supported by the fact that they contain a bHLH interaction signature (see above) and the results of the yeast two-hybrid assays showing that both MYBC2-L1 and MYBC2-L3 interact with the petunia bHLH PhAN1. Although only a weak interaction was detected with the grapevine bHLH MYC1, the hypothesis of participation in the MBW-dependent processes is conserved

in grapevine. It is possible that, in grapevine, MYBC2-L1 and MYBC2-L3 interact more strongly with other bHLH factors (e.g. MYCA1 described in Matus et al., 2010) that, in turn, may participate in the complex alone or by forming heterodimers with MYC1. The recruitment of MYB repressors by the MBW complex, including bHLH heterodimers, as recently proposed by Albert et al. (2014) seems crucial for the action of repressors, such as PhMYB27. The experimental evidence provided by Albert et al. (2014) suggests that PhMYB27 cannot directly bind DNA and that it is, therefore, unable to repress the transcription of targets by itself. Instead, PhMYB27 seems to act as a corepressor of the MBW complex by repressing genes normally targeted by the MBW activation complex. This mechanism should require an interaction between MYB activators, which specify the target genes, and MYB repressors bridged by bHLH factors. Subclade A and D repressors have been previously tested for their ability to interact with bHLH proteins (FaMYB1 with JAF13 and AN1 [Aharoni et al., 2001] and AtMYB4 and TT8 [Zimmermann et al., 2004]). Despite the fact that the requirement of such interactions in regulating the phenylpropanoid pathway still has to be proven, the fact that AtMYBL2 interacts with bHLH proteins within the MBW complex and directly modulates the expression of flavonoid target genes (Dubos et al., 2008) allows us to hypothesize that an MBW-dependent mode of action may occur with grapevine subclade D2 members.

LC-MS analyses revealed a reduced amount of hydroxycinnamic acid derivatives in MYBC2-L1- and MYBC2-L3-expressing petunia plants, indicating that the action of these negative regulators is not restricted to the anthocyanin pathway. In fact, in addition to the anthocyanin pathway genes, our results indicate that MYBC2-L1 and MYBC2-L3 also strongly reduced the mRNA levels of early biosynthetic genes, like *C4H* and *CHS*, which have expression in petunia that is independent of the MBW regulatory complex (Tornielli et al., 2009). This suggests a flexible mode of action of MYBC2-L1 and MYBC2-L3, which may regulate genes both independently and through the interaction with the MBW complex. These data suggest that R2R3-MYB C2 repressors belonging to subclade D share some functional characteristics with those of subclade A in the regulation of early steps of the phenylpropanoid pathway.

Other than their roles as anthocyanin repressors, MYBC2-L1 and MYBC2-L3 showed repressive action toward PA accumulation in petunia seeds. MYBC2-L1 was previously identified as a specific regulator of PA synthesis in grapevine (Huang et al., 2014), and here, we showed that its homolog MYBC2-L3 down-regulated all known PA-related genes when overexpressed in grapevine hairy roots, leading to a reduction of the total PA content. The negative correlation of expression of these genes with respect to MYBC2-L3 indicates that they might represent targets of this MYB repressor in grapevine. Because MYBC2-L1, MYBC2-L2, and MYBC2-L3 have intact EAR motifs (LxLxL) but

also, TLLLFR-like sequences, it is likely that both the intact EAR and TLLLFR motifs may confer repressive activity; the presence of both may enhance repression, or these motifs may act redundantly. This hypothesis may explain why MYB4b, although it is able to interact with MYC1, is unable to repress the anthocyanin pathway.

### The Complex Transcriptional Relationships between Repressors and Activators

Several lines of evidence indicate that positive and negative regulators of the phenylpropanoid pathway are part of a complex network involving different mechanisms of transcriptional hierarchy and regulatory loops (Albert et al., 2014). Restricting our consideration only to grapevine regulators functionally analyzed in this study or previous studies, there are several indicators suggesting that (1) activators induce repressors, (2) repressors repress activators, and (3) repressors repress repressors (Table II). Cutanda-Perez et al. (2009) found that the MYBC2-L1 repressor was induced after overexpressing the anthocyanin activator *VvMYBA1* in grapevine hairy roots. Similarly, Terrier et al. (2009) found the induction of the same repressor in grapevine hairy roots overexpressing the PA activators MYBPA1 or MYBPA2. Cavallini (2012) reported the induction of both MYBC2-L1 and MYBC2-L3 in grapevine leaves upon ectopic expression of MYB5a or MYB5b, known regulators of the general flavonoid pathway. The functional analysis of MYBA1 and MYB5b carried out in petunia revealed their ability to induce the endogenous repressor *PhMYB27* (Cavallini et al., 2014).

Concerning the transcriptional hierarchy of repressors on activators, we found that MYBC2-L1 and MYBC2-L3 act like the *PhMYB27* ortholog (Albert et al., 2014), down-regulating the expression of the anthocyanin bHLH *PhAN1*. Similarly, the ectopic expression of MYBC2-L3 in grapevine hairy roots led to the down-regulation of the flavonoid bHLH regulator *VvMYC1*. The repressive action could also be mediated by the down-regulation of MYB positive regulators like MYBPA1, which was shown by ectopic expression of MYBC2-L3 in hairy roots (this work) and also, for MYBC2-L1 (Huang et al., 2014). This ability was also proven in our MYBC2-L1- and MYBC2-L3-expressing petunias that revealed the down-regulation of the endogenous genes *PhAN2* and *PhPH4*, which are positive MYB regulators of the anthocyanin and acidification pathways, respectively (Quattrocchio et al., 1999, 2006). Therefore, in addition to an active EAR-mediated repression of structural genes, these repressors might act indirectly to restrain the abundance of bHLH and MYB activators, thus limiting the formation of the MBW transcriptional activation complex. The reciprocal control of expression levels between activators and repressors (Table II) indicates the existence of a regulatory loop, where these proteins collaborate to fine tune the synthesis of secondary metabolites during the development of

**Table II.** *Transcriptional relationships between MYB repressors and activators shown in this study and previous studies*

Gene	Action	Target	Plant System	Reference
<b>Activators</b>				
VvMYBA1	→	VvMYBC2-L1	Grapevine hairy roots	Cutanda-Perez et al.(2009)
VvMYBPA1	→	VvMYBC2-L1	Grapevine hairy roots	Terrier et al. (2009)
VvMYBPA2	→	VvMYBC2-L1	Grapevine hairy roots	Terrier et al. (2009)
VvMYB5a	→	VvMYBC2-L1 VvMYBC2-L3	Grapevine transgenic leaves	Cavallini (2012)
VvMYB5b	→	VvMYBC2-L1 VvMYBC2-L3	Grapevine transgenic leaves	Cavallini (2012)
VvMYBA1	→	PhMYB27	Petunia transgenic petals	Cavallini et al. (2014)
VvMYB5b	→	PhMYB27	Petunia transgenic petals	Cavallini et al. (2014)
<b>Repressors</b>				
VvMYBC2-L1	⊥	VvMYBPA1 VvMYBPA2	Grapevine hairy roots	Huang et al. (2014)
VvMYBC2-L1	⊥	PhAN2 PhAN1	Petunia transgenic petals	This work
VvMYBC2-L3	⊥	PhAN2 PhAN1	Petunia transgenic petals	This work
VvMYBC2-L3	⊥	VvMYBPA1 VvMYB5a VvMYC1 VvMYBC2-L1 VvMYB4a	Grapevine hairy roots	This work

many grapevine organs. Another level of action of C2 repressors possibly involves autorepression or repression of other members of the MYB C2 repressor clade. This does not seem to be an indirect regulation, because Zhao et al. (2007) showed that AtMYB4 binds to its own promoter and inhibits the level of its transcription in a negative autoregulatory loop that would explain the transient gene induction by diverse stimuli (Jin et al., 2000). The *MYBC2-L3* transgenic hairy roots characterized in our work show a down-regulation of *MYBC2-L1* and *MYB4a*. This could be the result of an indirect regulation mediated by the repression of the activators (i.e. *MYBPA1* and/or *MYC1*) or a direct repression by the transgene. These possibilities or other still unknown transcriptional relationships remain to be elucidated.

#### Function of R2R3-MYB C2 Repressors in Grapevine

The expression of repressors in response to environmental or developmental signals may reflect different roles (e.g. production of stress-related sunscreen compounds and fine-tuning regulation of specific metabolite accumulation in determined tissues or cell types, respectively). Considering the published work on FaMYB1, PhMYB27, and AtMYB4, these different roles seems associated with a different expression pattern behavior (i.e. [1] negative correlation with biosynthetic genes of the repressed pathway in the case of environmental stress regulation and [2] positive correlation with biosynthetic genes in the case of developmentally controlled pathways; Aharoni et al., 2001; Jin

et al., 2000; Colquhoun et al., 2011; Schaart et al., 2013; Albert et al., 2014).

The function of the R2R3-MYB C2 repressors studied through the aforementioned approaches together with the analysis of their expression pattern in grapevine organs at various developmental stages and leaves under UV-B treatment allow us to speculate about the biochemical pathways actually regulated in planta. Both MYB4a and MYB4b could be involved in the negative regulation of SWP accumulation mainly in reproductive tissues and in young vegetative organs. However, given their dissimilar expression profiles, MYB4a may have a role throughout all stages of pericarp development, whereas MYB4b may have a role before the onset of ripening. The expression of *MYB4a* is also increased dramatically during flower formation. Therefore, MYB4a may play a role in the late stages of flower development by blocking the synthesis of sporopollenin or polyamine conjugates in anthers by the time that the flowers are opened. Sporopollenin, the major part of the pollen exine, contains high amounts of phenylpropanoids and hydroxylated fatty acids (Dominguez et al., 1999). However, the highest expression of *MYB4b* was detected in tendrils before their lignification. This inflorescence homolog organ is an important reservoir of phenylpropanoids (mainly flavonols, tannins, and lignins), and its development is marked by a high level of transcription of genes related to secondary metabolism (Díaz-Riquelme et al., 2014); therefore, it is possible that MYB4b regulates the maturation of this organ. Because hierarchical clustering analysis showed a close correlation

between MYB4a and SWP-related genes, we propose that MYB4a is involved in the fine-tuning regulation of SWPs, whereas the physiological role of MYB4b seems more elusive and challenging to infer.

As previously shown, the expression of the MYB factors belonging to subgroup A depends on internal and external stimuli (Jin et al., 2000; Fornalé et al., 2014). Arabidopsis *AtMYB4* is down-regulated in response to UV-B treatment, resulting in enhanced *C4H* expression and increased production of phenolic compounds (synapate esters) acting as chemical sunscreens. Our experiments indicate that, like *AtMYB4*, MYB4a and MYB4b are involved in the response to UV-B stress. In fact, the exposure of grapevine 'Cabernet Sauvignon' plantlets to UV-B radiation caused a significant reduction of both *MYB4a* and *MYB4b* expressions together with a concomitant increment of expression of general phenylpropanoid pathway genes in leaves. Consistent with our results, a previous microarray analysis performed on leaves of grapevine 'Malbec' subjected to low- and high-intensity UV-B radiation revealed a down-regulation of *MYB4b* expression (Pontin et al., 2010).

Concerning MYB repressors belonging to subclade D, our data suggest that they have a repression capacity somewhat wider compared with subclade A members; they are able to strongly down-regulate flavonoid branch genes and those belonging to the acidification pathway. Considering the expression profile in grapevine, we propose that MYBC2-L1 acts as a fine-tuning regulator of the synthesis of many classes of phenylpropanoid compounds, including PAs in inflorescences, young berries, and seeds, as well as anthocyanins in berry skins during ripening. However, the general low-expression level of *MYBC2-L3* suggests a limited involvement as a repressor of SWPs and PAs in berry pericarp at early developmental stages, developing buds, and seedlings. The coexpression of *MYBC2-L1* and *MYBC2-L3* with the PA regulator *MYBPA1* or the anthocyanin regulator *MYBA1* supports the idea of subclade D repressors acting as corepressors in the MBW complex (Albert et al., 2014). Despite the fact that *MYBC2-L1* and *MYBC2-L3* were not significantly down-regulated in our UV-B experiment, their involvement in the response to UV-B cannot be excluded, because they may respond to a different dose or time of exposure.

## CONCLUSION

The central backbone of the phenylpropanoid pathway is highly conserved in plants, but its divergent branches transform this metabolic route into a biosynthetic grid of multiple outcomes. The end products of the pathway have gained divergent roles during the course of plant evolution. The productions of sinapate esters in Brassicaceae, phlobaphenes in monocot cereals, or resveratrol-derived stilbenes in Vitaceae plants constitute valuable cases of study for different expression regulatory mechanisms possibly related to the functional

divergence found between phenylpropanoids. In this work, we show that a group of grapevine R2R3-MYB C2 repressors distinctively regulate different branches of the phenylpropanoid pathway and may influence the phenolic composition of different grapevine organs, including fruit tissues at pre- and postripening stages. Our results indicate that MYB4a and MYB4b may play a key role in repressing SWP compound synthesis, whereas MYBC2-L1 and MYBC2-L3 directly fine tune flavonoid levels throughout development by balancing the inductive effects of transcriptional activators.

## MATERIALS AND METHODS

### Plant Material

Reproductive grapevine (*Vitis vinifera* 'Cabernet Sauvignon') organs were collected from a commercial vineyard in the Maipo Valley in Chile. Inflorescence clusters from different developmental stages were included as in Matus et al. (2010). In total, nine grape clusters were sampled from three plants every 2 weeks throughout fruit development beginning 2 to 3 weeks after fruit set (4 weeks before véraison) and ending at 6 or 8 weeks after véraison. Berries were immediately peeled and deseeded. Seeds and skins were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until required for RNA extraction (skins were also used for anthocyanin and SWP compound quantification).

Functional analysis was carried out in petunia (*Petunia hybrida*) plants derived from the collection of Amsterdam University. The V30  $\times$  M1 line used for the transformation did not harbor any mutations in the known anthocyanin loci. All petunia plants were cultivated under normal greenhouse conditions in Verona, Italy.

For grapevine agroinfiltration, plantlets of grapevine 'Sultana' were in vitro micropropagated and cultivated in a growth chamber at  $25^{\circ}\text{C}$  with a 16-h photoperiod.

For the genetic transformation of grapevine 'Maccabeu,' the in vitro plantlets were grown for 90 d under light- and temperature-controlled conditions.

### Bioinformatics

Oligonucleotides were designed using Primer3 ([http://biotoools.umassmed.edu/bioapps/primer3\\_www.cgi](http://biotoools.umassmed.edu/bioapps/primer3_www.cgi)) using grape sequences provided by Genoscope 8.4X or CRIBI 12XV1 genome predictions ([http://www.cns.fr/externe/English/corpus\\_anglais.html](http://www.cns.fr/externe/English/corpus_anglais.html) and <http://genomes.cribi.unipd.it>). Grapevine genes were aligned against the full predicted amino acid sequences of proteins belonging to Arabidopsis (*Arabidopsis thaliana*), maize (*Zea mays*), petunia, snapdragon (*Antirrhinum majus*), Eucalyptus (*Eucalyptus gunnii*), Panicum (*Panicum virgatum*), and *Fragaria*  $\times$  *ananassa*. 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  $\gamma$ -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 was used to analyze protein sequences and discover motifs with an expected value lower than  $2 \times 10^{-30}$  (<http://meme.nbcr.net/>; Bailey et al., 2009) using the following search parameters: four to eight residues (minimum to maximum length), zero or one repetition of motifs, and five motifs maximum. A second motif search included *AtMYBL2* and used the following parameters: six to 18 residues (minimum to maximum length), any number of repetitions, and five motifs to find. The following GenBank accession numbers were used: ABL61515 (VvMYB4a), ACN94269 (VvMYB4b), AAC83582 (AtMYB4), ADX33331 (PhMYB4), CAE09058 (EgMYB1), AEM17348 (PvMYB4a), NP\_001105949 (ZmMYB31), NP\_001106009 (ZmMYB42), NP\_179263 (AtMYB7), NP\_195225 (AtMYB32), ATIG22640 (AtMYB3), P81395 (AmMYB330), NP\_849749 (AtMYB8), NP\_192684 (AtMYB6), AC215912 (ZmMYB11), AAK84064 (FaMYB1), AHX24372 (PhMYB27), ABW34393 (VvMYBC2-L1), ACX50288 (VvMYBC2-L2), KM046932 (VvMYBC2-L3), CAB09172 (AtMYB12), FJ948477 (VvMYBF1), U26935 (AtMYB5), AAS68190 (VvMYB5a), Q58QD0 (VvMYB5b),



AM259485 (VvMYBPA1), ACK56131 (VvMYBPA2), Q9FJA2 (AtMYB123/TT2), AAG42001 (AtMYB75/PAP1), and BAD18977 (VvMYBA1).

## Gene Cloning and Genetic Transformation Procedures

The cDNA sequences of *MYB4a* and *MYBC2-L1* were amplified by PCR from cv Corvina cDNA synthesized from a pool of RNA isolated from mid-ripening and ripening berry skin. The *MYBC2-L3* cDNA sequence was amplified by PCR from cv Corvina cDNA synthesized from a pool of RNA isolated from postfruit set and midripening berry pericarp. The functional characterization of *MYB4b* was carried out using the cDNA sequence amplified by PCR from cv Cabernet Sauvignon inflorescence (after cap fall) RNA, and *MYBC2-L2 L3* cDNA sequence was amplified by PCR from cv Cabernet Sauvignon cDNA synthesized from RNA of a pea-sized berry pericarp. The primer sequences used for the isolation are listed in Supplemental Table S2.

The PCR products were purified and directionally inserted into the Gateway entry vector pENTR/D-TOPO (Invitrogen) aided by the presence of a 5'-CACC-3' leader sequence in each forward primer and verified by sequencing (Macrogen Inc.). For expression in petunia, the products were cloned into the binary overexpression vector pK7GW2.0 (Laboratory of Plant Systems Biology, Ghent University) by site-specific LR recombination. The constructs were then transferred to *Agrobacterium tumefaciens* strain EHA105 by electroporation. Petunia plants were transformed using the leaf disc method (van der Meer, 1999), and regenerated transgenic shoots were transferred to soil and hardened off in a temperature-controlled glasshouse.

For overexpression of *MYBC2-L3* in grapevine 'Maccabeu' hairy roots, the cDNA sequence was cloned into the binary vector pH2GW7 by site-specific recombination. The construct was then inserted into *A. tumefaciens* A4 by electroporation and used for grapevine transformation. The induction and culture of transgenic hairy roots in grapevine were performed as described by Torregrosa and Bouquet (1997), with modifications reported by Cutanda-Perez et al. (2009).

## pH Measurement of Petunia Petal Crude Extracts

The pH of petal extracts was measured by grinding a single-petal limb in 6 mL of distilled water as described by Quattrocchio et al. (2006). Each pH value represented the mean of 10 measurements.

## Quantification of Anthocyanins and SWP Compounds in Grapevine Berry Development

Berry skin samples were weighed and ground with 15 mL of distilled water, 20 mL of hydroalcoholic solution (ethanol:water [10:90, v/v]), and 2.5 g of tartaric acid adjusted to the final solution weight of 100 g. Extracts were macerated for 2 h at 30°C, centrifuged, and filtered with a glass microfiber. Samples were filtered through a 0.45- $\mu$ m membrane under vacuum at less than 35°C and used for HPLC with diode-array detection (DAD) anthocyanin analysis as described by Matus et al. (2009). The detection was carried out by scanning from 210 to 600 nm. SWP was analyzed from a total extraction of nonanthocyanin compounds. These were extracted from an aliquot (50 mL) of macerated and filtered berry skins by mixing the sample three times with 20 mL of diethyl ether and 20 mL of ethyl acetate. The organic fractions were then combined, and extracts were evaporated to dryness under vacuum at less than 35°C. The residue was dissolved in 1 mL of methanol:water (1:1, v/v) and analyzed by HPLC-DAD and HPLC-DAD-MS as in Matus et al. (2009). SWP detection was performed by scanning from 210 to 360 nm with an acquisition speed of 1 s. The identification of compounds was carried out by comparison of their spectra and retention times with those obtained from Peña-Neira et al. (2004, 2007). Quantitative determinations were performed using the external standard method with commercial standards.

## Phenylpropanoid Analysis of Petunia Petals

Petal limb tissues collected from three distinct flowers (representing three biological replicates) were collected from the wild type and each transgenic line. Powdered petal samples were extracted in 8 volumes (w/v) of methanol acidified with 0.1% (v/v) hydrochloric acid in an ultrasonic bath at room temperature at 40 kHz for 15 min. Total amount of anthocyanins was determined by a spectrophotometer at  $\lambda = 540$  nm using malvidin 3-glucoside as the standard. For the determination of phenylpropanoids, HPLC-electrospray

ionization-MS analysis was carried out using a Beckman Coulter Gold 127 HPLC System (Beckman Coulter) equipped with a System Gold 508 Beckman Coulter Autosampler as described in Cavallini et al. (2014). Metabolites were identified by comparing the mass-to-charge ratio values, fragmentation patterns (MS/MS and MS<sup>2</sup>), and retention times of each signal with those of available commercial standards and values reported in the literature. Chromatogram data extraction and alignment were carried out using MZmine software (<http://mzmine.sourceforge.net>), and the final data matrix was processed with Simca P+ 13.0 (Umetrics). The resulting models were statistically validated by performing a permutation test (200 permutations) and a cross validation ANOVA ( $P$  value < 0.01). For comparison between transformed genotypes and the respective wild type, the quantitative metabolite data were further analyzed with  $t$  tests.

## Analysis of PAs in Petunia Seeds and Grapevine Hairy Roots

PAs from petunia seeds were extracted and analyzed as described by Zenoni et al. (2011). Each biological replicate is represented by seeds obtained from one self-fertilized flower from the wild type and each transgenic line. For the analysis in grapevine hairy roots, PAs were quantified after phloroglucinolysis using an Agilent 1100LC HPLC-DAD-Fluorimeter System as described in Verries et al. (2008). In detail, 100 mg of frozen and powdered sample was mixed with 750  $\mu$ L of extraction solution (acetone:water [70:30, v/v] containing 0.05% [v/v] trifluoroacetic acid) and 50  $\mu$ L of internal standard solution (*p*-hydroxy methyl ester; 3 g L<sup>-1</sup> in methanol). After 1 h of incubation, the mixture was centrifuged (13,000g for 15 min at 4°C), and the supernatant was recovered; 200  $\mu$ L of supernatant was dried under vacuum at 35°C for 2 h (Genevac) and resuspended in 100  $\mu$ L of reagent solution (0.25 g of phloroglucinol, 0.05 g of ascorbic acid, and 5 mL of acidified methanol [0.2 N HCl]) for acid-catalyzed degradation in the presence of excess phloroglucinol. After incubation (50°C for 20 min), the reaction was stopped by adding 100  $\mu$ L of sodium acetate buffer (200 mM; pH 7.5). Samples were then centrifuged before injection into the HPLC 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 phloroglucinol adducts for extension units). PA polymer length, estimated by the mean degree of polymerization, was calculated as the molar ratio of the sum of all PA units to the sum of terminal 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 and epicatechins) were analyzed by injecting 5  $\mu$ L into a Waters Millennium HPLC System equipped with a diode-array detector (PDA 996; 190–700 nm) and a fluorimeter detector and then quantified using a W2475 Fluorimeter Detector ( $\lambda_{em} = 275$  nm and  $\lambda_{ex} = 322$  nm). Concentrations were determined from standard curves calculated from pure monomers. Results were expressed in milligrams per gram of fresh weight.

## Transient Transfection Experiments in Grapevine and Dual-Luciferase Assay in Tobacco

For the dual-luciferase promoter assay, the previously isolated *UFGT* promoter and the *Renilla luciferase* gene (*REN*) were cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) and then transferred by site-specific recombination into the binary vectors pPGWL7.0 and pK7WG2.0, respectively (Laboratory of Plant Systems Biology, Ghent University). The effector constructs (each one expressing 35S:MYBA1, 35S:MYC1, 35S:MYBC2-L1, 35S:MYB4a, and 35S:REN) and the construct expressing the Firefly *Luciferase* under control of *UFGT*prom were transferred to *A. tumefaciens* strain EHA105 by electroporation. Promoter assays were performed in *Agrobacterium* sp.-infiltrated tobacco (*Nicotiana benthamiana*) leaves as described by Espley et al. (2009).

For grapevine agroinfiltration, pK7WG2.0 vectors containing *MYBA1*, *MYBC2-L1*, and *MYB4a* coding sequences were transferred to *A. tumefaciens* strain C58C1 by electroporation. Five in vitro plants of grapevine 'Sultana' were immersed in each bacterial suspension and vacuum infiltrated (2  $\times$  2 min at 90 kPa). As control, plantlets transformed with pK7WG2.0 containing a noncoding sequence were used as control. After agroinfiltration, plantlets were rinsed with sterile water and allowed to recover in vitro for 6 d before collecting material for RNA extraction and anthocyanin quantification.

## Expression Analysis by qPCR Analysis

Total RNA was isolated using TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and 1- $\mu$ g aliquots were treated with DNase I (Promega) and then reverse transcribed using Improm-II Reverse Transcriptase (Promega) according to the manufacturer's instructions. The transcriptional profile was analyzed by qPCR using the SYBR Green PCR Master Mix (Applied Biosystems) and an Mx3000P Real-Time PCR System (Stratagene). Each expression value was normalized to *ACTIN* internal control cDNA.

For reproductive organ development and UV-B gene expression studies, total RNA was isolated according to the procedure by Reid et al. (2006) using a cetyl trimethyl ammonium bromide-spermidine extraction buffer. For cDNA synthesis, 1  $\mu$ g of total RNA was reverse transcribed with random primers in a 20- $\mu$ L reaction mixture using the SuperScript II Reverse Transcriptase (Stratagene) according to the manufacturer's instructions. Gene transcriptional profiles were analyzed by qPCR using the SensiMix SYBR Hi-ROX Kit (BIOLINE), and the Mx3000P Detection System (Stratagene) was used. *UBIQUITIN1* was used as the reference gene for normalization.

For grapevine 'Sultana' agroinfiltration experiments, total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) following the manufacturer's protocol, and 1- $\mu$ g aliquots were treated with DNase I (Promega) and then reverse transcribed using Improm-II Reverse Transcriptase (Promega) according to the manufacturer's instructions. The transcriptional profile was analyzed by qPCR using the SYBR Green PCR Master Mix (Applied Biosystems) and an Mx3000P Real-Time PCR System (Stratagene). *UBIQUITIN1* was used as the reference gene for normalization.

For grapevine hairy roots, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. cDNA synthesis was performed with the ImProm-II TM Reverse Transcription System. Gene transcriptional profiles were analyzed by qPCR using the SYBR Green PCR Master Mix (Applied Biosystems) and the model 7300 Sequence Detection System (Applied Biosystems). *UBIQUITIN1* was used as the reference gene for normalization.

In all qPCR analyses, gene expression data (cycle threshold values) were used to quantify relative gene expression by using the efficiency corrected method described by Pfaffl (2001). All qPCR measurements were performed with three technical replicates for the number of biological replicates reported in the respective figures. The primer sequences used for qPCR analysis are listed in Supplemental Table S2.

## Microarray Construction, Hybridization, and Data Analysis

Total RNA for microarray analysis was isolated from transgenic and wild-type hairy roots using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. The three biological replicates used for the experiment corresponded to three clones of both the highest overexpressor and the control line. RNA quality and quantity were determined using a Nanodrop 2000 Instrument (Thermo Scientific) and a Bioanalyzer Chip RNA 7500 Series II (Agilent). The cDNA synthesis, labeling, hybridization, and washing reactions were performed according to the NimbleGen Arrays User's Guide (version 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 Pro-7 software (Molecular Devices) according to the manufacturer's instructions. Images were analyzed using NimbleScan version 2.5 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 four probes for each gene. The normalized gene expression data were finally converted to  $\log_2$  values to process the data. A Pearson correlation analysis was carried out to evaluate the robustness of three biological replicates in each sample.

Correlation analysis of gene expression was performed on a reduced microarray expression data set obtained by removing: (1) low or nonexpressed genes (i.e. genes with normalized median expression values that were <150 in both set of transgenic and wild-type biological triplicates) and (2) genes with low variation of expression levels (i.e. genes with a coefficient of variation < 0.365 among six microarray analyses). Correlation analysis of this reduced data set was performed with *MYBC2-L3* as the query, calculating the Pearson correlation distance by the CorTo tool (<http://www.usadellab.org/cms/index.php?page=corto>).

To identify the genes significantly modulated between the 35S:*MYBC2-L3* and the wild-type hairy roots, a two-class unpaired comparison analysis was carried out using SAM with a false discovery rate of 4% (TMeV 4.3).

## Yeast Two-Hybrid Assay

The coding sequences of *MYB4a*, *MYB4b*, *MYBC2-L1*, *MYBC2-L3*, *MYC1*, and *PhAN1* previously cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) were transferred by site-specific recombination into the vectors pDEST22 (downstream of the *GAL4 AD* sequence) and pDEST32 (downstream of *GAL4 BD* sequence; Laboratory of Plant Systems Biology, Ghent University). A construct containing a short noncoding sequence fused to the *AD* was used as negative control. The yeast (*Saccharomyces cerevisiae*) strain PJ69 used (James et al., 1996) harbors *HIS3*, *ADE2*, and *LACz* reporter genes driven by distinct *GAL4*-responsive promoters. Yeast transformation was performed according to the lithium acetate method (Gietz and Woods, 2002). The vectors pDEST32 (baits) and pDEST22 (preys) were used to transform MAT $\alpha$  and MAT $\alpha$  strains of yeast, respectively. The baits and preys were systematically mated by spotting them on top of each other on a plate with nonselective medium. After overnight incubation, the spots were selected for diploid yeasts containing two plasmids through growth on selective medium (SD -Trp/-Leu). Growth was scored after 2 d at 30°C, and then, the yeasts were transferred onto two separate selection media (SD -Trp/-Leu/-His for weak interactions and SD -Trp/-Leu/-His/-Ade for strong interactions) to test for possible protein-protein interactions.

## UV-B Treatment

In vitro-grown grapevine 'Cabernet Sauvignon' (30–40 d old) plants with at least six to eight fully expanded leaves were exposed to artificial UV-B radiation. Four biological replicates were used, where plantlets were exposed to a background photosynthetic photon flux of 50 to 70  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>. Photosynthetic photon flux was monitored with an LQM 50-3 Quantum Meter (Apogee Instruments). For UV-B treatments, supplemental UV-B was provided by using Philips TL20W/12 RS SLV Tubes (Philips), which were suspended about 60 cm above the top of the in vitro glass pots. The tubes were covered with cellulose acetate filters. These cellulose acetate films do not remove any UV-B radiation but exclude wavelengths lower than 280 nm. Plants were exposed for 6 h at approximately 15  $\mu$ W cm<sup>-2</sup> irradiance. As a negative control, plantlets (four biological replicates) were exposed for 6 h to the same UV-B tubes but covered with a polyester filter (100- $\mu$ m clear safety polyester plastic film). This plastic absorbs total UV-B from the spectrum without affecting the photosynthetic active radiation. UV-B radiation was measured using a VLX-3.W UV Radiometer equipped with a CX-312 UV-B Sensor (Vilber Loumart).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers MYB4b (FJ792820), MYBC2-L2 (GQ903730), and MYBC2-L3 (KM046932).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Alignment of R2R3-MYB C2 repressors of different plant species.

**Supplemental Figure S2.** Sequence comparison between MYB factors showing the bHLH signature.

**Supplemental Figure S3.** Expression analysis of *MYBC2-L1* and *MYBC2-L3* by qPCR in inflorescence and seeds of grapevine 'Cabernet Sauvignon.'

**Supplemental Figure S4.** Branch of the phenylpropanoid pathway involved in the synthesis of small-weight phenolic compounds in grapevine and HPLC chromatograms for the identification of the most common hydroxycinnamic acid derivatives in berry skins.

**Supplemental Figure S5.** Flower phenotypes of transgenic petunia lines expressing *MYB4a*, *MYB4b*, *MYBC2-L1*, and *MYBC2-L3*, and expression level of the corresponding transgenes.

**Supplemental Figure S6.** Flower phenotypes in *MYB4b*- and *MYBC2-L1*-expressing petunia plants and seed phenotypes of wild-type and *MYB4a*-, *MYB4b*-, *MYBC2-L1*-, and *MYBC2-L3*-expressing plants.

- Supplemental Figure S7.** *CCoAOMT* expression levels by qPCR on grapevine ‘Sultana’ transiently overexpressing *MYBA1*, *MYBA1/MYBC2-L1*, and *MYBA1/MYB4a*.
- Supplemental Figure S8.** *MYBC2-L3* expression by qPCR and PA content by HPLC of *35S:MYBC2-L3* transgenic hairy roots of grapevine ‘Maccabeu.’
- Supplemental Figure S9.** Expression analysis of the *MYBC2-L1*, *MATE2*, *MYC1*, *MYBPA1*, *LDOX*, and *ANR* in wild-type and *MYBC2-L3*-overexpressing hairy roots of grapevine ‘Maccabeu.’
- Supplemental Table S1.** Gene models identified in the C2 repressor clade in the PN40024 grapevine 8.4X and 12X V1 genome accessions.
- Supplemental Table S2.** List of primers.
- Supplemental Data S1.** Results of metabolomic analyses by LC-MS in petal limbs of transgenic and wild-type petunia plants.
- Supplemental Data S2.** Results of transcriptomic analyses in *35S:MYBC2-L3* and wild-type hairy roots of grapevine ‘Maccabeu.’
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