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► **To cite this version:**

Christian Dubos, Jose-Sabrina Le Gourrierc, Antoine Baudry, Gunnar Huet, Elodie Lanet, et al.. MYBL2 is a new regulator of flavonoid biosynthesis in Arabidopsis thaliana. Plant Journal, 2008, 55, pp.940-953. 10.1111/j.1365-313X.2008.03564.x . hal-01203970

HAL Id: hal-01203970

<https://hal.science/hal-01203970>

Submitted on 31 May 2020

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MYBL2 is a new regulator of flavonoid biosynthesis in *Arabidopsis thaliana*

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Received 8 February 2008; revised 23 April 2008; accepted 7 May 2008; published online 11 July 2008.

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Summary

In *Arabidopsis thaliana*, several MYB and basic helix-loop-helix (BHLH) proteins form ternary complexes with TTG1 (WD-Repeats) and regulate the transcription of genes involved in anthocyanin and proanthocyanidin (PA) biosynthesis. Similar MYB-BHLH-WDR (MBW) complexes control epidermal patterning and cell fates. A family of small MYB proteins (R3-MYB) has been shown to play an important role in the regulation of epidermal cell fates, acting as inhibitors of the MBW complexes. However, so far none of these small MYB proteins have been demonstrated to regulate flavonoid biosynthesis. The genetic and molecular analyses presented here demonstrated that *Arabidopsis MYBL2*, which encodes a R3-MYB-related protein, is involved in the regulation of flavonoid biosynthesis. The loss of *MYBL2* activity in the seedlings of two independent T-DNA insertion mutants led to a dramatic increase in the accumulation of anthocyanin. In addition, overexpression of *MYBL2* in seeds inhibited the biosynthesis of PAs. These changes in flavonoid content correlate well with the increased level of mRNA of several structural and regulatory anthocyanin biosynthesis genes. Interestingly, transient expression analyses in *A. thaliana* cells suggested that *MYBL2* interacts with MBW complexes *in planta* and directly modulates the expression of flavonoid target genes. These results are fully consistent with the molecular interaction of *MYBL2* with BHLH proteins observed in yeast. Finally, *MYBL2* expression studies, including its inhibition by light-induced stress, allowed us to hypothesise a physiological role for *MYBL2*. Taken together, these results bring new insights into the transcriptional regulation of flavonoid biosynthesis and provide new clues and tools for further investigation of its developmental and environmental regulation.

Keywords: flavonoid, transcription, network, MYB, bHLH, TTG1.

Introduction

Flavonoids are secondary metabolites that fulfil important biological functions and provide useful metabolic and genetic models for plant research, including the analysis of transcriptional regulation of gene expression (Koes *et al.*, 2005; Lepiniec *et al.*, 2006; Peer and Murphy, 2007; Taylor and Grotewold, 2005; Winkel-Shirley, 2001). Flavonoids are involved in protection against various biotic and abiotic stresses, they play roles in the regulation of plant reproduction and development and act as signalling molecules

with the biotic environment. Besides these physiological functions, there is a growing interest in these secondary metabolites due to their potential benefits for human health (Halliwell, 2007; Luceri *et al.*, 2007); therefore, improving our understanding of the regulation of flavonoid biosynthesis is an important objective.

Although structural genes can be efficiently targeted for crop improvement, the use of regulatory genes seems to be at least as promising (Bovy *et al.*, 2007; Grotewold *et al.*,

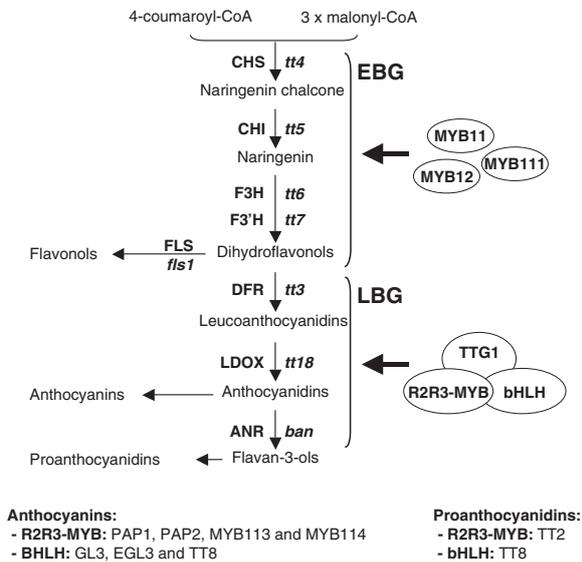


Figure 1. Simplified model of the flavonoid biosynthetic pathway. The pathway is initiated by the condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA by the chalcone synthase (CHS) enzyme. The subsequent steps in flavonoid biosynthesis are catalysed by a series of enzymes leading to the production of three types of final end-products: flavonols, anthocyanins and proanthocyanidins (CHI, chalcone isomerase; F3H, flavonol 3-hydroxylase; F3'H, flavonol 3'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase; ANR, anthocyanidin reductase). Enzymes are indicated in upper-case letters and genetic loci in lower-case italics. Dotted lines represent putative regulations. EBG, early biosynthetic gene; LBG, late biosynthetic gene.

1998). Indeed, in the different species studied so far, many structural genes have been found to be co-regulated (Koes *et al.*, 2005; Quattrocchio *et al.*, 2006). In *Arabidopsis thaliana*, two types of co-regulated structural genes can be distinguished in seedlings: (i) early biosynthetic genes (EBGs) which are common to the different flavonoid sub-pathways (Figure 1) and are induced prior to (ii) the late biosynthetic genes (LBGs; Pelletier and Shirley, 1996; Pelletier *et al.*, 1997; Shirley *et al.*, 1995; Winkel-Shirley, 2001). The patterns in other dicots such as antirrhinum and petunia are quite similar (Martin *et al.*, 1991; Quattrocchio *et al.*, 2006). Many regulatory proteins controlling the expression of the structural flavonoid genes have been identified in various model species such as maize, petunia, antirrhinum and *Arabidopsis* (Koes *et al.*, 2005; Lepiniec *et al.*, 2006; Morita *et al.*, 2006; Quattrocchio *et al.*, 2006; Schwinn *et al.*, 2006). Most of these regulators belong to the large R2R3-MYB and basic helix-loop-helix (BHLH) families of transcription factors (Heim *et al.*, 2003; Stracke *et al.*, 2001; Yanhui *et al.*, 2006).

Arabidopsis thaliana accumulates various and well-characterized flavonoids, including proanthocyanidins (PAs), anthocyanins and flavonols (Abrahams *et al.*, 2003; Kerhoas *et al.*, 2006; Lepiniec *et al.*, 2006; Routaboul *et al.*,

2006; Tohge *et al.*, 2005). Proanthocyanidins are flavan-3-ol polymers that specifically accumulate in the seed coat in the micropylar area, the endothelial cell layer and the chalazal strand (Debeaujon *et al.*, 2003). Loss-of-function mutants producing seeds affected in PA content have allowed the identification of six regulatory loci (Lepiniec *et al.*, 2006). These genes encode TT1 (TRANSPARENT TESTA1, a zinc-finger protein), TT2 (R2R3-MYB/MYB123), TT8 (BHLH subgroup IIIf/bHLH042), TT16 (a MADS box protein), TTG1 (TRANSPARENT TESTA GLABRA 1, a WDR protein) and TTG2 (a WRKY transcription factor; Johnson *et al.*, 2002; Nesi *et al.*, 2000, 2001, 2002; Sagasser *et al.*, 2002; Walker *et al.*, 1999). Three of these regulators, namely TT2, TT8 and TTG1, play a central role in the regulation of the LBGs and PA biosynthesis (Nesi *et al.*, 2000, 2001). They form a MYB-BHLH-WDR (MBW) complex that regulates the expression of the LBGs at the transcriptional level (Baudry *et al.*, 2004; Debeaujon *et al.*, 2003; Lepiniec *et al.*, 2006). Moreover, this MBW complex can also regulate *TT8* expression in a positive-feedback loop that ensures a strong accumulation of PAs in the endothelium (Baudry *et al.*, 2006). *TT2* expression is restricted to PA-accumulating cells in the seed coat, whereas *TT8* and *TTG1* are expressed in both seeds and vegetative tissues (Baudry *et al.*, 2004; Nesi *et al.*, 2001; Walker *et al.*, 1999). Both TTG1 and TT8 (as well as the two TT8-related BHLH proteins GL3/GLABRA 3/bHLH001 and EGL3/ENHANCER OF GLABRA 3/bHLH002) can interact, in yeast, with the MYB proteins PAP1/MYB75 and PAP2/MYB90 (PRODUCTION OF ANTHOCYANIN PIGMENT 1 and 2) to form MBW complexes and control anthocyanin LBGs (Borevitz *et al.*, 2000; Gonzalez *et al.*, 2008; Teng *et al.*, 2005; Zhang *et al.*, 2003; Zimmermann *et al.*, 2004). The two proteins MYB113 and MYB114, closely related to PAP1 and PAP2, may also be involved in this regulation, although their precise role remains to be determined (Gonzalez *et al.*, 2008). Interestingly, the ectopic expression of *PAP* genes also induces the accumulation of some EBG mRNA and flavonol contents (Borevitz *et al.*, 2000; Matsui *et al.*, 2004; Tohge *et al.*, 2005). However, analyses of loss-of-function mutants suggested that LBGs are the main targets of the MBW complexes (Cominelli *et al.*, 2008; Gonzalez *et al.*, 2008; Routaboul *et al.*, 2006). Consistent with this view, it has been demonstrated recently that three R2R3-MYB proteins (i.e. MYB11, MYB12 and MYB111), which are probably not involved in a MBW complex, control flavonol biosynthesis through the regulation of EBGs (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007; Zimmermann *et al.*, 2004).

The MBW complexes are not only involved in the regulation of flavonoid biosynthesis, but also in various aspects of epidermal cell patterning such as the formation of trichomes in aerial parts, non-hair cells (atrachoblasts) in roots and stomata in the embryonic stem, as well as in biosynthesis of seed mucilage (Broun, 2005; Lepiniec *et al.*, 2006; Ramsay and Glover, 2005; Serna and Martin, 2006). It is

worth noting that in *A. thaliana* a specific class of MYB genes encoding one-repeat MYB (small MYBs of the R3 type) proteins have been shown to be involved in the same epidermal patterning mechanisms (Esch *et al.*, 2004; Kirik *et al.*, 2004a,b; Schellmann *et al.*, 2007; Schnittger *et al.*, 1999; Simon *et al.*, 2007; Tominaga *et al.*, 2007; Wada *et al.*, 1997; Wang *et al.*, 2007). Five of these closely related MYB proteins, CPC (CAPRICE), TRY (TRIPTYCHON), ETC1 (ENHANCER OF CAPRICE AND TRIPTYCHON 1), ETC2 and ETC3 are functionally similar and can counteract the activity of the MBW complexes by sequestering its BHLH component (Schellmann *et al.*, 2007; Simon *et al.*, 2007). In addition, TCL1 (TRICHOMELESS1), a related small MYB (see Figure 7a), may act by inhibiting the expression of the MYB component of the MBW complex, through direct binding to DNA (Wang *et al.*, 2007). At the molecular level, it has been shown that CPC, TRY and ETC1 can interact with the BHLH proteins GL3, EGL3 and TT8 (demonstrated only for CPC), in yeast or/and *in vitro*, competing with R2R3-MYB (Bernhardt *et al.*, 2003; Esch *et al.*, 2003, 2004; Zhang *et al.*, 2003; Zimmermann *et al.*, 2004). A more distantly related small MYB protein named MYBL2, previously identified as a leaf-specific one-repeat MYB (Kirik and Baumlein, 1996), can also interact with GL3, EGL3 and TT8 (Sawa, 2002; Zimmermann *et al.*, 2004). The ectopic expression of MYBL2 suggested that, as for other small MYB proteins, MYBL2 may be involved in the control of trichome formation (Sawa, 2002). In this paper, the function and regulation of MYBL2 were analysed by genetic, molecular, biochemical and cytological approaches. The results obtained show that MYBL2

negatively regulates anthocyanin biosynthesis. Interestingly, a R3-MYB from petunia, PhMYBx, has recently been reported to downregulate anthocyanin synthesis (Koes *et al.*, 2005; Quattrocchio *et al.*, 2006), suggesting that such a regulatory mechanism is conserved, at least amongst dicots.

Results

Mutations in *mybl2-1* and *mybl2-2* increase anthocyanin accumulation in seedlings

The role of MYBL2 was investigated by reverse genetics. Two independent transgenic lines with putative T-DNA insertions in the MYBL2 gene were obtained from the SALK collection. Molecular analyses confirmed the presence of T-DNA insertions in the 5'-untranslated region (UTR) and 3'-UTR of the *mybl2-1* and *mybl2-2* lines, respectively (Figure 2a). Both mutations were sufficient to prevent the detection of MYBL2 transcript in mutant seedlings when using primers covering the coding sequence (Figure 2b). Interestingly, when comparing 3-day-old mutant seedlings with the wild type (WT), clear increases in the level of purple anthocyanin were observed for cotyledons and the upper part of the hypocotyl (Figure 2c, left panel). Norflurazon [(4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]-3(2H)-pyridazinone] treatment allowed a better visualization of anthocyanin by depleting carotenoid and chlorophyll pigments (Figure 2c, right panel). These observations were confirmed by the direct quantification of anthocyanins present in the seedlings of the three genotypes (Figure 2d).

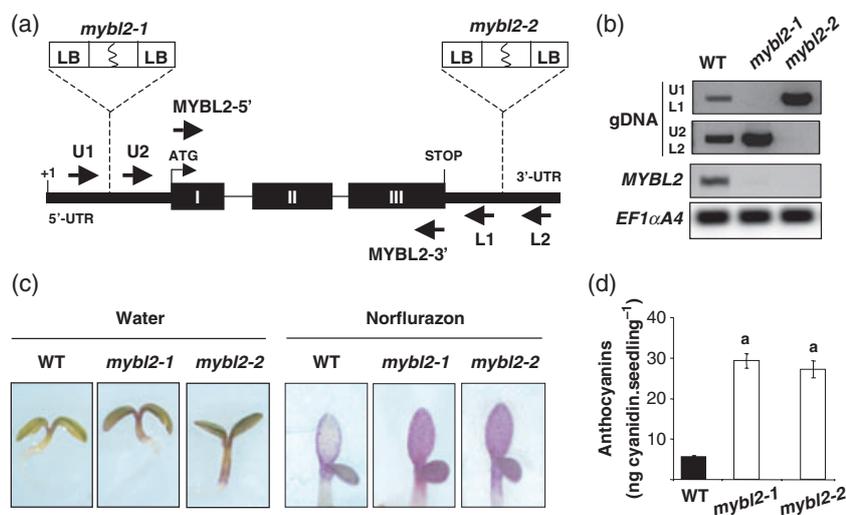


Figure 2. MYBL2 controls anthocyanin accumulation in Arabidopsis seedlings.

(a) Diagram of MYBL2 gene structure [thick lines, untranslated regions (UTRs); thin lines, introns; black boxes, exons] with the relative position of the two T-DNA insertions (white boxes) corresponding to *mybl2-1* (5'-UTR) and *mybl2-2* (3'-UTR) mutants. The positions of the U1/L1 and U2/L2 primer pairs used for the screening of homozygous *mybl2-1* and *mybl2-2* lines, respectively, are shown.

(b) Molecular characterization of the two *mybl2* mutant lines at the genomic (using U1/L1 and U2/L2 primer pairs for the *mybl2-1* and *mybl2-2* lines, respectively) and transcriptional (RT-PCR experiments using MYBL2-5' and MYBL2-3' primers; *EF1αA4* transcripts were used as control) levels.

(c) Phenotypic characterization of wild-type (WT) and *mybl2* 3-day-old seedlings, showing the over-accumulation of purple anthocyanins in the mutant lines.

(d) Quantitative measurement of anthocyanins in 3-day-old seedlings (*t*-test significant difference: a = $P < 0.001$). Error bars show SE. In (a–d) WT is Columbia.

Similar results were obtained in young siliques (1–2 days post-flowering) and young inflorescence stems (Figure S1). It is worth noting that under our normal growth conditions no modification of the PA or flavonol content was detected in either the seeds or vegetative tissues of the mutants (data not shown).

MYBL2 negatively regulates the expression of structural and regulatory anthocyanin genes in seedlings

In order to unravel the molecular mechanisms involved in the strong accumulation of anthocyanins in *mybl2* mutants, an analysis of flavonoid gene expression was carried out by quantitative RT-PCR. In *mybl2* seedlings, significant increases in *DFR* and *LDOX* mRNA were observed and, to a lesser extent, an increase in *F3H* and a decrease in *CHI* mRNA (Figure 3a). Interestingly, the expression of *DFR* and *LDOX* is known to be regulated by MBW complexes (Gonzalez *et al.*, 2008; Nesi *et al.*, 2000, 2001). Subsequently, therefore, expression of the regulatory genes was analysed (Figure 3b). Consistent with the results obtained with the structural genes, a significant and reproducible increase in transcript accumulation was detected for *GL3*, *TT8* and *PAP1*. It should be noted that the expression of *PAP2* and *TTG1* was not affected (data not shown). Altogether these results suggest that *MYBL2* negatively regulates the accumulation of anthocyanins in the vegetative tissues of the plant, at least indirectly through the expression of the genes of the MBW regulatory complexes.

Overexpression of MYBL2 in seeds is sufficient to inhibit PA biosynthesis and the expression of flavonoid genes

Since the MBW complex, consisting of *TT2*, *TT8* and *TTG1*, is involved in the regulation of PAs, we hypothesised that PA accumulation should be affected by expressing *MYBL2* in seeds under the control of the *TT8* promoter described

earlier (Baudry *et al.*, 2006). In order to check this hypothesis, wild-type *A. thaliana* were transformed with a *proTT8:MYBL2* construct. As expected, the 12 primary transformants harbouring this construct produced seeds with only small amounts of or no PAs (Figure 4a). Consistent with this result, drastic decreases in soluble and insoluble PAs were observed in the progenies of the primary transformants (Figure S2). As for the *mybl2* mutants, no variations in flavonol content were observed (data not shown). Other small MYB proteins (i.e. *CPC*, *TRY*, *ETC1*, *ETC2* and *ETC3*, but not *TCL1* isolated recently) were tested using the same approach. Interestingly, no abnormal flavonoid phenotypes were observed in any of the 12 transgenic lines analysed for each construct (data not shown). These results suggested that *MYBL2* is the only small MYB protein interfering with the flavonoid pathway.

To unravel how the specific overexpression of *MYBL2* can inhibit PA biosynthesis, the seeds of three independent overexpressing lines (OE lines H3, H6 and H12) showing a strong phenotype (i.e. yellow seeds; Figure 4a and Figure S2) were analysed by semi-quantitative and quantitative RT-PCR. Both analyses showed that although the accumulation of *TT2* and *TTG1* mRNA were not affected, *TT8* expression was significantly lower in the three transformants than in wild-type seed (Figure 4b). Consistent with this observation, a decrease in transcript accumulation was observed for most of the PA biosynthetic genes, *LDOX* and *DFR* again being the most affected (Figure 4c).

MYBL2 directly inhibits the expression of DFR by interacting with MBW complexes

The genetic analyses presented above suggested that *MYBL2* regulates flavonoid biosynthesis (i.e. PAs and anthocyanins) through negative control of the expression of regulatory genes (e.g. *TT8* and *GL3*). In addition, direct interactions of *MYBL2* with *TT8* and *GL3* have been

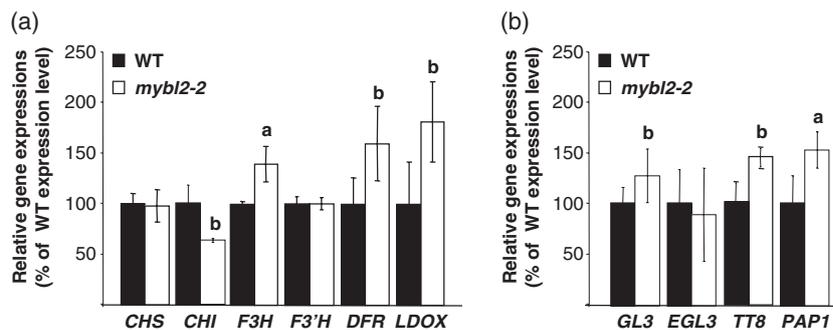


Figure 3. *MYBL2* affects accumulation of structural and regulatory gene transcript in Arabidopsis seedlings.

(a) Quantitative RT-PCR analysis of mRNA levels for anthocyanin biosynthetic genes showing an increased transcript accumulation for *F3H*, *DFR* and *LDOX*, in 3-day-old *mybl2* seedlings compared to wild type (WT), whereas the *CHI* mRNA level is decreased.

(b) Quantitative RT-PCR analysis of the mRNA levels for the regulatory genes involved in anthocyanin biosynthesis. Increased transcript accumulation was measured for *GL3*, *TT8* and *PAP1*.

t-test significant difference: a = $P < 0.01$ and b = $P < 0.05$. Error bars show SE. For (a) and (b) WT is Columbia.

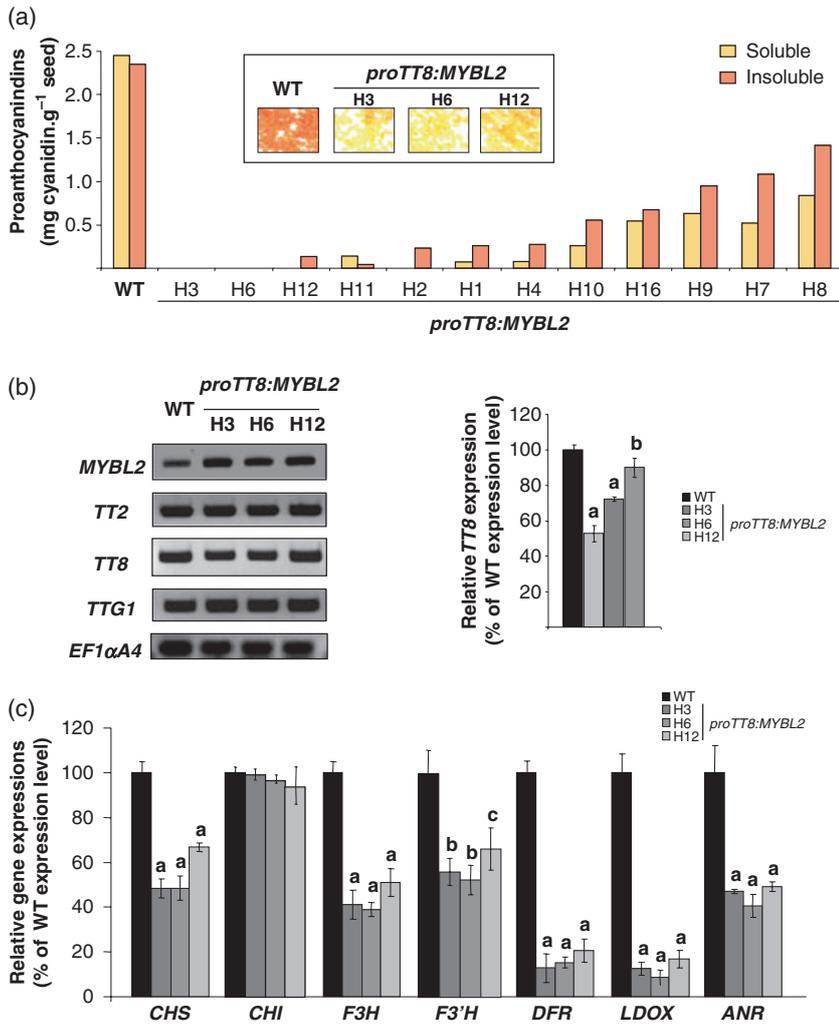


Figure 4. *MYBL2* overexpression affects the proanthocyanidin (PA) content in seeds.

(a) Mature seed PA content analysis of 12 independent primary transformant lines *proTT8:MYBL2* and corresponding wild type (WT). Inset: seed colour phenotype of the three strongest lines (i.e. lowest PA content, yellow seeds) compared to WT (brown seeds).

(b) Molecular characterization of 4-day post-fertilization siliques from three independent T₂ lines compared with WT. Left panel: RT-PCR analysis of transcript abundance for *MYBL2*, and the three genes of the MBW complex involved in PA biosynthesis: *TT2*, *TT8* and *TTG1*. *EF1αA4* transcript abundance was used as a control. Right panel: quantitative RT-PCR analysis of *TT8* mRNA levels in the three transgenic lines compared with WT. Measurements were expressed as the percentage of the WT level. *t*-test significant difference: a = *P* < 0.01 and b = *P* < 0.05. Error bars show SE.

(c) Quantitative RT-PCR analysis of the mRNA levels for the flavonoid biosynthesis genes in 4-day post-fertilization siliques in three transgenic lines compared with WT. Measurements are expressed as the percentage of the WT level. *t*-test significant difference: a = *P* < 0.001, b = *P* < 0.01 and c = *P* < 0.05. Error bars show SE. In (a–c) WT is Wassilewskija.

reported in yeast (Sawa, 2002; Zimmermann *et al.*, 2004), suggesting that *MYBL2* can also directly inhibit activity of the MBW complex. In order to test this hypothesis, a transient expression system was used. It has been demonstrated recently that the transient expression of both *TT2* and *TT8* is sufficient to induce expression of *DFR* in *A. thaliana* cells (Berger *et al.*, 2007). We took this oppor-

tunity to test the effect of the simultaneous and constitutive expression of *MYBL2* with *TT2* and *TT8* or *PAP1* and *EGL3* on *proDFR:GUS* expression. The quantification of *GUS* activity showed that the addition of *MYBL2* led to a decrease of approximately 36% and 60% of *GUS* activity for *TT2/TT8* and *PAP1/EGL3*, respectively (Figure 5). These experiments suggested that *MYBL2* directly interferes with

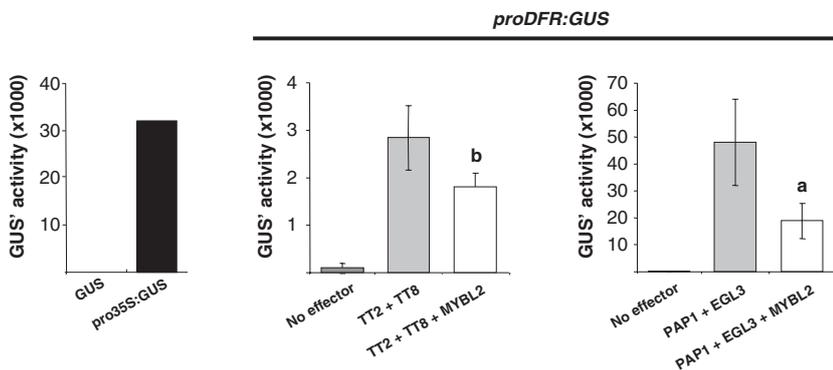


Figure 5. *MYBL2* inhibits *TT2-TT8* and *PAP1-EGL3* transcriptional activity.

Standardized specific β-glucuronidase (*GUS*) activity of the reporter *proDFR:uidA* (*GUS*) construct in transient co-transfection assays. *uidA* and *pro35S:uidA* were used as negative and positive controls, respectively. R2R3-MYB (*TT2*, *PAP1* and *MYBL2*) and BHLH factors were constitutively expressed using the CaMV 35S promoter. *t*-test significant difference: a = *P* < 0.01 and b = *P* < 0.05. Error bars show SE.

the MBW complexes *in vivo* to control the expression of flavonoid genes.

MYBL2 is expressed in both seeds and vegetative parts and is negatively regulated by high light

As expected from RNA gel-blot experiments (Kirik and Baumlein, 1996) and transcriptomic data (<http://bar.utoronto.ca/efp> and <https://www.genevestigator.ethz.ch>; Figure S5; Winter *et al.*, 2007; Zimmermann *et al.*, 2005), *MYBL2* mRNA was detected by RT-PCR in all the tissues tested, i.e. leaf, seedling and seed (data not shown). These analyses showed that *MYBL2* is expressed in all tissues, although in some cases at very low levels (e.g. in roots). Strong expression was detected in very young tissues (e.g. embryo) and in old and maturing tissues (e.g. leaves and siliques). In order to obtain further insights into the expression pattern of *MYBL2*, we investigated its regulation at the transcriptional level. A DNA fragment of about 1.5 kb upstream of the translational initiation start of *MYBL2* was fused to the GUS reporter gene (*proMYBL2:GUS*) and introduced into wild-type plants. Histochemical localization of GUS activity was carried out throughout plant development, from germinating seedlings to mature seeds (Figure 6). In seedlings, GUS activity was first detected at the cotyledon margins (2 days old), then mostly in the central part of the cotyledon (3 days old) and finally in the entire cotyledon, the emerging leaves and the upper part of the hypocotyl (4 days old; Figures 6a–c). In plants (5 weeks old), GUS activity was found in old leaves (Figure 6d) in the cells surrounding the vascular bundles (Figure 6e) and in the sub-epidermis of the adaxial side (Figure 6g), but not in trichomes (data not shown). We also detected GUS activity in the inflorescence (Figure 6g), in the sub-epidermis of the stem and the epidermis of the developing sepals (Figure 6h), as well as in young flowers (before fertilization, Figure 6i). In seeds, GUS activity was detected very early during development (i.e. starting before fertilization) in the innermost integument layer (endothelium) and the mucilage cell layer (MCL; Figure 6l). Aborted seeds displayed strong GUS activity, mainly in the endothelium (Figure 6k). During seed development, GUS activity was first found in the endothelium, the chalazal strand and the MCL (two-cell stage; Figure 6l), then extended to the suspensor (globular stage; Figure 6m) and was finally restricted to the chalazal strand, the MCL (torpedo to walking-stick stage; Figure 6n,o) and the funiculus epidermis (only at the walking-stick stage; Figure 6p).

Besides this developmental regulation, the modulation of *MYBL2* expression by light was also investigated. Under high-light conditions that trigger strong accumulation of anthocyanins in rosette leaves (Figure 6q), a decrease in *MYBL2* mRNA levels was detected after 3 h. Interestingly, a concomitant increase in *PAP1*, *PAP2* and, to a lesser extent,

TT8 mRNA was also detected. These results are consistent with a strong accumulation of mRNA encoded by the respective target genes such as *LDOX* and *DFR*.

Discussion

MYBL2 controls anthocyanin biosynthesis and gene expression in seedlings

Two null T-DNA insertion mutants, namely *mybl2-1* and *mybl2-2*, have been characterised. Interestingly, the two lines accumulated about four-fold more anthocyanins than wild-type seedlings (Figure 2c,d). Several structural as well as regulatory genes (i.e. *GL3*, *TT8* and *PAP1*) were upregulated in mutant seedlings (Figure 3). This suggested that *MYBL2* acts through the expression of the regulatory genes *MYB* and *BHLH*. Nevertheless, transient expression analyses in *A. thaliana* cells, using the *DFR* promoter as a target activated by the expression of regulatory factors (i.e. *TT2*, *TT8*, *PAP1* and *EGL3*), suggested that *MYBL2* can directly inhibit the activity of the MBW complexes (Figure 5). In support of this interpretation, it has been demonstrated that *MYBL2* can interact with *TT8*, *GL3* and *EGL3* in yeast (Sawa, 2002; Zimmermann *et al.*, 2004). These results are consistent with the hypothesis that R3-MYBs can prevent the formation of a MBW complex through interaction with the BHLH protein. Interestingly, a *MYBL2:VP16* chimeric protein (VP16 is a strong transcriptional activation domain from a virus) is unable to activate *DFR* expression in transient experiments (data not shown). Although this is a negative result, it suggests that, like CPC and unlike *TCL1*, *MYBL2* does not bind directly to DNA.

In both *mybl2* knock-out and *MYBL2* overexpressor lines, the two LBG genes, *DFR* and *LDOX*, were the most affected, although the transcript accumulation of some EBG, including *F3H* and *F3H*, were also slightly modified. The level of *CHI* mRNA was lower in *mybl2* mutants than in wild-type seedlings, but was not affected by the ectopic expression of *MYBL2*. Therefore, it is tempting to speculate that the expression of *CHI* may be negatively regulated by metabolic feedback due to the abnormal accumulation of anthocyanins in *mybl2* seedlings. It would be interesting to quantify *CHI* expression in a genetic background unable to synthesise flavonoids (e.g. *mybl2* × *tt4* double mutant) to address this point. Similar experiments should be conducted for the regulation of the different early biosynthetic genes.

MYBL2 can inhibit PA biosynthesis in seeds

Although the *mybl2* mutations have a drastic impact on anthocyanin accumulation in seedlings, no modifications of other flavonoids (i.e. flavonols and PAs) were detected. This result was not unexpected for flavonols, the biosynthesis of which is known to be mainly controlled by a specific family

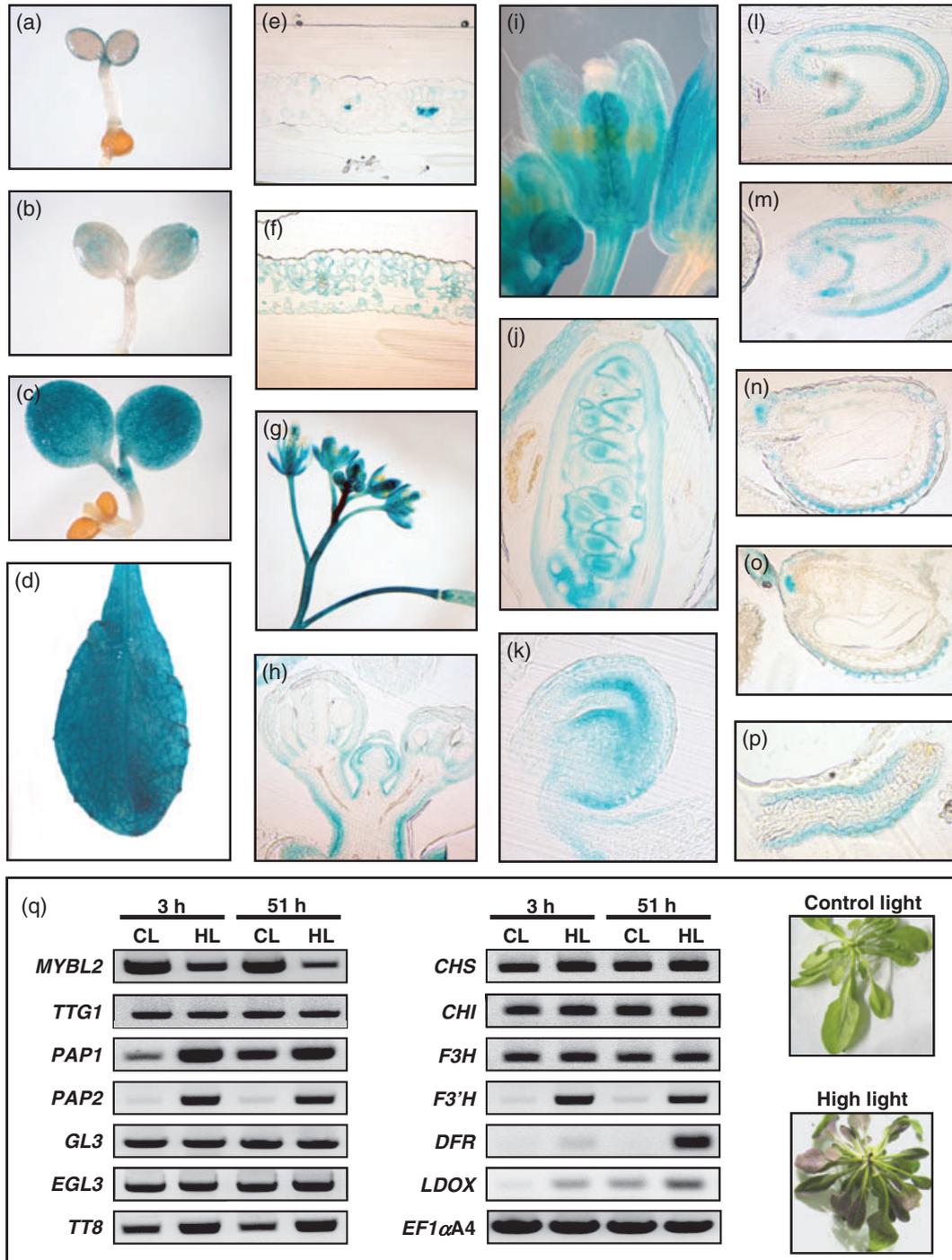


Figure 6. *MYBL2* promoter activity and light regulation of transcript accumulation of *MYBL2* and flavonoid genes.

(a)–(p) Histochemical analysis of the β -glucuronidase (GUS) activity generated by *proMYBL2:uidA* in wild-type (WT) plants: (a) 2-, (b) 3- and (c) 4-day-old germinating seedlings; (d) whole mount and (e, f) cross-section of mature rosette leaves; (g) inflorescence; (h) cross-section of inflorescence buds; (i) whole mount and (j) cross-section of unfertilised silique; (k) cross section of aborted seed; (l)–(o) cross section of developing seeds: (l) two-cell stage embryo, (m) globular stage embryo, (n) torpedo stage embryo and (o) walking-stick stage embryo; (p) cross section of seed funiculus. NB: 6 mM potassium ferricyanide/potassium ferrocyanide was used for (e, f, h, j, o and p), and 3 mM for the others. (q) Light regulation of *MYBL2* and flavonoid regulatory and biosynthesis genes, in rosette leaves. Transcript accumulation was analysed by RT-PCR using samples harvested after 3 and 51 h of control (CL, $150 \mu\text{mol m}^{-2} \text{sec}^{-1}$) or high (HL, $800 \mu\text{mol m}^{-2} \text{sec}^{-1}$) light treatments. *EF1αA4* transcript abundance was used as control.

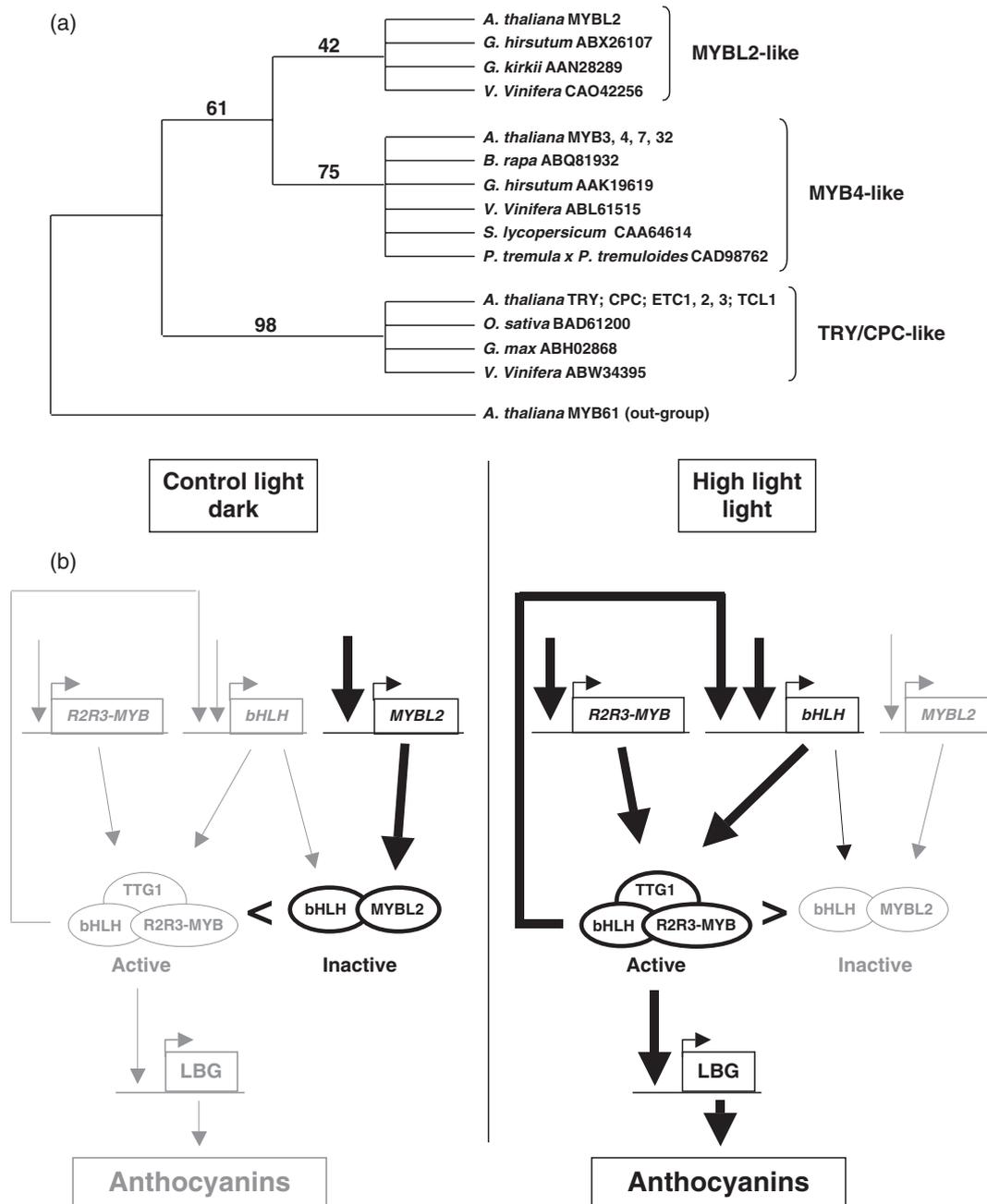


Figure 7. Role of *MYBL2* in the transcriptional control of flavonoid genes.

(a) Repressor MYB consensus dendrogram constructed from parsimony and distance analysis based on the R3 domain amino acid sequence (1000 bootstraps). The selected Arabidopsis MYBs and their homologues belong to the R3-MYB group (*MYBL2* and the TRY/CPC subgroup shown to control development of trichomes and atrichoblasts) and the MYB4 group (shown to negatively regulate part of the phenylpropanoid pathway; Jin *et al.*, 2000), and all present a basic helix-loop-helix (BHLH) interaction motif in their R3 domain (Zimmermann *et al.*, 2004). Arabidopsis *MYB61* was used as an out-group due to its distant relationship with the analysed MYBs and the absence of a BHLH interaction motif in its R3 domain (Stracke *et al.*, 2001).

(b) Proposed model for the transcriptional regulation of flavonoid genes by *MYBL2*. BHLH (*TT8*, *GL3* or *EGL3*), R2R3-MYB (*TT2*, *PAP1* or *PAP2*) and TTG1 form transcriptional MBW complexes that activate the expression of flavonoid biosynthetic genes, leading to the production of proanthocyanidin (PAs; *TT8* and *TT2*) and anthocyanins (*GL3*, *EGL3*, *TT8*, *PAP1* and *PAP2*) in seeds and vegetative parts, respectively. Interactions of *MYBL2* and BHLH might counteract the activity of MBW complexes. The balance between MBW complexes and the amount of *MYBL2* protein present in a cell type might be regulated by environmental signals such as light intensity and thereby modulate the level of target gene expression, which in turn controls the amount of flavonoids.

of MYB proteins acting independently of the MBW complexes (Stracke *et al.*, 2007). The slight increase in *F3H* mRNA apparently had no effect on flavonols, suggesting that it was not sufficient to increase F3H activity or that the FLS activity was limiting (hence, channelling additional flavonoids towards anthocyanins). This hypothesis could be tested by quantifying the amount of flavonols in *A. thaliana* mutants unable to synthesise anthocyanins (e.g. *mybl2* × *tt3* double mutant).

The result was more intriguing for PAs, the biosynthesis of which requires a MBW complex consisting of TT2, TT8 and TTG1 to direct the expression of *DFR* and *LDOX*. Furthermore, although there are no data at the protein level, *MYBL2* expression was detected in the endothelium (Figure 6). Nevertheless, the tremendous accumulation of PAs in these cells and the known limitations for their quantification (Debeaujon *et al.*, 2003; Routaboul *et al.*, 2006) may simply prevent the detection of higher amounts of PAs in the mutants. Therefore, in order to test if *MYBL2* can interfere with the TT2–TT8–TTG1 complex, transgenic plants that specifically overexpressed *MYBL2* in seed were generated. This set-up was found to be very efficient in inhibiting PA biosynthesis in the seed coat. Several of these transgenic plants produced yellow seeds with no detectable PAs (Figure 4). Molecular analyses showed that this reduction in PA correlated with a lower level of the mRNAs encoded by PA biosynthesis genes and *TT8*. These results are consistent with both the interaction of *MYBL2* with MBW complexes in yeast and the positive feedback of the TT8-containing MBW complex on *TT8* expression (Baudry *et al.*, 2006).

MYBL2 is not redundant with the other small MYB proteins

Here, we have shown that *MYBL2* is involved in the regulation of anthocyanin biosynthesis in seedlings and that it can inhibit PA accumulation when overexpressed in seed. Interestingly, it has previously been shown that the ectopic expression of *MYBL2* in leaves, under the control of a strong promoter, can also inhibit trichome formation (Sawa, 2002). These results are consistent with the demonstration that *MYBL2* can interact with TT8, GL3 and EGL3 in yeast (Sawa, 2002; Zimmermann *et al.*, 2004). Indeed, besides their role in trichome formation, GL3 and EGL3 are also involved in the regulation of anthocyanin biosynthesis in the vegetative parts of the plant (Zhang *et al.*, 2003). However, neither of the two *mybl2* mutants displayed abnormal trichome phenotypes. Similarly, no obvious modification of mucilage accumulation was detected by ruthenium red staining (data not shown). Instead, these results suggest that *MYBL2* is only involved in the regulation of the expression of flavonoid biosynthetic genes. Alternatively, functional redundancies may exist with the other small MYB proteins, as already demonstrated between TRY, CPC and the three ETC proteins

for epidermal patterning (Simon *et al.*, 2007). To the best of our knowledge, however, no abnormal flavonoid phenotype has been reported for the corresponding single and multiple mutants. Furthermore, although this is a negative result, the ectopic expression of these R3-MYBs did not affect anthocyanin or PA accumulation. Taken together, these results suggested that *MYBL2* is neither genetically nor functionally redundant with the other known members of the small MYB protein family.

Other negative regulators affecting flavonoid TTG1-dependent pathways may exist in *A. thaliana*. MYB4 is a R2R3-MYB (subgroup 4; Stracke *et al.*, 2001) that can interact with BHLH proteins controlling the expression of flavonoid structural genes (Zimmermann *et al.*, 2004). The three closely related proteins from the R2R3-MYB subgroup 4 (MYB3, MYB7 and MYB32) could also potentially interact with these BHLH (Stracke *et al.*, 2001; Zimmermann *et al.*, 2004). It has been shown that MYB4 represses the transcription of early genes in phenylpropanoid metabolism, primarily *C4H* (Jin *et al.*, 2000). MYB32 has been suggested to be involved in the regulation of LBG in pollen (Preston *et al.*, 2004). Interestingly, the overexpression of these four genes inhibited the accumulation of PAs in seeds (CD, unpublished results). Nevertheless, the physiological role of these proteins remains to be investigated in single and multiple mutants. In support of these conclusions, sequence and phylogenetic analyses showed that *MYBL2* is more distantly related than the other R3-MYBs within subgroup 4 of the R2R3-MYB family (Figure 7a, Figures S3 and S4). Interestingly, a R3-MYB from petunia, PhMYBx, has been recently reported to downregulate anthocyanin synthesis (Koes *et al.*, 2005; Quattrocchio *et al.*, 2006). PhMYBx can bind PhBHLHs in yeast cells and would be part of an autoregulatory loop that modulates flavonoid BHLH activity. This latter report suggests that a similar regulatory mechanism is conserved, at least amongst dicots.

Developmental and environmental regulation of MYBL2

Flavonoid biosynthesis is under both developmental and environmental control (Hartmann *et al.*, 2005). In *A. thaliana* seedlings, the transient accumulation of anthocyanins observed in the cotyledons and the upper part of the hypocotyls (3 days after germination), coincides with increased transcript accumulation from the corresponding biosynthetic genes and overlaps with the expression of MBW genes (Baudry *et al.*, 2004, 2006; Gonzalez *et al.*, 2008; Kubasek *et al.*, 1998; Zhang *et al.*, 2003). This pattern of regulation is fully consistent with the negative role of *MYBL2* in the control of MBW activity and its expression profile. In seeds, and in agreement with the hypothesized role of *MYBL2* in PA biosynthesis, *MYBL2* promoter activity was no longer detected in PA-accumulating cells after the torpedo

stage when mRNA levels of the biosynthetic genes have decreased. Moreover, further analyses are required to firmly establish the role of MYBL2 in PA biosynthesis, including looking for different environmental conditions that may modify PA accumulation in seeds. Nevertheless, MYBL2 is a good candidate for biotechnological applications aimed at producing seeds lacking PAs without affecting the flavonol content that may be necessary for plant fitness.

Interestingly, it has been shown that the MBW factors play important roles in regulating anthocyanin biosynthesis in response to different nutritional (sugar, nitrogen or phosphorus) and environmental (e.g. light) conditions (Cominelli *et al.*, 2008; Lea *et al.*, 2007; Lillo *et al.*, 2008; Scheible *et al.*, 2004; Solfanelli *et al.*, 2006; Teng *et al.*, 2005). The negative correlation observed in this study between light intensity (triggering anthocyanin accumulation) and the level of MYBL2 mRNA strongly supports the hypothesis that MYBL2 expression is inhibited under conditions of stress. These results are confirmed by transcriptomic data available online (<http://www.bar.utoronto.ca>). Moreover, the repression of MYBL2 expression by light is fully consistent with its reported regulation by circadian rhythm (<http://diurnal.cgrb.oregonstate.edu>; Figure S6). However, the use of proMYBL2:GUS transformed plants grown under various biotic and abiotic stresses would be helpful in order to investigate further the environmental regulation of MYBL2 expression.

Fine-tuned induction of flavonoid biosynthesis in Arabidopsis; model and prospects

Here, we propose a model in which MBW complexes (PAP1–EGL3–TTG1 and TT2–TT8–TTG1 for the anthocyanins and PAs, respectively) activate the expression of the flavonoid biosynthetic genes, and where MYBL2 acts as a transcriptional inhibitor (Figure 7b). In this model, MYBL2 might counter-balance the activity (i.e. the formation) of MBW complexes, thus controlling the level and/or the tissue specificity of flavonoid biosynthesis during plant development and in response to growth conditions (i.e. light intensity; Figure 6q). Given that PAP1 overexpressors and MYBL2 knock-out seedlings show the same anthocyanin phenotype (Figure 2c and Figure S1) and that both proteins can interact *in vivo* with the same BHLH partners (Sawa, 2002; Zimmermann *et al.*, 2004), an attractive hypothesis is that the expression of the flavonoid biosynthesis genes is controlled by the balance between (PAP-containing) MBW and MYBL2–BHLH complexes. This hypothesis is reinforced by the negative correlation observed at the mRNA level between the MYB (PAP1 and PAP2) and BHLH (TT8) partners in the MBW complex compared with MYBL2, in relation to the light intensity provided (TTG1 transcript accumulation remaining unchanged; Figure 6q). In addition, Baudry *et al.* (2006) showed that the TT2–TT8–TTG1 complex not only regulates

the expression of biosynthetic flavonoid genes directly but also controls the expression of TT8 in a positive-feedback loop. In this context, such a negative mechanism would be an efficient way of rapidly downregulating flavonoid accumulation by repressing the expression of both transcriptional activators (i.e. TT8) and structural genes. Interestingly, a locus that may be involved in the same regulatory pathway, ICX1 (INCREASED CHALCONE SYNTHASE EXPRESSION1), has been described. ICX1 would be a negative regulator of various cell patterning pathways and flavonoid biosynthesis (Wade *et al.*, 2003). However, gene cloning and functional characterization of the encoded protein remain to be carried out.

We also showed that MYBL2 is regulated by light. However, the regulation of its expression by other environmental stimuli (i.e. biotic stresses), nutrient availability (i.e. sugar, nitrogen, phosphate) or other conditions inducing anthocyanin accumulation requires further investigation. Indeed, the regulation of MYBL2 expression itself will also have to be elucidated. For these purposes, the availability of both the proMYBL2:GUS construct and the flavonoid biosynthesis mutants (metabolic feedback) will be useful tools. Finally, the different putative targets of MYBL2 described in this work will have to be confirmed and an exhaustive search should be carried out using, for instance, inducible systems (Baudry *et al.*, 2004).

Experimental procedures

Plant material

Arabidopsis thaliana accessions Wassilewskija (WS) and Columbia (Col-0) were used as wild-type controls where appropriate. All methods and conditions used for plant growth, plant transformation and selection for transgenic lines were as previously reported by Nesi *et al.* (2000). The MYBL2 T-DNA insertion lines *mybl2-1* (SALK_107780) and *mybl2-2* (SALK_126807) derived from the SALK collection (Ecker reference) were ordered from the Nottingham Arabidopsis Stock Centre (NASC). Homozygous *mybl2* T-DNA lines were obtained by PCR screening using the U1 and L1 primers for *mybl2-1*, and U2 and L2 primers for *mybl2-2* (Table S1).

Constructs

All the PCR products were obtained using high-fidelity Phusion DNA polymerase (Finnzymes, <http://www.finnzymes.com/>) and the constructs were sequenced to ensure their integrity. *attB1* and *attB2* correspond to the Gateway[®] recombination sequences (Invitrogen, <http://www.invitrogen.com/>).

proMYBL2:GUS. The MYBL2 promoter (1511 bp) used in this study corresponds to the region –1522 to –11 relative to the translation initiation site, and was amplified from WS genomic DNA using the proMYBL2-B1 and the proMYBL2-B2 primers (Table S2). The PCR product was introduced by a BP recombination reaction into the pDONR207 vector (Gateway[®]), and then introduced into the

binary vector pBI-101GUS containing the Gateway[®] cassette, by a LR recombination (Baudry *et al.*, 2006).

proTT8:MYBL2. This construct is based on the pBIB-Hyg binary vector in which the Gateway[®] recombination cassette has been *EcoRV*-inserted at the multiple cloning site, giving the pBIB-Hyg-GTW vector. The orientation of the Gateway[®] recombination cassette was verified by *SalI* digestion. The 1518-bp *TT8* promoter fragment was amplified from the pDONR207 vector (Gateway[®]) described in Baudry *et al.* (2006), with the primers proTT8-5'-*HindIII* and proTT8-3'-*XbaI* (Table S3). The DNA fragment obtained was subsequently cloned into the pBIB-Hyg-GTW vector digested by *HindIII* and *XbaI*, giving the pBIB-Hyg *proTT8:GTW* vector. The *MYBL2* cDNA was PCR-amplified from WS genomic cDNA using the cMYBL2-B1 and cMYBL2-B2 primers (Table S2), introduced into the pDONR207 vector (Gateway[®]), and then recombined into the pBIB-Hyg *proTT8:GTW* vector, resulting in a pBIB-Hyg vector containing the *proTT8:MYBL2* fusion.

The same approach was carried out for the *CPC* (*At2g46410*), *TRY* (*At5g53200*), *ETC1* (*At1g01380*), *ETC2* (*At2g30420*), *ETC3* (*At4g01060*), *MYB3* (*At1g22640*), *MYB4* (*At4g38620*), *MYB7* (*At2g16720*) and *MYB32* (*At4g34990*) cDNA fusions with *proTT8*. The primer pairs used to amplify the corresponding cDNAs are described in Table S2.

Histochemical detection of GUS activity

The GUS staining, resin embedding and sectioning for the plant material expressing the *proMYBL2:GUS* construct were performed as described by Debeaujon *et al.* (2003), in the presence of 3 or 6 mM potassium ferricyanide/potassium ferrocyanide. *ProDFR:GUS* activity tests in transient assays using *A. thaliana* cells were carried out as described in Berger *et al.* (2007).

Biochemical analysis

Extraction and analysis of seed flavonoids were carried out using a modified protocol adapted from Routaboul *et al.* (2006). Modifications were as follows: (i) seeds were ground in 1 ml methanol/acetone/water/trifluoroacetic acid (30/42/28/0.05; v/v/v/v; Mane *et al.*, 2007) and (ii) the final extract was not concentrated. The PA polymers were hydrolysed into coloured anthocyanidin, prior to quantification. Anthocyanin qualitative differences between WT, *mybl2-1* and *mybl2-2* seedlings were observed after the following treatments: seeds were germinated on filter paper imbibed with distilled water and grown for 3 days, in the presence or absence of 100 µM Norflurazon. The amount of anthocyanin accumulated in 3-day-old WT and *mybl2* mutant lines was determined as follows: 50 seedlings per sample were incubated in 150 µl of methanol containing 1% HCl overnight at 4°C in the dark, with shaking. After extraction, 100 µl of water and 250 µl of chloroform were added and the samples centrifuged at 17 000g for 3 min. Absorbance was read at 530 (Abs530) and 657 (Abs657) nm, and the quantity of anthocyanin was determined by calculating Abs530–Abs657. For the measurements of both PAs and anthocyanidins, a calibration curve was made using commercial cyanidin chloride (Extrasynthese, <http://www.extrasynthese.com/>).

Expression analysis

Total RNAs were extracted from plant material, treated with DNase and then reverse transcribed into cDNA as described (Baudry *et al.*,

2006). Semi-quantitative RT-PCRs for the genes of interest were carried out using a Perkin-Elmer 2720 thermocycler (<http://www.perkinelmer.com/>). The following cycling parameters were used: 4-min preliminary denaturation at 94°C, then 35 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 55°C) and extension (1–2 min, 72°C) and a final 5-min extension at 72°C. Ten microlitres of PCR products was then separated on 2% ethidium-bromide-stained agarose gel. The primer pairs used for *MYBL2* (*At1g71030*), *TT2* (*At5g35550*), *PAP1* (*At1g56650*), *PAP2* (*At1g66390*), *GL3* (*At5g41315*), *EGL3* (*At1g63650*), *TT8* (*At4g09820*) and *TTG1* (*At5g24520*) are described in Table S4. The primer pairs used for the *CHS* (*At5g42800*), *CHI* (*At3g55120*), *F3H* (*At3g51240*), *F3'H* (*At5g07990*), *DFR* (*At5g42800*), *LDOX* (*At4g22880*) and *ANR* (*At1g61720*) are available at the following URL: <http://www.catma.org/database/simple.html>. The primers used for *EF1α4* are described in Nesi *et al.* (2000).

Real-time RT-PCR were carried out as described in Baudry *et al.* (2004). Primer sets used in this study for *TT8*, *CHS*, *DFR*, *ANR* and *EF1α4* analysis are described in Baudry *et al.* (2004, 2006). QuantiTect Primers from Qiagen (<http://www.qiagen.com/>) were purchased in order to analyse the *F3H* (QT00789544), *F3'H* (QT00825181) and *LDOX* (QT00813176) transcript accumulation.

Transfection experiments

The *A. thaliana* cell line At7, which was used for transient expression analysis, the protoplast isolation and the transfection experiments were described previously (Mehrtens *et al.*, 2005). The *DFR* reporter construct and the *TT2*, *PAP1*, *TT8* and *EGL3* effector constructs have been described by Baudry *et al.* (2004), Mehrtens *et al.* (2005) and Zimmermann *et al.* (2004). In the co-transfection experiments, a total of 25 µg of pre-mixed plasmid DNA was transfected, which consisted of 10 µg of the reporter plasmid, 1 µg of each of the used effector plasmids and 5 µg of the luciferase (LUC) standardization plasmid. The determination of LUC and GUS enzyme activities as well as the calculation of the standardized specific GUS activity (GUS') was performed essentially as previously described (Sprenger-Haussels and Weisshaar, 2000).

Acknowledgements

This work was supported by the European Commission (FOOD-CT-2004-513960 FLAVO). CD was supported by a post-doctoral fellowship from FLAVO. JL-G was supported by a post-doctoral fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche (France) and AB was supported by a post-doctoral fellowship from the GABI-Géno plante project NO2002-00045. We thank Bertrand Dubreucq, Nathalie Berger and Sébastien Baud for technical help and useful comments during this work and Helen North for correcting the manuscript.

Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Complementary phenotypic characterization of *mybl2* mutants.

Figure S2. Seed PA content analysis of the three independent secondary transformant lines (*proTT8:MYBL2*) studied compared with wild type.

Figure S3. Parsimony analysis of the MYB R3 domain amino acid sequences.

Figure S4. Distance analysis of the MYB R3 domain amino acid sequences.

Figure S5. Transcriptomic analyses of *MYBL2* accumulation in different tissues.

Figure S6. Transcriptomic analyses of *MYBL2* accumulation in plants grown under long-day conditions (diurnal variation).

Table S1. Primers used for screening of *mybl2* T-DNA lines.

Table S2. Primer used for PCR-amplified target MYB promoter and cDNA prior to BP recombination into pDONR207 vector (Gateway®).

Table S3. Primers used in the pBIB-Hyg proTT8:GTW vector cloning.

Table S4. Primers used for expression analysis.

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