The R2R3-MYB Transcription Factors MYB14 and MYB15 Regulate Stilbene Biosynthesis in *Vitis vinifera*[W]

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Plant stilbenes are phytoalexins that accumulate in a small number of plant species, including grapevine (*Vitis vinifera*), in response to biotic and abiotic stresses and have been implicated in many beneficial effects on human health. In particular, resveratrol, the basic unit of all other complex stilbenes, has received widespread attention because of its cardio-protective, anticarcinogenic, and antioxidant properties. Although stilbene synthases (STSs), the key enzymes responsible for resveratrol biosynthesis, have been isolated and characterized from several plant species, the transcriptional regulation underlying stilbene biosynthesis is unknown. Here, we report the identification and functional characterization of two R2R3-MYB–type transcription factors (TFs) from grapevine, which regulate the stilbene biosynthetic pathway. These TFs, designated *MYB14* and *MYB15*, strongly coexpress with *STS* genes, both in leaf tissues under biotic and abiotic stress and in the skin and seed of healthy developing berries during maturation. In transient gene reporter assays, *MYB14* and *MYB15* were demonstrated to specifically activate the promoters of *STS* genes, and the ectopic expression of *MYB15* in grapevine hairy roots resulted in increased *STS* expression and in the accumulation of glycosylated stilbenes in planta. These results demonstrate the involvement of MYB14 and MYB15 in the transcriptional regulation of stilbene biosynthesis in grapevine.

INTRODUCTION

Plant stilbenes are a small group of phenylpropanoid (PP) compounds that have been detected in a limited number of unrelated plant species and accumulate in response to biotic and abiotic stresses. Stilbenes have been detected in at least 72 plant species distributed among 31 genera and 12 families, including the Fagaceae, Liliaceae, Moraceae, Myrtaceae, Papilionaceae, Pinaceae, and Poaceae (Sotheeswaran and Pasupathy, 1993; Yu et al., 2005; Counet et al., 2006). Recently, stilbenes, and in particular *trans*-resveratrol (3,5,4'-trihydroxy-trans-stilbene), synthesized in grapevine (*Vitis vinifera*), cranberry (*Vaccinium oxycoccos*), blueberry (*Vaccinium myrtillus*), and peanut (*Arachis hypogaea*), have elicited particular interest within the scientific community, not only because of their role in plant defense, but also because of their pharmacological properties. The role of stilbenes in the prevention of and protection from cardiovascular and neurodegenerative diseases, cancer, and diabetes have been studied and validated in many laboratory models, and a growing body of evidence indicates that these compounds have health-protecting properties in mammals, although the bioavailability and long-term effects of their supplementation in the human diet have not yet been confirmed (Baur and Sinclair, 2006; Kalantari and Das, 2010). The interest in stilbenes has been growing since these compounds were found to accumulate in significant amounts not only in vegetative parts of plants, such as leaves and roots, but also in fruits, which in turn, are widely used in the production of mass industrial derivatives, such as juices and, in the case of grapevine, wines (Keen and Ingham, 1976; Langcake and Pryce, 1976; Oleszek et al., 2001; Yu et al., 2005; Counet et al., 2006; Chong et al., 2009).

Most stilbene-producing plants, including grapevine, contain only low concentrations of these PPs under normal growth conditions, but these compounds rapidly accumulate in response to a wide range of abiotic and biotic stresses, as a result of an increased transcription of stilbene biosynthetic genes and the coordinate activation of upstream genes belonging to the PP pathway, such as phenylalanine ammonia-lyase (*PAL*) and cinnamate 4-hydroxylase (*C4H*). A vast range of abiotic stress treatments lead to stilbene biosynthesis, including mechanical damage (Chiron et al., 2000; Pezet et al., 2003), exposure to UV-C light (Bais et al., 2000), treatment with chemicals, such as aluminum ions, cyclodextrins, and ozone (Rosemann et al., 1991; Adrian et al., 1996; Zamboni et al., 2009), and the application of plant hormones like ethylene and jasmonates (Belhadj et al., 2008a,

2008b; D'Onofrio et al., 2009). In terms of biotic stresses, the synthesis of stilbenes is particularly well documented in grapevine, where it has been shown to be induced upon infection with various pathogens, including *Plasmopara viticola* (Langcake and Pryce, 1976), *Erisyphe necator* (Fung et al., 2008; Schnee et al., 2008), and *Botrytis cinerea* (Adrian et al., 1997). The biosynthetic pathway leading to the production of stilbenes is a side branch of the general PP pathway and can be considered as an extension of the flavonoid pathway (Vannozzi et al., 2012). All higher plants are able to accumulate compounds like *p*-coumaroyl-CoA and cinnamoyl-CoA through the action of ubiquitous enzymes such as PAL, C4H, and 4-coumarate:CoA ligase, and in all plants, these compounds represent substrates for chalcone synthases, fundamental enzymes leading to the biosynthesis of the flavonoids. However, in stilbene-synthesizing plant species, these compounds are also substrates for stilbene synthases (STSs), which compete with chalcone synthases and represent the key enzymes responsible for the biosynthesis of resveratrol and its derivatives (Figure 1) (Schöppner and Kindl, 1984; Lanz et al., 1991).

To date, *STS* genes have been cloned from peanut (Schröder et al., 1988), Scots pine (*Pinus sylvestris*) (Preisig-Müller et al., 1999), Eastern white pine (*Pinus strobes*) (Raiber et al., 1995), Japanese red pine (*Pinus densiflora*) (Kodan et al., 2002), grapevine (Sparvoli et al., 1994), and sorghum (*Sorghum bicolor*) (Yu et al., 2005); apart from the latter, for which only one *STS* has been identified, these genes appear to exist as families of closely related members. In grapevine, a recent analysis of the *STS* multigenic family based on both the PN40024 and PN ENTAV 115
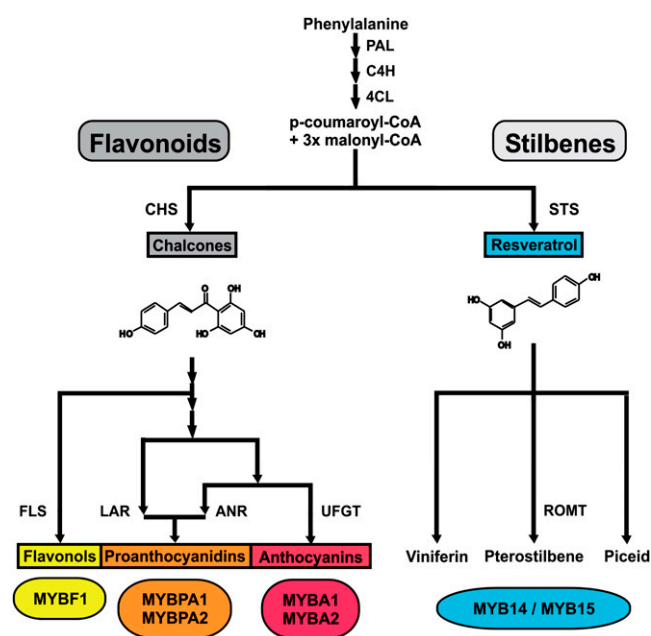
genomes (Jaillon et al., 2007; Velasco et al., 2007) led to the identification of 48 putative Vv-*STS* gene sequences, with at least 33 of these encoding full-length proteins (Vannozzi et al., 2012). To date, transcription factors (TFs) regulating the expression of structural genes for stilbene synthesis have not yet been identified. However, recently, a growing number of TFs have been demonstrated to be responsible for the regulation of PP biosynthesis in a range of plant species, including grapevine, which represents one of the most studied crop plants in terms of the regulation of the flavonoid biosynthetic pathway (Figure 1; reviewed in Czemmel et al., 2012). For example, the grapevine MYB TFs MYB5a and MYB5b were found to modulate several branches of the flavonoid pathway, regulating structural genes, such as *LEUCOANTHOCYANIDIN REDUCTASE* (*LAR*), *ANTHO-CYANIDIN REDUCTASE* (*ANR*), *FLAVONOID 3'-HYDROXYLASE*, and *CHALCONE ISOMERASE* (Deluc et al., 2006, 2008). MYBA1 and MYBA2 regulate *UDP-Glc:FLAVONOID-3-0-GLUCOSYL-TRANSFERASE* (*UFGT*), which encodes an enzyme responsible for the conversion of anthocyanidins to anthocyanins (Kobayashi et al., 2002; Walker et al., 2007). MYBPA1 regulates the expression of the flavonoid branch pathway, leading to the production of proanthocyanidins (PAs; condensed tannins) (Bogs et al., 2007), and the grape At-*MYB12*–like gene, *MYBF1*, was shown to complement the flavonol-deficient mutant *myb12*, underlining its role in the regulation of this branch of the flavonoid pathway and in the transcriptional regulation of the flavanol aglycone biosynthetic gene *FLAVONOL SYNTHASE1* (*FLS1*) (Czemmel et al., 2009). Besides *MYBF1*, all of the MYB TFs shown to date to regulate the flavonoid biosynthetic pathway in grapevine, belong to the R2R3-MYB subgroup, and require interaction with a basic helix-loop-helix (bHLH) protein (enhancer of glabra3 [*Arabidopsis thaliana*] and MYC1 [grapevine]) to activate gene expression. Based on a recent analysis of the PN40024 8.4X coverage assembly performed by Matus et al. (2008), grapevine appears to possess at least 108 R2R3-MYB TFs (Matus et al., 2008; Hichri et al., 2010). We now demonstrate that two closely related R2R3-MYB–type TFs, designated MYB14 and MYB15, play an important role in the transcriptional regulation of the stilbene biosynthetic pathway in grapevine. Transcript analyses revealed a close correlation between the expression of *MYB14* and *MYB15* and selected members of the *STS* gene family and the subsequent accumulation of stilbenes under normal and stressed conditions. Transient gene reporter assays and ectopic expression of *MYB15* in grapevine hairy roots also strongly supported their key roles in the transcriptional control of *STS* expression and accumulation of stilbene derivatives in grapevine.

RESULTS

Identification of *MYB* Genes Coexpressed with *STSs* in Stressed Grapevine Tissues

To identify TFs potentially involved in the regulation of *STS* genes in grapevine, mRNA-Seq transcriptional data obtained from *V. vinifera* cv Pinot Noir leaf discs exposed to UV-C, which was shown to induce high levels of *STS* gene expression (Vannozzi et al., 2012), were used to analyze the expression patterns of all putative grape R2R3-MYB TFs (see Supplemental Figure 1A

Figure 1. Simplified Representation of the Grapevine Phe/Polymalonate Pathway, Which Leads to the Biosynthesis of Flavonoids and Stilbenes.

Transcriptional regulation of the general enzymes is conducted by the R2R3 MYB TFs MYBPA1, MYBA, MYBF1, MYB14, and MYB15. 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; ROMT, resveratrol-*O*-methyltransferase.

online). Of the 108 grape *R2R3-MYB* TFs examined, only two genes displayed similar expression patterns to that of the inducible *STS* genes. These two genes, corresponding to the identifiers VIT_07s0005g03340 and VIT_05s0049g01020 on the 12X V1 genome assembly of the PN40024 genotype (http://genomes.cribi.unipd.it/gb2/gbrowse/public/vitis_vinifera/) (Jaillon et al., 2007), were previously assigned the gene names *MYB14* and *MYB15*, respectively, by Matus et al. (2008) based on their homology with the *Arabidopsis* genes At-*MYB14* and At-*MYB15*, respectively (Stracke et al., 2001).

To confirm these mRNA-Seq observations, the transcript levels of two highly responsive group-B–type *STS* genes, *STS29* and *STS41* (Vannozzi et al., 2012), and of the *MYB14* and *MYB15* TFs, were monitored using quantitative RT-PCR in wounded, UV-C–irradiated, and *P. viticola*–infected *V. vinifera* cv Shiraz leaf discs (Figure 2). As the *STS* family contains 48 closely related genes (Vannozzi et al., 2012), it was not possible to design sequence-specific primers for the detection of only one *STS* isoform. Thus, the primers *STS41-F/R* detect the isoforms *STS41* and *STS45*, while the primers *STS29-F/R* quantify the combined expression levels of *STS29*, *STS25*, and *STS27*.

The results of the quantitative PCR analysis confirmed the findings of the mRNA-Seq analysis, showing a strong coinduction between the *STS* genes and *MYB14/15* in response to different stress treatments (Figure 2). In all treatments analyzed, *MYB14* and *MYB15* expression was found to correlate with the expression of the highly responsive group B *STS* genes (Figures 2A to 2C). Thus, whenever *STS* genes were induced by biotic or abiotic stress, *MYB14* and/or *MYB15* were also found to be induced. In a second wounding experiment, the expression of other *R2R3-MYB* genes known to be involved in the regulation of flavonoid synthesis (i.e., *MYBA*, *MYB5a*, *MYB5b*, and *MYBPA1*) were also monitored along with *MYB14* and *MYB15* (see Supplemental Figure 1B online). Apart from *MYB14* and *MYB15*, only *MYBA* showed a transient increase in transcription 1 h after wounding.

The level of *MYB14* and *MYB15* transcription was also correlated with the observed level of *STS* transcription. For example, exposure of grape leaf discs to UV-C light (Figure 2B) increased the transcription of *MYB14* and *MYB15* by 7- and 20-fold, respectively, within 8 h of treatment compared with untreated discs (wounding only). Similarly, *STS41/45* and *STS25/27/29* expression was increased by 20- and 40-fold, respectively, 8 h after UV-C treatment compared with transcript levels in untreated discs (Figure 2B). It is also important to note that the induction of *MYB14* and *MYB15* expression preceded the initial induction of *STS* transcription after wounding and UV-C treatment (Figures 2A and 2B). Taken together, these results confirm a strong coinduction of selected *STS* and *MYB14/MYB15* genes in stressed grapevine tissues. Furthermore, the temporal expression profiles strongly suggest that these TFs might be involved in the induction of *STS* transcripts in grape leaves in response to biotic and abiotic stress.

Expression of *MYB14* and *MYB15* Correlates with Stilbene Accumulation in Skins and Seeds of Developing Grape Berries

Although the majority of previous studies on stilbene biosynthesis in grapevine have focused on their accumulation in
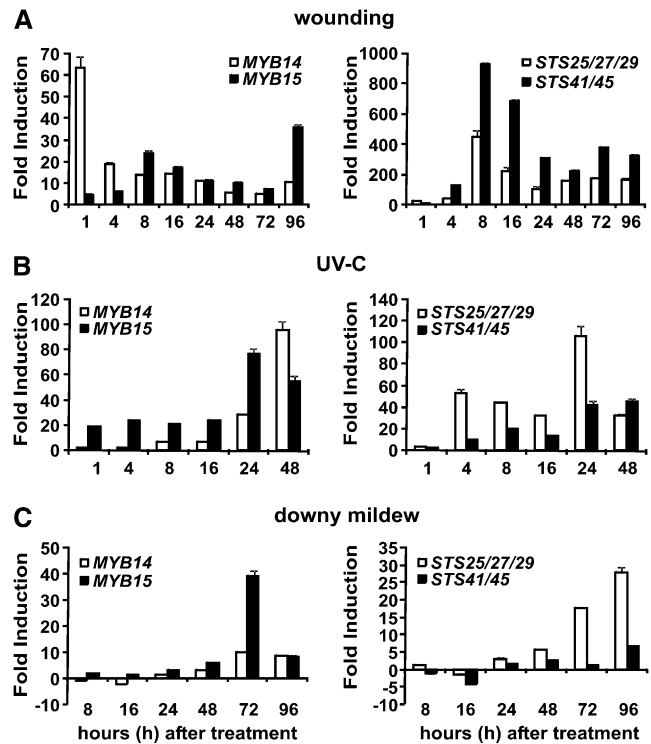

Figure 2. Expression of *MYB14*, *MYB15*, and Selected *STS* Group B-Type Genes (*STS25/27/29* and *STS41/45*) in Response to Different Abiotic and Biotic Stresses.

(A) *V. vinifera* cv Shiraz leaf discs were subjected to wounding,
(B) UV-C light irradiation.
(C) Downy mildew infection.
Rapidly expanding leaves collected from nodes 5 to 8 of new shoots harvested at different time points following treatments (hours after treatment). Transcript levels were normalized to the expression of elongation factor *EF1-α* and plotted as fold change. Fold change for wounded discs was calculated relative to the untreated sample (0 h), whereas fold change for UV-C–treated and downy mildew infected discs was obtained by calculating the ratio between treated (UV-C or downy infected) and untreated (i.e., wounded discs) samples at the same time point. The experiment was repeated twice with the same results. Data show the results of one of these experiments. Bars indicate SE of three technical replicates.

response to stress treatments, several studies have also reported that healthy grape berries synthesize stilbenes under natural growing conditions (Versari et al., 2001; Hall and De Luca, 2007; Gatto et al., 2008). To confirm if the observed correlation between *MYB14/MYB15* and *STS* expression also holds for expression during the period of stilbene accumulation in developing grape berries, we compared transcript levels of selected *STS* genes, *MYB14* and *MYB15* TFs, and the accumulation of stilbene metabolites during berry development (Figure 3).

Quantitative RT-PCR analyses revealed both *MYB14* and *MYB15* to be expressed in berry skins and seeds and their strong induction coincided with the onset of ripening (véraison) (Figures 3A and 3C), which correlated closely with the pattern of transcript

accumulation of selected *STS* transcripts. In agreement with these observations, HPLC fluorometric determination indicated a significant accumulation of the glycosylated resveratrol derivative, *trans*-piceid, in skins and seeds with the onset of ripening (Figures 3B and 3D). Stilbene accumulation, therefore,
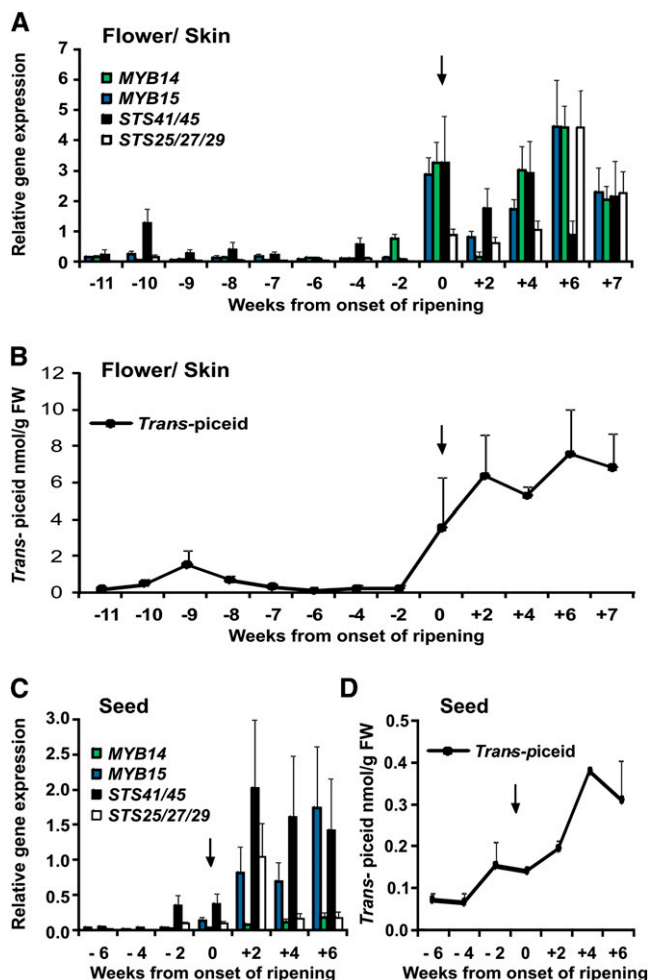

Figure 3. Expression of *MYB14*, *MYB15*, *STSs*, and Stilbene Accumulation during Grape Berry Development of *V. vinifera* cv Pinot Noir.

(A) Columns represent *MYB* and *STS* transcript profiles in flower/skins.
(B) Stilbene accumulation in flower/skins.
(C) *MYB* and *STS* transcript profiles in seeds.
(D) Stilbene accumulation in seeds.

Data points are given as weeks from the onset of ripening (véraison; labeled with an arrow), with expression values as means of three replicate PCRs (*n* = 6) and error bars indicating SE. Note that from 6 weeks before véraison (-6), berry skin has been separated from seeds. Flowering occurred 8 weeks before véraison. Accumulation of stilbenes was measured as methanol extracts of developing grape tissues using reverse-phase HPLC analysis. **(B)** and **(D)** show relative stilbene contents of *trans*-piceid in developing berries and in seeds as the mean of three different experiments, with error bars indicating SE. Note that each time point during developmental series is a pool of >100 berries collected from ~20 plants growing as described in Methods. FW, fresh weight.

closely correlates with the expression of *MYB14/MYB15* and *STS* isoforms during grape berry development. Interestingly, *trans*-piceid accumulation was not only detected in berry skins after véraison, but also at flowering and fruit set (9 to 10 weeks before véraison), which correlates with an early increase in the expression of *STS41*, but not *STS29*, *MYB14*, or *MYB15* (Figures 3A and 3B).

To confirm the presence of *trans*-piceid and search for other stilbenoid compounds in seeds, we performed a liquid chromatography–hybrid quadrupole time-of-flight–mass spectrometry (LC-QTOF-MS) analysis. Supplemental Table 1 online lists the range of stilbenoid compounds that were confirmed to be present in grape seeds.

MYB14 and MYB15 Contain a Putative C-Terminal Stress-Response Domain

Analysis of the deduced amino acid sequences of *MYB14* and *MYB15* revealed that the two genes encode proteins of 272 and 253 amino acids in length, respectively, and confirmed that both of them belong to the R2R3-MYB subfamily, based on the presence of the highly conserved R2R3-MYB DNA binding domains within the N-terminal region of the protein (see Supplemental Figure 2A, Supplemental Methods 1, and Supplemental References 1 online) (Matus et al., 2008). In contrast with the highly conserved N terminus, C-terminal regions of R2R3-MYBs are less conserved with only a few motives allowing subgrouping of proteins into separate clades (Stracke et al., 2001). Alignment of the predicted protein products of Vv-*MYB14* and Vv-*MYB15* with the R2R3-MYB family from *Arabidopsis*, together with several TFs functionally characterized from tobacco (*Nicotiana tabacum*) and carrot (*Daucus carota*), shows that both MYB14 and MYB15 cluster with a group of proteins including At-MYB13, At-MYB14, At-MB15, Nt-LBM1/Nt-LBM2, Nt-MYBJS1, and Dc-MYB1 (see Supplemental Figure 2B, Supplemental Data Set 1, Supplemental Methods 1, and Supplemental References 1 online), which share one of these conserved domains within the C terminus of the protein. This domain, designated as SG2 DxSFW–MxFWFD (see Supplemental Figure 2A, Supplemental Methods 1, and Supplemental References 1 online) appears to be associated with stress responses in *Arabidopsis* (Stracke et al., 2001). Indeed, all of the *Arabidopsis*, tobacco, and carrot TFs belonging to the Vv-MYB14/15 cluster have been shown to respond to abiotic stresses (Maeda et al., 2005; Chen et al., 2006; Gális et al., 2006). Interestingly, homologous MYB-factors from other stilbene-producing plants, such as *Poaceae* (Sb_06g022660, Sb_04g026480, and Sb_01g019270) and *Pinaceae* (Pg-MYB11 and Pg-MYB12), have been identified and also cluster with Vv-MYB14/MYB15 (see Supplemental Figure 2A, Supplemental Methods 1, and Supplemental References 1 online).

Although lacking any obvious nuclear localization signal motif, analysis of the predicted cellular localization of Vv-MYB14 and Vv-MYB15 using WOLF PSORT (Horton et al, 2006) predicted these TFs to be targeted to the nucleus, and this was confirmed for Vv-MYB14 by bombardment of a MYB14:GFP (for green fluorescent protein) fusion into onion (*Allium cepa*) epidermal cells (see Supplemental Figure 3 online).

MYB14 and MYB15 Activate *STS* Promoter Activity

To determine the capacity of both MYB14 and MYB15 to regulate the transcription of structural genes of the stilbene biosynthetic pathway, transient expression assay were conducted using Chardonnay grapevine suspension cell cultures and a dual luciferase reporter system (Horstmann et al., 2004). Promoter fragments corresponding to ~2 kb upstream of the start codon of *STS29* and *STS41* (Vannozzi et al., 2012) were first isolated and sequenced and found to contain several putative MYB binding and stress-related *cis*-elements using the plant DNA *cis*-element database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo et al., 1998).

To confirm the in silico analysis and investigate the specificity of MYB14 and MYB15 for the activation of stilbene biosynthetic genes, the activation potentials of both TFs with the promoters of *STS29*, *STS41*, and selected flavonoid pathway branch genes *UFGT* (anthocyanins) and *ANR* (PAs) were analyzed and compared with previously characterized positive acting flavonoid-specific MYB TFs (Boss et al., 1996; Kobayashi et al., 2002; Bogs et al., 2007). Chardonnay cell suspensions transiently expressing *STS* luciferase reporter constructs showed significant increases in the promoter activity of 5.6-fold for *STS41* and 3.5-fold for *STS29* when cotransformed with *MYB15* and 3.0-fold for *STS41* and *STS29* when cotransformed with *MYB14* in comparison to the control bombardments in the absence of the MYB TFs (Figure 4A; see Supplemental Figure 4 and Supplemental References 1 online). By contrast, the flavonoid-specific MYB TFs MYBA2 and MYBPA1 had little or no effect on *STS* promoter activity. Conversely, coexpression of *MYB14* or *MYB15* with the promoters of *ANR* or *UFGT* resulted in only minor increases in promoter activity when compared with that obtained with the TFs MYBA2 and MYBPA1 (Figures 4B and 4C; see Supplemental Figure 4 and Supplemental References 1 online). For example, although the *UFGT* promoter was induced ~17-fold by MYB14 and 11-fold by MYB15, this is much lower than the 137-fold induction obtained with the anthocyanin pathway-specific MYB factor MYBA2 (Figure 4C; see Supplemental Figure 4 and Supplemental References 1 online) and is comparable to activation rates observed with the *UFGT* promoter with other nonspecific MYB TFs such as MYBPA1 (2.5-fold; Czemmel et al., 2009).

Hichri et al. (2010) previously showed that MYBA and MYBPA1 were not able to induce their target gene promoters without the presence of bHLH and WD40 cofactors (Figure 4D; see Supplemental Figure 4 and Supplemental References 1 online). By contrast, the induction of *STS29* promoter activity by MYB14 and MYB15 was not dependent on the presence of the bHLH factor MYC1. Analysis of the deduced protein sequences of MYB14 and MYB15 indicates that these TFs lack the (D/E)L×2(R/K)×3L×6L×3R amino acid motif in their R3 repeat (see Supplemental Figure 2A, Supplemental Methods 1, and Supplemental References 1 online), which is necessary for the interaction with bHLHs (Grotewold et al., 2000; Zimmermann et al., 2004).

To exclude any grape cultivar–specific effects on the induction of *STS* promoters by MYB14/MYB15, transient expression assays were also undertaken in Pinot Noir grape suspension cell cultures (see Supplemental Figure 5A online). MYB15 was found to increase *STS29* and *STS41* promoter activity by 19- and 6.8-fold,

respectively, confirming the results obtained with Chardonnay cell cultures.

One factor contributing to the lower apparent induction of the *STS* promoters by *MYB14* and *MYB15* (Figure 4A) compared with that observed for MYB TF activation of the *ANR* and *UFGT* promoters (Figures 4B and 4C) is the much higher background activity of the *STS* promoters in this transient assay–system (Figure 4E). To investigate this further, we measured the endogenous transcript levels of MYB14, MYB15, and MYBA2 in Chardonnay and Pinot Noir grape suspension cell cultures. Quantitative PCR analysis revealed that the basal transcript level of *MYB14* and *MYB15* was 20- to 35-fold higher than the level of expression of *MYBA* (see Supplemental Figure 5B online), which could explain the much higher levels of background activity of the *STS* promoters compared with the *UFGT* promoter. The high level of endogenous *MYB14* and *MYB15* expression in suspension cell cultures significantly limits the dynamic range of further *STS* promoter induction by *MYB14/15* in this transient promoter assay.

The promoter induction assay was also conducted in non-grape tissue, lacking an endogenous stilbene biosynthetic pathway, in an attempt to reduce the background *STS* promoter activity. Transient coexpression of *MYB14* or *MYB15* with the *STS29* promoter in *Nicotiana benthamiana* leaf discs was found to increase *STS29* promoter activity by 3- and 13-fold, respectively, compared with control bombardments in the absence of the *MYB* TFs (see Supplemental Figure 5C online), which was not significantly different to the fold increase observed in grape suspension cells (Figure 4; see Supplemental Figure 4A and Supplemental References 1 online). However, in this assay system, the fold induction of the *STS29* promoter by *MYB15* approximated the fold induction of *UFGT* promoter by *MYBA2* (see Supplemental Figure 5D online).

Taken together, the results of these transient promoter experiments, in grapevine suspension cells and tobacco leaves, strongly support the hypothesis that MYB14 and MYB15 have a role in the transcriptional regulation of stilbene biosynthesis in grapevine.

Overexpression of *MYB15* in Grapevine Hairy Roots Leads to Increased *STS* Expression and Stilbene Accumulation

To confirm the role of *MYB14/15* in the regulation of the stilbene biosynthetic pathway in planta, *MYB15* was ectopically expressed in *V. vinifera* cv Chardonnay hairy roots under the control of the constitutively active cauliflower mosaic virus (CaMV) 35S promoter. Grapevine hairy roots expressing GFP coupled to β-glucuronidase (GUS) (Karimi et al., 2002) were used as a negative control. Four *MYB15*-overexpressing transgenic lines (*MYB15* 1-1, 2-2, 2-3, and 5-1) were identified by quantitative PCR, based on comparison with the level of endogenous *MYB15* transcripts in various *GFP:GUS*-expressing control lines (GFP:GUS77, GFP:GUS78, GFP:GUS79, and GFP:GUS80) (Figure 5A). Selected *MYB15* transgenic lines displayed increased levels of transcription of *STS* genes (Figure 5A) and accumulated higher levels of stilbenes compared with the *GFP:GUS* transformed lines (Figure 5B; see Supplemental Figure 6 online). On average, transgenic *MYB15* transgenic lines accumulated a fivefold higher level of *trans*-piceid compared with *GFP:GUS* transformed lines
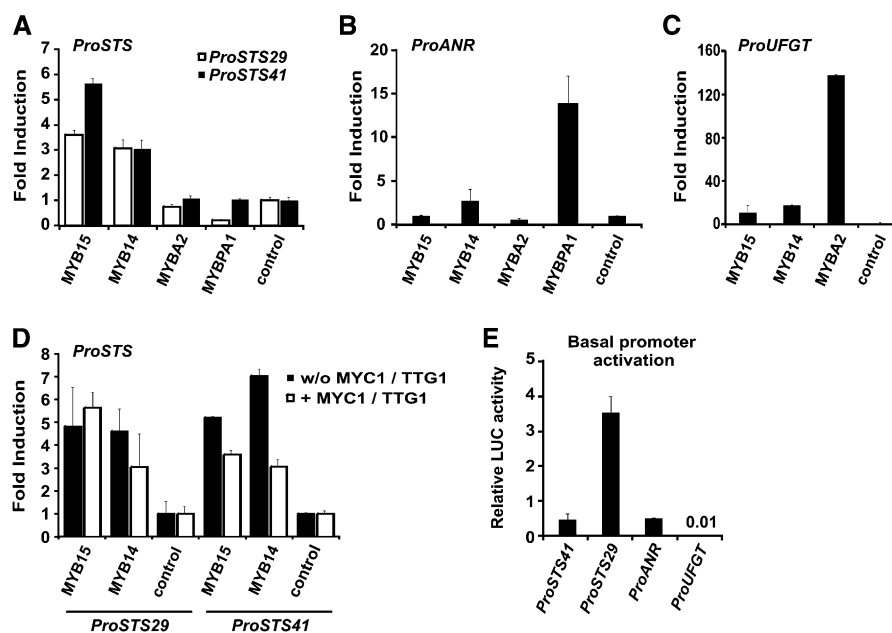
Figure 4. MYB14 and MYB15 Specifically Induce the Promoter Activity of *STS41* and *STS29*, Which Are Involved in the Biosynthesis of Stilbenes.

(A) to **(C)** Fold induction of promoter activity in the presence of a MYB TF relative to promoter activity in the absence of the MYB TF. Control columns show a fold induction of 1.0, indicating no effect on promoter activity.

(D) Fold induction of *STS* promoter activity by MYB14 and MYB15 with and without (w/o) bHLH and WD40 cofactors Vv-MYC1 and At-TTG1.

(E) Basal activity of *V. vinifera* promoters in suspension cell culture in the absence of added MYB TFs.

Transient expression in *V. vinifera* cv Chardonnay suspension cell culture following particle bombardment. Specific promoters linked to a firefly luciferase gene were cobombarded into cells with pART7-MYB TF constructs or with a pART7 (empty vector) control. Each transfection contained the *Renilla* luciferase plasmid pRluc as an internal control (Horstmann et al., 2004) and the cofactors Vv-MYC1 (bHLH type) and At-TTG1 (WDR type) (except for the experiment shown in **[D]**). The columns represent relative LUC activity (Firefly/Renilla) of the corresponding promoter plus MYB factor relative to the respective control (without the MYB factor) of six independent experiments with error bars indicating se.

(Figure 5B) and smaller increases in the level of *trans*-resveratrol, 2,3,4′,5-tetrahydroxystilbene-2-glucoside and other complex stilbenes (see Supplemental Figure 6 online) when measured by fluorimetric HPLC detection. The presence of *trans*-piceid and other stilbenoid compounds in *MYB15* transgenic hairy roots was confirmed by LC-QTOF-MS (see Supplemental Table 1 online).

In addition to activation of *STS* expression, transgenic MYB15-overexpressing hairy root lines were also found to have elevated levels of *PAL* expression (Figure 5A). The average level of *PAL* expression in the four *MYB15* transgenic lines was found to be 13-fold higher than the average level of *PAL* expression in the *GFP:GUS* transformed lines

The increase in *STS* expression and stilbene production in these transgenic hairy roots confirms the role of MYB15 as a regulator of stilbene biosynthesis in grapevine cells. Our data also demonstrate that MYB15 may have a role in the transcriptional regulation of the core PP pathway.

DISCUSSION

STS Transcription in Grapevine Is Correlated with *MYB14* and *MYB15* Expression

It is well known that stilbenes are synthesized in plant tissues as a result of the induction of *STS* genes in response to biotic and

abiotic stresses (Versari et al., 2001; Borie et al., 2004; Vannozzi et al., 2012). But to date, no information is available regarding the identity of the TFs responsible for the transcriptional regulation of structural genes of the stilbene biosynthetic pathway. Considering the close relationship between the stilbene and flavonoid biosynthetic pathways and considering that the latter appears to be regulated exclusively by TFs belonging to the R2R3-MYB subgroup, we postulated that stilbene biosynthesis may also be under similar transcriptional control. The analysis of transcriptional data from grapevine leaf discs treated with various abiotic and biotic stresses led to the identification of two R2R3-MYB TFs, *MYB14* and *MYB15*, that were coinduced with *STS* genes across a range of stress conditions. In all treatments analyzed, *MYB14* and *MYB15* expression was found to correlate with the expression of highly responsive *STS* genes in terms of pattern and timing. Thus, whenever *STS* genes were induced, either *MYB14* and/or *MYB15* was also found to be induced, and, most importantly, their induction preceded the initial induction of *STS* genes. By contrast, no evidence was found for any significant involvement of other flavonoid-specific R2R3-MYB TFs in the regulation of stilbene biosynthesis (see Supplemental Figure 1B online).

Our results indicate that MYB14 and MYB15 are not only involved in the induction of *STS* transcription in grape tissues in response to biotic and abiotic stress but are also responsible for
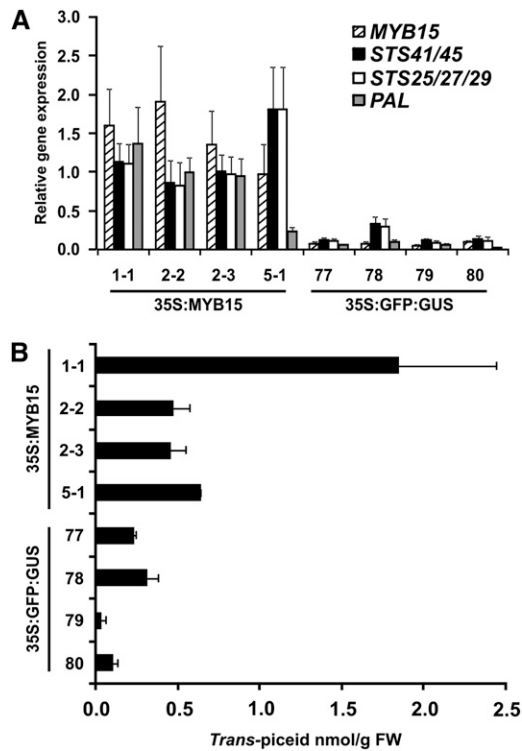
Figure 5. Expression of *STSs* and *PAL* and Accumulation of *trans*-Piceid in Grapevine Hairy Roots Ectopically Expressing *MYB15*.

(A) Transcript levels of *MYB15*, *STS*, and *PAL* were determined by quantitative PCR in *V. vinifera* cv Chardonnay hairy root lines transformed with pART27-MYB15 compared with control lines transformed with GFP:GUS (pKGWFS7; Karimi et al., 2002). Each column in shows transcript levels corrected to *Ubiquitin1*, *EF1-α*, and *GAPDH* (Pfaffl et al., 2002), expressed as mean values of three replicate PCRs (*n* = 6) and error bars indicating SE. Note that expression levels can only be compared between *MYB15* and *GFP:GUS* control lines and not between the individual genes using this method.
(B) Accumulation of *trans*-piceid *MYB15*-overexpressing hairy root lines compared with GFP transformed controls expressed as relative content in nmol/g fresh weight. *Trans*-piceid levels shown represent the mean of three different experiments with error bars indicating SE. FW, fresh weight.

stilbene biosynthesis observed in developing grape berries (Figure 3). Gatto et al. (2008) previously reported that stilbenes accumulate in red and white grapevine varieties after the onset of véraison. While the levels of stilbenes produced in ripening berries are only 1 to 5% that observed in both berries and leaves subjected to biotic and abiotic stress (Langcake, 1981; Douillet-Breuil et al., 1999; Bais et al., 2000), our data confirm that the induction of *STS* transcription in berry skins at véraison also coincides with an increase in *MYB14/15* transcription (Figure 3). The induction of stilbenes with the onset of ripening may provide some protection to the berry from fungal pathogens, such as *B. cinerea*.

Stilbene biosynthesis shares common substrates with the synthesis of the three major flavonoid subgroups (i.e., PAs, flavonols,

and anthocyanins; Figure 1), raising the question as to whether common MYB TFs might regulate both the stilbene and flavonoid pathway. However, previous studies have demonstrated that PA and flavonol accumulation during grape berry development does not correlate with stilbene accumulation. PAs accumulate in developing Shiraz grape berry skin and seeds until véraison (Downey et al., 2003a), correlating with the expression of the major PA pathway genes *LDOX*, *ANR*, *LAR1*, and *LAR2* and their transcriptional regulators MYBPA1/2 (Bogs et al., 2005, 2007; Terrier et al., 2009), whereas stilbenes are induced after véraison. Downey et al. (2003b) reported that flavonols are present in high levels at flowering and early berry development of cv Chardonnay and Shiraz and in the late-maturation phase. Thus, the expression of *FLS1* (Downey et al., 2003b) and its regulating TF MYBF1 correlates with flavonol synthesis (Czemmel et al., 2009) but not with the synthesis of stilbenes. Anthocyanin accumulation and the regulating TFs MYBA1/2 are induced in grape berry skin at véraison in red berry cultivars, and this does correlate with stilbenes biosynthesis (Figure 3). However, in white berry cultivars in which anthocyanins are not synthesized at véraison because of mutations in MYBA1/2 (Walker et al., 2007), the biosynthesis of stilbenes is still observed (Gatto et al., 2008), which argues against a direct control of *STS* genes by MYBA1/2. When considered together with the results of our transient promoter assays (Figure 4), these results suggest that *STS* transcription is regulated by MYB14 and MYB15 and that there is little or no direct involvement of the respective *MYB* regulators of flavonol, anthocyanin, and PA synthesis in the regulation of stilbene production during grape berry development.

MYB14 and MYB15 Contain a C-Terminal Amino Acid Motif Conserved in R2R3-MYB TFs Involved in Biotic and Abiotic Stress Responses

MYB14 and MYB15 not only share the highly conserved N-terminal R2R3 domain, which characterizes all R2R3-MYB TFs, but also share a small amino acid motif, designated as SG2, within the C-terminal region (see Supplemental Figure 2A, Supplemental Methods 1, and Supplemental References 1 online). This amino acid motif has been previously described as a stress-related motif (Stracke et al., 2001; Dubos et al., 2010) and is also a common feature of R2R3-MYB TFs from other plant species, such as At-MYB13, At-MYB14, and At-MYB15 from *Arabidopsis* and Nt-LBM1-4 from tobacco, which cluster with Vv-MYB14/15 and respond to environmental stresses (see Supplemental Figure 2B, Supplemental Methods 1, and Supplemental References 1 online). Little information is available regarding the function of At-MYB13, At-MYB14, or At-MYB15, apart from a few reports, which showed that ectopic expression of At-*MYB15* improved drought and salt tolerance in *Arabidopsis* by enhancing the expression of genes involved in abscisic acid biosynthesis and signaling (Ding et al., 2009; Dubos et al., 2010). While conserved amino acid motifs suggest proteins have a common evolutionary origin, they do not always indicate a conserved functional similarity. For example, Ph-PH4, the homolog of the At-MYB5 and Vv-MYB5 TFs, is not involved in activation of the general flavonoid pathway in petunia (*Petunia* × *hybrida*) (Quattrocchio et al., 2006).

Nt-LBM2, Nt-MYBJS1, and Dc-MYB1 have also been shown to be involved in regulating upstream genes in the core PP pathway, such as *PAL* and *C4H* (Sugimoto et al., 2000; Gális et al., 2006), which are key genes in the production of *p*-coumaroyl-CoA, the main substrate for flavonoid and stilbene biosynthesis. Grapevine hairy roots ectopically expressing Vv-*MYB15* showed a marked increase in the expression of Vv-*PAL* (Figure 5A). This suggests that, in addition to their role in the regulation of stilbene biosynthesis, Vv-MYB15 (and possibly Vv-MYB14) may also be involved in the regulation of upstream PP pathway genes to ensure the availability of substrates for stilbene biosynthesis.

The introduction of Vv-*STS41* (formerly *pVst1*) into other plant species, such as tobacco, under the control of its own promoter, induced the production of stilbenes, suggesting there are common TFs present in these other plant species, which do not contain *STS* genes (Hain et al., 1990; Stark-Lorenzen et al., 1997; Thomzik et al., 1997; Zhu et al., 2005). A number of MYB factors orthologous to Vv-*MYB14* and Vv-*MYB15*, which have been detected in other known stilbene-producing plants, such as sorghum and *Picea glauca* (see Supplemental Figure 2B, Supplemental Methods 1, and Supplemental References 1 online; i.e., SORBIDRAFT_06g022660; PgMYB12), would also be interesting candidates for studying the regulation of stilbene biosynthesis in these plants.

It has previously been reported that *STS* transcription in grape cells following stress or elicitor treatment is often biphasic in nature (Wiese et al., 1994; Borie et al., 2004; Wang et al., 2010). Vannozzi et al. (2012) demonstrated that this is most likely a result of the differential timing of the stress response of members of the different subgroups within the *STS* gene family. Similarly, our results demonstrate that *MYB14* and *MYB15* are differentially expressed in response to various biotic and abiotic stresses (Figure 2), which mirrors the *STS* subgroup–specific expression, indicating the specificity of the interaction of the two TFs (MYB14 or MYB15) with certain *STS* genes. The observed differences in expression patterns of *MYB14* and *MYB15* also raises the question as to whether their own transcription is regulated by different upstream stress response pathways, such as the jasmonic-acid and ethylene-signaling pathways (Belhadj et al., 2006, 2008a, 2008b; Vezzulli et al., 2007).

MYB14 and MYB15 Specifically Activate *STS* Transcription in the Absence of bHLH

The transcriptional control of the anthocyanin and PA pathway genes is accomplished, in most plant species, by a complex in which R2R3-MYB TFs, bHLH proteins, and WD40 proteins all interact to activate gene transcription (Mol et al., 1998; Winkel-Shirley, 2001; Hichri et al., 2010). By contrast, the induction of the Vv-*STS* promoter activity by Vv-MYB14 and Vv-MYB15 was independent of the Vv-MYC1 bHLH cofactor (Figure 4D) as was previously observed for Vv-MYBF1, a TF involved in the regulation of flavonol biosynthetic genes (Czemmel et al., 2009). In agreement with these observations, Vv-MYB14, Vv-MYB15, and Vv-MYBF1 lack the bHLH interaction amino acid motif [D/E]Lx2[R/K]x3Lx6Lx3R (see Supplemental Figure 2A, Supplemental Methods 1, and Supplemental References 1 online) (Grotewold et al., 2000; Zimmermann et al., 2004), indicating

that these R2R3-MYB TFs do not require bHLH TFs for their activity.

Ectopic Expression of *MYB15* in Grapevine Hairy Roots Results in the Induction of *STSs* and Their Metabolic Products

In grapevine, ectopic gene expression in hairy roots has previously been used for functional characterization and target gene identification of the flavonoid TFs MYBA1, MYBPA1, and MYBPA2 (Cutanda-Perez et al., 2009; Terrier et al., 2009). This approach was also used in this study to provide in planta evidence for the role of MYB15 in regulating stilbene biosynthesis in grapevine. Transgenic grapevine hairy root lines ectopically expressing *MYB15* exhibited increased levels of transcription of *STS41* and *STS29* and an increased accumulation of stilbenes (Figure 5). Surprisingly, the level of stilbene production in hairy roots overexpressing MYB15, measured as *trans*-piceid accumulation, was found to be only fivefold higher, on average, than the control lines (Figure 5B). One explanation for this observation might be the stress-related induction of endogenous *MYB14/15* and *STS* genes in control hairy root lines. Indeed, similar results were reported for grapevine hairy roots ectopically expressing *MYBPA2*, which regulates the PA pathway. Accumulation of PAs in the *MYBPA2* overexpressing lines was found to be only fivefold higher than the control lines because of a high PA background level in the control roots (Terrier et al., 2009). Another explanation may be the possible phytotoxic effect of stilbene accumulation on hairy-root growth and development as reflected in the relatively low number of regenerating *MYB15* hairy-root transformants and the loss of several lines showing high *MYB15* expression and putative high stilbenoid levels during the culturing process (see Methods).

The ectopic expression of *STS* genes in a range of plant species, including *Arabidopsis*, rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), alfalfa (*Medicago sativa*), apple (*Malus domestica*), barley (*Hordeum vulgare*), wheat (*Triticum* spp), kiwifruit (*Actinidia* spp), and strawberry (*Fragaria* spp), has revealed that, together with an increased resistance to pathogen attack, there is commonly an accumulation of *trans*- and *cis*-piceid or *trans*-resveratrol (reviewed in Delaunois et al., 2009). Similar results were observed in *MYB15* overexpressing hairy roots (Figure 5B; see Supplemental Figure 6 online). It has been demonstrated that stilbenes, and in particular δ-viniferin, an oxidative resveratrol dimer, and pterostilbene, the 3,5-dimethoxy analog of resveratrol, have fungitoxic properties (Pezet et al., 2003, 2004; Schmidlin et al., 2008) and that the glycosylation of stilbenes may occur in plant cells both to protect cells from their potential toxic effects and to protect resveratrol from degradation (Jeandet et al., 1997; Hipskind and Paiva, 2000). This might explain the high amount of *trans*-piceid in the *MYB15* transgenic hairy roots in comparison with the relatively low amounts of other modified stilbenes (see Supplemental Figure 6 online).

The vast majority of the genes responsible for stilbene diversity, which has been predicted to influence bioavailability, antioxidant capacity underlying phytoalexin function, and stability of the resulting polyphenols, are still unknown. The accumulation of different modified stilbenes in *MYB15* transgenic

lines suggests that the MYB factors MYB15, and presumably its paralog MYB14, regulate enzymes catalyzing downstream modification and transport processes of the stilbene biosynthetic pathway. Therefore, a comparative transcriptome analysis of transgenic MYB15 and GFP:GUS grapevine hairy roots could lead to the identification of genes involved in stilbene modification and transport. The analysis of these unknown enzymes would expand our knowledge of the stilbene biosynthetic pathway in plants and would be valuable tools for the pharmacological industry exploring modified resveratrol derivatives and their beneficial effect on human health (Mikulski and Molski, 2010; Agarwal and Baur, 2011; Kapetanovic et al., 2011). Quantitative variation of polyphenol levels, including anthocyanins and stilbenes, has been detected for different grapevine cultivars and susceptibility to fungal diseases has been correlated to the content and composition of the stilbene derivatives (Pezet et al., 2004). The variation in anthocyanin content in grape cultivars was recently explained by polymorphisms of a single gene cluster of three *MYBA* genes (Fournier-Level et al., 2010). Similarly, investigating the genetic differences in *MYB14* and *MYB15* expression and/or transcriptional activity in different grapevine cultivars might lead to the development of molecular markers for breeding of grapevines with optimized stilbene content and composition.

METHODS

Plant Material

For mRNA-Seq analysis, leaves of grape (*Vitis vinifera* cv Pinot Noir) were obtained from field-grown vines at the Lucio Toniolo experimental farm of the University of Padova (Legnaro, Italy) as described by Vannozzi et al. (2012). The quantitative RT-PCR analyses after stress treatment were conducted using *V. vinifera* cv Shiraz rapidly expanding leaves collected from nodes 5 to 8 of new shoots and samples from potted glasshouse vines at the Waite Campus (Adelaide, South Australia, latitude 34° 56' south, longitude 138° 36' east). Grapevine tissues of the developmental series (*V. vinifera* cv Pinot Noir) were collected from a commercial vineyard during the 2007 to 2008 season in Schrießheim, Germany (49° 28' 40'' south, 8° 40' 22'' east) with a moderate climate and mean daily temperature of 22°C during grape berry development. Sampling was performed from the start of floral initiation until harvest, with ~100 berries from 20 different plants and bunches collected at weekly intervals, as previously described (Downey et al., 2003a, 2003b). *Nicotiana benthamiana* plants used for particle bombardment were grown under green house conditions for 6 to 8 weeks.

The grape cell suspension cultures used in the luciferase transient transfection assay were established from Chardonnay and Pinot Noir petiole callus culture and maintained in Grape Cormier medium as previously described (Czemmel et al., 2009). Transgenic hairy roots were generated as described below.

Analysis of mRNA-Seq Expression Data Sets for Identifying R2R3-MYB TFs Coexpressed with Vv-*STS* Genes

Generation and analysis of mRNA-Seq data from UV-C–treated *V. vinifera* cv Pinot noir leaf discs was performed as described previously (Vannozzi et al., 2012). *STS* and coexpressed MYB TFs expression patterns were analyzed and graphically expressed using mean of Multi Experiment Viewer software (Saeed et al., 2006). The mRNA-Seq data were deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) as series GSE37743.

Bioinformatic Analysis

Multiple sequence alignments were conducted using the ClustalW program (Chenna et al., 2003), and the editing of aligned sequences was performed using GeneDoc 1.6 software. An unrooted phylogenetic tree showing selected plant R2R3-MYB TFs was generated with a neighbor joining method using MEGA 3.1 software (Kumar et al., 2004). Protein sequences were identified after a BLASTp search with the protein sequence from Vv-MYB14/15. Selected protein sequences from TFs were downloaded from the GenBank or EMBL databases (http://www.ensembl.org/index.html; The EMBL Nucleotide Sequence Database), and their accession numbers are listed below.

The Vv-*STS29* and Vv-*STS41* promoter regions were screened for plant *cis*-acting regulatory DNA elements both manually and using the Plant *cis*-Element database (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999).

Quantitative RT-PCR Expression Analyses of Vv-*MYB14*, Vv-*MYB15*, and Selected Vv-*STSs*

Total RNA extraction and cDNA synthesis from *V. vinifera* cv Shiraz leaf discs subjected to stress treatments was performed as described previously (Vannozzi et al., 2012). Total grapevine RNA from the various tissues of the developmental stages was isolated according to Downey et al. (2003b). RNA of grapevine hairy roots and suspension cell cultures was isolated with the EURx GeneMATRIX Universal RNA purification kit (Roboklon) following the manufacturer's protocol. For cDNA synthesis, 1 μg of grapevine total RNA was reverse transcribed in 20 μL for 1 h at 42°C using oligo(dT)18 primer, RNase inhibitor, and thermo-stable avian myeloblastosis virus reverse transcriptase (dART cDNA synthesis kit; Roboklon).

Expression analysis of *MYB* and *STS* genes after treatment of *V. vinifera* cv Shiraz leaves with abiotic (UV-C irradiation, wounding) and biotic (*Plasmopara viticola* infection) stress was performed by quantitative PCR as described previously (Vannozzi et al., 2012). Transcript levels of *MYB14*, *MYB15*, and selected *STS* genes during grape berry development and in transgenic hairy roots were determined by quantitative PCR with the SYBR Green method on a Rotor-Gene Q (Qiagen). The PCR reaction mix (12.5 μL) contained 1 μL of cDNA (diluted 1:10), 6.25 μL of Maxima SYBR Green qPCR Master Mix (2×) (Fermentas), 5 μM each primer, and sterile water. The thermal cycling conditions used were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 58°C (Vv-*MYBs*) or 60°C (Vv-*STS*) for 30 s, and 72°C for 20 s, followed by a melt cycle with 1°C increments (5 s) from 56 to 96°C. The primer efficiency was tested in preliminary experiments with dilutions of pART7-MYB14/pART7-MYB15 plasmid or cDNAs of samples maintaining a value of $r^2 \geq 0.90$. The cycle threshold values of the developmental series and the transgenic hairy roots are corrected to Vv-*Ubiquitin1*, Vv-*EF1-α*, and Vv-*GAPDH*, which were previously shown to be appropriate reference genes during berry development (Reid et al., 2006). Normalization against three reference genes was conducted as described previously (Pfaffl et al., 2002). The analysis of melting curves, measurement of primer pair efficiencies, and determination of cycle threshold values, including the calculation of the mean normalized expression of the genes, were conducted using the Rotor-Gene Q Series Software Q 2.0.2 (Qiagen) and the Q-Gene software (Muller et al., 2002). The expression of Vv-*MYB* TFs was determined using the following gene-specific primers: MYB14-F (5′-TCTGAGGCCGGATAT-CAAAC-3′), MYB14-R (5′-GGGACGCATCAAGAGAGTGT-3′), MYB15-F2 (5′-CAAGAATGAACAGATGGAGGAG-3′), MYB15-R2 (5′-TCTGCGACTGC-TGGGAAA-3′), Vv-MYB5a-F (5′-CTAGAACTGTCTGGGAACCT-3′), Vv-MYB5a-R (5′-TGCAAGGATCCATTTCACATAC-3′), Vv-MYB5b-F (5′-TGACAGCC-GGTGTTCTTTAAT-3′), Vv-MYB5b-R (5′-AGCATACTAACACAACAACA-CAACC-3′), Vv-MYBPA1-F (5′-AGATCAACTGGTTATGCTTGCT-3′), and Vv-MYBPA1-R (5′-AACACAAATGTACATCGCACAC-3′). To measure the

expression of the *STS* genes Vv-*STS41* and Vv-*STS29*, it was not possible to design gene-specific quantitative PCR primers because of the high sequence homology between different family members (Vannozzi et al., 2012). Thus, STS primers Vv-STS41-F (5′-GAGTACTATTTGGTTTTG-GACCT-3′) and Vv-STS41R (5′-AACTCCTATTTGATACAAAACAACGT-3′) also detect the isoforms Vv-*STS41* and Vv-*STS45*, and the primers Vv-STS29-F (5′-GGTTTTGGACCAGGCTTGACT-3′) and Vv-STS29-R (5′-GAGATAAA-TACCTTACTCCTATTCAAC-3′) quantify the expression levels of Vv-*STS29*, Vv-*STS25*, and Vv-*STS27*. Expression of *PAL* in grapevine hairy roots was measured with the primers Vv-PAL_qF (5′-GTTGTCGTGAAAAACCAGCTT-3′) and Vv-PAL_qR (5′-GGATCACTCACGACGAAACTC-3′). The primers for Vv-MYBA (Vv-MYBA-F (5′-CTTTTCGGCTTCTGGAGAGA-3′), and Vv-MYBA-R (5′-CTGTGTTGCAGTTTCTTCTGTC-3′) detect the isoforms Vv-MYBA1, Vv-MYBA2, and Vv-MYBA3. All quantitative PCR products were verified by agarose gel electrophoresis and by determination of melting curves at the end of each run. Amplified gene products were sent for sequence analysis to Eurofins MWG Operon.

Cloning of Vv-*MYB14*, Vv-*MYB15*, and Vv-*STS* Promoter Constructs

The complete coding sequences of *MYB14* and *MYB15* were amplified using proofreading Taq polymerase from Shiraz cDNA obtained from UV-C–irradiated grape berry skins (*MYB14*) or grape berry cDNA (*MYB15*). Sequence-specific oligonucleotides binding into the putative 5′ and 3′ untranslated regions of the target genes were designed using Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) on grape sequences provided by Genoscope (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis). Gene-specific primers Vv-MYB14-ATG-Xho (5′-GCGCTCGAGAAAATGGGGAGAGCTCCTTG-3′) and Vv-MYB14-STOP-BamHI (5′-CGCGGATCCTTCTTTCCCTCATATTTCTGATAATT-3′) introduced *Xho*I and *Bam*HI restriction sites to the complete coding sequence of MYB14. Additional restriction sites for *Xba*I and *Bam*HI were attached to *MYB15* using sequence-specific primers MYB15-F (5′-TGATCTAGAGCCAGAGG TTA-GAGTAGTGGTG-3′) and MYB15-R (5′-TGAGGATCCTTCCGCTTTTTGC-TTTCTTG-3′). The generated PCR fragments were purified, digested with the above-mentioned restriction enzymes, and cloned in the vector pART7 (Gleave, 1992) to give pART7-MYB14 and pART7-MYB15, where both MYB factors were under the control of the CaMV 35S promoter. The nucleotide sequence of the *MYB14* open reading frame (ORF) with 836 bp and *MYB15* ORF with 926 bp in pART7 was determined by DNA sequencing. The expression cassette present in pART7-MYB14 and pART7-MYB15 was isolated as a *Not*I fragment and cloned into the *Not*I site of the binary vector pART27 (Gleave, 1992). For hairy root transformation, pART27-MYB15 was transferred into *Agrobacterium rhizogenes* strain ATCC 15834 by electroporation.

To conduct the transient expression assay, promoter regions related to the 8.4× grape genome sequences of the STSs *STS29* (1255 bp) and *STS41* (1436 bp) (Vannozzi et al., 2012) were isolated from genomic DNA (cv Shiraz) using Phusion DNA polymerase (Finnzymes). The specific primers STS41pF (5′-TATGAGCTCCGTCCATAGGAAGTAGAGTAAAATG-3′) and STS41pR (5′-TATCTCGAGACGATGCCAGATACGTTCTGAAATTG-3′) were used to introduce the restriction sites *Sac*I-*Xho*I to the chosen promoter sequence of STS41 and STS29pF (5′-TATGAGCTCAAAATG-TCGAAACACTTTGTATTAAA-3′) and STS29pR (5′-TATCTCGAGTGATCCC AGCTACGTACTCAA-3′) added this motive to *STS29*. The promoter regions of *STS29* and *STS41* were ligated into the luciferase vector pLuc (Horstmann et al., 2004) and confirmed by DNA sequencing. The cloning of control promoter fragments of *ANR* and *UFGT* into pLuc as well as the coding regions of *MYBPA1* and *MYBA2* into pART7 was described previously (Bogs et al., 2007).

Localization of Vv-*MYB14* in Onion Epidermal Strips

A modified version of shuttle vector pART7 (Gleave, 1992), termed pN'gfp (Selth et al., 2005), was used to produce the MYB14:GFP fusion construct. The *MYB14* ORF was amplified and cloned into the *Xho*I-*Bam*HI

sites of pN'gfp to generate an in-frame C-terminal fusion to the GFP gene downstream of the CaMV 35S promoter. Onion (*Allium cepa*) epidermal strips were prepared and bombarded with MYB14:GFP as described previously (Selth et al., 2005). Onion strips were sampled after 48 h and mounted in 1× PBS, and GFP was visualized with a Zeiss Axio Imager M1 microscope, GFP filter (470/40-nm excitation; 525/50-nm emission) under ×20 magnification. Nuclei were visualized using 1 µg/mL of 4′,6-dia-midino-2-phenylindole dissolved in 1× PBS, using a 4′,6-diamidino-2-phenylindole filter (365-nm excitation; 445/50-nm emission).

Transformation and Induction of Transgenic Grapevine Hairy Roots

The *A. rhizogenes* strain ATCC 15834 containing either pART27-MYB15 or pKGWFS7 (Karimi et al., 2002), which both express their respective gene of interest, *MYB15* or *GFP*:*GUS*, under control of the CaMV 35S promoter, was grown with shaking at 200 rpm and 28°C for 24 h on MGL medium (Mathews et al., 2003).

Transgenic hairy roots were generated as previously described by Torregrosa and Bouquet (1997) with the following modifications: Leaves from in vitro–grown Chardonnay plants with attached petioles were cut and placed upside down with the petiole facing upwards on hairy root (HR) medium agar plates (1% agar). HR medium was prepared with macro-elements (1.6 g/L NH_4NO_3, 4.4 g/L $CaCl_2$, 3.7 g/L $MgSO_4$, and 23.25 g/L KNO_3) and Murashige and Skoog microelements (3.1 g/L H_3BO_3, 8.45 g/L $MnSO_4$, 4.3 g/L $ZnSO_4$, 0.414 g/L KI, 0.125 g/L Na_2MoO_4, 12.4 mg/L $CuSO_4$, and 12.5 g/L $CoCl_2$) modified as previously described by Murashige and Skoog (1962), B5 vitamins (Gamborg et al., 1968), FeEDTA (7.44 g/L Na_2EDTA and 1.86 g/L $FeSO_4$), and 1.5% Suc, pH 5.7 (1 N KOH). The freshly cut petioles were inoculated with one drop of the overnight *Agrobacterium tumefaciens* culture on the top of the stem and were co-cultivated for 5 d at 22°C in the dark. Leaves were transferred onto HR medium containing 400 µg/mL of timentin with the petioles and the lower surface of the leaf in contact with the agar. Leaves were incubated at 28°C in the light. Newly emerging root tips were removed once they reached ~2 cm in length and were placed onto HR medium containing 400 µg/mL of timentin. Stable transgenic roots were cultivated at 24°C in the dark and subcultured every 4 to 5 weeks on the same medium.

Due to low transformation/regeneration and slow growth of MYB15 transgenic hairy roots and to avoid possible gene silencing effects, 1 to 2 cm of roots were sampled from each line during the subculturing process at weeks 4 and 5 and frozen at −80°C. The remaining root material was divided and transferred to new media. To harvest enough root material, each line was harvested over a period of 1 year and the samples combined for HPLC, quantitative RT-PCR, and LC-QTOF-MS analysis. In addition, several MYB15 transformation experiments were undertaken, each generating several independent lines showing ectopic *MYB15* expression. However, those lines showing the highest levels of *MYB15* expression were found to become necrotic during the culturing process, and it was not possible to sample enough root material to carry out a complete analysis. Therefore, the independent transgenic hairy root lines MYB15 1-1, 2-2, 2-3, and 5-1 described in Figure 5 represent those lines with intermediate levels of *STS* induction. Control root lines showed no necrotic phenotype during growth.

Transient Promoter Assays

Transient promoter assays were developed using a cell suspension of *V. vinifera* cv Chardonnay and Pinot Noir petiole callus cultures and *N. benthamiana* as previously described (Bogs et al., 2007; Walker et al., 2007). For transient expression in *N. benthamiana*, leaf discs (d = 3 cm) were used. The Dual Luciferase Assay protocol was modified based on Czemmel et al. (2009). The *Renilla* luciferase plasmid pRluc was used as an internal control in each transfection experiment (Horstmann et al., 2004). Transient assays were performed with and without the bHLH and

WD40 cofactors Vv-MYC1 and At-TTG1 (Figure 4D). All transfection experiments were repeated six times. Mean values of firefly and *Renilla* luciferase ratios are reported as relative luciferase activity with error bars indicating SE.

Analysis of Stilbenoids in Grapevine via HPLC

HPLC analysis of stilbene compounds was performed on a reverse-phase HPLC device (Kontron Instruments; 322 pump system/360 autosampler/ 335 HPLC detector) with a Symmetry C18 column (3.5 μm, 4.6 mm × 150 mm [WAT200632]; Waters) protected by a guard column (Czemmel et al., 2009). Buds, flowers, berries, and seeds of the developmental series and the transgenic hairy roots overexpressing Vv-MYB15 or GFP (pKGWFS7) were frozen in liquid nitrogen and homogenized, and 50-mg samples were extracted by adding 200 μL of 50% (v/v) methanol (HPLC grade) in water and sonicated for 20 min in an ice water bath. Extracts were incubated at room temperature for 15 min and centrifuged for 10 min at 13,000 rpm to obtain a clear supernatant, and 150 μL was used for HPLC analysis. Separation was performed with a binary gradient of solvent A (10% [v/v] formic acid in water) to solvent B (100% [v/v] methanol; HPLC grade). The gradient conditions were 0 min, 17% solvent B; 30 min, 35% solvent B; 41 min, 37% solvent B; 43 min, 100% solvent B; 51 min, 100% solvent B; 52 min, 17% solvent B; and 59 min, 17% solvent B. The column was maintained at 40°C, and the flow rate was 1.0 mL/min. Fluorimetric detection with a maximum excitation wavelength at 330 nm and emission at 374 nm was used to detect stilbenes as described previously (Pezet et al., 1994). Data acquisition and processing were performed using Kroma System 2000 software (Kontron) (Czemmel et al., 2009). Concentrations were calculated from calibration curves prepared from commercial stilbene standard *trans*-piceid (PhytoLab). The stilbene concentrations were quantified relative to the *trans*-piceid calibration curve in each sample and expressed in nmol/g fresh weight. Columns in Figures 3B, 3D, and 5B show three HPLC measurements, and error bars indicate SE. *Trans*-piceid was identified and retention times were verified in all HPLC extracts by spiking with the commercial standard (0.4 μg *trans*-piceid), and acidic hydrolysis was performed to cleave the glycosyl group for a shift to the aglycon by the addition of 50 μL 3 N HCL and 150 μL 50% (v/v) methanol in water and incubation for 3 h at 95°C.

Identification of Stilbenoids via LC-QTOF-MS Analysis

LC-QTOF-MS was performed to identify flavonoids and stilbenoids in skin and seed during grape berry development and within grapevine hairy roots overexpressing MYB15 and GFP:GUS control lines. Extraction of flavonoids and stilbenoids was performed as described by for HPLC analysis as methanolic extracts from 50 mg of tissue. LC-QTO-MS was performed as previously described (Broecker et al., 2011a, 2011b) with the following modifications: Liquid chromatography was performed using an Agilent 1260 Infinity (Agilent Technologies) with 1260 degasser, 1260 high performance autosampler, binary pump 1260 (maximum 600 bar), and 1260 TCC column oven. The Agilent Zorbax Eclipse Plus RRHT C18 column (100 mm × 2.1 mm, 1.8 μm; Agilent Technologie) was used at 40°C with the following separation conditions: eluent (A) 94.8% water, 5% methanol, and 0.2% CH_2O_2 and eluent (B) 5% water, 94.8% methanol, and 0.2% CH_2O_2 with the time program: 0 min, 100% A; 2 min, 100% A; 10 min, 0% A; 15 min, 0% A; 15.5 min, 100% A; 19.5 min, 100% A (post) with the flow rate of 0.4 mL/min. The injection volume was 2 μL. For mass spectrometry analysis, a Q-TOF 6530 time-of-flight mass spectrometer (Agilent Technologies) was used with ion source electrospray ionization+ Agilent Jet Stream in positive (protonated molecules $[M+H]^+$) and negative (deprotonated molecules $[M-H]^-$) ionization modes. Quadrupole was used as described by previously (Broecker et al., 2011b) for selection of precursor ions within a mass window $\Delta m/z$ (mass-to-charge ratio) of 1.3

until 4 in tandem mass spectrometry (MS-MS) mode. MS-MS spectra were generated in product-ion scan at collision-induced dissociation energies of 10, 20, and 40 eV. The QTOF conditions are as follows: drying gas, 325°C; 6 L/min; sheath gas, 380°C; 11 L/min; nebulizer pressure, 35 p.s.i.; Vcap voltage, 3000 V; fragmentor voltage, 150 V; mass rage (mass spectrometry and MS-MS), 100 to 1700 *m/z*, acquisition rate 1 spectra/s; and reference ions for mass calibration 112.985587 $[M-H]^-$ and 966.000725 $[M+H]^+$. Software tools for measurement, identification, and analysis were previously described by Broecker et al. (2011b). The stilbenoid database was based on over 100 forms reviewed by Pawlus et al. (2012). For *trans*-piceid identification, retention time was additionally verified using a commercial standard, and fragmentation of the precursor ion *m/z* 389.123 led to the identification of *trans*-resveratrol *m/z* 227.077 in skin and seed.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative (http://www.Arabidopsis.org), GenBank, or EMBL database (The EMBL Nucleotide Sequence Database) under the following accession numbers: AM489345 (Vv-*STS41*), AM463938 (Vv-*STS29*), Vv-*PAL* (AEX32784), TC32075 (Vv-*Ubiquitin1*), EC959059 (Vv-*EF1-α*), CB973647 (Vv-*GAPDH*), CAD91911 (Vv-*ANR*), AY955269 (Vv-*UFGT*), EU447172 (Vv-MYC1), AT5G24520 (At-TTG1; NM_180738), BAE54312 (Dc-MYB1), BAE93149 (Nt-MYBJS1), AT2G31180 (At-MYB14; AAD20663), ABW34392 (Vv-MYB14), AT1G06180 (At-MYB13; AAF80215), KC514110 (Vv-MYB15), XP_002448188 (hypothetical protein SORBIDRAFT_06g022660 [*Sorghum bicolor*]), AT3G23250 (At-MYB15; NP_188966), XP_002454197 (hypothetical protein SORBIDRAFT_04g026480 [sorghum]), XP_002464484 (hypothetical protein SORBIDRAFT_01g019270 [sorghum]), ACN40772 (unknown [*Picea sitchensis*]), ABQ51228 (Pg-MYB12), ABQ51227 (Pg-MYB11), ACK56131 (Vv-MYBPA2), AM259485 (Vv-MYBPA1), P27898 (Zm-P), AAS68190 (Vv-MYB5a), Q58QD0 (Vv-MYB5b), BAD18977 (Vv-MYBA1), BAD18978 (Vv-MYBA2), and FJ948477(Vv-MYBF1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Coinduction of Selected *MYB* TFs and *STS* Genes in Grapevine Leaves in Response to Abiotic Stress.

Supplemental Figure 2. Phylogenetic Analysis of Putative Phenylpropanoid Transcriptional Regulators.

Supplemental Figure 3. Targeting of a MYB14:GFP Fusion Protein within Onion Cells.

Supplemental Figure 4. MYB14 and MYB15 Specifically Induce the Activity of *STS41* and *STS29* Promoters Involved in the Biosynthesis of Stilbenes.

Supplemental Figure 5. STS Promoter Induction and Expression Analysis of MYBs in *V. vinifera* Cell Culture and Promoter Induction Analysis in *N. benthamiana* Leaf Discs.

Supplemental Figure 6. Chromatogram of Putative Stilbenoids Extracted from *MYB15*-Overexpressing Hairy Root Line 1-1 Compared with GFP:GUS Control Line 77.

Supplemental Table 1. Identification of Stilbenoids in *V. vinifera* Tissues via LC-QTOF-MS.

Supplemental Methods 1. Accession Numbers Used for Phylogenetic Analysis.

Supplemental References 1. References for Supplemental Figures 2 and 4.

Supplemental Data Set 1. Alignment Used to Generate the Phylogeny Presented in Supplemental Figure 2B.

AUTHOR CONTRIBUTIONS

J.H., A.V., S.C., M.L., I.B.D., and J.B. designed the research. J.H. and A.V. performed research. J.H., A.V., C.D., T.R., P.K.B., I.B.D., and J.B. analyzed data. J.H., A.V., S.C., A.R.W., and P.K.B. contributed new analytic/computational tools. J.H., A.V., I.B.D., and J.B. wrote the article.

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