MYBL2 is a new regulator of flavonoid biosynthesis in Arabidopsis thaliana

Christian Dubos1,2, José Le Gourrierec1,†, Antoine Baudry1,‡, Gunnar Huep3, Elodie Lanet4, Isabelle Debeaujon1,2, Jean-Marc Routaboul1,2, Alessandro Alboresi4, Bernd Weisshaar3 and Loïc Lepiniec1,2,*
1INRA and 2AgroParisTech, UMR204, Seed Biology Laboratory, IJPB, Route de Saint-Cyr 78026 Versailles, France,
3Department of Biology, Bielefeld University, D-33594 Bielefeld, Germany, and 4Laboratoire de Génétique et de Biophysique des Plantes (LGBP), UMR 6191 CNRS-CEA-Université de la Méditerranée, Faculté des Sciences de Luminy, France

Received 8 February 2008; revised 23 April 2008; accepted 7 May 2008; published online 11 July 2008.
*For correspondence (fax +33 130 83 3111; e-mail lepiniec@versailles.inra.fr).
†Present address: Laboratoire de Morphogenèse des Ligneux, UMR A-462 SAGAH Université d’Angers-INRA-INH, UFR Sciences, 2 Boulevard Lavoisier, 49045 Angers Cedex 01, France.
‡Present address: S. Kay Laboratory, Cell and Developmental Biology, University of California San Diego, 9500 Gilman Drive, 92037 La Jolla, CA, USA.

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.,...
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 subpathways (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 arrhinum and petunia are quite similar (Martin et al., 1991; Quattrocchio et al., 2000). Many regulatory proteins controlling the expression of the structural flavonoid genes have been identified in various model species such as maize, petunia, arrhinum 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; Matsu 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 (atrichoblasts) 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; Schnitter et al., 1999; Simon et al., 2007; Tomininga 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](image-url)  
**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; *EF1α* 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

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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.

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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 opportunity 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
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 subepidermis 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 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 Arabidopsis 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 F3′H, 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 siliques; (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 μmol m⁻² sec⁻¹) or high (HL, 800 μmol m⁻² 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 between TRY, CPC and the three ETC proteins that MYBL2 can interact with TT8, GL3 and EGL3 in yeast (Sawa, 2002). 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.
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 \textit{PAP1} overexpressors and MYBL2 knock-out seedlings show the same anthocyanin phenotype (Figure 2c and Figure S1) and that both proteins can interact \textit{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 \textit{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 \textit{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. \textit{TT8} and structural genes. Interestingly, a locus that may be involved in the same regulatory pathway, ICX1 (INCREASED CHALCONE SYNTHASE EXPRESSION), 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

\textit{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 \textit{mybl2-1} (SALK_107780) and \textit{mybl2-2} (SALK_126807) derived from the SALK collection (Ecker reference) were ordered from the Nottingham Arabidopsis Stock Centre (NASC). Homozygous \textit{mybl2} T-DNA lines were obtained by PCR screening using the U1 and L1 primers for \textit{mybl2-1}, and U2 and L2 primers for \textit{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. \textit{attB1} and \textit{attB2} correspond to the Gateway \textsuperscript{®} recombination sequences (Invitrogen, http://www.invitrogen.com/).

proMYBL2:GUS. The \textit{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 pro\textit{MYBL2-B1} and the pro\textit{MYBL2-B2} primers (Table S2). The PCR product was introduced by a BP recombination reaction into the pDONR207 vector (Gateway\textsuperscript{®}), and then introduced into the
The MYB2 gene described in Baudry et al. (2007) using the cMYBL2-B1 and cMYBL2-B2 primers (Table S2) was subsequently cloned into the pBIB-Hyg-GTW vector digested with 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-GTW vector containing the proTT8:MYBL2 fusion. The same approach was carried out for the proTT8:MYBL2 construct which 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’T-XbaI and proTT8-3’-XbaI (Table S3). The DNA fragment obtained was subsequently cloned into the pBIB-Hyg-GTW vector digested with 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-GTW vector, resulting in a pBIB-Hyg vector containing the proTT8:MYBL2 fusion. The primers used to amplify the corresponding cDNAs are listed 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 (30/42/8/0.05; v/v/v/v; Mane et al., 2007) and (ii) the final extract was not concentrated. The PA polyphenol oxidase activity (GUS¢) was performed essentially as previously described (Debeaujon et al., 2005). The GUS¢ activities as well as the calculation of the standardized specific GUS¢ activity (GUS') were performed essentially as previously described (Sprenger-Haussens and Weisshaar, 2000).

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 were 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 (At1g38600), TT8 (At4g09620) and TTG1 (At5g24520) are described in Table S4. The primer pairs used for the CHS (At5g42000), CHI (At3g55120), F3H (At2g51240), F3’H (At2g79800), DFR (At5g42800), LDOX (At4g22980) and ANR (At1g61720) are available at the following URL: http://www.catma.org/database/simple.html. The primers used for EF1α::GUS 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 EF1α::GUS 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 (QT00813178) 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-Haussens 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 fromextrasynthese.com/). The same approach was carried out for the proTT8:MYBL2 constitutive promoter was amplified from the pDONR207 vector (Gateway®) and then recombined into the pBIB-Hyg-GTW vector, resulting in a pBIB-Hyg vector containing the proTT8:MYBL2 fusion. The primers used to amplify the corresponding cDNAs are listed in Table S2. 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-Ganoplante project N02002-00045. 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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References


