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Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings

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Summary

In all higher plants studied to date, the anthocyanin pigment pathway is regulated by a suite of transcription factors that include Myb, bHLH and WD-repeat proteins. However, in *Arabidopsis thaliana*, the Myb regulators remain to be conclusively identified, and little is known about anthocyanin pathway regulation by TTG1-dependent transcriptional complexes. Previous overexpression of the *PAP1* Myb suggested that genes from the entire phenylpropanoid pathway are targets of regulation by Myb/bHLH/WD-repeat complexes in Arabidopsis, in contrast to other plants. Here we demonstrate that overexpression of *Myb113* or *Myb114* results in substantial increases in pigment production similar to those previously seen as a result of overexpression of *PAP1*, and pigment production in these overexpressors remains TTG1- and bHLH-dependent. Also, plants harboring an RNAi construct targeting *PAP1* and three Myb candidates (*PAP2*, *Myb113* and *Myb114*) showed downregulated Myb gene expression and obvious anthocyanin deficiencies. Correlated with these anthocyanin deficiencies is downregulation of the same late anthocyanin structural genes that are downregulated in *ttg1* and bHLH anthocyanin mutants. Expression studies using GL3:GR and TTG1:GR fusions revealed direct regulation of the late biosynthetic genes only. Functional diversification between GL3 and EGL3 with regard to activation of gene targets was revealed by GL3:GR studies in single and double bHLH mutant seedlings. Expression profiles for Myb and bHLH regulators are also presented in the context of pigment production in young seedlings.

Keywords: Myb, bHLH, *TTG1*, anthocyanins, flavonoids, Arabidopsis.

Introduction

The branch of the phenylpropanoid pathway yielding flavonoid-based pigments in plants is an excellent model for the study of transcriptional regulation. Flavonoid pigment biosynthetic genes are regulated developmentally and in response to various biotic and abiotic stresses by a combination of transcription factors. These transcriptional regulators include members of the Myb, bHLH and WD-repeat families (Carey *et al.*, 2004; Morita *et al.*, 2006; Quattrocchio *et al.*, 1999; Schwinn *et al.*, 2006; Spelt *et al.*, 2000; Taylor and Briggs, 1990; de Vetten *et al.*, 1997; Walker *et al.*, 1999; Zhang *et al.*, 2003). Various transcription factor combinations can specify the class of flavonoid pigment produced, where it will be produced, production in response to a particular stimulus, and whether transcriptional regulation of structural genes is positive or negative (Aharoni *et al.*, 2001; Baudry *et al.*, 2004; Burr *et al.*, 1996; Hartmann *et al.*, 2005; Lepiniec *et al.*, 2006; Piazza *et al.*, 2002; Solfanelli *et al.*, 2006; Taylor and Briggs, 1990; Winkel-Shirley, 2001).

This model for transcriptional regulation of the flavonoid pigment pathway emerged over 20 years ago with the first cloning of a plant transcription factor, an R2R3 Myb from maize (*Zea mays*) known as *colorless1* (*C1*) (Cone *et al.*, 1986; Paz-Ares *et al.*, 1987). Soon after, bHLH transcription factors, such as *R*, were identified that regulate flavonoid pigments in parallel with the Myb proteins (Chandler *et al.*, 1989; Goff *et al.*, 1992; Ludwig *et al.*, 1989). Since then, a general WD-repeat/Myb/bHLH model for regulation of the anthocyanin biosynthetic pathway was found to operate in

all plant species studied including snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*) and *Arabidopsis thaliana* (Morita *et al.*, 2006; Quattrocchio *et al.*, 1999; Schwinn *et al.*, 2006; Spelt *et al.*, 2000; de Vetten *et al.*, 1997; Walker *et al.*, 1999; Zhang *et al.*, 2003). Based (partly) on the genes regulated by WD-repeat/Myb/bHLH transcriptional complexes, the flavonoid biosynthetic pathway is subdivided into 'late' steps that are dependent on these complexes and 'early' steps that are not (Martin *et al.*, 1991; Pelletier and Winkel-Shirley, 1996; Pelletier *et al.*, 1997; Quattrocchio *et al.*, 1993; Winkel-Shirley *et al.*, 1995; Zhang *et al.*, 2003) .

Despite the wealth of knowledge regarding the WD/bHLH/Myb regulatory model governing the phenylpropanoid pathway in many species, and various cell fate pathways in Arabidopsis (Bernhardt *et al.*, 2003, 2005; Koornneef, 1981; Lee and Schiefelbein, 1999; Lepiniec *et al.*, 2006; Payne *et al.*, 2000; Serna and Martin, 2006; Winkel-Shirley, 2001; Zhang *et al.*, 2003), little is known about anthocyanin pathway targets of transparent testa glabra1 (TTG1)-dependent complexes and the relative contributions of specific bHLH and Myb elements to structural gene regulation. Recently, the *production of anthocyanin pigment1* (*PAP1*) Myb has been shown to be an anthocyanin regulator in seedlings (Teng *et al.*, 2005). However, three other Arabidopsis Myb candidates (*PAP2*, *Myb113 and Myb114*) exist, based on several criteria: sequence similarity to *PAP1* and other homologous pigment regulators (Figure 1), their ability to interact with the Arabidopsis bHLH anthocyanin regulators glabra3 (GL3), enhancer of glabra3 (EGL3) and transparent testa8 (TT8) in yeast, and, in the case of *PAP2*, its ability to upregulate the phenylpropanoid pathway when overexpressed (Borevitz *et al.*, 2000; Stracke *et al.*, 2001; Zhang *et al.*, 2003; Zimmermann *et al.*, 2004). *PAP1* and *PAP2* were originally identified through activation-tagging experiments, with plants overexpressing either of these Mybs showing enhanced accumulation of anthocyanin pigment. This increase in flavonoid pigment production is due to upregulation of the entire phenylpropanoid pathway, as evidenced by increases in late structural gene expression and more modest increases in early structural gene expression in *PAP1*-overexpressing leaves (Borevitz *et al.*, 2000; Tohge *et al.*, 2005a). This observation is surprising, as it suggests that Myb/bHLH/TTG1 complexes in Arabidopsis possibly regulate more than just a late subset of flavonoid pathway genes, contrary to pathway regulation by bHLHs and Mybs in other plant species and contrary to studies showing that *GL3*, *EGL3*, *TT8*, *TT2* and *TTG1* regulate late genes such as *dihydroflavonol reductase* (*DFR*) and the proanthocyanidins-specific structural gene *Banyuls* (*BAN*) but not the early gene *chalcone synthase* (*CHS*) nor the general phenylpropanoid genes *phenylalanine ammonia lyase* (*PAL*) and *cinnamate 4-hydroxylase* (*C4H*) (Baudry *et al.*, 2004; Nesi *et al.*, 2000, 2001; Zhang *et al.*, 2003).

In this study, we attempt to better understand flavonoid pathway regulation by Myb/bHLH/TTG1 transcriptional complexes of Arabidopsis. *Myb113* and *Myb114* are identified as able to regulate the anthocyanin pathway. Overexpression of either gene results in enhanced accumulation of anthocyanin pigments similar to *PAP1* and *PAP2* overexpressors. Myb113 overexpression in seedlings gives increased expression of the 'late' gene *leucoanthocyandin dioxygenase* (*LDOX*) but not the early gene *CHS*. Pigment overaccumulation in Myb overexpressors is bHLH/TTG1-dependent, underscoring the requirement for all members of the complex for gene activation. Plants harboring a silencing construct targeting all four Mybs show decreased Myb gene expression and strong visible decreases in pigmentation throughout the plant life cycle (but produce seeds of normal color). These Myb-silenced plants show decreased expression of the same late anthocyanin biosynthetic genes that are reduced in bHLH anthocyanin and *ttg1* mutants; early pathway gene expression remains unaffected in Myb-silenced mutants and bHLH and *ttg1* mutants. Studies utilizing *35S:GL3:GR* and *35S:TTG1:GR* fusions in seedlings reveal that late anthocyanin biosynthetic genes (but not early biosynthetic genes) are direct targets of TTG1-dependent transcriptional complexes. In addition, GL3:GR studies in single and multiple bHLH mutant seedlings demonstrate a greater contribution by EGL3 than by GL3 in anthocyanin structural gene regulation. Seedling expression profiles via promoter:GUS and RT-PCR analysis are presented for Arabidopsis Myb and bHLH anthocyanin regulators.

Results

Overexpression of Myb113 *or* Myb114 *upregulates the anthocyanin pathway and is bHLH-dependent*

Previous phylogenetic analysis revealed that *Myb113* and *Myb114* of Arabidopsis are closely related to the Arabidopsis flavonoid pigment regulators *PAP1* and *PAP2*, and to other plant pigment regulators (Stracke *et al.*, 2001). To further examine *Myb113* as a potential regulator of the anthocyanin pathway in Arabidopsis, the Columbia genomic locus from the start to the stop codons was placed under the control of the CaMV 35S promoter. *Myb113* overexpression in Col wild-type plants resulted in overaccumulation of anthocyanin pigments. Indeed, primary transformants harvested from floral-dipped plants were identifiable as black seeds due to strongly pigmented embryos visible through the seed coat (Figure 2a). During germination, the seedlings showed anthocyanin pigmentation in the cotyledons and hypocotyls, and some faint, inconsistent pigmentation in roots (Figure 2b,c). However, as the seedlings developed over 3–5 days, pigmentation became restricted to non-hair cell files of the root epidermis and the corresponding cell files in the hypocotyls, resulting in a striped purple pigment pattern

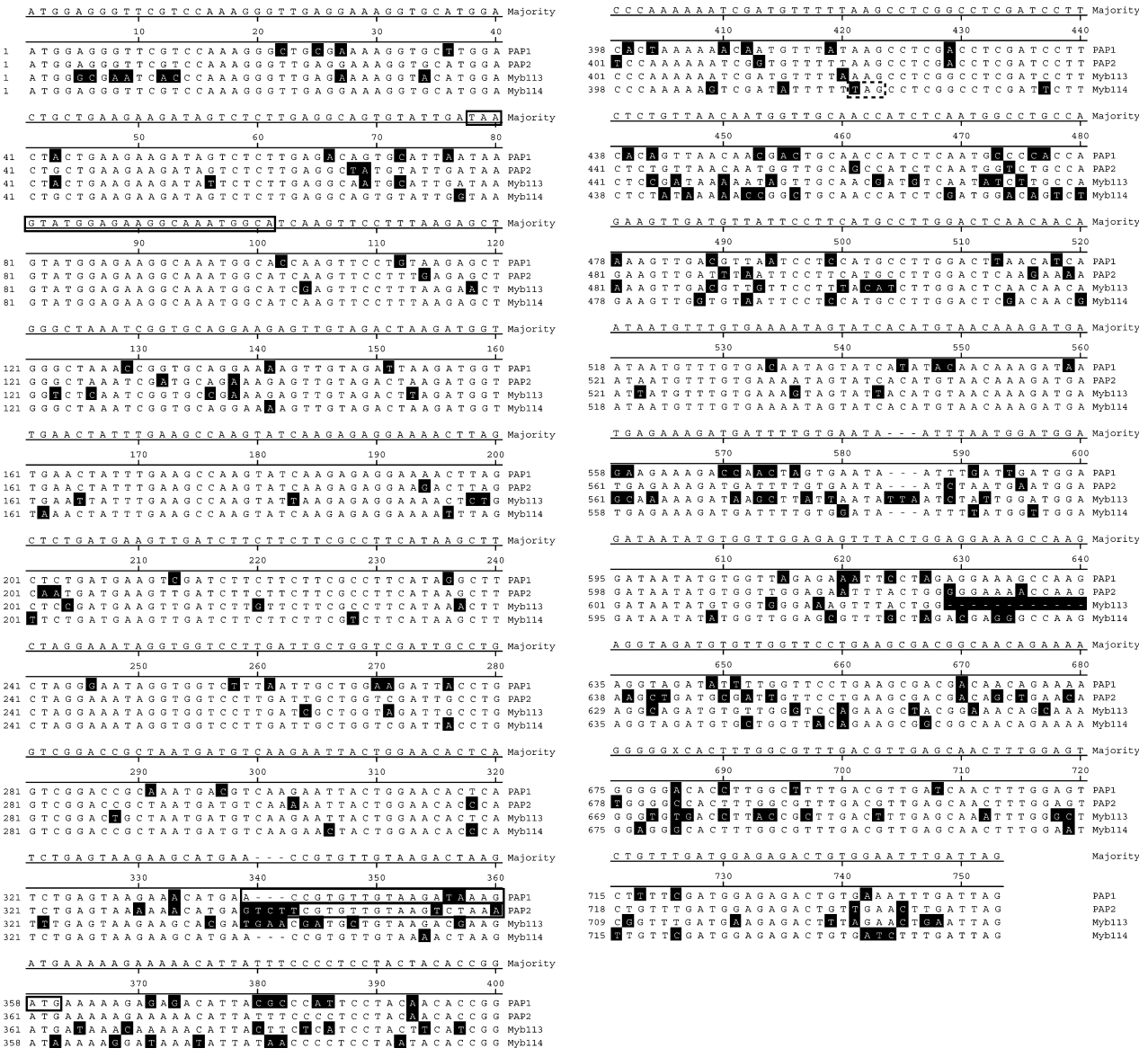

Figure 1. Alignment of *PAP1*, *PAP2*, *Myb113* and *Myb114* coding regions.
The outlined box in the Myb majority sequence indicates the 24 nt sequence used for multi-RNAi vector construction. The outlined boxes in the *PAP1* and *PAP2* sequences indicate the 22 nt sequences used for specific silencing of *PAP1* and *PAP2*, respectively. The box with a dashed outline indicates the early stop codon in the Col *Myb114* allele. Black boxes indicate mismatches. The *Myb114* sequence up to the early stop codon is from the Col allele. The sequence shown after the early stop codon is identical for both the Col and L*er* alleles.

(Figure 2d,e). These are in which GL3 bHLH protein accumulates, as previously shown (Bernhardt *et al.*, 2005). As the seedlings continued to develop, anthocyanins generally disappeared from hypocotyls and fully expanded cotyledons but persisted in root epidermis and appeared in emerging leaf primordial tissue (Figure 2f).

This dynamic pigmentation pattern during the first week of seedling development overlaps with *GL3* and *EGL3* bHLH expression patterns (compare Figure 2b,c with Figure 3d,f and Figure 2f with Figure 3e,g). In general, as bHLH expression becomes refined and restricted to various organs of the growing seedling, so does anthocyanin production, illustrating the requirement for bHLH transcription factor activity as well as Myb activity for positive regulation of the anthocyanin pathway. This bHLH requirement is more obvious when *Myb113* is overexpressed in bHLH and *ttg1* mutants. Newly germinated *egl3* seedlings overexpressing *Myb113* show a very reduced, patchy pattern of anthocyanin accumulation in the hypocotyls where *EGL3* is normally expressed, but anthocyanins accumulate in cotyledon, root and leaf primordia epidermis where *GL3* is still expressed (compare Figure 3a,b with Figure 3d). *gl3 egl3* double

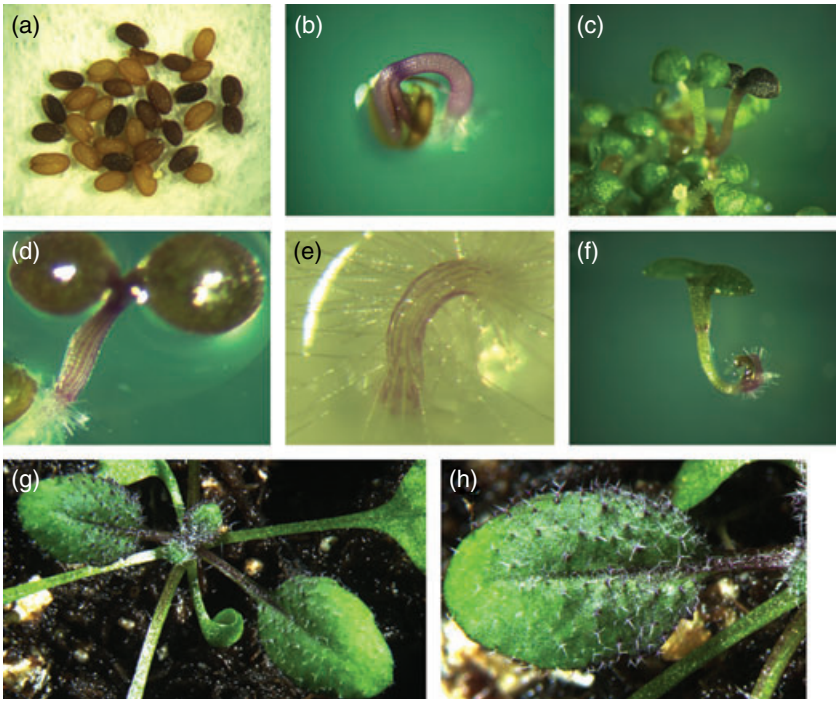

Figure 2. Transgenic phenotypes of *Myb113* and *Myb114L* overexpression.
(a)–(f) *Myb113* overexpression in Col. (a) Dark T_1 individuals are identifiable among Col seed.
(b) Newly germinated *35S:Myb113* seedling. (c) Comparison of a Col seedling with a *35S:Myb113* seedling.
(d) Seedling showing striped anthocyanin pattern in hypocotyls.
(e) Root showing anthocyanin accumulation in non-hair cell files only.
(f) Anthocyanin accumulation in an older seedling (approximately 7 days old).
(g, h) bHLH-dependent anthocyanin expression in young rosette leaves of *35S:Myb114L* plants in the L*er* background.

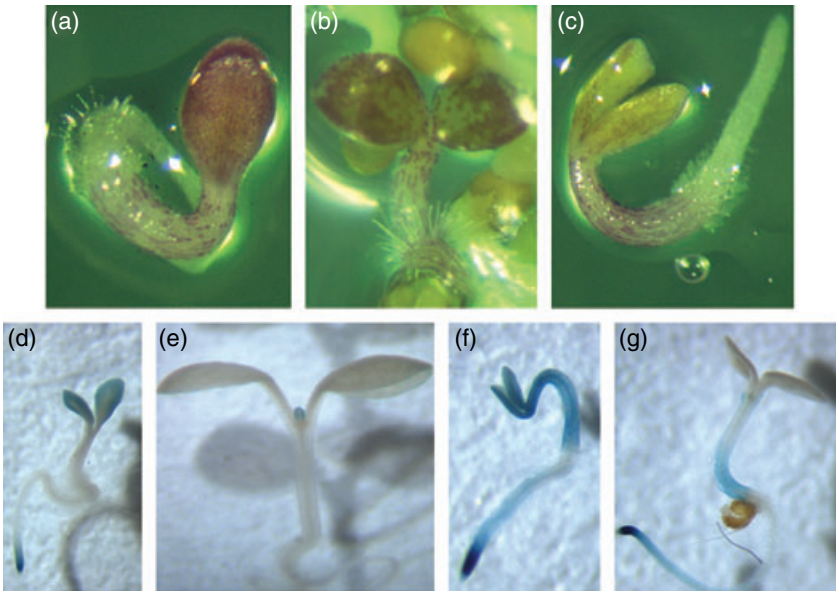

Figure 3. Comparison of *Myb113* overexpression in bHLH mutants and the bHLH expression pattern.
(a)–(c) *Myb113* overexpression in bHLH mutants. (a) *Myb113* overexpressed in *egl3-1.*
(b) *Myb113* overexpressed in the *egl3* T-DNA insertion mutant. (c) *Myb113* overexpressed in the *egl3 gl3* double insertion mutant.
(d)–(g) ProGL3:GUS and ProEGL3:GUS expression in Col seedlings.
(d) ProGL3:GUS expression in a young seedling (approximately 3 days old).
(e) ProGL3:GUS expression in an older seedling (approximately 7 days old).
(f) ProEGL3:GUS expression in a young seedling (approximately 3 days old).
(g) ProEGL3:GUS expression in an older seedling (approximately 7 days old).

mutant seedlings overexpressing *Myb113* show an even greater reduction in pigment production, with loss of anthocyanins in cotyledons, consistent with loss of *GL3* in this organ (compare pigment in cotyledons in Figure 3a,b with that in Figure 3c). *ttg1* mutant and *gl3 egl3 tt8* triple bHLH mutant plants overexpressing *Myb113* show the least anthocyanin pigmentation, consistent with a nearly complete lack of TTG1-dependent bHLH activity in these plants (data not shown).

Interestingly, the *Myb114* gene from the Columbia (Col) accession encodes a putative protein that lacks a transcriptional activation domain due to a stop codon just after the Myb domains (at amino acid 140). The Landsberg *erecta* (L*er*) *Myb114* allele encodes a full-length gene (Figure 1). Other than this stop codon, the L*er* and Col *Myb114* coding sequences are identical, suggesting that the mutation leading to the premature stop in Col occurred very recently. To examine *Myb114* as a potential anthocyanin regulator in

Arabidopsis, the L*er* genomic locus from the start to the stop codons was placed under the control of the CaMV 35S promoter and introduced into L*er* and Col wild-types. Primary transformants were again identifiable as very dark seed due to pigmented embryos visible through the seed coat, and germinated as purple seedlings similar to *Myb113* overexpressors (data not shown). Again, the *Myb114* requirement for bHLH proteins in pigment production was evident in early rosette leaves of 35S:*Myb114* plants. Under normal growth conditions, early rosette leaves exhibit no anthocyanins. Strikingly, pigment production in *Myb114* overexpressing leaves overlapped well with bHLH expression in the context of trichome development (Zhang *et al.*, 2003); anthocyanins accumulated on the basal and lateral portions of leaves and in more basally located trichomes (Figure 2g,h).

Col *Myb114* overexpression resulted in dominant repression of TTG1-dependent epidermal cell fate pathways. Overexpressors exhibited trichome and anthocyanin deficiencies (compare trichomes and anthocyanins in stems in Figure S1A with that in Figure S1B), and perhaps a mild effect on seed coat mucilage production (data not shown; root hair phenotype was not examined).

Multiple silencing of Arabidopsis anthocyanin Myb *candidate genes results in downregulation of the anthocyanin pathway*

High sequence similarity between *PAP1*, *PAP2*, *Myb113* and *Myb114* suggested functional redundancy, and thus multiple mutants might be required to uncover the full scope of anthocyanin pathway regulation by these Mybs. However, generating multiple Myb mutants by recombination is not practical given the very tight linkage of these genes: Myb113 (At1g66370), Myb114 (At1g66380) and PAP2 (At1g66390) occur in tandem on chromosome 1. In an attempt to create plants that were deficient for all four Myb gene functions, we adopted an RNAi approach, taking advantage of the conserved sequence among the Myb transcripts (Figure 1). A construct overexpressing a 24 bp hairpin loop (with sequence perfectly conserved in all four Mybs) was introduced into Col wild-type and *pap1-Dominant* (*pap1D*) plants. The *pap1-D* background serves as a positive control, in that pigment reduction caused through silencing should be immediately obvious. Indeed, *pap1-D* Myb RNAi transformants showed a very strong reduction in the purple pigmentation that these plants normally exhibit (Figure 4a). Col Myb RNAi and *pap1-D* myb RNAi seedlings observed on medium containing 3% sucrose showed an obvious loss of anthocyanins in the hypocotyls and cotyledons (Figure 4a,b). The relative anthocyanin content in Col Myb RNAi seedlings compared to Col seedlings was greatly reduced as determined by spectrophotometric measurements of anthocyanin extracts (Figure S2). As transformants continued to develop, it was clear that a strong reduction in anthocyanin production had been achieved by the silencing construct (Figure 4c). However, production of pro-anthocyanidins (PAs) in the seed coat remained unaffected.

BLAST searches using the 24 nt RNAi sequence identifies only the four PAP-related Mybs, suggesting that it is unlikely that the RNAi transgene targets additional Mybs.

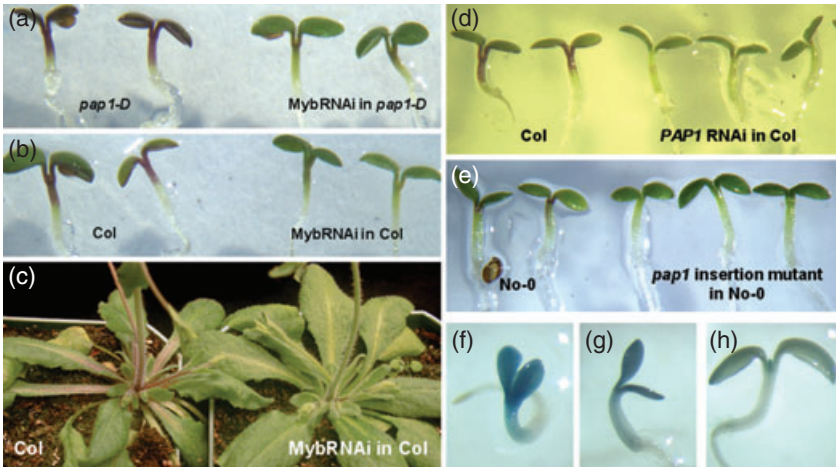

Figure 4. Myb mutant anthocyanin phenotypes and *PAP1* expression pattern.
(a) Comparison of *pap1-D* versus *pap1-D* Myb RNAi seedlings (approximately 4 days old) germinated on medium containing 3% sucrose.
(b) Comparison of Col versus Col Myb RNAi seedlings (approximately 4 days old) germinated on medium containing 3% sucrose.
(c) Comparison of Col versus Col Myb RNAi plants in soil.
(d) Comparison of Col seedlings versus *PAP1* RNAi Col seedlings (approximately 4 days old) germinated on medium containing 3% sucrose.
(e) Comparison of No-0 seedlings versus *pap1* insertion mutant seedlings (approximately 4 days old).
(f)–(h) *ProPAP1*:GUS expression pattern in Col seedlings. (f, g) Expression in very young seedlings (2–3 days old).
(h) Expression in an older seedling (approximately 7 days old).

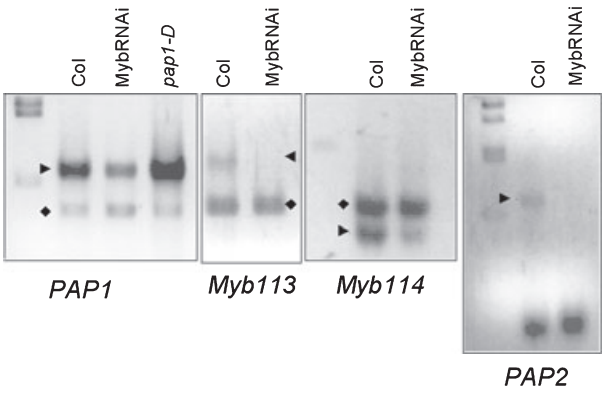

Figure 5. Standard RT-PCR experiments comparing Myb expression in Col seedlings versus Col myb RNAi seedlings.
Triangles indicate the Myb target and diamonds indicate the *APRT* loading control. The *APRT* control was omitted from *PAP2* lanes (see text).

Also, RNAi lines showing strong anthocyanin deficiencies are phenotypically normal for trichome development (regulated by the Myb GL1; Oppenheimer *et al.*, 1991) and PA production (regulated by the Myb TT2; Nesi *et al.*, 2001), indicating that closely related TTG1-dependent Mybs are not targeted.

We performed standard RT-PCR experiments to verify that expression of the targeted Myb genes was reduced in seedlings. Of the four Mybs, *PAP1* was the most highly expressed in Col seedlings, but *PAP2* was barely detectable (see Figure 5: 10 μl after 35 cycles of PCR for *PAP1* cDNA amplification, compared with 15 μl after 40 cycles of PCR for *PAP2*). In fact, we could only amplify *PAP2* cDNA if *APRT* endogenous control primers were excluded from the reaction, so it appears to be close to the limit of detection by this method. Col seedlings also showed low expression levels for *Myb113* and *Myb114* (Figure 5, both 15 μl after 40 cycles). In Col Myb RNAi seedlings, expression of all four Mybs was strongly reduced or undetectable. Thus, it appears that all four Myb genes were successfully targeted by a single silencing construct.

Next we determined which anthocyanin structural genes might be downregulated in Col Myb RNAi plants using a

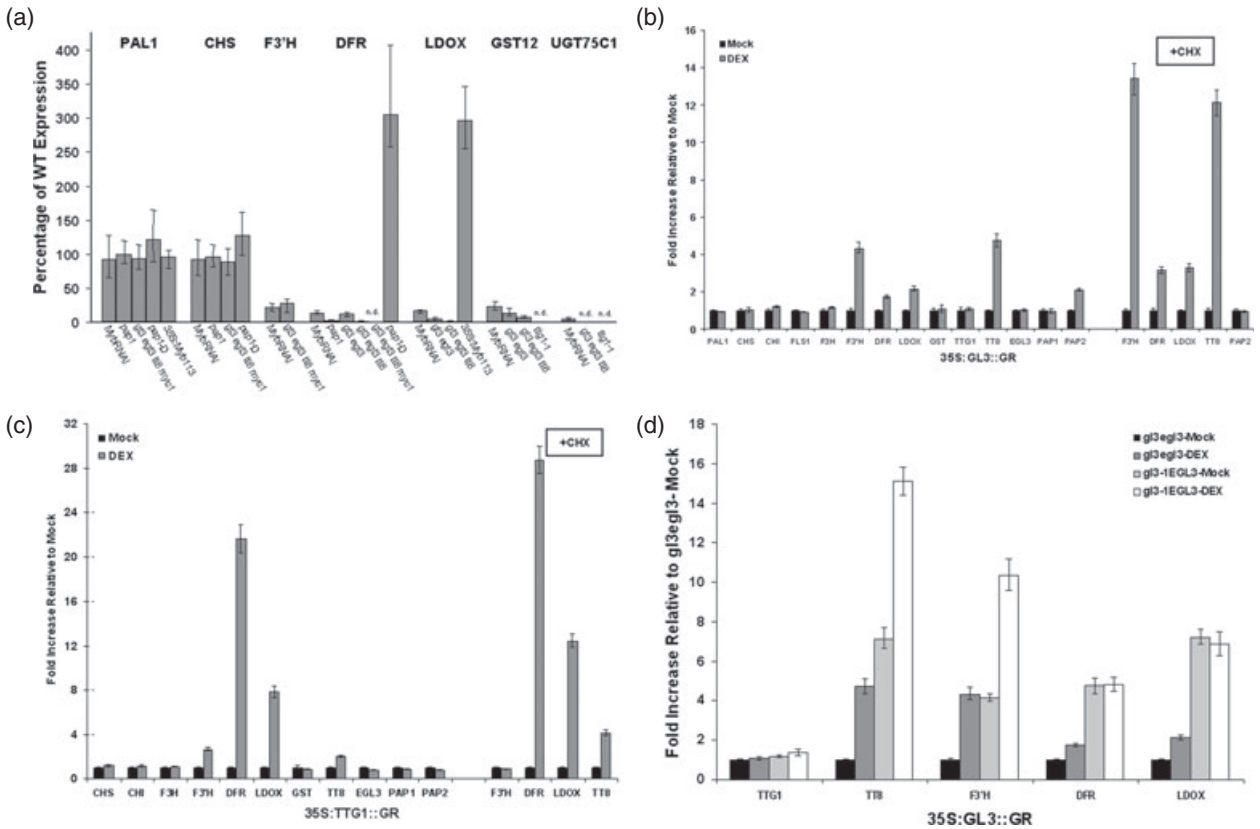

Figure 6. Anthocyanin structural and regulatory gene expression.
(a) Anthocyanin gene expression in Myb, bHLH and *ttg1* mutant seedlings reported as a percentage of wild-type expression. n.d., not detected.
(b) Anthocyanin gene expression in *gl3 egl3* mutant seedlings expressing *GL3::GR*. Expression changes in DEX-treated and DEX+CHX-treated plants are reported as fold increases compared with mock treatment.
(c) Anthocyanin gene expression in *ttg1* mutant seedlings expressing *TTG1::GR*. Expression changes in DEX-treated and DEX+CHX-treated plants are reported as fold increases compared to mock treatment.
(d) Anthocyanin gene expression in *gl3* and *gl3 egl3* mutant seedlings expressing *GL3::GR*. Expression changes are reported as fold increases compared to mock-treated *gl3 egl3* transgenic seedlings germinated on medium containing 3% sucrose.

quantitative PCR approach. We compared expression levels in Col versus Myb RNAi seedlings of the early genes *PAL1* and *CHS*, the late genes *DFR* and *LDOX*, a glutathione *S*-transferase (*GST12*), and *flavonoid 3′-hydroxylase* (*F3′H*) and the glycosyl transferase gene, *UGT75C1*. *UGT75C1* was of interest as a possible late pathway gene because glycosylation of anthocyanins during biosynthesis is thought to determine variations in pigment intensity and color (Kroon *et al.*, 1994; Yamazaki *et al.*, 1999). Moreover, it was recently shown that *UGT75C1* is upregulated by *PAP1* overexpression (Tohge *et al.*, 2005a). *F3′H* and *GST12* were of interest as pathway genes whose regulation had never been investigated in *ttg1* or TTG1-dependent Myb and bHLH loss-of-function mutants in Arabidopsis (Kitamura *et al.*, 2004; Schoenbohm *et al.*, 2000).

Our results indicate that expression of *PAL1* and *CHS* remain unchanged in seedlings knocked down for Myb expression, compared with Col seedlings (Figure 6a). *PAL1* and *CHS* were also expressed to wild-type levels in the *pap1* insertion line pst16228 from the Riken Biological Resource Center (Figure 6a) (Kuromori *et al.*, 2004; Teng *et al.*, 2005). However, reduced Myb expression did result in reductions in expression for the late genes *DFR*, *LDOX* and *GST12* (Figure 6a), as well as for *F3′H* and *UGT75C1*. Together, these results demonstrate that the TTG1-dependent Mybs of Arabidopsis regulate transcription of genes comprising the late steps of the anthocyanin pathway, and that *F3′H* and *UGT75C1* are coordinately regulated with the late genes.

PAP1 *expression overlaps bHLH expression in young seedlings*

We created ProPAP1:GUS, ProPAP2:GUS, ProMyb113:GUS and ProMyb114:GUS lines to determine Myb expression patterns in developing seedlings. Overall results from the analysis of these reporter lines were similar to the RT-PCR results. *PAP1* is predominantly expressed over the other three Myb genes in young seedlings (very little to no expression was detected in seedlings of the ProPAP2:GUS, ProMyb113:GUS and ProMyb114:GUS lines even after overnight staining).

The *PAP1* expression pattern as revealed by the ProPAP1:GUS lines overlaps well with bHLH expression patterns both spatially and temporally (compare Figure 3d–f with Figure 4f–h) (Baudry *et al.*, 2006), except that *PAP1* is not expressed in the root (Figure 4f). *PAP1* is well expressed in the hypocotyls and cotyledons of very young seedlings (2–3 days old) but quickly fades over the following days and is ultimately restricted to young emerging leaf tissue/primordia of 5- to 7-day-old seedlings. This pattern, like the bHLH patterns, is consistent with the developmental expression pattern of anthocyanins in young seedlings, peaking in the hypocotyls and cotyledons of 3- to 5-day-old seedlings but quickly fading from these tissues over the following few days before reappearing in young emerging leaves. The expression patterns given in the AtGenExpress database (Schmid *et al.*, 2005) for PAP1 and EGL3 verify the promoter:reporter results in young seedlings, namely expression peaks in cotyledons and hypocotyls. Thus the overall dynamic expression pattern of *PAP1* is consistent with the expression patterns of bHLH anthocyanin regulators *GL3* and *EGL3* (this study) as well as pigment production in young seedlings (Kubasek *et al.*, 1992).

PAP1 *single mutant seedlings show loss of anthocyanins*

Because *PAP1* is the predominant Myb expressed in seedlings, we speculated that a loss of *PAP1* function only might result in an obvious anthocyanin deficiency, while loss of *PAP2* would not. To this end, we created *PAP1*- and *PAP2*-specific RNAi lines and visually inspected seedlings germinated on 3% sucrose for anthocyanin deficiencies. Col seedlings specifically silenced for *PAP1* showed strong reductions in pigment production compared to Col wild-type controls (Figure 4d and Figure S2). By contrast, Col seedlings harboring a *PAP2*-specific silencing construct appeared essentially wild-type for anthocyanin content (Figure S2). In the course of this work, *pap1* insertion mutant seedlings (pst16228) in the No-0 ecotype from the Riken Biological Resource Center (Kuromori *et al.*, 2004) were shown to be anthocyanin-deficient (Teng *et al.*, 2005). We also found that pst16228 seedlings displayed an anthocyanin-deficient phenotype similar to the *PAP1* RNAi line (Figure 4e), further demonstrating that a loss of *PAP1* function alone is sufficient for loss of anthocyanins in young seedlings.

TTG1-dependent bHLH transcription factors of Arabidopsis regulate 'late' flavonoid pathway genes

It has been shown previously that *ttg1* and bHLH multiple mutant seedlings have reduced expression of the late flavonoid pathway genes *DFR* and *LDOX*, but not the early genes *CHS* and *PAL* (Pelletier *et al.*, 1997; Winkel-Shirley *et al.*, 1995; Zhang *et al.*, 2003). To better characterize the extent of pathway regulation by Arabidopsis bHLHs, and for comparison with pathway regulation by the Arabidopsis Mybs studied here, we compared expression levels of various anthocyanin structural genes in bHLH mutants, *ttg1* mutants and the wild-type by quantitative PCR. Because *Myc1* has been shown to interact with TTG1-dependent Mybs and is paralogous with *GL3*, *EGL3* and *TT8* (Zimmermann *et al.*, 2004), we created a quadruple TTG1-dependent bHLH mutant to represent the strongest bHLH loss-of-function line (although preliminary investigations indicate little or no role for *Myc1* in anthocyanin biosynthesis).

F3′H, *LDOX*, *GST12* and *UGT75C1* are downregulated in bHLH multiple mutant seedlings as well as in *ttg1* seedlings (Figure 6a). *PAL1* and *CHS* expression did not differ compared to wild-type in any of the mutants evaluated (including the bHLH quadruple mutant; Figure 6a), indicating that early gene expression is not affected by a severe loss in bHLH function. These results indicate that the bHLH transcription factors, like the TTG1 protein, are regulators of late-acting flavonoid pathway genes, and that *F3′H* and *UGT75C1* are coordinately regulated with these genes.

GL3:GR and TTG1:GR studies in seedlings reveal direct regulation of anthocyanin genes by the TTG1-dependent complex

To better define the flavonoid biosynthetic gene set regulated by TTG1/bHLH/Myb transcriptional complexes, GL3 and TTG1 were fused to the glucocorticoid receptor (Baudry *et al.*, 2006; Lloyd *et al.*, 1994) and placed under the control of the CaMV 35S promoter. These fusion proteins, constitutively expressed in plants, are inactive without dexamethasone (DEX) treatment. Addition of DEX allows the chimeric transcription factors to regulate their primary and secondary targets. Simultaneous treatment with DEX and cycloheximide (CHX) blocks *de novo* protein synthesis, allowing the identification of primary targets (Sablowski and Meyerowitz, 1998). Recovery of the *gl3 egl3* double mutant and the *ttg1* mutant by GL3:GR and TTG1:GR fusions, respectively, indicated that these chimeric proteins were functional (Figure S3) (Baudry *et al.*, 2004).

35S:GL3:GR transgenic *gl3 egl3* seedlings were assayed by quantitative PCR for gene expression changes after DEX induction for a range of flavonoid biosynthetic and regulatory genes. This experiment identified the structural genes *F3′H*, *DFR* and *LDOX*, and the regulatory genes *TT8* and *PAP2*, as upregulated by GL3 overexpression (Figure 6b). The experiment was repeated with a DEX plus CHX treatment to determine whether any of these loci are directly regulated by GL3. All three structural genes were again upregulated in response to GL3::GR induction (Figure 6b). Also, TT8 was directly upregulated by GL3, consistent with the findings of Baudry *et al.* (2006) identifying TT8 as a direct target of TT2 and TTG1. Interestingly, PAP2 appears to be a secondary target of GL3.

35S:TTG1:GR transgenic *ttg1* seedlings were similarly analyzed for expression changes in flavonoid biosynthetic and regulatory genes after DEX induction. DEX induction of TTG1:GR resulted in upregulation of *F3′H*, *DFR*, *LDOX* and *TT8* in transgenic *ttg1* seedlings (Figure 6c). Induction with DEX plus CHX identified *DFR*, *LDOX* and (as previously shown by Baudry *et al.*, 2006) *TT8* as direct targets of TTG1. Although identified as a direct target of GL3, *F3′H* appears to be a secondary target of TTG1.

GL3 *and* EGL3 *differentially regulate anthocyanin genes*

We next determined the effects on gene regulation of DEX induction in *35S:GL3:GR* transgenic *gl3* single versus transgenic *gl3 egl3* double mutants. Genes tested included *TTG1*, *TT8*, *F3′H*, *DFR* and *LDOX*, and expression changes are reported as fold increases relative to mock-treated *35S:GL3:GR* transgenic *gl3 egl3* seedlings.

TT8 is expressed to higher levels in non-induced *gl3* mutant seedlings than in DEX-induced *gl3 egl3* double mutant seedlings, suggesting that *EGL3* contributes more to *TT8* regulation than *GL3* even when *GL3* is overexpressed (Figure 6d). Moreover, *TT8* expression is highest in DEX-induced *gl3* single mutant seedlings (to about twice the levels in DEX-induced double mutant and non-induced single mutant seedlings), suggesting that both *GL3* and *EGL3* can additively contribute to the regulation of *TT8*. Similarly, *F3′H* reaches its highest expression levels in DEX-induced *gl3* single mutant seedlings, with only about half as much expression observed in the DEX-induced double mutant and non-induced *gl3* single mutant (Figure 6d). Again, this suggests additive effects by these bHLH proteins for *F3′H* regulation, with perhaps a greater contribution by *EGL3* considering that *GL3* is driven by a strong promoter.

Interestingly, this experiment suggests a different mechanism for the regulation of *DFR* and *LDOX* compared with *TT8* and *F3′H*. For either *DFR* or *LDOX*, the highest levels of expression are achieved in both non-induced and DEX-induced *gl3* single mutant seedlings (Figure 6d); overexpressing *GL3* has no effect as long as the wild-type *EGL3*

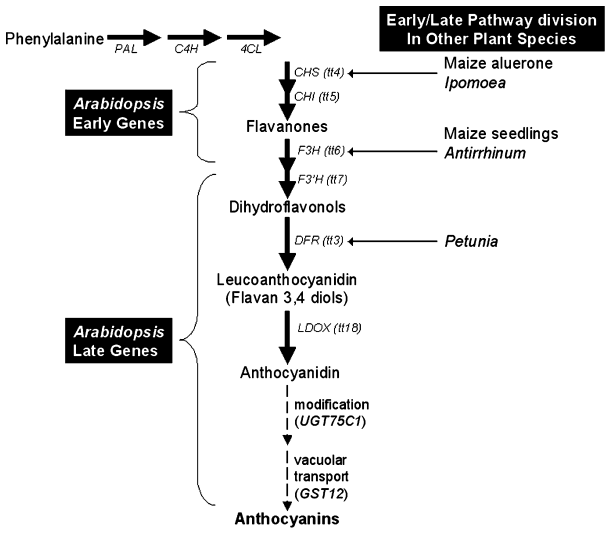

Figure 7. The branch of the phenylpropanoid biosynthetic pathway yielding anthocyanins.
Brackets indicate the early and late divisions of the flavonoid pathway in Arabidopsis, with the late genes being those regulated by Myb, bHLH and WD-repeat proteins. Thin horizontal arrows indicate the first structural gene in the pathway that is regulated by Myb/bHLH/WD-repeat transcriptional complexes in other plant species.

locus is present. This suggests a greatly reduced role for *GL3* in *DFR* and *LDOX* regulation, consistent with the observation that fold increases in DEX-induced over non-induced double mutant seedlings for *DFR* and *LDOX* are only about half as high as the increases observed for *TT8* and *F3′H* in the double bHLH mutant. This finding also provides molecular verification for phenotypic differences between *gl3*, *egl3* and *gl3 egl3* mutant seedlings, which similarly suggests a greater role for *EGL3* in anthocyanin biosynthesis (Figure S2) (Zhang *et al.*, 2003).

Discussion

The TTG1-dependent Mybs and bHLHs of Arabidopsis regulate late anthocyanin pathway genes, beginning with F3′H

Overexpression of *PAP* Mybs suggested that these genes may normally regulate transcription at all stages of the phenylpropanoid pathway (Figure 7) (Borevitz *et al.*, 2000; Tohge *et al.*, 2005a). We addressed whether observations in *PAP1* overexpressors accurately reflect native phenylpropanoid pathway regulation by *PAP* Mybs in Arabidopsis. If it does, this is contrary to pathway regulation by orthologous Mybs in other plant species where the anthocyanin pathway has been examined. In all cases, only a subset of the phenylpropanoid pathway genes are affected by loss-of-function mutations in Myb or bHLH regulatory loci, although the particular set of genes may differ between species and between tissues within a species (Figure 7; Borovsky *et al.*, 2004; Brugliera *et al.*, 1999; Deboo *et al.*, 1995; Martin *et al.*, 1991; Morita *et al.*, 2006; Quattrocchio *et al.*, 1993; Schwinn *et al.*, 2006; Taylor and Briggs, 1990). Thus a general trend may be noted across the plant species studied, in which a WD-repeat/Myb/bHLH transcriptional complex predominantly regulates late genes over early genes, with the particular pathway steps comprising the late and early sets and the degree of regulation of the sets differing between species and tissues (Figure 7).

It should be noted that, where examined, genes of the general phenylpropanoid pathway before *CHS* (such as *PAL*, *C4H* and *4CL*) are never downregulated in WD-repeat or WD-repeat-dependent regulatory mutants (Martin *et al.*, 1991; Nesi *et al.*, 2000; Quattrocchio *et al.*, 1993; Winkel-Shirley *et al.*, 1995). However, it has been observed that *PAP1* overexpression results in upregulation of genes across the entire phenylpropanoid pathway, including genes such as *PAL1* (Borevitz *et al.*, 2000; Tohge *et al.*, 2005a). Without analysis of loss-of-function mutants, however, the question remained as to whether the Arabidopsis Mybs are such broad regulators of the phenylpropanoid pathway. Based on the observations made in the Arabidopsis multiple Myb knockdown and *pap1* insertion mutants in this study, it appears that flavonoid pathway regulation by Arabidopsis TTG1-dependent Mybs is consistent with pathway regulation by orthologous Mybs in other plant species. Analysis of Myb mutant seedlings indicates that, in Arabidopsis, these Mybs are regulators of late anthocyanin structural genes (beginning at *F3′H*).

Careful inspection of the literature reveals little or in some cases no upregulation of early phenylpropanoid pathway genes by PAP1 overexpression, particularly when compared to the large increases in late gene expression. Microarray experiments have reliably shown strong upregulation of late genes by *PAP1* overexpression, but only weak upregulation of early genes that is not consistently observed among experimental treatments or duplicates (Tohge *et al.*, 2005a). The data presented in this study demonstrate a similar trend, with Myb overexpression resulting in slight or no increases in early gene expression. However, we found expression of late genes in Myb overexpressing seedlings at about 300% of the wild-type levels. In the study by Borevitz *et al.* (2000), Northern analysis showed early and late structural gene increases in 6-week-old *pap1-D* plants, but the degree of upregulation was difficult to assess as no bands on the RNA gel blot were detected for *PAL1*, *CHS* and *DFR* when wild-type total RNA preparations were probed.

In excess, PAP1 and/or TTG1-dependent transcriptional complexes may have the ability to directly regulate early flavonoid genes. Alternatively, late anthocyanin pathway genes may be direct targets of Myb/bHLH/TTG1 complexes in Arabidopsis, with the early gene expression changes observed in *pap1-D* being due to secondary effects such as a metabolite feedback phenomenon resulting from strong upregulation of the late pathway genes and increased flux through the flavonoid pathway (Jorgensen *et al.*, 2005); at the protein level, it has been shown that decreasing the activity of the later part of the pathway, as in *tt3* and *ttg1* mutants, can alter the levels of early flavonoid enzymes (Pelletier *et al.*, 1999). The fact that early gene expression is not responsive to *GL3* or *TTG1* overexpression nor reduced in TTG1-dependent Myb mutants, together with the observation that early gene expression in response to *Myb* overexpression does not seem to reliably increase in all instances and organs examined that overaccumulate pigment, suggests that these transcription factors do not regulate early flavonoid biosynthetic genes. Also, reductions in early gene expression were not detected in *pap1* insertion mutant seedlings even when grown on 3% sucrose (this study), a treatment previously shown to induce *PAP1* transcription in wild-type seedlings by over fourfold compared to 1% sucrose (Solfanelli *et al.*, 2006). Thus the biological/physiological relevance of *PAP1* as an early gene regulator is questionable.

Some TTG1-independent Mybs have been demonstrated to be regulators of the early steps of the flavonoid pathway, such as the flavonol-specific activators of phenylpropanoid biosynthesis *Myb11*, *Myb12* and *Myb111* (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007). Overexpression of these *Myb*

genes in co-transfected Arabidopsis protoplasts activates promoters of early genes (*CHS*, *CHI*, *F3H* and *FLS*) but not of late genes (*F3′H* and *DFR*). Target gene expression analysis in *myb11 myb12 myb111* triple mutant seedlings generally indicated downregulation of early genes but not late genes. Although the *F3′H* promoter was unresponsive to *Myb11*, *Myb12* and *Myb111* in co-transfection assays, *F3′H* expression did appear to be downregulated in microarray-based expression analysis of the *myb11 myb12 myb111* triple mutant (Stracke *et al.*, 2007). The authors suggest that this could be due to metabolic feedback, or, alternatively, F3′H is a target of these Mybs. Interestingly, while expression of other late genes such as *DFR* and *LDOX* is nearly off or is undetectable in ttg1 and strong bHLH loss-of-function mutants (Winkel-Shirley *et al.*, 1995; Pelletier *et al.*, 1997; Zhang *et al.*, 2003; this study), we have measured *F3′H* expression that is as high as 30% of wild-type in strong bHLH loss-of-function multiple mutant seedlings. This suggests that *F3′H* may be dually regulated by TTG1-dependent and -independent mechanisms, consistent with its requirement in the production of both quercetin-based flavonols and cyanidin-based anthocyanins.

Curiously, *myb11 myb12 myb111* triple mutant seedlings still accumulate wild-type levels of anthocyanins (Stracke *et al.*, 2007). This suggests residual expression levels of early genes such as *CHS* that are required for anthocyanin biosynthesis. A likely mechanism for residual early gene expression in *myb11 myb12 myb111* triple mutant seedlings might be via light activation of flavonoid biosynthetic genes during photomorphogenesis; light-dependent *CHS* expression is mediated by regulators (such as HY5 and CIP7) that can bind to a minimal light-responsive region of the *CHS* promoter (Ang *et al.*, 1998; Yamamoto *et al.*, 1998).

Thus there generally seems to be a division of labor among regulators of the flavonoid pathway, with TTG1-dependent transcriptional complexes regulating the production of anthocyanins by regulating late steps of the pathway, and leaving the synthesis of flavonols to a class of co-activator-independent Mybs (such as *Myb11*, *Myb12* and *Myb111*) that regulate early biosynthetic genes. The light-dependent regulation of flavonoid pathway genes by regulators such as HY5 and CIP7 may represent a third distinct regulatory mechanism, although its relation to the other mechanisms is not entirely clear.

PAP1, PAP2, Myb113 *and* Myb114 *do not regulate the proanthocyanidin branch of the flavonoid pathway*

Previously, the *transparent testa2* (*TT2*) Myb gene of Arabidopsis was identified as necessary for PA production, as *tt2* mutants produce yellow seeds and are downregulated for genes specifically required for this branch of the flavonoid pathway, such as *BAN* and *TT12* (Nesi *et al.*, 2001). Based on the observation of transparent testas resulting from overexpression of a dominant-negative PAP1 chimera, it has been suggested that *PAP1* is a positive regulator of PA production in the inner endothelial layer of developing seed coats (Hiratsu *et al.*, 2003; Matsui *et al.*, 2004). However, neither Col Myb RNAi mutants nor the *PAP1* insertion line produce seeds with transparent testas, suggesting that the *PAP* Mybs have no role in PA production. In addition, TT2 is predominantly expressed in developing wild-type siliques (Matsui *et al.*, 2004). Also, *PAP1* overexpression results in a loss of PAs and a gain of anthocyanins in the seed coat (Tohge *et al.*, 2005b). Finally, *PAP1* overexpression does not upregulate key structural genes such as *BAN*, *TT12* and *AHA10* in the pro-anthocyanidin branch of the flavonoid pathway (Sharma and Dixon, 2005; Tohge *et al.*, 2005a), consistent with the observation that *PAP1* overexpression causes a loss of PAs and a gain of anthocyanins in the seed coat. It is therefore unlikely that *PAP1*, *PAP2*, *Myb113* and *Myb114* play a regulatory role in PA production, and more likely that the transparent testa phenotype caused by the ubiquitously expressed dominant-negative PAP1 chimera is due to suppression of structural and regulatory targets, such as *DFR*, *LDOX* or *TT8*, during seed coat differentiation.

Interesting questions remain, however, as to the distinct functions of the four Arabidopsis anthocyanin Myb regulators. It is clear that *PAP1* is largely sufficient for anthocyanin production in seedlings, but the relative contributions of *PAP2*, *Myb113* and *Myb114* later in plant development remain unknown. Based on the regulation of anthocyanin production in other genera, it has been proposed that gene duplication and subsequent functional diversification of Myb loci is a primary cause of natural variation of this trait. Three *Antirrhinum* Mybs were recently shown to differentially regulate structural genes of the anthocyanin pathway, and striking differences in floral pigment patterns and intensity observed in six *Antirrhinum* species were attributable to variations in the activity of this Myb set (Schwinn *et al.*, 2006). Functional diversification has been genetically demonstrated for *GL3* and *EGL3* in trichome and seed coat development, as well as in anthocyanin biosynthesis (Zhang *et al.*, 2003). Interestingly, GL3 and EGL3 bHLH proteins contribute differentially to anthocyanin target gene activation (this study), molecularly verifying functional diversification between these regulators that could possibly contribute to phenotypic variation. Given this context, it will be interesting to determine the full nature of any functional diversification among the Arabidopsis Myb set.

Experimental procedures

Arabidopsis accessions

All lines used in this study are in the Columbia (Col), Landsberg *erecta* (L*er*) or No-0 backgrounds as noted. The *egl3-1* (L*er* background) and *gl3 egl3 tt8* bHLH mutants have been described

previously (Zhang *et al.*, 2003). *pap1-D* plants (Col background) (Borevitz *et al.*, 2000) were kindly provided by J. Borevitz, University of Chicago. The *egl3* insertion line SALK 019114, the *tt8* insertion line SALK 030966 and the *myc1* insertion line SALK 057388 (Col background) were acquired from the Arabidopsis Biological Resource Center (Alonso *et al.*, 2003). The GL3 insertion line WiscDsLox412G05 (Col background) was obtained from the Arabidopsis Knockout Facility at the University of Wisconsin-Madison (Sussman *et al.*, 2000). The *pap1* insertion line pst16228 (No-0 background) was obtained from the Riken Biological Resource Center (Kuromori *et al.*, 2004). Myb RNAi plants (Col-0 background) express a small silencing RNA hairpin targeting *PAP1*, *PAP2*, *Myb113* and *Myb114* transcripts. *PAP1* RNAi plants (Col background) express a small silencing RNA hairpin targeting the *PAP1* message. The transgenic line expressing *35S:TTG1:GR* in *Ler* (Baudry *et al.*, 2006) was generously provided by L. Lepiniec, Institut Jean-Pierre Bourgin. Plants were grown in soil at 21°C in continuous white light, or seedlings were grown on germination medium containing 3% sucrose as described by Zhang *et al.* (2003).

Plasmid construction

pGWQM. A 24 nt sequence that is perfectly conserved among *PAP1*, *PAP2*, *Myb113* and *Myb114* (Figure 1) was used to design a hairpin and 4 bp loop RNAi silencing construct targeting all four transcripts. The sense and antisense oligos 5′-attB1-taagtatggagaaggcaaatggcaCGAAtgccatttgccttctccatactta-attB2–3′ and 5′-attB1-attcatacctcttccgtttaccgtGCTTacggtaaacggaagaggtatgaat-attB2–3′, respectively, which are flanked by Gateway recombination sites attB1 and attB2 (Invitrogen, http://www.invitrogen.com/), were annealed, and the double-stranded form was recombined into pDONOR222 (Invitrogen).

pLBJ17-rfb. pLBJ17 (Payne *et al.*, 2000) was converted into a Gateway destination vector as follows: *Xho*I and *Sst*I sites were introduced on the flanks of the rfb cassette B by PCR, and the product was cloned using TOPO cloning (Invitrogen). The rfb B *Xho*I/*Sst*I fragment was ligated into *Xho*I/*Sst*I-cut pLBJ17, producing pLBJ17-rfb.

p35QM. pGWQM was used in a Gateway LR recombination reaction to subclone the Myb silencing fragment into the plant overexpression vector pLBJ17-rfb. *pGWQPAP1.* A 22 nt coding sequence specific to *PAP1* was used as the basis for an RNAi silencing construct targeting only the *PAP1* message. The sense and antisense oligos 5′-attB1-accgtgttgtaagataaagatgCGAAcatctttatcttacaacacggt-attB2–3′ and 5′-attB1-tggcacaacattctatttctacGCTTgtagaaatagaatgttgtgcca-attB2–3′, respectively, which are flanked by Gateway recombination sites, were annealed, and the double-stranded form was recombined into pDONOR222 (Invitrogen).

p35QPAP1. pGWQPAP1 was used in a Gateway LR recombination reaction to subclone the Myb silencing fragment into the plant overexpression vector pK7WG2 (Karimi *et al.*, 2002). *pGWQPAP2.* A 22 nt coding sequence specific to *PAP2* was used as the basis for an RNAi silencing construct targeting only the *PAP2* message. The sense and antisense oligos 5′-attB1-gtcttcgtgttgtaagtctaaaCGAAattagacttacaacacgaagac-attB2–3′ and 5′-attB1-cagaagcacaacattcagatttGCTTaaatctgaatgttgtgcttctg-attB2–3′, respectively, which are flanked by Gateway recombination sites, were annealed, and the double-stranded form was recombined into pDONOR222 (Invitrogen).

p35QPAP2. pGWQPAP2 was used in a Gateway LR recombination reaction to subclone the Myb silencing fragment into the plant overexpression vector pH7WG2 (Karimi *et al.*, 2002). *p35Myb113.* The 971 bp Col *Myb113* genomic locus from the start codon to the stop codon was amplified using the forward primer 5′-attB1-atgggcgaatcacccaaagggttg-3′ and the reverse primer 5′-attB2-ctaattcagttctaaagtctcttc-3′, and recombined into pDONOR222 to produce pGWMyb113. *Myb113* was recombined from pGWMyb113 into pH7WG2 (Karimi *et al.*, 2002).

p35Myb114C. The 1078 bp truncated Col *Myb114* genomic locus from the start codon to the (internal) stop codon was amplified using the forward primer 5′-attB1-atggagggttcgtccaaagggttg-3′ and the reverse primer 5′-attB2-ctaaaaaatatcgagtttttggg-3′, and recombined into pDONOR222 to produce pGWMyb114C. The Col *Myb114* gene was recombined from pGWMyb114C into the pLBJ17-rfb plant overexpression vector.

p35Myb114L. The 1551 bp L*er Myb114* genomic locus from the start codon to the stop codon was amplified using the forward primer 5′-attB1-atggagggttcgtccaaagggttg-3′ and the reverse primer 5′-attB2-ctaaaaaatatcgagtttttggg-3′, and recombined into pDONOR222 to produce pGWMyb114L. The L*er Myb114* gene was recombined from pGWMyb114L into pH7WG2 (Karimi *et al.*, 2002).

pPAP1pG. An approximately 2.2 kb fragment upstream of the *PAP1* start codon was amplified from Col genomic DNA using the forward primer 5′-attB1-cattgcgttttctgggattcg-3′ and the reverse primer 5′-attB2-ggaacaaagatagatacgtaa-3′, and recombined into pDONOR222 to produce pGWPAP1p. pGWPAP1p was then used to recombine the *PAP1* promoter fragment into pKGWFS7 GUS vector (Karimi *et al.*, 2002).

ProGL3:GUS and ProEGL3:GUS constructs have been previously described (Zhang *et al.*, 2003).

pB35GL3GR. pD2L-2 (Payne *et al.*, 2000) was modified for subsequent cloning (as described by Bernhardt *et al.*, 2005) to provide a *GL3* genomic fragment. The *GR* coding region was amplified from pRGR (Lloyd *et al.*, 1994) using the forward primer 5′-gcggccgcgggagctcgggaggaggagaagctcgaaaaacaaag-3′ and the reverse primer 5′-tctagagtcgactcatttttgatgaaacag-3′, *Sac*I/*Sal*I-digested and ligated to the *Sac*I/*Sal*I fragment of the modified pD2L-2 vector, resulting in an in-frame fusion of the *GR* coding fragment to the 3′ end of *GL3* (pGL3GR).

The GL3:GR fusion coding sequence was amplified from pGL3GR using the forward primer 5′-attB1-atggctaccggacaaaacag-3′ and the reverse primer 5′-attB2-ctgtcaattttaactaag-3′, and recombined into pDONOR222 to produce pGWGL3GR. pGWGL3GR was then used to recombine the *GL3:GR* fusion into pB7WG2 (Karimi *et al.*, 2002) to produce pB35GL3GR.

Flavonoid biosynthetic gene expression in bHLH and Myb mutants

Light upon extension (LUX) RT-PCR was performed as described previously (Zhang *et al.*, 2003). Seedlings were grown on germination medium containing 3% sucrose at 21°C under continuous white light. Five reactions were run per target in three independent experiments with varying RNA or cDNA preparations (from 4-day-old seedlings) with consistent results. The results of a representative experiment are presented.

Additionally, a previously described tailed-primer strategy (Boutin-Ganache *et al.*, 2001) was used for PCR, in which the target gene-specific forward primer is 5′-tailed with a generic sequence and used with a 10-fold excess of a LUX FAM fluorophore-labeled forward primer (Invitrogen) of the same sequence as the tail. The tailing scheme was adopted because one fluorophore-labeled forward primer can be used with any unlabeled target gene primer pair instead of independently labeling all target gene-specific forward primers for RT-PCR experiments. The tail sequence primer used was 5′-CTGCTTGCCGAATATCATGGTG-3′and the LUX tail

Table 1 Primer pairs (Invitrogen) which were used for target gene amplification

PAL1-RT-F: 5′TCCTCTCTCCTACATCGCcG 3′
PAL1-RT-R: 5′GCTTCACCGTTGGGACCAGTAG 3′
CHI(TT5)-RT-F: 5′CTTCGCTCTCTCCCCTACcG 3′
CHI(TT5)-RT-R: 5′GATCACAGCGATCCCGGTTT 3′
F3H-RT-F: 5′GACCAAGTCGGTGGATTACAAGC 3′
F3H-RT-R: 5′tTCCTTCAACAGGCTGAACcG 3′
F3′H-RT-F: 5′ACCCGAGAGATTCTTACCcG 3′
F3′H-RT-R: 5′TCGAAATCGCTTCCTTTCACAT 3′
DFR(TT3)-RT-F: 5′CATGTGTTAAGGCAAAGACcG 3′
DFR(TT3)-RT-R: 5′ATTAACGGTTCCGGCAGATGAA 3′
LDOX-RT-F: 5′GGAGCGTGTCAAGAAAGCcG 3′
LDOX-RT-R: 5′TCTTTCCAGTGGCTTGATCGTTT 3′
TT4-RT-F(CHS): 5′TTACCTTCCGTGGTCCCTCTG 3′
TT4-RT-R(CHS): 5′CACTGAAAAGAGCCTGACcG 3′
EGL3-RT-F: 5′CACTAGACGAGCTTCCGCcG 3′
EGL3-RT-R: 5′AGTACCACTCGGTGTCGGTGAG 3′
TT8-RT-F: 5′GTCCTCAACAACGGGTCTTGG 3′
TT8-RT-R: 5′TTCCTCGTCTTTATTGCACcG 3′
GST-RT-F: 5′TCAAGAATGCAGCAAAGGAAGA 3′
GST-RT-R: 5′AACCAATGTCGATGAACCCG 3′
FLS1-RT-F: 5′GCATAGGACGACGGTGGATAAA 3′
FLS1-RT-R: 5′GGGAGGCTCCAAGAAAACcG 3′
TTG1-RT-F: 5′TCCTCGAAGATTACAACAACCG 3′
TTG1-RT-R: 5′CGGGAGAGGCTTAACGGTCAT 3′
PAP1-RT-F: 5′AGAAGGCAAATGGCACCAAGTT 3′
PAP1-RT-R: 5′TCTACAACTTTTCCTGCACCG 3′
PAP2-RT-F: 5′ACCAAGAAGCTGATGCGATTG 3′
PAP2-RT-R: 5′AACGTCAAACGCCAAAGTGG 3′

primer was 5′-CACCATCTGCTTGCCGAATATCATGG**T**G-3′ (FAM-labeled nucleotide in bold).

The following primer pairs (Sigma-Genosys, http://www.sigma-aldrich.com/) were used for target gene amplification: *LDOX* (*TT18*) tailed forward (5′-CTGCTTGCCGAATATCATGGTGGGTTAGGATTT-CTTGGGCTGTG-3′) and *LDOX* (*TT18*) reverse (5′-CATCTCCGGCAA-CGGCTTA-3′); *GST12* (*TT19*) tailed forward (5′-CTGCTTGCCGAA-TATCATGGTGCGTCAGCCATTTGGTCAAGTTC-3′) and *GST12* (*TT19*) reverse (5′-GCGATGGCTCGTGATTCAAA-3′); *PAL1* tailed forward (5′-CTGCTTGCCGAATATCATGGTGCGAGCGTGAGATTA-ACTCCG-3′) and *PAL1* reverse (5′-CGCCTTGTTCCTCGAAACATC-3′); *UGT75C1* tailed forward (5′-CTGCTTGCCGAATATCATGGTGA-GAGTTTAGAGAGTGGTGTTCCG-3′) and *UGT75C1* reverse (5′-CCT-CCACGTATCCTCCACAAGC-3′). Primer sequences for *DFR* (*TT3*) and *CHS* (*TT4*) have been described previously (Zhang *et al.*, 2003).

Flavonoid biosynthetic gene expression in GL3::GR and TTG1::GR transgenics

Seedlings were grown on germination medium containing 3% sucrose at 21°C under continuous white light. Four-day-old seedlings were treated with 20 μM dexamethasone or mock-treated with 0.001% ethanol for 4 h, then washed with water and frozen in liquid nitrogen. Total RNA was prepared using the Qiagen plant RNeasy mini kit (http://www.qiagen.com/). Aliquots (4 μg) of RNA were used in 20 μl reverse transcription reactions containing 250 nM actin and target gene-specific reverse primers. Parallel PCR reactions using reverse transcription reactions as template were set up in a total volume of 25 μl with 12.5 μl 2 × SuperPower Syber mixture (ABI; http://www.appliedbiosystems.com), and run on a spectrofluorometric thermal cycler (ABI 7900HT). For each target, five PCR reactions containing 400 nM primer and 3 μl first-strand cDNA template were performed alongside four actin control PCR reactions containing 200 nM actin primers and 1 μl first-strand actin cDNA. The PCR cycling parameters used were as follows: 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The comparative cycle threshold method was used to analyze the results of quantitative PCR (user bulletin 2, ABI PRISM sequence detection system). This experiment was performed twice for each target with consistent results. The results of a representative experiment are presented. The primer pairs used for target gene amplification are shown in Table 1.

Standard RT-PCR

RNA and first-strand cDNA were prepared from Col wild-type, *pap1-D* and Col myb RNAi 4-day-old seedlings using the same method as for the LUX RT-PCR experiments described previously by Zhang *et al.* (2003). PCR reactions (50 μl) were performed using 1 μl cDNA, 20 nM *APRT* start–stop loading control primers and 200 nM start–stop target primers (*PAP1*, *PAP2*, *Myb113* or *Myb114*). PCR products were run on 1.7% agarose gels. For *PAP1*, 10 μl of PCR product were obtained after 35 PCR cycles had been run. For *Myb113* and *Myb114*, 15 μl of PCR product were obtained after 40 PCR cycles had been run. *PAP2* could only be amplified if *APRT* control primers were omitted from reactions, and 15 μl of PCR product were obtained after 40 PCR cycles had been run. The approximate product sizes are as follows: 750 bp for *PAP1*, *PAP2* and *Myb113*, 420 bp for *Myb114*, and 500 bp for *APRT*. Several reactions were run for each target using varying RNA or cDNA preparations with consistent results. The results from a representative experiment are presented.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Overexpression of *Myb114.*

Figure S2. Relative anthocyanin content.

Figure S3. Recovery of anthocyanins in *gl3 egl3* seedlings expressing GL3::GR.

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