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Phenylpropanoid MYB repressors in grapevine

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The phenylpropanoid pathway is controlled at different branches by a set of R2R3-MYB C2 repressors in grapevine

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Abstract

Due to the vast range of functions that phenylpropanoids possess, their synthesis requires precise spatio-temporal 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 bHLH protein families. We characterized four grapevine R2R3-MYB proteins from the C2 repressor motif clade, all of which harbor the EAR repression domain but differ in the presence of an additional TLLLFR repression motif found in the strong flavonoid repressor AtMYBL2. Constitutive expression of \textit{VvMYB4a} and \textit{VvMYB4b} in petunia repressed general phenylpropanoid biosynthetic genes and selectively reduced the amount of small-weight phenolic compounds. Conversely, transgenic petunia lines expressing \textit{VvMYBC2-L1} and \textit{VvMYBC2-L3} showed a severe reduction in petal anthocyanins and in 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 leaves and grapevine plantlets. Finally, \textit{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 UV-B radiation. Our results indicate that \textit{VvMYB4a} and \textit{VvMYB4b} may play a key role in negatively regulating the synthesis of small-weight phenolic compounds, while \textit{VvMYBC2-L1} and \textit{VvMYBC2-L3} may additionally fine-tune flavonoid levels, balancing the inductive effects of transcriptional activators.
INTRODUCTION

The phenylpropanoid pathway is regulated at the level of transcription by developmental, environmental and stress-related cues (reviewed by Jaakola, 2013). Phenylpropanoids act as free radical scavenging or UV-absorbing compounds, as pigments of co-evolutionary traits or as 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 (Xu et al., 2014, Albert et al., 2011). 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, bHLH and WD40 proteins, the main components of a regulatory complex (MBW complex) which 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) or separately, regulating the so-called ‘late’ structural genes like in petunia, snapdragon and Arabidopsis (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 (Xu et al., 2014; Tornielli et al., 2009). In some cases (e.g. flavonol or phlobaphene synthesis), a simpler mechanism just involving MYB factors has been described (Mehrtens et al., 2005; Stracke et al., 2007; Grotewold et al., 1994; Grotewold et al., 2000).

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 (ERF)-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., 2013), or the anthocyanin-related FaMYB1 and PhMYB27, which are either restricted to reproductive organs (FaMYB1; Aharoni et al., 2001; Schaar et al., 2013) or ubiquitous but differentially regulated by light or by developmental signals through the MBW complex (PhMYB27; Albert et al., 2011; Albert et al., 2014).

The scenario for the regulation of flavonoid synthesis depends on the plant species and the origin of its genome structure, influenced by natural variation and crop domestication episodes. In the case of grapevine (Vitis vinifera L.), 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, 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, in contrast to the five and six genes found in rice 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 though a repressive role may also be inferred.

The aim of the present 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 expressing these genes in a colored genotype of Petunia hybrida and also by 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 as 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 non-pigmented 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 ORFs of VIT_04s0023g03710 and VIT_17s0000g02660 from cv. Cabernet Sauvignon pre-bloom inflorescence 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 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 (lsrGIDPxT/NHR) and C2 (pdLNLD/EL) motifs, characteristic of Subgroup 4 (Kranz et al., 1998) were also present in all 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, while MYB4b, with 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 MEME bioinformatic tool (Fig. 1) allowed us to divide the C2 repressor motif clade in further sub-clades and to redefine motifs previously classified by Kranz et al. (1998) and Aharoni et al. (2001). MYB4a and MYB4b were closely related to AtMYB4 and PhMYB4, while 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 domain (C3), also present in other Arabidopsis and maize homologs, but absent in AtMYB6, AtMYB8 and in 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^f/RxLEMK amino acid signatures (Fig. 1B). By including in the motif search analysis the single-repeat MYB and flavonoid repressor AtMYBL2 (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 in four sub-clades (A to D) according to the presence of C1, C2, C3 and C4 motifs. Sub-clade D was further sub-grouped based on the presence of the TLLLFR (C5) motif. Despite the fact that FaMYB1 does not possess a proper C1 motif, all members of sub-clade D are more closely-related than the other sub-clades due to the occurrence of similar amino acid changes in the conserved R2R3 domain (depicted with vertical bars in protein diagrams of Fig. 1B and red crosses in the alignment of 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 for mediating response specificity, but is also in charge of the interaction with other co-factors. C2 repressors showed differences in one of the two elements that in MYB activators mediate the specificity for either the anthocyanin or proanthocyanidin (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 sub-clades A, B and C, the sequence of this element is DNEI, in sub-clades 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 are 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 by Zimmermann et al. (2004) (Supplemental Fig. S1), suggesting a putative interaction with bHLH co-factors.

**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 *V. vinifera* cv. Corvina global gene expression atlas (Fasoli et al., 2012). In order 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 PHENYLALANINE AMMONIA LYASE (PAL), C4H, 4CL, CAFFEYL-CoA O-METHYLTRANSFERASE (CCoAOMT) and CAFFEIC ACID 3-O-METHYLTRANSFERASE (COMT) were selected as small-weight phenolics (SWPs)-related genes. Additionally, the anthocyanin-related genes MYBA1, UDP GLUCOSE: FLAVONOID-3-O-GLUCOSYLTRANSFERASE (UFGT) and GLUTATHIONE S-TRANSFERASE 4 (GST4) (Walker et al., 2007; Conn et al., 2008), and the PA-related genes MYBPA1, LEUCOANTHOCYANIDIN REDUCTASE 1 and 2 (LAR1 and LAR2) and ANTHOCYANIDIN REDUCTASE (ANR) (Bogs et al., 2005; Bogs et al., 2007) were included, together with the bHLH regulator of the flavonoid pathway MYC1 (Hichri et al., 2010).

The constructed heat map allows visualizing 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 in seeds after fruit set, while 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) and in all leaf developmental stages, in latent buds, and in 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, in young leaves and in buds close to bud-break period. Similarly, MYBC2-L3 was expressed weakly in berry tissues at post-fruit set and also in 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 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). On the other hand MYB4b, MYBC2-L1, MYBC2-L2 and MYBC2-L3 clustered together with PA-related genes, whose expression 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 spatio-temporal 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 establishing 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 may control multiple branches of
the flavonoid pathway.

Given the high level of identity among sub-clade 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, while MYB4b and MYBC2-L3 were mainly expressed during early berry development
(Fig. 3A).

In order 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 onwards, while SWP compounds all tended to
decline towards ripening (Fig. 3B), as largely proven in several previous works (e.g. 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, while MYB4b and MYBC2-L3 expression coincides
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 *P. hybrida* 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, 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. In order 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
further 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 to 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 were 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 in comparison to the wild type, while a strong reduction was observed in
MYBC2-L1 and MYBC2-L3 expressing petals.

Since in petunia the anthocyanin and the vacuolar acidification pathways share a common
regulatory 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 in comparison to the control, while significantly increased values were detected in MYBC2-
L1 and, more substantially, in MYBC2-L3 lines.

The metabolic profiles of transgenic petals were investigated by liquid chromatography-mass
spectrometry (LC-MS) (Fig. 4B; Supplemental Dataset S1). In order 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, while only in \textit{MYB4b} plants benzoic acid derivatives were reduced (Fig. 4B). In \textit{MYBC2-L1} and \textit{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 \textit{35S:MYBC2-L3} petals (Fig. 4B; Supplemental Dataset S1).

To evaluate the effect of each repressor on the PA branch of the flavonoid pathway, we used the seeds of self-fertilized transgenic and wild type lines. The seed coat pigmentation appeared similar to the wild type for \textit{35S:MYB4b} line, slightly less colored for \textit{35S:MYB4a}, and paler for \textit{35S:MYBC2-L1} and \textit{35S:MYBC2-L3} seeds (Supplemental Fig. S6C). However, the analysis of PA content showed that only \textit{35S:MYBC2-L1} and \textit{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 \textit{MYBC2-L1} and \textit{MYBC2-L3} are able to affect anthocyanin and proanthocyanidin accumulation and the vacuolar acidification pathway in petunia.

\textbf{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 \textit{PAL}, \textit{C4H} and \textit{4CL}, the ‘early’ flavonoid gene \textit{CHALCONE SYNTHASE (CHS)}, the ‘late’ flavonoid gene \textit{DIHYDROFLAVONOL REDUCTASE (DFR)} and the proton P-ATPase encoding gene \textit{PH5}.

The expression of \textit{MYB4a} and, to a lesser extent, \textit{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 \textit{PAL}, \textit{C4H} and \textit{4CL}, in agreement with the decreased amount of SWPs revealed in the metabolomic analysis (Fig. 4B). The slight reduction of \textit{CHS}, \textit{DFR} and \textit{PH5} expression by \textit{MYB4a} and \textit{MYB4b} is consistent with the non-significant changes in anthocyanin accumulation and vacuolar acidification in these plants (Fig. 4A and B), indicating that the impact of these MYBs on these pathways is very limited. Regarding \textit{MYBC2-L1} and \textit{MYBC2-L3} expressing lines, we observed a strong down-regulation of \textit{C4H}, which represents a crucial point in the metabolism of hydroxycinnamic acids, and that 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 \textit{CHS}, \textit{DFR}, and \textit{PH5}, which correlates with the anthocyanin reduction and the pH increase of the \textit{MYBC2-L1} and \textit{MYBC2-L3} expressing petals (Fig. 4A 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 AN2, the vacuolar pH MYB regulator PH4 and the bHLH regulator of both pathways AN1 (Quattrocchio et al., 1999; Spelt et al., 2000; Quattrocchio et al., 2006) (Fig. 5B). In both 35S:MYBC2-L1 and 35S:MYBC2-L3 a strong reduction of AN2 and PH4 gene expression was observed, while AN1 down-regulation was less pronounced.

These results indicate that in the petunia heterologous system, sub-clade 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 (Fig. 2 and 3), we tested their ability to repress this flavonoid branch by approaching a transient transformation assay in V. vinifera. Plantlets of cv. Sultana were vacuum-infiltrated with *Agrobacterium tumefaciens* to overexpress the anthocyanin activator MYBA1 (i) independently, (ii) in combination with MYBC2-L1 or (iii) in combination with MYB4a. Among five plantlets agro-infiltrated for each combination we selected two independent lines overexpressing the respective MYB genes to a similar extent (Fig. 6A 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 increase for MYBA1 and MYBA1/MYB4a stems with respect to MYBA1/MYBC2-L1 and control lines, consistently with the observed pigmentation phenotypes (Fig. 6A). The expression analysis of the anthocyanin structural gene UFGT, that is directly regulated by MYBA1 (Walker et al., 2007), showed it was clearly down-regulated in MYBA1/MYBC2-L1 lines compared to 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, whose expression profile 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 in MYBA1/MYBC2-L1 plantlets in comparison with control lines (Supplemental Fig. S7).
The functional diversification of MYB4a and MYBC2-L1 in repressing the accumulation of anthocyanins was further evaluated analyzing their ability to interfere in the activation of the *UFGT* promoter, by performing a dual luciferase assay in agroinfiltrated leaves of *Nicotiana benthamiana* (Fig. 6C). The MYBA1/MYC1-directed activation of *UFGT* promoter was significantly lower when MYBC2-L1 was co-transfected 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 Fig. S1 and S2). Their ability to interact with bHLH factors was therefore investigated in yeast two-hybrid assays using two 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 (BD) and the different MYB proteins were fused to the GAL4 Activation Domain (AD). A construct containing a short non-coding sequence fused to the AD was used as negative control. Yeasts co-transformed 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 growing the transformants on media lacking tryptophan, leucine and histidine (weak) or tryptophan, leucine, histidine 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 via the participation of the MBW complex. They also bound the grapevine MYC1, but the strength of these interactions appeared very weak.

Albeit 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 sub-clade A C2-repressors

Several R2R3-MYB proteins from Subgroup 4 have been previously shown to be involved in UV radiation protective responses (Jin et al., 2000; Fornalé et al., 2010). The Arabidopsis myb4 mutants were more UV-B tolerant than wild type plants, whilst 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 six hours 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 µW cm\(^{-2}\) irradiance with a dose of 1.6 J cm\(^{-2}\) d\(^{-1}\)) and followed gene expression changes after six hours of radiation. MYB4a and MYB4b were significantly down-regulated upon radiation (Fig. 8A). MYBC2-L1 and MYBC2-L3 had a slight tendency towards repression but at least in our experimental setup (6 hours 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 ultraviolet radiation and suggest a set of putative targets.

Ectopic expression of MYBC2-L3 in grapevine hairy roots reduces proanthocyanidin accumulation and represses a narrow set of flavonoid-related genes

Recently, it was demonstrated 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 to further investigate its role in grapevine, we ectopically expressed its coding sequence in hairy roots of V. vinifera cv. Maccabeu. Of the ten independent transgenic hairy root clones obtained, we selected two lines with the highest expression of MYBC2-L3, with 185- and 181- fold compared to the control, respectively (Supplemental Fig. S8A). The PA amount was reduced in both transgenic lines (0.70 and 0.77 mg g\(^{-1}\) FW) in comparison to the control (2.53 mg g\(^{-1}\) FW) (Supplemental Fig. S8B), suggesting that, similarly 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 to 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 number: 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 co-expression-based approach (Usadel et al., 2009), to identify genes showing negatively-correlated profiles respect to MYBC2-L3, and a two-class unpaired comparison using Significance Analysis of Microarray (SAM) (Table I; Supplemental Dataset 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 Dataset S2). These included a PAL, a cinnamoyl-CoA reductase, CHS1, CHS2, CHS3 (Goto-Yamamoto et al., 2002; Harris et al., 2013), F3H1, LAR, ANR, the putative PA transporters MATE1 and MATE2 (Perez-Diaz 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 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; Deluc et al., 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- and 17 up-regulated genes (Supplemental Dataset 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 whose expression was negatively-correlated with MYBC2-L3 expression (Table I; Supplemental Dataset 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-regulation of (i) several transcripts related to ion and sugar transport, (ii) several members of the family of patatin-like phospholipases and (iii) 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, originally described by Kranz et al. (1998). Subgroup 4 was originally defined by the
presence of C1, C2 and Zinc Finger (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 (Sub-clades A to 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 demonstrated by chimeric
protein fusion experiments (Matsui and Ohme-Takagi, 2010, Hiratsu et al., 2003). 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, while
PhMYB27 has a DLNSPP signature. MYBC2-L1 is only different from this pattern in the last
residue of the core sequence (Valine replacing Leucine). However, both are non-polar 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 Sub-clade 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), in switchgrass PvMYB4, and in maize ZmMYB31 and ZmMYB42
negative regulators of the early phenylpropanoid and lignin pathways (Shen et al., 2012; Sonbol et
al., 2009; Fornalé et al., 2010). 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 sub-clade A members (data not shown).

Sub-clades 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 as FaMYB1, which lacks this motif, strongly inhibits flavonoid synthesis (Aharoni et al., 2001; Paolocci et al., 2011). The D3 sub-clade 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 sub-clade 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 sub-clade 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 TFs to have a TLLLFR motif, giving support to their strong repression activity found in our work for anthocyanin and proanthocyanidin 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 R3α1 and R3α2 helices forms a characteristic surface-exposed pattern of charged residues which constitute the structural basis for the MYB-bHLH interaction (Zimmermann et al., 2004). While 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 (reviewed by Mol et al., 1998). Grotewold et al. (2000) demonstrated that four residues from C1 in the R3α1 helix were enough to confer P the ability to interact with R, and two additional residues in R3α2 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 towards 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), even though this down-regulation might have not 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 towards 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 V. vinifera revealed that MYB4a down-regulates the expression of CCoAOMT, 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) demonstrated that maize COMT and CCoAOMT genes were directly bound and transcriptionally regulated by ZmMYB31, 42 and 11 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. Besides a common impact on caffeic acid derivatives, MYB4a expression caused the reduction of coumaric acid derivatives, while 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 proanthocyanidin pathways

Our phylogenetic analysis showed that MYBC2-L1 and MYBC2-L3 clustered separately from sub-clade 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 sub-clade D genes since 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 due to a decrease in anthocyanin and PA content and a down-regulation of flavonoid structural genes. A role as anthocyanin negative regulator was confirmed for MYBC2-L1 by its transient expression in grapevine and tobacco, demonstrating 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, as 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 by the results of the yeast two-hybrid assays showing that both MYBC2-L1 and MYBC2-L3 interact with the petunia bHLH PhAN1. Even though 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 by 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 these authors 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 co-repressor 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. Sub-clade 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; 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 to hypothesize that a MBW-dependent mode of action may occur with grapevine sub-clade D 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 indicates that MYBC2-L1 and MYBC2-L3 also strongly reduced the mRNA levels of ‘early’ biosynthetic genes like C4H and CHS, whose expression in petunia 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 sub-clade D share some functional characteristics with those of sub-clade A in the regulation of early steps of the phenylpropanoid pathway.

Besides their role as anthocyanin repressors, MYBC2-L1 and MYBC2-L3 showed repressive action towards PA accumulation in petunia seeds. MYBC2-L1 was previously identified as specific regulator of PA synthesis in grapevine (Huang et al., 2014) and here we demonstrated 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. As 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, resulting in that 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 or in previous studies, there are several indicators suggesting that: (i) activators induce repressors, (ii) repressors repress activators and (iii) repressors repress repressors (Table II). Cutanda-Perez et al. (2009) found that the MYBC2-L1 repressor was induced after overexpressing the anthocyanin activator VlMYBA1 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 et al. (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, as demonstrated by ectopic expression of MYBC2-L3 in hairy roots (this work) and shown 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, positive MYB regulators of the anthocyanin and acidification pathways, respectively (Quattrocchio et al., 1999; Quattrocchio et al., 2006). Therefore, in addition to an active EAR-mediated repression of structural genes, these repressors might act indirectly restraining 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 many grapevine organs. A further level of action of C2 repressors possibly involves auto-repression, or repression of other members of the MYB C2 repressor clade. This seems not to be an indirect regulation, as Zhao et al. (2007) demonstrated 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 of 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. (i) negative correlation with biosynthetic genes of the repressed pathway in the case of environmental stress regulation and (ii) positive correlation with biosynthetic genes in the case of developmentally controlled pathways (Aharoni et al., 2001; Albert et al., 2014; Jin et al., 2000; Colquhoun et al., 2011; Schaart et al., 2013).

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 in 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 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 flowers are opened. Sporopollenin, the major part of the pollen exine, contains high amounts of phenylpropanoids and hydroxylated fatty acids (Domínguez et al., 1999). On the other hand, the highest expression of *MYB4b* was detected in tendrils prior to 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), so it is possible that MYB4b regulates the maturation of this organ. Since 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 while 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; Fornalè 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 _V. vinifera_ cv. Cabernet Sauvignon plantlets to UV-B radiations caused a significant reduction of both _MYB4a_ and _MYB4b_ expression 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 _V. vinifera_ cv. 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 sub-clade D, our data suggest that they have a repression capacity somewhat wider compared to sub-clade A members, being 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. On the other hand, the general low expression level of _MYBC2-L3_ suggests a limited involvement as repressor of SWPs and PAs in berry pericarp at early developmental stages, in developing buds and in seedlings. The co-expression of _MYBC2-L1_ and _MYBC2-L3_ with the PA regulators _MYBPA1_ or the anthocyanin regulator _MYBA1_, supports the idea of sub-clade D repressors acting as co-repressors in the MBW complex (Albert et al., 2014). Despite _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 as 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 production of sinapate esters in Brassicacea, phlobaphenes in monocot cereals or resveratrol-derived stilbenes in Vitaceous 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 fruits tissues at pre- and post-ripening stages. Our results indicate that MYB4a and MYB4b may play a key role in repressing small-weight phenolic compound synthesis, while 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 organs (Vitis vinifera L. cv. Cabernet Sauvignon) were collected from a commercial vineyard in the Maipo Valley (Chile). Inflorescence clusters from different developmental stages were included as in Matus et al. (2010). A total of nine grape clusters were sampled from three plants every two weeks throughout fruit development, beginning two-three weeks after fruit set (four weeks before véraison) and ending at six or eight weeks after véraison. Berries were immediately peeled and deseeded. Seeds and skins were frozen in liquid nitrogen, and stored at -80 °C until required for RNA extraction (skins were also used for anthocyanin and SWP compound quantification).

Functional analysis was carried out in Petunia hybrida plants derived from the collection of Amsterdam University. The V30 x 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 V. vinifera cv. Sultana were in vitro micropropagated and cultivated in a growth chamber at 25°C with a 16 h photoperiod.

For the genetic transformation of V. vinifera cv. Maccabeu, the in vitro plantlets were grown for 90 days under light and temperature controlled conditions.

Bioinformatics
Oligonucleotides were designed using Primer3 (http://biotools.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/corps_anglais.html; http://genomes.cribi.unipd.it). Grape genes were aligned against the full predicted amino acid sequences of proteins belonging to Arabidopsis thaliana, Zea mays, Petunia hybrida, Antirrhinum majus, Eucalyptus gunnii, Panicum virgatum and Fragaria x 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 1000 replicates. The trees obtained were generated in MEGA5 and visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree). The online MEME (Multiple EM for Motif Elicitation) Suite was employed to analyze protein sequences and discover motifs with an expected value lower than \(2 \times 10^{-30}\) (http://meme.nbcr.net/; Bailey and Elkan, 1994) and using the following search parameters: 4-8 residues (min-max length), zero or one repetition of motifs and 5 motifs maximum. A second motif search included AtMYBL2 and used the following parameters: 6-18 residues (min-max length), any number of repetitions and 5 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), AT1G22640 (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 post-fruitset and mid-ripening 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 synthetized from RNA of pea-size 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. South Korea). For expression in *P. hybrida*, the products were cloned into the binary overexpression vector pK7GW2.0 (Laboratory of Plant Systems Biology, Ghent University, Belgium) by site-specific LR recombination. The constructs were then transferred to *Agrobacterium tumefaciens* strain EHA105 by electroporation. The Petunia plants were transformed using the leaf disc method (van der Meer et al., 1999), and
regenerated transgenic shoots were transferred to soil and hardened off in a temperature-controlled
glasshouse.

For overexpression of MYBC2-L3 in V. vinifera cv. 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 in 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 small-weight phenolic compounds (SWP) in grapevine
berry development.**
Berry skin samples were weighed and ground with 15 ml distilled water, 20 ml hydroalcoholic
solution (EtOH:H2O, 10:90 v/v) and 2.5 g tartaric acid, adjusting the final solution weight to 100 g.
Extracts were macerated for 2 h at 30 ºC, centrifuged and filtered with glass microfibre. Samples
were filtered through a 0.45 µm membrane under vacuum at <35 ºC and used for HPLC-DAD
anthocyanin analysis as described by Matus et al. (2009). The detection was carried out by scanning
from 210 to 600 nm. SWP were analysed from a total extraction of non-anthocyanin compounds.
These were extracted from an aliquot (50 ml) of macerated and filtered berry skins, by mixing the
sample three times with 20 ml diethyl ether and 20 ml ethyl acetate. The organic fractions were then
combined and extracts were evaporated to dryness under vacuum at <35 ºC. The residue was
dissolved in 1 ml methanol/water (1:1, v/v), and analysed 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 time with those obtained by 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 wild type and each transgenic line. Powdered petal samples were extracted in 8
vols. (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
spectrophotometer at \( \lambda = 540 \text{ nm} \) using malvidin 3-glucoside as standard. For the determination of phenylpropanoids, HPLC-electrospray ionization-mass spectrometry (ESI-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 \( m/z \) values, fragmentation patterns (MS/MS and MS\(^3\)) and retention times of each signal with those of available commercial standards and by comparison with values reported in the literature. 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, USA). 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 in grapevine hairy roots

Proanthocyanidins (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 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 Verriès et al. (2008). In détail, 100 mg of frozen and powdered sample was mixed with 750 µl of the extraction solution (acetone:water [70:30, v/v] containing 0.05% trifluoroacetic acid) and 50 µl of an internal standard solution (p-hydroxy methyl ester, 3 g l\(^{-1}\) in methanol). After 1 h incubation, the mixture was centrifuged (13,000 g, 15 min, 4°C), and the supernatant recovered. 200 µl of supernatant were dried under vacuum at 35°C for 2 h (Genevac) and resuspended in 100 µl 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, 20 min), the reaction was stopped by adding 100 µl 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 as phloroglucinol adducts for extension units). PA polymer length, estimated by the mean degree of polymerisation (mDP), 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, epicatechins) were
analyzed by injecting 5 μl into a Waters Millennium HPLC system equipped with a diode-array
detector [PDA 996 (190-700nm)] and a fluorimeter detector, and then quantified using a W2475
fluorimeter detector (λ.em=275nm and λ.ex=322nm). Concentrations were determined from standard
curves calculated from pure monomers. Results were expressed in milligrams per gram 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 pPGW7.0 and
pK7WG2.0 (Laboratory of Plant Systems Biology, Ghent University, Belgium), respectively. 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 (*LUC*) under control of *UFGT* prom
were transferred to *Agrobacterium tumefaciens* strain EHA105 by electroporation. Promoter assays
were performed in *Agrobacterium*-infiltrated *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 *Agrobacterium tumefaciens* strain C58C1 by electroporation.
Five *in vitro* plants of *Vitis vinifera* cv. Sultana were immersed in each bacterial suspension and
vacuum infiltrated (2× 2 min, 90 kPa). As control, plantlets transformed with pK7WG2.0
containing a non-coding sequence were used as control. After agroinfiltration, plantlets were rinsed
with sterile water and allowed to recover *in vitro* for six days 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 μg 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 a 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 of Reid et al. (2006), using a CTAB-Spermidine extraction buffer. For
cDNA synthesis, 1 μg of total RNA was reverse transcribed with random primers in a 20 μl reaction
mixture using the SuperScript® II reverse transcriptase (Stratagene, USA) 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) were used. *UBIQUITIN1* was used as the reference gene for normalization.

For grapevine cv. Sultana agroinfiltration experiments, total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich) following the manufacturer’s protocol and 1 µg 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 a 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 (Ct values) were employed 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 figure legends. 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 (V 3.2). Each hybridization was carried out on a NimbleGen microarray 090818 Vitis exp HX12 (Roche, NimbleGen Inc.), representing 29,549 predicted genes on the basis of the 12X grapevine V1 gene prediction version ([https://urgi.versailles.inra.fr/Species/Vitis/Annotations](https://urgi.versailles.inra.fr/Species/Vitis/Annotations)). The microarray was scanned using a ScanArray 4000XL (Perkin-Elmer) at 532 nm (Cy-3 absorption peak) and GenePix Pro7 software (Molecular Devices) according to the manufacturer’s instructions.
Images were analyzed using NimbleScan v2.5 software (Roche), which produces Pair Files containing the raw signal intensity data for each probe and Calls Files with normalized expression data derived from the average of the intensities of the four probes for each gene. The normalized gene expression data were finally converted to log2 values to process the data. A Pearson correlation analysis was carried out to evaluate the robustness of the three biological replicates in each sample.

Correlation analysis of gene expression was performed on a reduced microarray expression dataset obtained by removing (i) low- or non-expressed genes (i.e. genes whose normalized median expression values were < 150 in both set of transgenic and wild-type biological triplicates) and (ii) genes with low variation of expression levels (i.e. genes with a Coefficient of Variation < 0.365 among the six microarray analysis). Correlation analysis of this reduced data set was performed with MYBC2-L3 as 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 Significance Analysis of Microarray (SAM) with a false discovery rate (FDR) of 4% (TMeV 4.3).

**Yeast two-hybrid assay**

The coding sequences of MYB4a, MYB4b, MYBC2-L1, MYBC2-L3, MYC1, and PhANI 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, PSB; Ghent University, Belgium). A construct containing a short non-coding sequence fused to the AD was used as negative control. The yeast 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 & Woods, 2002). The vectors pDEST32 (baits) and pDEST22 (preys) were used to transform MATα and MATα strains of yeast, respectively. The baits and preys were systematically mated by spotting them on top of each other on a plate with non-selective medium. After overnight incubation, the spots were selected for diploid yeasts containing the 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 plants (cv. Cabernet Sauvignon, 30-40 day old), with at least 6-8 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 (PPF) of 50-70 μmol m⁻² s⁻¹. PPF was monitored with a LQM 50-3 quantum meter (Apogee instruments, Logan, USA). For UV-B treatments, supplemental UV-B was provided by using Philips TL20W/12 RS SLV tubes (Philips, Eindhoven, The Netherlands), 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 hours at ~15 μW cm⁻² irradiance. As a negative control, plantlets (four biological replicates) were exposed for 6 hours to the same UV-B tubes but covered with a polyester filter (100 μm clear safety polyester plastic film). This plastic absorbs total UV-B from the spectrum without affecting PAR. UV-B radiation was measured using a VLX-3.W UV radiometer equipped with a CX-312 UV-B sensor (Vilber Loumart, Germany).
SUPPLEMENTAL MATERIAL

Supplemental Figure S1
Alignment of R2R3-MYB C2 repressors showing the positions of the DNA-binding domain and C1, C2, C3, C4 and C5 motifs. Sub-clade members are shown with their names in different colors (A-black, B-blue, C-green, D-red). Sub-clade specific features are highlighted by red crosses and boxes (these are shown as vertical bars inside motif diagrams in Figure 1B). The R-single AtMYBL2 is also included. The amino acid signature of Element 3 (Heppel et al., 2013) is shown in a black box within the R3 repeat. bHLH-interacting residues are shown by asterisks while the complete interaction signature is underlined.

Supplemental Figure S2
A. Sequence comparison between MYB factors and the anthocyanin-related C1 from maize, showing the residues that contribute to the specificity of the interaction with the bHLH cofactor R (Grotewold et al., 2000). As shown in the top of the figure, all interacting residues form part of the signature described by Zimmermann et al. (2004). Residue numbers are based on the sequences of ZmP and ZmC1. Black shading indicates identical residues to ZmP, grey shading indicates identical residues to ZmC1. B. Models of some grapevine R2R3-MYB domains on the basis of the NMR structure of c-MYB, obtained through comparative modeling by Matus et al. (2008). Amino acids which form part of the motif [DE]Lx2[RK]x3Lx6Lx3R are highlighted in colors. VvMYB14 is shown as a non bHLH-interacting example.

Supplemental Figure S3
Expression analysis of MYBC2-L1 and MYBC2-L3 by qPCR in inflorescence and seeds of cv. Cabernet Sauvignon at different stages of development. The data correspond to the means ± SE of three biological replicates normalized against the UBIQUITIN1 control.

Supplemental Figure S4
A. Portion of the Phenylpropanoid Pathway that is involved in the synthesis of small-weight phenolic (SWP) compounds found in grapevine. Berries accumulate in their skin and flesh large quantities of hydroxycinnamic acids, some of which undergo esterification with organic acids to form other cinnamates such as caftaric and chlorogenic acid. Abbreviations are as follow: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; C3H, coumarate 3-hydroxylase; COMT, caffeate 3-O-methyltransferase; CCoAOMT, caffeoyl-CoA 3-O-
methyltransferase; CHS, chalcone synthase. B. HPLC Chromatograms for the identification of the most common hydroxycinnamic acids (and their derivatives) in berry skins. The chromatogram also shows the identification of flavan-3-ol monomers and flavonol derivatives as described by Matus et al. (2009).

**Supplemental Figure S5**
Flower phenotypes of transgenic lines expressing MYB4a, MYB4b, MYBC2-L1 and MYBC2-L3 in relation to the expression level of the corresponding transgenes. Each value corresponds to the mean ± SE of three biological replicates (MNE) relative to an ACTIN housekeeping control and normalized against the highest transgene expressor (arbitrary set to 100) of each line. We selected lines L3 (35S:MYB4a), L1 (35S:MYB4b), L2 (35S:MYBC2-L1) and L2 (35S:MYBC2-L3) for further metabolic and gene expression analyses.

**Supplemental Figure S6**
Flower phenotypes observed in MYB4b (A) and MYBC2-L1 (B) expressing plants and seed phenotypes (C) of wild type and MYB4a, MYB4b, MYBC2-L1 and MYBC2-L3 expressing plants.

**Supplemental Figure S7**
CCoAOMT expression levels by qPCR on two selected lines of Vitis vinifera cv. Sultana expressing MYBA1, MYBA1/MYBC2-L1 and MYBA1/MYB4a. The data correspond to the mean ± SE of three technical replicates normalized against the UBIQUITIN1 control.

**Supplemental Figure S8**
MYBC2-L3 expression levels determined by qPCR (A) and the relative PA content by HPLC (B) of the two selected lines of 35S:MYBC2-L3 transgenic hairy roots of Vitis vinifera cv. Maccabeu. The expression data correspond to the mean ± SD of three technical replicates relative to the UBIQUITIN1 control.

**Supplemental Figure S9**
Expression analysis of the MYBC2-L1, MATE2, MYC1, MYBPA1, LDOX and ANR in wild type and MYBC2-L3 overexpressing hairy roots. The data correspond to the mean ± SD of three biological replicates relative to the UBIQUITIN1 control and normalized against the wild type value.

**Supplemental Table S1**
Gene models identified as part of the C2 repressor clade in the PN40024 grapevine 8.4X and 12X V1 genome accessions.

**Supplemental Table S2**

List of primers.

**Supplemental Dataset S1**

Results of metabolomic analyses by LC-MS in petal limbs of transgenic and wild type plants. Sheet 1 reports the secondary metabolites that are significantly differently accumulated in at least one comparison between lines (ANOVA, $P < 0.01$). For each molecule, the identification number (ID), the retention time (RT), the mass to charge ratio value in negative mode (m/z), the tentative identification, the amount relative to each biological replicate, the average and the standard deviation of the replicates are reported. For each transgenic line, the p value (t-test) was calculated against the wild type to show significant differences. Sheet 2 reports for each ID, the m/z, the RT, the tentative identification and fragmentation (ms/ms of the molecular ion and ms3 of the fragment highlighted in red). The sugars were determined by neutral loss, while the aglycones were determined by comparison with m/z and fragmentation trees of authentic standards.

**Supplemental Dataset S2**

Results of transcriptomic analyses in 35S:MYBC2-L3 and wild type hairy roots. Sheet 1 reports the top 2000 genes whose expression is most highly correlated or negatively-correlated to MYBC2-L3 expression. For each gene, the Seq ID, the description and the Pearson’s correlation coefficient is indicated. If present in the last column, the asterisk indicates that the gene is significant modulated in 35S:MYBC2-L3 line compared to wild type. Sheet 2 reports the complete list of differentially-expressed genes in 35S:MYBC2-L3 line resulted from the SAM analysis. For each gene, the Seq ID, the description and the Fold Change is indicated.
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FIGURE LEGENDS

Figure 1
Relationships between R2R3-MYB activators and repressors of the phenylpropanoid pathway from grapevine and other plant species. A. Phylogenetic tree showing selected plant MYB transcription factors. Functions of characterized proteins are included next to each name. C2 repressors are classified in different sub-clades. Accession numbers are listed in “Materials and Methods”. B. Protein domain organization within each subclade represented by colored boxes. Sub-clade specific features are highlighted with vertical bars inside each box and can be found in Supplemental Figure S1. The consensus sequence of the repression motifs C2 and C5 as well as of the other motifs (C1, C3 and C4) identified by MEME Suite is reported.

Figure 2
Expression analysis in the V. vinifera cv. 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 highlighted), the regulators MYBA1 (VIT_02s0033g00410), MYBPA1 (VIT_15s0046g00170) and MYC1 (VIT_07s0104g00090), the small-weight phenolic (SWP)-related genes PAL1 (VIT_08s0040g01710), PAL2 (VIT_16s0039g01170), C4H (VIT_06s0004g08150), 4CL (VIT_11s0052g01090), CCoAOMT (VIT_07s0031g00350) and COMT (VIT_16s0098g00850), the proanthocyanidin (PA) structural genes ANR (VIT_00s0361g00040), LARI (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 log2 and genes were hierarchically clustered based on average Pearson’s distance metric. Abbreviations after organ correspond to: FS, fruit set; PFS, post-fruit set; V, véraison; MR, mid-ripening; R, ripening; PHWI, PHWII, PHWIII, post-harvest withering (1st, 2nd and 3rd month, respectively), Bud - L, latent bud; Bud - W, winter bud; Bud - S, bud swell; Bud - B, bud burst; Bud - AB, bud after burst; Inflorescence - Y, young; Inflorescence - WD, well developed; Flower - FB, flowering begins; Flower - F, flowering; Tendril - Y, young; Tendril - WD, well developed; Tendril - FS, mature; Leaf - Y, young; Leaf - FS, mature; Leaf - S, senescing leaf; Stem - G, green; Stem - W, woody. Gene abbreviations correspond to: PAL, PHENYLALANINE AMMONIA LYASE; C4H, CINNAMATE 4-HYDROXYLASE; 4CL, 4-COUMARATE-COA LIGASE; CCoAOMT, CAFFEIC ACID O-METHYLTRANSFERASE; COMT, CAFFEIC ACID 3-O-METHYLTRANSFERASE; ANR, ANTHOCYANIDIN REDUCTASE; LARI and LAR2, LEUCOANTHOCYANIDIN REDUCTASE 1 and 2; UFGT, UDP GLUCOSE: FLAVONOID-3-O-GLUCOSYLTRANSFERASE; and GST4.
GLUTATHIONE S-TRANSFERASE 4. Red and green boxes indicate high and low expression levels, respectively, for each gene.

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

Figure 4
Phenotypic analysis of transgenic petunia plants (line V30xM1) expressing MYB4a, MYB4b, MYBC2-L1 and MYBC2-L3. A. Flower phenotype of untransformed plant in comparison to 35S:MYB4a, 35S:MYB4b, 35S:MYBC2-L1 and 35S:MYBC2-L3 lines. The relative anthocyanin content determined spectrophotometrically at 540 nm represents the means ± SD of three biological replicates (pink bars). The pH of crude petal extracts represents the means ± SD of ten biological replicates (white bars). Asterisks indicate significant differences compared to the wild type (*P < 0.05). B. Comparison of benzoic acid, hydroxycinnamic acid and anthocyanin levels in petunia petals of 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 ± SD of three biological replicates. Asterisks indicate significant differences of the total amounts compared to 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 ± SD of three biological replicates. Asterisks indicate significant differences compared to the wild type (**P < 0.01).

Figure 5
Expression analysis of phenylpropanoid- and pH-related genes in petunia petals by qPCR.
A. Expression of structural genes PAL, C4H, 4CL, CHS, DFR and PH5 in the wild type and in all transgenic lines.
B. Expression of regulatory genes AN2, PH4 and AN1 in wild type and in 35S:MYBC2-L1 and 35S:MYBC2-L3 lines. The data correspond to the means ± SE of three biological replicates relative to an ACTIN housekeeping control and normalized against the wild type value. Asterisks indicate
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**Figure 6**

*Agrobacterium*-mediated transient expression of *MYBA1*, *MYBA1/MYBC2-L1* and *MYBA1/MYB4a* in *Vitis vinifera* cv. 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 right of each image. Values correspond to the means ± 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 ± SE of three technical replicates. C. Dual Luciferase assay of the *UFGT* promoter activation in *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves. Firefly luciferase (LUC) values are reported relative to the Renilla luciferase (REN) control and normalized on the negative control (empty). Each value represents the mean ± SD of three biological replicates. Asterisks indicate significant differences against *MYBA1* line (*P < 0.05).

**Figure 7**

Interaction of the MYB repressors MYB4a, MYB4b, MYBC2-L1 and MYBC2-L3 with the bHLH MYC1 of *Vitis vinifera* and AN1 of *Petunia hybrida* by yeast two-hybrid analysis. Yeasts were co-transformed with different combinations of plasmids expressing the MYB proteins fused to the Activation Domain (AD) and bHLHs fused to Binding Domain (BD), and selected for activation of the HIS and ADE reporter genes. The negative control contains a short non-coding sequence fused to the AD.

**Figure 8**

Expression analysis by qPCR in young grape plantlets after UV-B radiation exposure of (A) the C2 repressors *MYB4a*, *MYB4b*, *MYBC2-L1* and *MYBC2-L3* and (B) the phenylpropanoid structural genes *PAL1, C4H, 4CL, COMT, CCoAOMT*. Asterisks indicate significant differences against the control (*P < 0.05).*
The most negatively-correlated genes with *MYBC2-L3* expression (with Pearson’s Correlation Coefficient, PCC < -0.98) associated with their fold change (FC) in 35S:*MYBC2-L3* grapevine hairy roots. For each gene, the gene ID, the description, the PCC and the FC are reported. Asterisks indicate genes significantly modulated by SAM analysis.

<table>
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<tr>
<th>GENE_ID</th>
<th>DESCRIPTION</th>
<th>PCC</th>
<th>FC</th>
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</thead>
<tbody>
<tr>
<td>VIT_12S0028G01150</td>
<td>Transparent testa 12-like (VvMATE2)</td>
<td>-0.999</td>
<td>-3.69*</td>
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<td>VIT_01S0127G00800</td>
<td>Polyamine oxidase precursor</td>
<td>-0.999</td>
<td>-1.84*</td>
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<td>VIT_14S0083G00320</td>
<td>Cinnamoyl-CoA reductase</td>
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<td>Photosystem I reaction center subunit III (PSAF)</td>
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Table II

Transcriptional relationships between MYB repressors and activator shown in this and in previous studies.

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<th>Target</th>
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<tr>
<td>VvMYBA1</td>
<td>VvMYBC2-L1</td>
<td>Grapevine hairy roots</td>
<td>Cutanda-Perez et al., 2009</td>
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Activators

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<td>Grapevine transgenic leaves</td>
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<td>PhMYB27</td>
<td>Petunia transgenic petals</td>
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<tr>
<td>VvMYB5b</td>
<td>PhMYB27</td>
<td>Petunia transgenic petals</td>
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Repressors

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<th>VvMYBPA1</th>
<th>Grapevine hairy roots</th>
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<td>PhAN2</td>
<td>PhAN1</td>
<td>Petunia transgenic petals</td>
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<tr>
<td>PhAN2</td>
<td>PhAN1</td>
<td>Petunia transgenic petals</td>
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VvMYBC2-L3  VvMYBPA1  This work

VvMYB5a

VvMYBC2-L3  VvMYC1  Grapevine hairy roots

VvMYBC2-L1

VvMYB4a
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