

# The Grapevine Transcription Factor VvMYBPA1 Regulates Proanthocyanidin Synthesis during Fruit Development<sup>1[OA]</sup>

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Proanthocyanidins (PAs; or condensed tannins) can protect plants against herbivores, contribute to the taste of many fruits, and act as dietary antioxidants beneficial for human health. We have previously shown that in grapevine (*Vitis vinifera*) PA synthesis involves both leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR). Here we report the characterization of a grapevine MYB transcription factor VvMYBPA1, which controls expression of PA pathway genes including both LAR and ANR. Expression of VvMYBPA1 in grape berries correlated with PA accumulation during early berry development and in seeds. In a transient assay, VvMYBPA1 activated the promoters of LAR and ANR, as well as the promoters of several of the general flavonoid pathway genes. VvMYBPA1 did not activate the promoter of VvUFGT, which encodes the anthocyanin-specific enzyme UDP-glucose:flavonoid-3-O-glucosyltransferase, suggesting VvMYBPA1 is specific to regulation of PA biosynthesis in grapes. The Arabidopsis (*Arabidopsis thaliana*) MYB transcription factor TRANSPARENT TESTA2 (TT2) regulates PA synthesis in the seed coat of Arabidopsis. By complementing the PA-deficient seed phenotype of the Arabidopsis *tt2* mutant with VvMYBPA1, we confirmed the function of VvMYBPA1 as a transcriptional regulator of PA synthesis. In contrast to ectopic expression of TT2 in Arabidopsis, constitutive expression of VvMYBPA1 resulted in accumulation of PAs in cotyledons, vegetative meristems, leaf hairs, and roots in some of the transgenic seedlings. To our knowledge, this is the first report of a MYB factor that controls genes of the PA pathway in fruit, including both LAR and ANR, and this single MYB factor can induce ectopic PA accumulation in Arabidopsis.

Proanthocyanidins (PAs), also known as condensed tannins, are polyphenolic secondary metabolites synthesized via the flavonoid biosynthetic pathway. They are present in many plants and act in defense against plant diseases and in seed dormancy (Debeaujon et al., 2000; Peters and Constabel, 2002). The increase of PAs in important forage crops like alfalfa (*Medicago sativa*) could protect ruminants against pasture bloat, reduce greenhouse gas, and increase plant disease resistance (Dixon et al., 1996; McMahon et al., 2000). Dietary PAs are present in many fruits and plant products like wine, fruit juices, and teas and contribute to their taste

and health benefits. PAs act as potential dietary antioxidants with beneficial effects for human health, including protection against free radical-mediated injury and cardiovascular disease (Bagchi et al., 2000; Middleton et al., 2000; Cos et al., 2004). There is also considerable interest in the PAs found in grapes (*Vitis vinifera*) because of their importance for the taste and astringency of red and white wine (Glories, 1988). For these reasons, there is a growing interest in metabolic engineering strategies aimed at developing agronomically important food crops and fruits with optimized levels and composition of flavonoids.

The biosynthesis of PAs, anthocyanins, and flavonols share common steps in the flavonoid pathway, and the genetics and biochemistry of this pathway (Fig. 1) have been characterized in several plant species including Arabidopsis (*Arabidopsis thaliana*) and grapes (Shirley et al., 1992; Holton and Cornish, 1995; Boss et al., 1996; Winkel-Shirley, 2001).

In Arabidopsis, the biosynthetic pathway leading to PA accumulation has been characterized by using the *transparent testa (tt)* and *tannin-deficient seed (tds)* mutants that fail to accumulate PAs in their seed coat (Shirley et al., 1995; Abrahams et al., 2002). Some of the identified TT and TDS loci correspond to enzymes of the general flavonoid pathway and others to enzymes, transporters, and regulators specifically involved in

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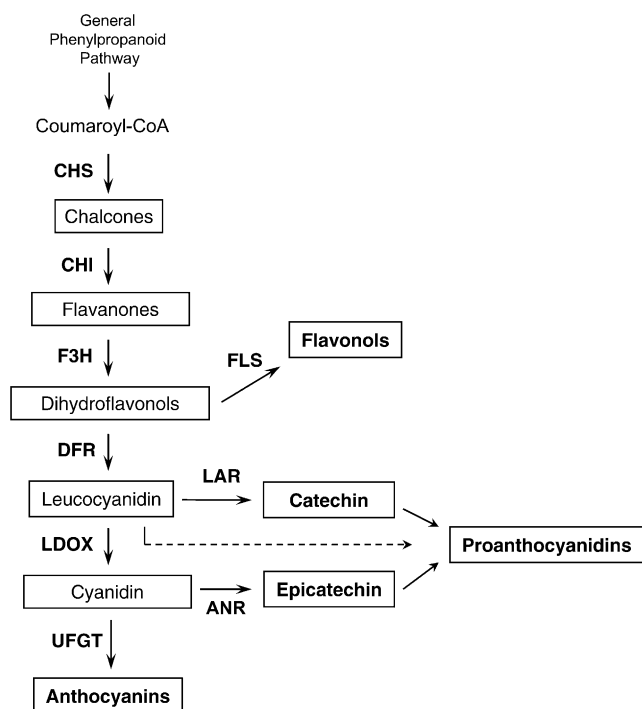
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**Figure 1.** Scheme of the flavonoid pathway leading to synthesis of anthocyanins, flavonols, and PAs. The enzymes involved in the pathway are shown as follows: CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid-3', 5'-hydroxylase; F3H, flavanone-3 $\beta$ -hydroxylase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; and UFGT, UDP-Glc:flavonoid-3-O-glucosyltransferase. The F3'H and F3'5'H enzymes, which hydroxylate flavanones and dihydroflavonols, were omitted to clarify the scheme of the flavonoid pathway (see Bogs et al., 2006). Note that Arabidopsis lacks LAR.

PA accumulation. The structural genes include *anthocyanidin reductase* (ANR; also called *BANYULS*) that catalyzes the synthesis of flavan-3-ols such as (–)-epicatechin (Xie et al., 2003), *TT19*, *TT12*, and *Autoinhibited H<sup>+</sup>-ATPase isoform 10*, which are involved in transport processes of PAs (Debeaujon et al., 2001; Kitamura et al., 2004; Baxter et al., 2005) and *TT10* encoding a laccase-type polyphenol oxidase involved in polymerization of flavonoids (Pourcel et al., 2005).

Regulation of flavonoid synthesis occurs mostly via coordinated transcriptional control of the structural genes by the interaction of DNA-binding R2R3 MYB transcription factors, MYC-like basic helix loop helix (bHLH) and WD40 proteins (Mol et al., 1998; Nesi et al., 2000, 2001; Winkel-Shirley, 2001). This has been shown for several regulators of anthocyanin synthesis isolated from maize (*Zea mays*), Arabidopsis, Antirrhinum, and petunia (*Petunia hybrida*; Mol et al., 1998).

The Arabidopsis genes *TT8*, *TT2*, and *TRANSPARENT TESTA GLABRA1* (*TTG1*), necessary for PA accumulation in the seed coat, were found to encode bHLH, MYB, and WD40 repeat proteins, respectively. By forming a transcription complex, they regulate the

expression of several flavonoid structural genes including *ANR* and *TT12* (Walker et al., 1999; Nesi et al., 2000, 2001; Baudry et al., 2004). Although these structural genes were also induced in roots after ectopic expression of *TT2* and *TT8*, the tissue failed to accumulate PAs, suggesting additional factors are required for ectopic PA accumulation in Arabidopsis (Nesi et al., 2001). The transcription factors *TT16*, *TT1*, and *TTG2* have been shown to influence expression of the PA-specific genes and the organ and cell development important for PA deposition (Johnson et al., 2002; Nesi et al., 2002; Sagasser et al., 2002).

However, the specific regulation of PA synthesis in plant species other than Arabidopsis is not well characterized and until now no MYB factor functionally similar to *TT2* has been identified from other plants. Whereas PA synthesis in Arabidopsis is exclusively epicatechin based and limited to the seed coat, many other plants produce both epicatechin- and catechin-based PAs of various amounts and compositions and in a range of different tissues (Dixon et al., 2005). As Arabidopsis lacks a functional *leucoanthocyanidin reductase* (*LAR*) gene, it is not known what regulates expression of *LAR* in PA synthesis in other plants. In grape berries, the first committed steps in PA biosynthesis are catalyzed by *LAR* and *ANR* by converting anthocyanidins to flavan-3-ols such as catechin and epicatechin, respectively. Grapevine synthesizes PAs in various compositions in the seeds and skin of the fruit where their accumulation occurs during the early stages of grape berry development, prior to the onset of ripening (termed *véraison* by viticulturists). At *véraison*, the flavonoid pathway switches to production of anthocyanins in the skin of red grapes and the grapevine transcription factor *VvMYBA*, which is only expressed after *véraison*, has been shown to regulate anthocyanin synthesis in grapes during ripening (Kobayashi et al., 2002; Walker et al., 2007). The tissue and temporal-specific expression of *ANR* and *LAR* correlates with PA accumulation prior to *véraison* in grapes (Bogs et al., 2005), suggesting a separate transcriptional regulator controls PA synthesis in grapevine. Another grapevine transcription factor, *VvMYB5a*, was recently shown to be expressed in grapes prior to *véraison* but ectopic expression of this regulator in tobacco (*Nicotiana tabacum*) affected the metabolism of anthocyanins, flavonols, lignins, and PAs, suggesting it controls a number of different branches of the phenylpropanoid pathway (Deluc et al., 2006). It is not yet clear what transcription factors regulate expression of *ANR* and *LAR* in developing grape berries nor how PA synthesis is specifically regulated during fruit development.

In this article, we isolated and characterized a grapevine gene *VvMYBPA1* that encodes a MYB transcription factor that is expressed when PAs accumulate during early grape berry development and in seeds. *VvMYBPA1* is able to activate the promoters of both of the PA branch genes *VvANR* and *VvLAR1* but also several of the general flavonoid pathway genes of

grapevine. VvMYBPA1 did not activate anthocyanin synthesis, suggesting it may be specific for the PA pathway in grapes. Constitutive expression of this transcription factor in *Arabidopsis* complemented the PA-deficient seed coat phenotype of the *Arabidopsis tt2* mutant and also induced ectopic PA accumulation in other tissues including cotyledons, stems, and roots. This grapevine regulator may provide the potential to alter PA synthesis in fruits and crops by metabolic engineering.

## RESULTS

### The Grapevine Gene *VvMYBPA1* Encodes a MYB Transcription Factor

We identified the Tentative Consensus (TC) sequence TC46393 by searching the grape gene index of the The Institute for Genomic Research (TIGR) expressed sequence tag database (Quackenbush et al., 2000; <http://www.tigr.org/tdb/tgi/>) for MYB transcription factors expressed during early flower and berry development, when PAs are accumulating. The 861-bp open reading frame (ORF) of TC46393 was amplified by PCR from cDNA isolated from developing Shiraz berries sampled 1 week after flowering. The isolated ORF was named *VvMYBPA1* (accession no. AM259485) and encoded a protein of 286 amino acid residues with a predicted mass of 32.2 kD and a calculated pI of 9.47. Analyses of the deduced amino acid sequence revealed that VvMYBPA1 contains an N-terminal R2R3 repeat that corresponds to the DNA-binding domain of plant MYB-type proteins (Fig. 2A). Similar to the over 100 members of the MYB protein family in *Arabidopsis*, the R2R3 repeat region of VvMYBPA1 is highly conserved and contains the motif [D/E]L<sub>x2</sub>[R/K]<sub>x3</sub>L<sub>x6</sub>L<sub>x3</sub>R for interaction with bHLH proteins, whereas the C-terminal region shows little homology to other MYBs (Fig. 2A; Stracke et al., 2001). Phylogenetic analysis revealed the similarity of VvMYBPA1 to other plant MYB proteins (Fig. 2B). The R2R3 DNA-binding domain of VvMYBPA1 is most closely related to PmMBF1 (AAA82943) of *Picea mariana* with 81% identical amino acid residues. The PmMBF1 protein has not yet been functionally characterized. The similarity between the R2R3 domain of VvMYBPA1 and AtTT2 (Fig. 2A), which has been shown to regulate PA synthesis in the seed coat of *Arabidopsis* (Nesi et al., 2001), is clear with 72% amino acid identity. Besides VvMYBPA1 and TT2, the maize MYB factor C1 was also shown to activate the *Arabidopsis ANR* promoter, which is the first gene leading specifically to PA synthesis (Baudry et al., 2004). Figure 2A shows the comparison of these MYB proteins with the anthocyanin-specific factors VvMYBA2 and AtPAP1 (production of anthocyanin pigment) as well as with AtMYB12, a transcription factor controlling flavonol synthesis (Mehrtens et al., 2005). There are no conserved amino acids in the sequences of

VvMYBPA1, TT2, and C1 that are not also present in the other MYB factors (Fig. 2A). Sequence similarity between MYB proteins is generally restricted to the R2R3 domain, but some MYB factors share conserved motifs in their C-terminal domains that may indicate similarities in function (Stracke et al., 2001). However, the C-terminal sequences of VvMYBPA1 (amino acids 116–286) and any other plant MYB factors showed no significant homology and none of the conserved motifs identified by Stracke et al. (2001) were present. The motif V<sub>x2</sub>IRTKA[I/L]RC[S/N] conserved between TT2 and OsMYB3 (Nesi et al., 2001) was not found in the sequence of VvMYBPA1 (Fig. 2A).

Taken together, the VvMYBPA1 protein sequence shows the typical features of a plant MYB transcription factor. However, we were not able to detect conserved amino acid homologies between the PA regulators TT2 and VvMYBPA1, which could be used to identify PA-specific MYB regulators from other plant species.

### Expression of *VvMYBPA1* Correlates with PA Accumulation during Fruit Development

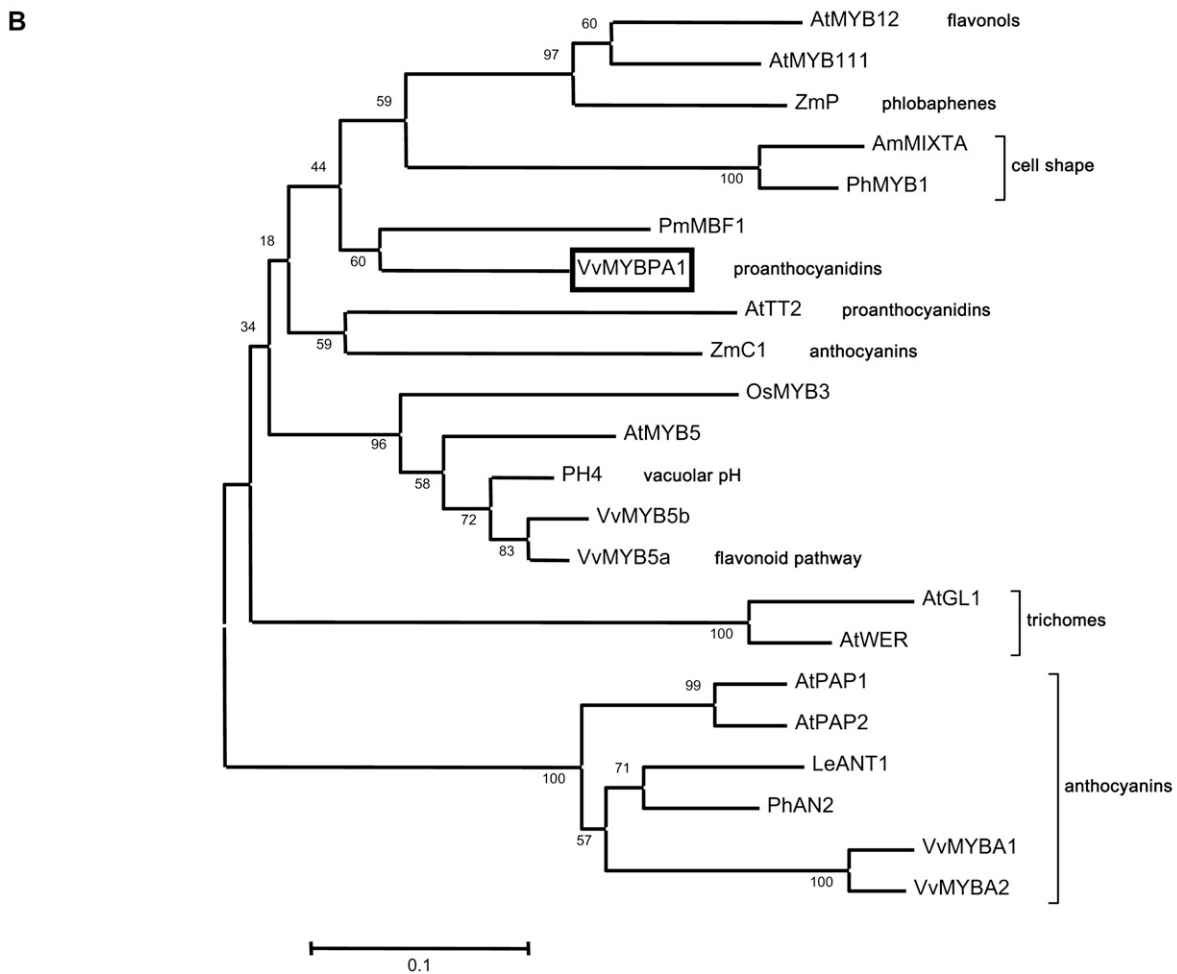
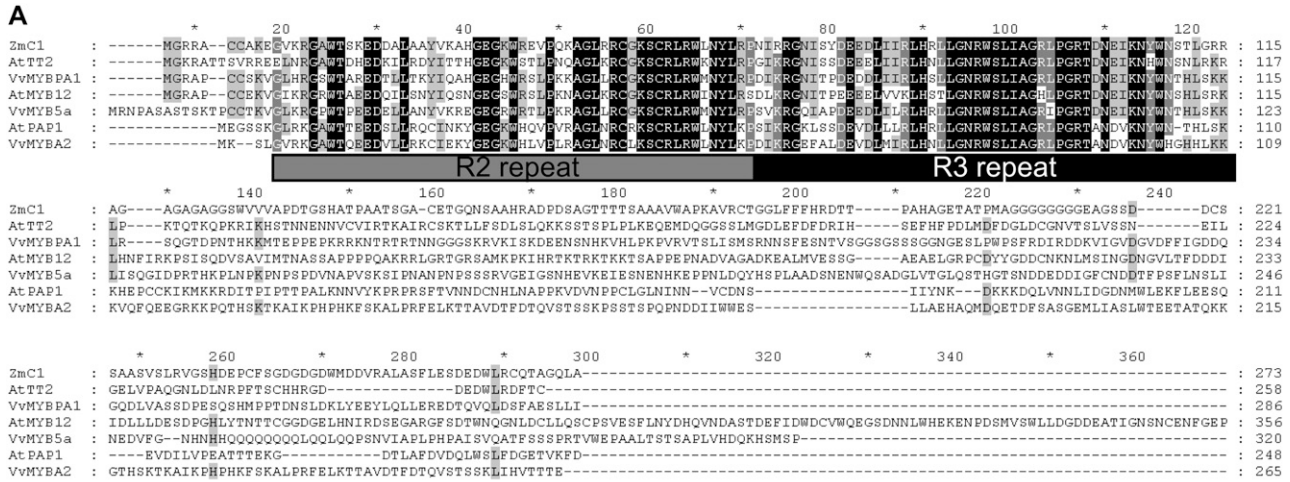
To confirm that *VvMYBPA1* is expressed when PAs are accumulating in grape berries, we investigated transcript levels of *VvMYBPA1* throughout grape berry (cv Shiraz) development during the season 2000 to 2001 by real-time PCR. *VvUbiquitin1* (BN000705) was chosen for normalization of gene expression because it was found to be relatively constant throughout grape berry development (Downey et al., 2003b; Bogs et al., 2005).

Figure 3 shows *VvMYBPA1* is expressed in flowers and grapes early in berry development from 10 to 6 weeks before onset of ripening. This early expression of VvMYBPA1 in developing flowers and grape berries correlates with the accumulation of PAs and the expression of the structural genes *leucoanthocyanidin dioxygenase* (*VvLDOX*), *VvANR*, and *VvLAR1* that are involved in PA synthesis in grapevine (Bogs et al., 2005).

In grape berry skins, transcript levels of *VvMYBPA1* were relatively low before véraison, which is the onset of ripening, increased to a maximum 2 weeks after véraison, and then declined to a low level (Fig. 3). The concentration of PAs in skins increased from 5 weeks before véraison, reaching a maximum around the time ripening commenced and then declined during ripening (Bogs et al., 2005). In seeds, *VvMYBPA1* is expressed before véraison (Fig. 3) when PAs start to accumulate. The expression pattern of *VvMYBPA1* in seeds (Fig. 3) correlates with PA synthesis and the expression of *VvLAR2* that continued in the seed up until 4 weeks after véraison with a maximum at véraison (Kennedy et al., 2000; Bogs et al., 2005).

### Promoter Isolation and Analysis of Grapevine Flavonoid Pathway Genes

To determine which genes of the flavonoid pathway are controlled by VvMYBPA1, we isolated the



**Figure 2.** A, Alignment of the deduced amino acid sequences of the MYB-type transcriptional regulators ZmC1 (maize), AtTT2, AtPAP1, AtMYB12 (Arabidopsis), and the grapevine regulators VvMYBPA1, VvMYBA2, and VvMYB5a. The R2 and R3 repeats of the MYB domain are indicated below the alignment. Identical amino acids are indicated in black, similar amino acids in gray.

promoter regions of the genes *flavonoid 3',5' hydroxylase* (*VvF3'5'H1*; 1,136 bp; accession no. AM259482), *chalcone isomerase* (*VvCHI*; 935 bp; accession no. A M259483), *VvANR* (1,034 bp; accession no. AM259484), and *VvLAR1* (1,342 bp; accession no. AM259481) by genome walking (see experimental procedures). These promoter regions were analyzed using the plant DNA cis-elements (PLACE) database (Higo et al., 1999; <http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html>) and contained the consensus sequences of the core DNA-binding sites of MYB (CNGTTR, PLACE accession no. S000176) and bHLH-type (CANNTG, PLACE accession no. S000407) transcription factors. The core MYB site CNGTTR is recognized by the plant transcription factor MYB Ph3 from petunia (Solano et al., 1995), which is involved in regulation of flavonoid biosynthesis and is present in a 86-bp promoter region of the Arabidopsis *AtANR* promoter necessary for expression in PA-accumulating cells (Debeaujon et al., 2003). Additionally, we cloned the promoter regions of *VvLDOX* (2,174 bp of accession no. AF290432), UDP-Glc:flavonoid-3-O-glucosyltransferase (*VvUFGT*; 1,674 bp of accession no. AY955269), and *AtANR* (also called *AtBAN*; 1,533 bp of accession no. AT1G61720), which also contain the core DNA-binding sites for MYB and bHLH factors (Gollop et al., 2001; Kobayashi et al., 2001). There are different target recognition sites for different groups of MYB proteins (Jin and Martin, 1999) and in addition to the core MYB DNA-binding site, all promoters contained putative cis-acting regulatory elements for different MYB proteins (data not shown).

#### VvMYBPA1 Activates Promoters of the Flavonoid Pathway Genes Involved in PA Synthesis

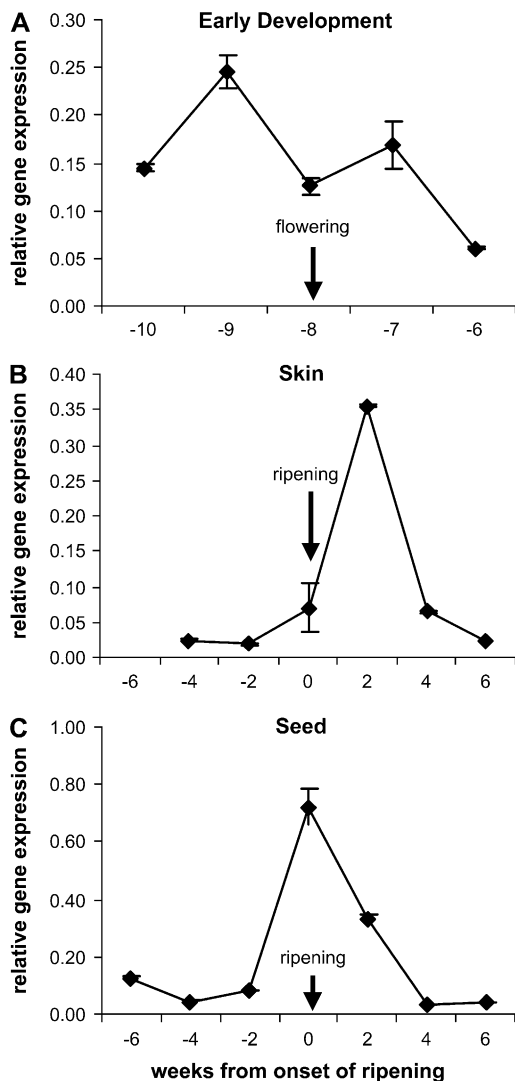
To investigate which structural genes of the flavonoid pathway are activated by VvMYBPA1, we established a transient expression method using grape cells grown in suspension culture and the dual-luciferase assay system. In this system, the cotransfection of effectors (transcription factors) and dual-luciferase reporter plasmids allows quantification of promoter activity by measuring firefly (*Photinus pyralis*) luciferase activity (promoter of interest inserted into pLuc), which is normalized by measuring *Renilla reniformis*

luciferase activity (pRluc; Horstmann et al., 2004). Therefore, *VvMYBPA1* was ligated to pART7 for expression under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV). The promoters controlling *VvCHI*, *VvF3'5'H1*, *VvLDOX*, *VvANR*, *AtANR*, *VvLAR1*, and *VvUFGT* were ligated to pLuc to control the expression of the firefly luciferase reporter gene. The *VvCHI*, *VvF3'5'H1*, and *VvLDOX* promoters were chosen as examples for general flavonoid pathway genes involved in synthesis of flavonols, anthocyanins, and PAs (Fig. 1). In contrast, *VvUFGT* is specifically required for anthocyanin synthesis, whereas *VvANR* and *VvLAR1* encode the branch point enzymes leading to the synthesis of PAs (Fig. 1). Except for *VvLAR1* and *VvF3'5'H1*, these genes are present as a single copy in the grapevine genome (Sparvoli et al., 1994; Bogs et al., 2005). The grapevine promoters and the Arabidopsis *AtANR* promoter were then tested as potential targets for VvMYBPA1 (Fig. 4). As controls, we also tested the promoters with the MYB transcription factor VvMYBA2 that specifically activates *VvUFGT*, controlling anthocyanin synthesis in grapes (Boss et al., 1996; Kobayashi et al., 2002) and TT2 from Arabidopsis that was shown to control *AtANR* expression and PA synthesis in Arabidopsis seed coat (Nesi et al., 2001). Similar results as for VvMYBA2 were obtained with its isoform VvMYBA1 when transfected with the *VvUFGT* or *VvANR* promoter and ENHANCER OF GLABRA3 (EGL3) in the transient expression system (Walker et al., 2007; data not shown).

VvMYBPA1 strongly activated the promoters of the genes *VvANR* (approximately 135-fold), *AtANR* (approximately 70-fold), and *VvLAR1* (approximately 72-fold), showing its ability to induce the PA-specific branch point genes of Arabidopsis and grapevine (Fig. 4, A–C). VvMYBPA1 also induced the promoters of the general flavonoid pathway genes *VvCHI* (approximately 16-fold), *VvF3'5'H1* (approximately 38-fold), and *VvLDOX* (approximately 125-fold), suggesting it can activate the whole pathway leading to PA synthesis (Fig. 4, D–F). The anthocyanin-specific promoter of *VvUFGT* was not affected by VvMYBPA1, whereas VvMYBA2 strongly activated (approximately 600-fold) this promoter (Fig. 4G) but did not activate the other genes. In comparison to VvMYBPA1, the activation of the *VvF3'5'H1*, *VvLDOX*, *VvCHI*, *VvANR*, *VvLAR1*,

#### Figure 2. (Continued.)

Sequences were aligned with the ClustalW program and displayed using the GeneDoc program (Version 2.6.002). B, Phylogenetic tree showing selected plant MYB transcription factors from GenBank or EMBL database. Functions of some of the proteins are given in bold. The ClustalW multiple sequence alignment was formed using the domain of the MYB proteins and the default parameters of the MEGA package (Kumar et al., 2004). The tree was constructed from the ClustalW alignment using the neighbor-joining method by the MEGA program. The scale bar represents 0.1 substitutions per site and the numbers next to the nodes are bootstrap values from 1,000 replicates. The GenBank accession numbers of the MYB proteins are as follows: VvMYBPA1 (AM259485), AtGL1 (P27900), ZmP (P27898), ZmC1 (AAA33482), VvMYBA1 (BAD18977), VvMYBA2 (BAD18978), AtPAP1 (AAG42001), PhAN2 (AAF66727), LeANT1 (AAQ55181), OsMYB4 (T02988), AtMYB5 (U26935), PhMYB1 (Z13996), AmMixta (CAA55725), AtMYB12 (CAB09172), AtMYB111 (AAK97396), PmMBF1 (AAA82943), AtTT2 (Q9FJA2), PH4 (AAY51377), AtPAP1 (AAG42001), AtPAP2 (AAG42002), AtWER (CAC01874), VvMYB5a (AAS68190), and VvMYB5b (Q58QD0).



**Figure 3.** Transcript levels of *VvMYBPA1* during grape flower and berry development. Gene expression was determined by real-time PCR and is shown relative to expression of *VvUbiquitin1* in each sample. All data is presented as mean of three replicates with error bars indicating  $\pm$ SEs.

and the *AtANR* promoter by *VvMYBA2* was absent or relatively low (Fig. 4). Similar to *VvMYBPA1*, the Arabidopsis PA regulator TT2 activated the *ANR* genes of Arabidopsis and grapevine (Fig. 4, A and B) but TT2 was not able to substantially induce the promoters of *VvLAR*, *VvF3'5'H1*, *VvLDOX*, and *VvCHI* (Fig. 4, C and F). These results suggest *VvMYBPA1* is a specific regulator of PA synthesis, potentially regulating the entire general flavonoid pathway and both of the PA branch genes *ANR* and *LAR*, leading to PA formation. Similar to other MYB transcription factors, *VvMYBPA1* requires a bHLH protein for promoter activation (Fig. 4E; *VvMYBPA1/w/o*). Therefore, all standard transfections included a construct expressing *EGL3* which encodes a bHLH protein involved in flavonoid pathway regulation in Arabidopsis (Ramsay et al., 2003;

Walker et al., 2007). Figure 4 also shows that using *VvMYBPA1* without a bHLH protein in our transfection experiments can induce the *VvLDOX* promoter up to 3-fold compared to the control without the MYB factor, and similar results (3- to 5-fold inductions) were obtained for other promoters and MYB factors (data not shown). However, in comparison to the 125-fold induction of the *VvLDOX* promoter by *VvMYBPA1* and *EGL3* we considered a 3-fold induction as insubstantial and possibly provoked by the large amounts of MYB factor and promoter DNA in the transfection assay.

In Arabidopsis, the bHLH protein TT8 has been shown to interact with TT2 and to be required for PA accumulation in the seed coat (Nesi et al., 2000). Therefore, we also tested the ability of TT2 and *VvMYBPA1* to interact with TT8 and to activate the *ANR* promoters of Arabidopsis and grapevine. We found that the *ANR* promoter activities were not substantially altered when we exchanged *EGL3* with TT8 in the transient assays (data not shown). This redundancy of *EGL3* and TT8 was also found for their ability to interact with the MYB factors TT2, PAP1, or PAP2 and to activate the *dihydroflavonol-4-reductase* (*AtDFR*) promoter of Arabidopsis (Zimmermann et al., 2004).

#### ***VvMYBPA1* Complements the Arabidopsis *tt2* Mutant PA-Deficient Phenotype**

The MYB transcription factor TT2 has been shown to regulate PA synthesis in the seed coat of Arabidopsis and the seeds of *tt2* mutants appear yellow due to the lack of PAs (Nesi et al., 2001). To confirm the function of *VvMYBPA1* as a regulator of PA synthesis, *VvMYBPA1* under the control of the CaMV 35S promoter was introduced into the *tt2* mutant. The ORF of *VvMYBPA1* was amplified from cDNA by PCR and inserted into the binary vector pART27 to give pART27MYBPA1, which contains kanamycin resistance for selection in planta. This construct was used to transform homozygous *tt2* plants by *Agrobacterium tumefaciens*-mediated transformation.

Only around 20% of the kanamycin-resistant  $T_1$  seedlings survived on soil and these were grown on to produce seed. Nine kanamycin-resistant *tt2* plants independently transformed with pART27MYBPA1 showed wild-type phenotype and developed brown seeds which stained blue for accumulation of PAs when stained with dimethylaminocinnamaldehyde (DMACA; Fig. 5, A–F). DMACA is a useful reagent for detection of molecules of the PA pathway because it reacts with both PA monomers and polymers to form a blue chromophore but does not react with anthocyanidin derivatives (Nagel and Glories, 1991). This demonstrated that constitutive expression of the transgene *VvMYBPA1* can complement the *tt2* mutant seed phenotype. From the independent *tt2* 35S::MYBPA1 lines 10 and 17,  $T_2$  plants were generated and analyzed for expression of *VvMYBPA1* and PA accumulation. Expression of *VvMYBPA1* by the *tt2* 35S::MYBPA1 lines 10F, 10-2,

10A, 17D, 17B, and 17 K was confirmed by reverse transcription-PCR (Fig. 6A). Blue PA staining was observed in hypocotyls, roots, seeds, bases of the rosette leaves (Fig. 5), and stipules of leaves (data not shown) after these plants were treated with DMACA, whereas Columbia-0 (Col-0) wild-type plants accumulated PAs exclusively in the developing seeds (Fig. 5). HPLC analysis of the developing siliques revealed that the PA levels of *tt2* plants complemented with *VvMYBPA1* were 3- to 8-fold higher than in the *tt2* background and reached about half of the PA concentration we detected in the Col-0 siliques (Fig. 6B).

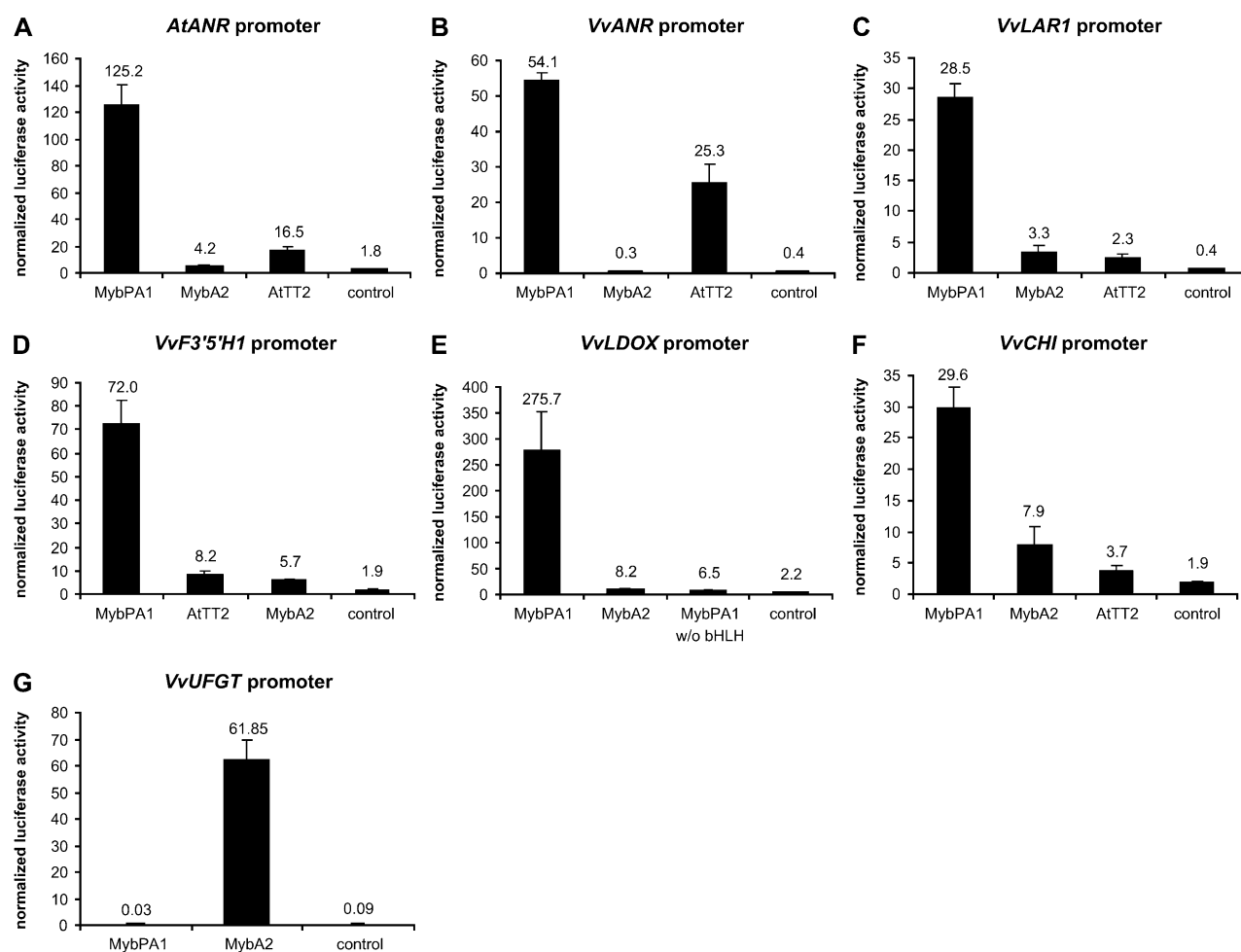
About 80% of the transgenic  $T_1$  seedlings showed growth abnormalities with bleaching and necrosis of the first leaves during their development. These plants showed a dwarf phenotype and died about 2 weeks after transferring them to soil. When these seedlings were stained with DMACA we observed accumulation

of PAs in cells of cotyledons, hypocotyls, and its apical meristem, roots, trichomes, and basal cells of trichomes indicated by their blue staining (Fig. 7). Control plants (*tt2* and Col-0) stained with DMACA did not show any blue staining, indicating their inability to accumulate significant amounts of PAs (Fig. 7, A, I, and J).

## DISCUSSION

### *VvMYBPA1* Encodes a MYB-Type Transcriptional Regulator

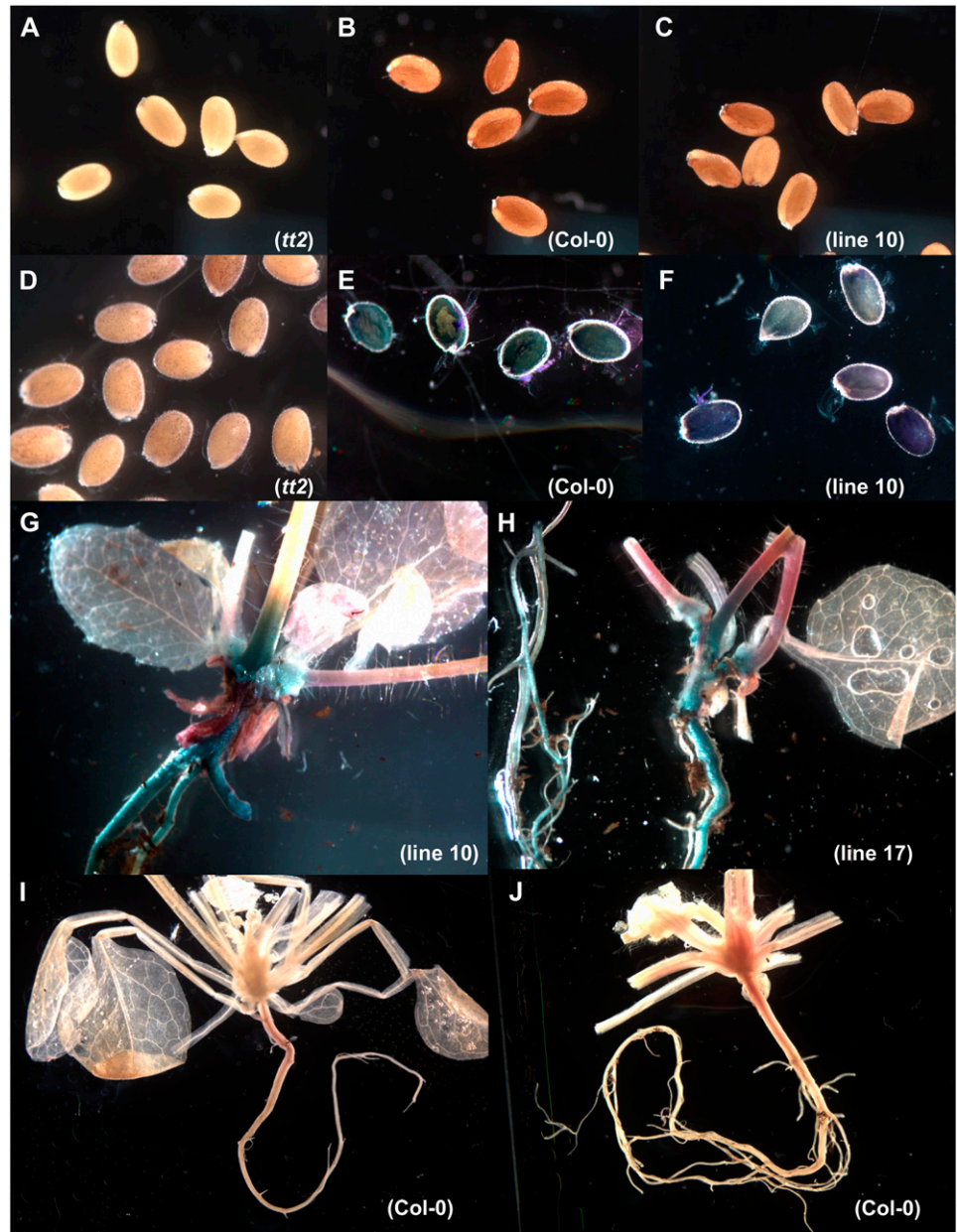
In this study, we have identified the transcriptional regulator *VvMYBPA1* from grapevine, which has some functional similarity to *AtTT2*, the only other regulator of PA synthesis isolated so far. Constitutive expression of *VvMYBPA1* in *Arabidopsis* can complement a



**Figure 4.** *VvMYBPA1* activates promoters of flavonoid pathway genes involved in PA synthesis. The MYB transcription factors and promoters used for transfection of grape cell cultures are indicated. Control indicates the activity of the respective promoter transfected without a MYB factor. Each transfection (except E, MybPA1 without bHLH) contained the 35S::EGL3 construct encoding the bHLH protein EGL3 (GenBank accession no. NM20235) from *Arabidopsis* and as internal control the Renilla luciferase plasmid pRLuc (Horstmann et al., 2004). The normalized luciferase activity was calculated as the ratio between the firefly and the Renilla luciferase activity. Each column represents the mean value of three independent experiments with error bars indicating  $\pm$ SES.



**Figure 5.** Complementation of the PA-deficient *tt2* mutant seed phenotype by expression of *VvMYBPA1* and ectopic PA formation of these plants. At least three independent lines showed a similar pattern of PA accumulation. A, Pale Arabidopsis *tt2* seeds. B, Brown Col wild-type seeds. C, Brown *tt2* 35S::*MYBPA1* seeds. D to H, PAs were localized by staining the seeds or plants with DMACA, which specifically stains PAs blue. D, *tt2* seeds. E, Col wild-type seeds. F, *tt2* 35S::*MYBPA1* seeds. G and H, PA formation in the hypocotyls, roots, and bases of the rosette leaves of *tt2* 35S::*MYBPA1* plants. PA formation in Arabidopsis wild-type plants (Col-0) after DMACA staining was exclusively observed in seeds (I and J).



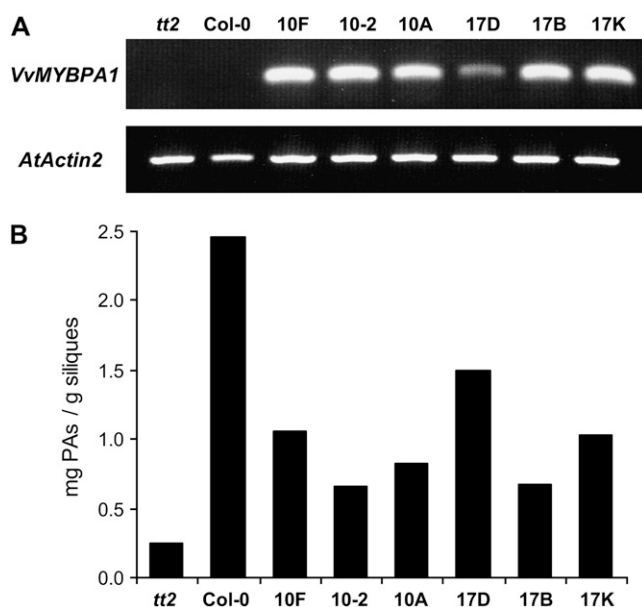
mutation in *tt2* and experimental evidence suggests that *VvMYBPA1* regulates PA synthesis during grape berry development. The protein sequence of *VvMYBPA1* shows homology to the R2R3 domain of various MYB transcription factors (Fig. 2). However, we were not able to find any of the conserved motifs described by Stracke et al. (2001) in the C-terminal domain of *VvMYBPA1* that may indicate similarities in function (Fig. 2). *VvMYBPA1* does not display significantly more similarity to TT2 or C1 from maize, which have been shown to activate the *ANR* promoter (Nesi et al., 2001), than to other functionally unrelated MYB regulators (Fig. 2). Therefore, we were not able to identify conserved amino acid motifs, which could be used to identify PA-specific MYB regulators from other plant

species. However, our transient expression assays and complementation experiments indicate that *VvMYBPA1* and TT2 share some common functions, suggesting that *VvMYBPA1* and TT2 are similar MYB factors. Similar findings were described for AN2 from petunia and C1 from maize, where the function of the proteins in regulating anthocyanin synthesis was not reflected in specific amino acid similarities (Quattrocchio et al., 1998, 1999).

#### Expression of *VvMYBPA1* Correlates with PA Synthesis in Grapes

The functional role of *VvMYBPA1* in the regulation of PA synthesis in grape berries is supported by its





**Figure 6.** *VvMYBPA1* expression in Arabidopsis *tt2* mutant induced PA accumulation in developing siliques. A, Detection of *VvMYBPA1* transcript in *tt2* mutant, Col-0 wild-type and *tt2* 35S::*MYBPA1* lines 10F, 10-2, 10A, 17D, 17B, and 17 K by reverse transcription-PCR. Expression of the Arabidopsis *Actin2* gene was used as a positive control. B, Quantification of PAs (total epicatechins) after acid-catalyzed hydrolysis of PA polymers in siliques of Arabidopsis *tt2*, Col-0 wild-type, and *tt2* 35S::*MYBPA1* lines 10F, 10-2, 10A, 17D, 17B, and 17 K by HPLC. Technical replicates could not be performed due to the limited amount of silique tissue.

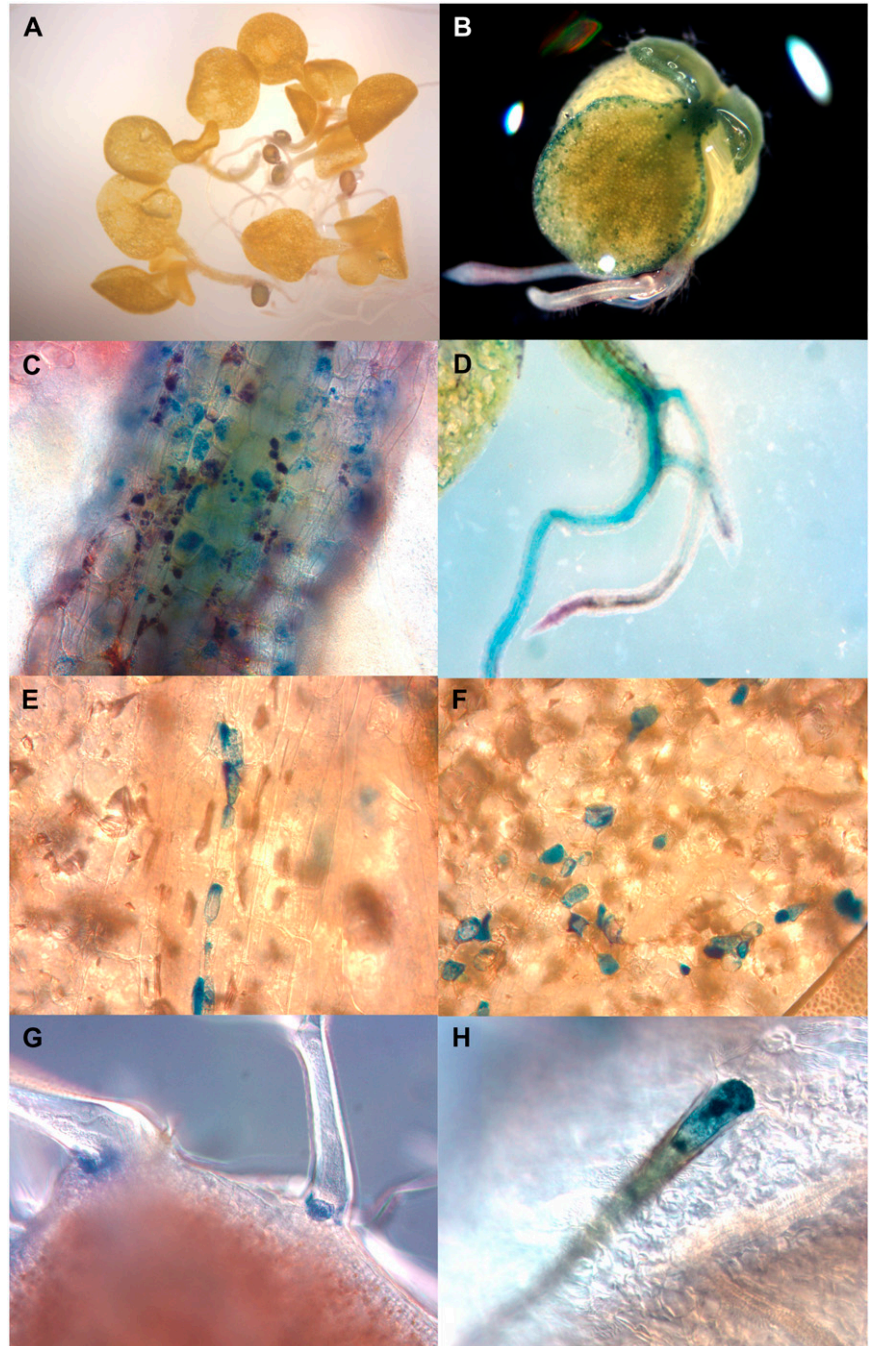
gene expression pattern during grape berry development (Fig. 3). Development of the grape berry occurs in two successive growth phases and the synthesis of flavonols, anthocyanins, and PAs and the expression of different flavonoid pathway genes is temporally separated during berry development. The first phase, from flowering until véraison (the onset of ripening in grapes), coincides with flavonol and PA synthesis and the second phase, starting with the onset of ripening of the berry, coincides with anthocyanin biosynthesis (Robinson and Davies, 2000; Downey et al., 2003a, 2003b; Bogs et al., 2005). The biosynthesis of PAs, anthocyanins, and flavonols share common steps in the flavonoid pathway, whereas the activities of enzymes specific for PAs, anthocyanins, or flavonols lead exclusively to the synthesis of the respective flavonoid (Fig. 1). Therefore, regulation of this pathway must occur to coordinate synthesis of different flavonoids during grape berry development. Regulation of flavonoid synthesis occurs mostly via coordinated transcriptional control of the structural genes by the interaction of DNA-binding MYB transcription factors and bHLH proteins (Mol et al., 1998). We have demonstrated that the *VvMYBPA1* gene, expressed during flower and early berry development and in seeds before ripening, encodes a MYB transcription factor (Fig. 3). The expression pattern correlates with PA accumulation and expression of the PA-specific biosynthetic genes *VvLAR1*,

*VvLAR2*, and *VvANR* (Bogs et al., 2005). PA synthesis appears to continue in the seed up until 2 to 4 weeks after véraison (Kennedy et al., 2000), which coincides with the expression pattern of *VvLAR2* (Bogs et al., 2005) and *VvMYBPA1* in seeds (Fig. 3). Both *VvLAR2* and *VvMYBPA1* expression reached their maximum in seeds at véraison and this corresponds to the peak of PA monomer accumulation at this time in seeds (Bogs et al., 2005). In grape skins, PA accumulation appeared to be complete by véraison and maximum transcript levels of *VvANR* and *VvLAR2* were detected 4 weeks before véraison (Bogs et al., 2005). The transcript level of *VvMYBPA1* in skins was relatively low before véraison, increased to a maximum 2 weeks after véraison, and then declined to a low level (Fig. 3). It is unclear if *VvMYBPA1* expression before véraison is sufficient to induce PA synthesis in skins or whether another regulator activates PA synthesis in this tissue. However, a maximum of *VvMYBPA1* expression in skins could take place earlier than 4 weeks before véraison, where we have no expression data because it was not possible to separate seeds and skins from very small berries. This would explain *VvMYBPA1* expression in skins before véraison, but the reason for its relatively high transcript level 2 weeks after véraison remains unclear. It is possible that in the skin of ripening grapes *VvMYBPA1* activates the shared genes of the flavonoid pathway required for anthocyanin synthesis, as the grape color regulator *VvMYBA* appears to be relatively specific for UFGT (Fig. 4). Taken together, the expression pattern of *VvMYBPA1* suggests that the encoded protein is involved in regulation of PA biosynthesis in grapevine at least during early fruit development and in seeds.

#### ***VvMYBPA1* Activates the Promoters of General Flavonoid Pathway and PA Branch Genes**

As well as activating the promoters of the two PA-specific genes, *VvANR* and *VvLAR*, *VvMYBPA1* also activated the promoters of general flavonoid pathway genes, whereas the Arabidopsis TT2 regulator appeared to be more specific for ANR in this transient assay (Fig. 4). Anthocyanin and PA biosynthesis share the general flavonoid pathway enzymes until LDOX catalyzes the synthesis of anthocyanidins (Abrahams et al., 2003), which are substrates for both anthocyanin synthesis via UFGT and PA synthesis catalyzed by ANR and LAR (Fig. 1). Therefore, UFGT and ANR/LAR are branch enzymes leading to anthocyanin or PA accumulation, respectively, and represent possible control points between the two flavonoid branches. Our results obtained with the transient promoter assay revealed the ability of *VvMYBPA1* to activate the PA-specific branch genes *VvANR* and *VvLAR1*, showing its capacity to control PA synthesis in grapevine. Furthermore, *VvMYBPA1* was not able to induce the *VvUFGT* promoter, suggesting it specifically regulates PA biosynthesis. In addition, we determined that *VvMYBA2*, which was shown to induce anthocyanin accumulation in

**Figure 7.** PA accumulation of Arabidopsis *tt2* seedlings expressing *VvMYBPA1*. Plants died before full development of their first leaves. PAs were localized by staining the plants with DMACA, which specifically stains PAs blue. At least five independent lines showed a similar pattern of PA accumulation. A, Arabidopsis *tt2* seedlings as negative control. B, Whole *tt2* 35S::*MYBPA1* seedlings with PAs in the apical meristem and the cotyledons. C, Hypocotyl. D, Root. E and F, Cells of cotyledons. G, Basal cells of trichome. H, Trichome.



grapes (Kobayashi et al., 2002; Walker et al., 2007), activates the *VvUFGT* promoter but not the *VvANR* or *VvLAR1* promoters, showing it specifically controls anthocyanin synthesis. These results suggest that in grapes the transcription factors *VvMYBA2/VvMYBA1* and *VvMYBPA1* control whether anthocyanins or PAs are synthesized by regulating expression of *VvUFGT* and *VvANR/VvLAR*, respectively. However, we cannot exclude that there are additional MYB transcription factors involved in regulation of anthocyanin and/or PA synthesis in grapevine.

In maize, most of the genes involved in anthocyanin synthesis are controlled by the MYB factor C1 interacting with the bHLH protein R (Goff et al., 1992), whereas the synthesis of phlobaphene, a pigmented polymeric flavonoid compound, is controlled by the MYB factor P1, independent of a bHLH protein, and MYB factor P1 activates only a subset of flavonoid biosynthetic genes (Grotewold et al., 1994). Interestingly, the change of six amino acids in the MYB domain of P1 allows the protein to interact with R and activate all the anthocyanin genes, resulting in

anthocyanin formation (Grotewold et al., 2000). These results emphasize the importance of the interaction between MYB and bHLH proteins for the regulatory specificity of transcription factors with very similar DNA-binding domains.

For the grapevine MYB factors VvMYBA and VvMYBPA1 the interaction with a bHLH protein (EGL3) was essential for their ability to activate gene expression (Fig. 4E). However, as we used EGL3 as bHLH protein in all our promoter experiments, their specificity to control either the anthocyanin-specific gene *UFGT* or the PA-specific genes *ANR* and *LAR* seems not to depend on the identity of the interacting bHLH protein (Fig. 4). The difference in specificity of VvMYBA, VvMYBPA1, and TT2 is likely to be due to their ability to bind to different sites in the promoters of grapevine and Arabidopsis genes. For example, differences of the MYB factors would change their ability to bind the promoters and/or other unidentified factors which are present in the grapevine cell cultures.

Another grapevine MYB, VvMYB5a, recently identified by Deluc et al. (2006) is also expressed early in berry development when PAs are synthesized. Ectopic expression of VvMYB5a in tobacco increased expression of a number of structural genes in the phenylpropanoid pathway and modified the levels of anthocyanins, PAs, flavonols, and lignins in the flowers. The transgenic tobacco flowers had increased levels of epicatechin but not catechin but it is not yet clear whether VvMYB5a can activate the genes specifically involved in PA synthesis, such as *ANR* and *LAR*, or whether it controls the pathway generally, leading to an increase in all flavonoids.

Control of the pathway in grapevine is thus analogous to that in Arabidopsis, where the MYB transcription factor TT2 controls PA synthesis, whereas PAP1/PAP2 control anthocyanin biosynthesis. However, the Arabidopsis flavonoid MYB factors seem to control different steps in the flavonoid pathway than the similar factors from grapevine. In previous studies it was shown that TT2 controls expression of the flavonoid late biosynthetic genes including *DFR*, *LDOX*, *AtANR* (*BAN*), and *TT12*, whereas transcript levels of the flavonoid early biosynthetic genes like *CHS*, *CHI*, *F3'H*, or *F3H* were not affected by TT2 (Nesi et al., 2001). In contrast, our quantitative analysis of *VvCHI*, *VvF3'5'H1*, *VvLDOX*, *VvANR*, and *VvLAR1* promoter activities revealed that VvMYBPA1 is able to induce promoters of both early and late flavonoid biosynthetic genes (Fig. 4). Thus, regulation of the flavonoid pathway in developing and ripening grape berries appears to differ from that in the Arabidopsis seed coat. The grapevine regulator of anthocyanin synthesis, VvMYBA2, appears to substantially activate only the promoter of *UFGT* and other factors appear to be required to activate the other structural genes of the pathway to synthesize anthocyanins. In contrast, VvMYBPA1 appears capable of activating the shared genes of the pathway as well as both of the PA-specific

steps (*ANR* and *LAR*) and may be sufficient to induce PA synthesis in both the seeds and skin of the fruit. These different transcription factors appear to contribute to the temporal and spatial regulation of the flavonoid pathway in grapes to provide for PA synthesis in the skin and seeds of the developing berry and only anthocyanin synthesis in the skin of the ripening fruit.

#### VvMYBPA1 Complements the *tt2* Seed Phenotype and Can Induce Ectopic PA Accumulation in Arabidopsis

The complementation of the PA-deficient seed phenotype of the *tt2* mutant by VvMYBPA1 indicates that this grapevine transcription factor is capable of substituting for the function of the Arabidopsis PA regulator TT2 (Fig. 5). Although TT2 controls PA synthesis in the Arabidopsis seed coat and its ectopic expression was shown to induce expression of the *AtANR* promoter, Arabidopsis 70S::TT2 plants failed to accumulate PAs in any tissue other than seeds (Nesi et al., 2001). In contrast, Arabidopsis 35S::MYBPA1 plants accumulated PAs in their cotyledons, hypocotyls, roots, trichomes, and the bases of rosette leaves as well as in seed coats (Figs. 5 and 7). A similar organ- and cell-specific pattern of PA accumulation was observed in Arabidopsis plants simultaneously overexpressing TT2 and *PAP1*, which reflected the *ANR* promoter activity in Arabidopsis (Sharma and Dixon, 2005). Our promoter studies (Fig. 4) and analysis of the 35S::MYBPA1 Arabidopsis plants (Figs. 5 and 7) suggest that in grapevine VvMYBPA1 regulates the whole flavonoid pathway branch leading to PA synthesis (early and late flavonoid biosynthetic genes), whereas in Arabidopsis TT2 controls only the late flavonoid biosynthetic genes (Nesi et al., 2001). Therefore, coexpression of TT2 and *PAP1* was needed for induction of the early and late flavonoid biosynthetic genes and ectopic formation of PAs in Arabidopsis (Sharma and Dixon, 2005), whereas the grapevine MYBPA1 appears capable of activating PA synthesis without additional MYB genes. The majority of our 35S::MYBPA1 transgenic seedlings developed growth abnormalities and accumulated relatively high levels of PAs in roots, hypocotyl, and apical meristem, which could have led to the death of these plants before they fully developed their first leaves. Presumably, these plants expressed much higher levels of VvMYBPA1 transcript than those lines (e.g. lines 10 and 17) that grew past this stage. Similar toxic effects for Arabidopsis were observed by constitutive coexpression of TT2, *PAP1*, and the maize regulatory gene *leaf color*, which resulted in PA formation in roots and leaves and death of the plants (Sharma and Dixon, 2005). As Arabidopsis wild-type plants accumulate PAs only in the seed coat, it may be that only these specific cells are equipped to accumulate PAs. Transgenic plants ectopically synthesizing PAs in other cells may not be able to process the PA or compartmentalize it into specific cell types or vacuoles so the PAs or their precursors could become toxic in these cells, resulting

in death of the plants. Alternatively, overexpression of *VvMYBPA1* may induce formation of other cytotoxic metabolites or could lead to other dysfunction in these plants.

It would be interesting to determine whether ectopic expression of *VvMYBPA1* in plants like alfalfa and clover (*Trifolium repens*) can induce PA formation in leaves or other tissue, because it is of great interest to engineer PAs in forage crops to reduce the risk of pasture bloat for ruminants (Dixon et al., 2005). However, our present results showing that PA formation after overexpression of *VvMYBPA1* is limited to certain cell types suggest that additional factor(s) may be needed for ectopic accumulation of PAs, at least in Arabidopsis. Recently, Xie et al. (2006) have shown that expression of *ANR* in alfalfa or coexpression of *ANR* and *PAP1* in tobacco can induce ectopic PA accumulation. It would be interesting to test whether *VvMYBPA1* is also able to induce PA accumulation in these plants.

Unlike Arabidopsis, grapevine synthesizes PAs of different polymer lengths and composition in leaves, flowers, and in the skin and seeds of the developing fruit (Kennedy et al., 2001; Bogs et al., 2005). It appears that regulation of PA synthesis in grapes differs from the regulation of PA in Arabidopsis, which is restricted to the developing seed coat. In grapes, *VvMYBPA1* appears capable of activating the both the early and late shared genes of the flavonoid pathway as well as the PA-specific genes encoding both *ANR* and *LAR*. There is considerable interest in grape PAs because of their importance for the color and taste of wine and their antioxidant capacity promoting health benefits in a number of model cell and animal systems (Bagchi et al., 2000). Our data suggest that *VvMYBPA1* regulates PA formation in grapes and alteration of *VvMYBPA1* expression may have the potential to manipulate the amount and composition of PAs in grape berries and possibly in other fruits.

## MATERIALS AND METHODS

### Plant Material

Grapevine (*Vitis vinifera*) tissues of cultivar Shiraz were collected from a commercial vineyard during the 2000 to 2001 season. Approximately 100 berries from at least 20 bunches were collected at weekly intervals throughout berry development from floral initiation until harvest, as described in Downey et al. (2003a). All samples were frozen in liquid nitrogen upon collection in the field and stored at  $-80^{\circ}\text{C}$  until analyzed.

Arabidopsis (*Arabidopsis thaliana*) Col-0 and *tt2* (SALK\_005260) seeds were provided by The Arabidopsis Biological Resource Center.

### Preparation of cDNAs

Total grapevine RNA was isolated from the various plant tissues as described in Downey et al. (2003b). Arabidopsis RNA was isolated from leaves with RNeasy kit (Qiagen) following the suppliers protocol. The quality of RNA was verified by demonstration of intact ribosomal bands following agarose gel electrophoresis in addition to the absorbance ratios (A<sub>260</sub>/A<sub>280</sub>) of 1.8 to 2.0. For cDNA synthesis, 4  $\mu\text{g}$  of grapevine or 1  $\mu\text{g}$  of Arabidopsis total RNA were reverse transcribed using oligo d(T)<sub>18</sub> and SuperScript III reverse transcriptase (Invitrogen Life Technologies) following the protocol of the supplier.

## Cloning of *VvMYBPA1* and Plant Transformation

The ORF of *VvMYBPA1* was inserted into the binary vector pART27 for expression of the gene in Arabidopsis. Therefore, the *VvMYBPA1* ORF was amplified by PCR from grapevine (cv Shiraz) cDNA (from RNA isolated 10 weeks before véraison) using *PfuTurbo* polymerase (Stratagene) and the primers MybPAartF (5'-TGAGGTACCGAGAGAGATATGGGCAGAGCAC-3') and MybPAartR (5'-TGAGGATCCTGATCTTTGGTCTCTCTGCAA-3'). The generated PCR fragment was purified, digested with *Bam*HI and *Kpn*I, and cloned in the vector pART7 (Gleave, 1992) to give pART7MYBPA1 where *VvMYBPA1* is under the control of the CaMV 35S promoter. The nucleotide sequence of the *VvMYBPA1* ORF (accession no. AM259485) in pART7MYBPA1 was determined by DNA sequencing. The expression cassette present in pART7MYBPA1 was isolated as *Not*I fragment, cloned into the *Not*I site of the binary vector pART27 (Gleave, 1992), and transferred into *Agrobacterium tumefaciens* (AGL1) by electroporation. Arabidopsis *tt2* (SALK005260) ecotype Col was transformed using the floral-dip method (Clough and Bent, 1998). T<sub>1</sub> transgenic plants were selected on one-half-strength Murashige and Skoog media containing 8 g/L agar and 35 mg/L kanamycin. Kanamycin-resistant T<sub>1</sub> seedlings were transferred to soil and grown at 20°C in a growth chamber (Phoenix Biosystems) with a 16-h day length and a light intensity of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Seeds of individual self-fertilized T<sub>2</sub> lines were collected and single-copy insertion lines were selected based on a Mendelian segregation ratio.

## HPLC Analysis and DMACA Staining of PAs

Immature Arabidopsis siliques were finely ground and 80 mg were extracted in 400  $\mu\text{L}$  70% (v/v) acetone containing 0.1% (w/v) ascorbate for 18 h at room temperature on a rotating wheel in darkness. Samples were centrifuged and 300  $\mu\text{L}$  aliquots of the supernatant were transferred to fresh tubes and vacuum dried at 35°C for 60 min. The pellet was resuspended in 100  $\mu\text{L}$  phloroglucinol buffer (0.25 g ascorbate, 1.25 g phloroglucinol, 215  $\mu\text{L}$  concentrated HCl, 25 mL methanol) and incubated at 50°C for 20 min, then neutralized with 100  $\mu\text{L}$  sodium acetate (200 mM, pH 7.5) for the analysis of PAs. Reverse-phase HPLC was used for analysis of PAs as described by Downey et al. (2003a).

The presence of PAs in plant tissue was detected by staining the tissues with DMACA solution (1% DMACA, 1% 6 N HCl in methanol). Dried seeds were stained for 6 to 14 h and seedlings for 10 to 30 min. The tissues were then transferred to distilled water and blue staining of the tissue was visualized with a microscope and documented using a digital camera.

## Cloning of the Reporter and Effector Constructs for the Transient Promoter Assays

The Universal GenomeWalker kit (CLONTECH) was used to isolate promoter fragments of *VvCHI*, *VvF3' 5' H1*, *VvANR*, and *VvLARI*. Four libraries of adaptor-ligated genomic fragments were constructed from grapevine (Shiraz) genomic DNA restricted by *Dra*I, *Eco*RV, *Pvu*II, or *Stu*I endonucleases and generated according to the GenomeWalker protocol. These genomic DNA libraries served as templates for the promoter isolation. Outer and nested gene-specific primers were designed to the 5' ends of the cDNA sequences of *VvCHI* (accession no. X75963), *VvF3' 5' H1* (accession no. AJ860356), *VvANR* (accession no. CAD91911), and *VvLARI* (accession no. AJ865336) and primary and secondary PCRs were performed with the outer adapter primer AP1 and the nested adapter primer AP2, respectively. Primer design and PCR conditions for genome walking were performed according to the manufacturer's instructions. The amplified promoter fragments of the nested PCRs were cloned into pDrive (Qiagen) and sequenced. These DNA sequences were then used to design specific primers for the amplification of the respective promoter from grapevine (Shiraz) genomic DNA using *PfuTurbo* polymerase (Stratagene). The primers used for these PCR reactions contained restriction sites (in bold) for cloning the promoters into the luciferase reporter vector pLuc (Horstmann et al., 2004) as a *Bam*HI/*Xho*I fragment. Their DNA sequences were as follows: CHIF (5'-ATAGGATCCTGGAATTATGGAAGACAAATAGTCAA-3'), CHIR (5'-TTACTCGAGGATATGGCTGCAGAGAAACGA-3'), ANRf (5'-CGAGG-ATCCCATTACATAGTCAAATTACAAAATCAA-3'), ANRr (5'-ATACTCG-AGATATGCCCTCACTTCCAAATTC-3'), F35Hf (5'-CGAGGATCCCAAAA-AGAGTTGGAAATACAACGA-3'), F35Hr (5'-ATACTCGAGTGAGTATAG-GATAGTGAAGGTGGCTAT-3'), LARf (5'-CGAGGATCCTCGAATAATTT-CATAGGGCTTT-3'), and LARr (5'-ATACTCGAGTCTGATGATGCTTCTT-CTCTACTACTC-3').

A 1,674-bp DNA fragment of the *VvUFGT* promoter (accession no. AY955269) was amplified by PCR from the plasmid pART7UFGT:GFP (gift from Paul Boss, Commonwealth Scientific and Industrial Research Organization, Australia) using *Pfx* polymerase (Invitrogen) with the primers UFGTp2F (5'-ACGGGATCCTCATGCGTCCACCTATTATCAA-3') and UFGTpR (5'-GTA-CTCGAGGGTTGGAATGGGGGATGTTA-3'). A 1,533-bp DNA fragment of the *AtANR* promoter was amplified with *PfuTurbo* polymerase using the primers AtANRf (5'-CGAGGATCCCTGGGAAGACAATCGCTTTA-3') and AtANRr (5'-ATCTCGAGTTGAAATTACAGAGATAGAGATTTAGTTG-3'). A 2,174-bp DNA fragment of the *VvLDOX* promoter was amplified with *PfuTurbo* polymerase using the primers LDOXf (5'-CGAGGATCCGTTTGCTTCCATCCCAATCTCACT-3') and LDOXr (5'-TGTCTCGAGAAATACACTGATCTACTTGTTC-3'). These PCR fragments were gel purified, digested with *Bam*HI and *Xho*I, and cloned between the respective sites of the vector pLuc. All described grapevine promoters were amplified from grapevine (Shiraz) genomic DNA.

For transient expression of *TT2* and *VvMYBA2* their ORFs were amplified from cDNA by PCR using *PfuTurbo* polymerase and cloned into the vector pART7, which contains the CaMV 35S constitutive promoter. Therefore, *TT2* was amplified using the primers TT2F (5'-AGGTCGACATGGGAAAGAGCAACTACTAGTG-3') and TT2R (5'-TACTCGAGTCAACAAGTGAAGTCTCGGAGC-3') from cDNA of Col-0 siliques. The PCR fragment was digested with *Sal*I and *Xho*I and ligated into pART7, digested with the same enzymes. The ORF of *VvMYBA2* was amplified from grapevine postvéraison berry skin cDNA using the primers MybAF (5'-CGCCTCGAGCTCGATGAGAGCTTAGGAGTTAG-3') and MybAR (5'-CGCTCTAGATAAATCAGATCAAATGATTACTT-3'). The PCR fragment was digested with *Xho*I and *Xba*I and ligated into pART7, digested with the same enzymes. All described PCR fragments were subjected to DNA sequencing before analysis in the transient assay system.

### Transient Transfection Experiments and Dual Luciferase Assay

A transient assay was developed using a cell suspension of a Chardonnay petiole callus culture, using the methods detailed in Takos et al. (2006), modified from Torregrosa et al. (2002), and utilizing a dual luciferase system (Horstmann et al., 2004). In brief, gold particles were coated with a mixture of DNA constructs (150 ng of the respective plasmid, giving a total plasmid concentration of 750 ng/shot) and used to bombard immobilized grape cells. After incubation in the dark at 27°C for 48 h, the harvested cells were assayed for the luciferase activities using the dual-luciferase reporter assay system (Promega) measured with TD-20/20 Luminometer (Turner Design). The relative luciferase activity was calculated as the ratio between the firefly (*Photinus pyralis*) and the Renilla (control) luciferase activity. All transfection experiments were performed in triplicate and each set of promoter experiments was repeated with similar relative ratios to the respective control.

### Expression Analysis of *VvMYBPA1*

Transcript levels of *VvMYBPA1* in grapevine were measured by real-time PCR, using SYBR green method on a Rotor-Gene 2000 (version 4.2) real-time cyclor (Corbett Research). Each PCR reaction (15  $\mu$ L) contained: 266 nM primer (each), cDNA (diluted 1:60), and 1 $\times$  Absolute QPCR SYBR Green ROX mix (ABgene House). The thermal cycling conditions were 95°C for 15 min followed by 95°C for 30 s, 58°C for 25 s, and 72°C for 25 s for 30 or 35 cycles, followed by a melt cycle from 50°C to 96°C.

The expressed sequence tag clone TC46393 (TIGR database) was used to design the primers MYBPA1F (5'-AGATCAACTGGTTATGCTTGCT-3') and MYBPA1R (5'-AACACAAATGTACATCGCACAC-3') that were used to detect the transcript level of *VvMYBPA1* in grapevine and amplified a 190-bp PCR fragment from the 3' untranslated region of the gene. With all cDNAs used the primer set gave a single PCR product that was verified by determining the melt curves for the product at the end of each run, by analysis of the product using gel electrophoresis, and by comparing the DNA sequence of the PCR product with the gene sequence. The efficiency of the primers was tested in preliminary experiments with dilutions of the purified PCR product and maintained an  $r^2$  value  $\geq$  0.98. The expression of genes was normalized to *VvUbiquitin1* (TC32075, TIGR database), which transcripts were detected by amplifying a 182-bp product with the primers VvUbiquitin Forward (5'-GTGGTATTATTGAGCCATCCTT-3') and VvUbiquitin Reverse (5'-AACCTCAATCCAGTCATCTAC-3'). All samples were measured in triplicate.

The difference between the cycle threshold (Ct) of the target gene and the Ct of Ubiquitin,  $\Delta Ct = Ct_{\text{target}} - Ct_{\text{Ubiquitin}}$ , was used to obtain the normalized expression of target genes, which corresponds to  $2^{-\Delta Ct}$ . The Rotor Gene 2000 software (Corbett Research) and the Q-Gen software (Muller et al., 2002) were used to calculate the mean normalized expression of the genes.

For detection of the *VvMYBPA1* transcript in Arabidopsis (Fig. 7), PCR reactions were performed as described above and analyzed on a 1.5% agarose gel containing ethidium bromide. The primers MYBf (5'-CAACTGACAACTCTCTGGACAA-3') and MYBr (5'-GATCTTTTGGTCTCTCTGCAAC-3') were used to amplify a 146-bp PCR from the 3' translated region of the gene. To determine whether the similar amounts of cDNA were applied to all samples, a 268-bp PCR fragment from the Arabidopsis *Actin2* gene (accession no. NM\_112764) was amplified with the primers ACT2F (5'-ATTCAGATGCCAGAAAGTCTTGTTC-3') and ACT2R (5'-ACCACCGATCCAGACTGTACTTCC-3').

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AM259485.

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