PCP
PLANT & CELL PHYSIOLOGY

AtMYB7, a New Player in the Regulation of UV-Sunscreens in *Arabidopsis thaliana*

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(Received July 26, 2013; Accepted December 3, 2013)

The phenylpropanoid metabolic pathway provides a wide variety of essential compounds for plants. Together with sinapate esters, in *Brassicaceae* species, flavonoids play an important role in protecting plants against UV irradiation. In this work we have characterized *Arabidopsis thaliana* AtMYB7, the closest homolog of AtMYB4 and AtMYB32, described as repressors of different branches of phenylpropanoid metabolism. The characterization of *atmyb7* plants revealed an induction of several genes involved in flavonol biosynthesis and an increased amount of these compounds. In addition, *AtMYB7* gene expression is repressed by AtMYB4. As a consequence, the *atmyb4* mutant plants present a reduction of flavonol contents, indicating once more that AtMYB7 represses flavonol biosynthesis. Our results also show that *AtMYB7* gene expression is induced by salt stress. Induction assays indicated that AtMYB7 represses several genes of the flavonoid pathway, *DFR* and *UGT* being early targets of this transcription factor. The results obtained indicate that AtMYB7 is a repressor of flavonol biosynthesis and also led us to propose AtMYB4 and AtMYB7 as part of the regulatory mechanism controlling the balance of the main *A. thaliana* UV-sunscreens.

Keywords: Flavonoids • Phenylpropanoids • R2R3-MYB factors • UV-sunscreens.

Abbreviations: ALDH, aldehyde dehydrogenase; CaMV, *Cauliflower mosiac virus*; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHX, cycloheximide; 4CL, 4-coumarate-CoA ligase; DEX, dexamethasone; DFR, dihydroflavonol reductase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; GR, glucocorticoid receptor; GUS, β-glucuronidase; MS, Murashige and Skoog; PAL, phenylalanine ammonia-lyase; RT–PCR, reverse transcription–PCR; SGT, sinapate UDP-glucose sinapoyltransferase; SMT, sinapoylglucose malate sinapoyltransferase; UGT, UDP sugar glycosyltransferase.

Introduction

The phenylpropanoid metabolic pathway generates a wide variety of compounds that are essential for plants, including lignin, flavonoids and the brassicaceae-specific sinapate esters (Dixon et al. 2002, Milkowski and Strack, 2010, Vogt 2010).

While lignin plays a structural role within the secondary cell walls (Vanholme et al. 2010), sinapate esters and flavonoids act as sunscreens that protect plants against the oxidative damage produced by UV-B radiation (Li et al. 1993, Landry et al. 1995, Sheahan et al. 1996, Dixon and Pasinetti 2010, Emiliani et al. 2013).

As both classes of compounds are produced by the phenylpropanoid pathway and have similar functions, it is likely that a tight regulation of their levels occurs in *Arabidopsis thaliana*.

Thus, it can be hypothesized that the relative amount of each one of these compounds is the result of the balanced expression of several interacting regulators, as already suggested (Hemm et al. 2001, Dubos et al. 2010).

During the last years, many efforts have been made to understand how the phenylpropanoid pathway is regulated to synthesize every phenolic compound precisely, and an increasing amount of evidence indicates that the family of R2R3-MYB transcription factors plays a key role in this regulation (Hemm et al. 2001, Bomal et al. 2008, Zhong and Ye 2009, Dubos et al. 2010, Feller et al. 2011, Gray et al. 2012). This large family of transcriptional regulators can be divided into subgroups based on the presence of conserved motifs in the C-terminal domain (Stracke et al. 2001, Dubos et al. 2010), and several studies showed that members of the same subgroup share similar functions. This is the case for subgroup 4, which clusters the only R2R3-MYB factors acting as transcriptional repressors of different branches of phenylpropanoid metabolism (Tamagnone et al. 1998, Jin et al. 2000, Preston et al. 2004, Fornalé et al. 2006, Sonbol et al. 2009, Legay et al. 2007, Fornalé et al. 2010).

In *A. thaliana*, subgroup 4 is composed of four members: AtMYB3, AtMYB4, AtMYB7 and AtMYB32 (Dubos et al. 2010). AtMYB4 is involved in the repression of the biosynthesis of sinapate esters (Jin et al. 2000) and AtMYB32 was characterized as a repressor of lignin biosynthesis specifically in pollen (Preston et al. 2004); however, nothing is known about the function of AtMYB7, the closest AtMYB4 and AtMYB32 homolog.

Plant Cell Physiol. 55(3): 507–516 (2014) doi:10.1093/pcp/pct187, available online at www.pcp.oxfordjournals.org

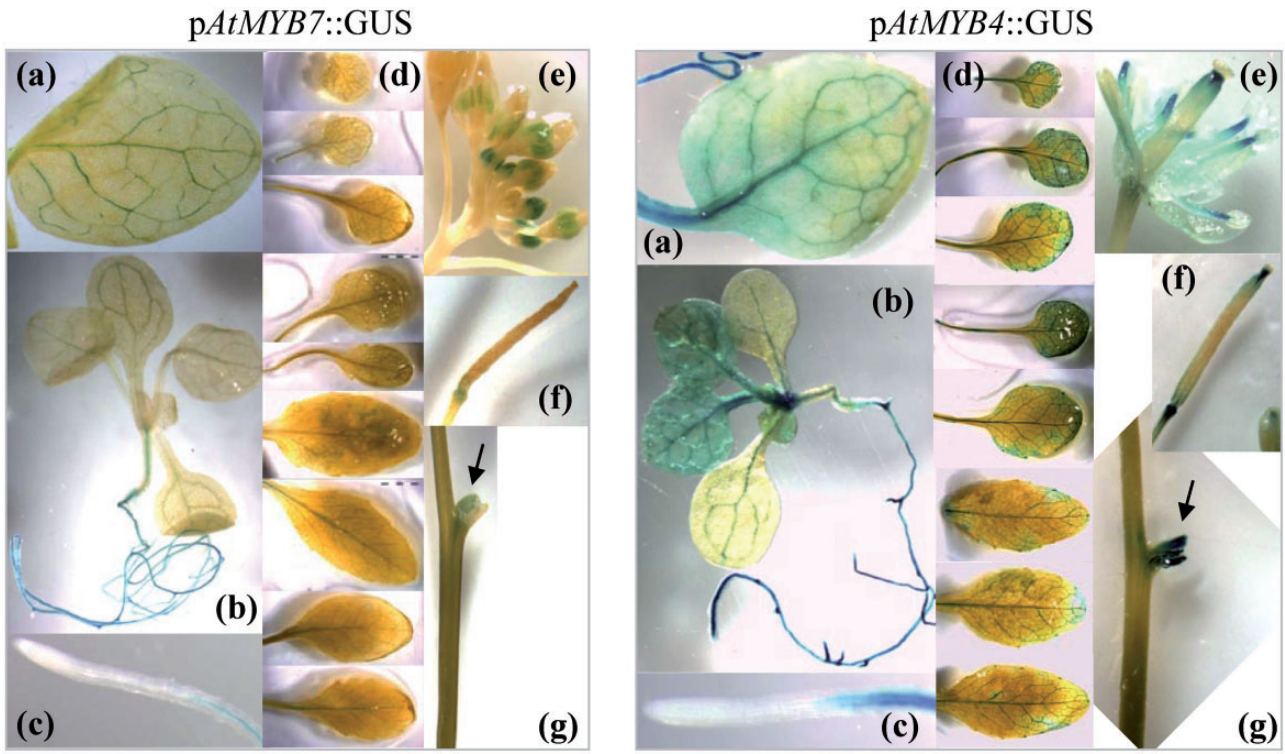

Fig. 1 Expression pattern of AtMYB7 and AtMYB4. Arabidopsis plants expressing the GUS reporter gene under the control of the *AtMYB7* gene promoter (left panel) or *AtMYB4* gene promoter (right panel). (a) Detail of a rosette leaf from a 10-day-old seedling. (b) Whole seedling. (c) Detail of an apical root from a 10-day-old seedling. (d) Rosette leaves from 3-week-old plants, (e) florets, (f) silique, (g) stalk. Arrows indicate excision points.

Therefore, we investigated the role of AtMYB7 in the regulation of the main branches of phenylpropanoid biosynthesis. In this work, we characterized the roles of AtMYB7 and we showed that it is mainly involved in the repression of flavonol biosynthesis. In addition, *AtMYB7* gene expression is controlled, at least partially, by AtMYB4 and induced by salt stress conditions.

Results

The expression of AtMYB7 is controlled by AtMYB4

To obtain a global picture of the gene expression of AtMYB7 and AtMYB4, we generated *A. thaliana* plants expressing β-glucuronidase (GUS) under the control of the *AtMYB7* and *AtMYB4* gene promoters, respectively (**Fig. 1**). The results show that *AtMYB7* and *AtMYB4* are both expressed in root tissues (with the exception of the root tips) and rosette leaves, and the expression of both factors decreases as leaves mature. The expression of *AtMYB7* in leaves is generally lower and more localized than that of *AtMYB4*. In fact, the *AtMYB7* promoter drives the expression of GUS mainly in the vasculature, while *AtMYB4* is expressed throughout the leaf (**Fig. 1**). These two factors present a non-overlapping pattern of expression in flowers, *AtMYB4* being mainly detected in filaments and in the style, and *AtMYB7* being detected exclusively in anthers (**Fig. 1**). In addition, the excised stems of plants expressing *GUS* under the control of the *AtMYB4* and *AtMYB7* gene promoters show that *AtMYB4*, but not *AtMYB7*, is induced by wounding (**Fig. 1**).

As *AtMYB7* is naturally expressed at a low level in nearly all the tissues in which *AtMYB4* is also expressed, we investigated whether AtMYB4 controls the expression of *AtMYB7*. Thus, we followed the expression of *AtMYB7* in the *atmyb4* mutant background, and vice versa, by both *GUS* gene reporter analyses and endogenous gene expression analyses (**Fig. 2**). The results show that in the absence of AtMYB4, the expression of *AtMYB7* is induced and can be detected throughout the leaf, while the expression of *AtMYB4* is not modified by the absence of AtMYB7 (**Fig. 2a**).

To confirm these data further, we generated *A. thaliana* plants overexpressing *AtMYB4* and *AtMYB7*, respectively, and we analyzed the effect on the expression of both factors on each genotype. The result obtained indicated that the overexpression of *AtMYB4* reduces the levels of *AtMYB7*, while *AtMYB4* expression is not influenced by *AtMYB7* (**Fig. 2b**).

AtMYB7 represses flavonol biosynthesis

Both AtMYB4 and AtMYB32 have been described as transcriptional repressors of the phenylpropanoid pathway. The characterization of the *atmyb4* mutant led to the demonstration that AtMYB4 acts as a main repressor of sinapate esters (Jin et al. 2000), while the characterization of *atmyb32* mutant plants

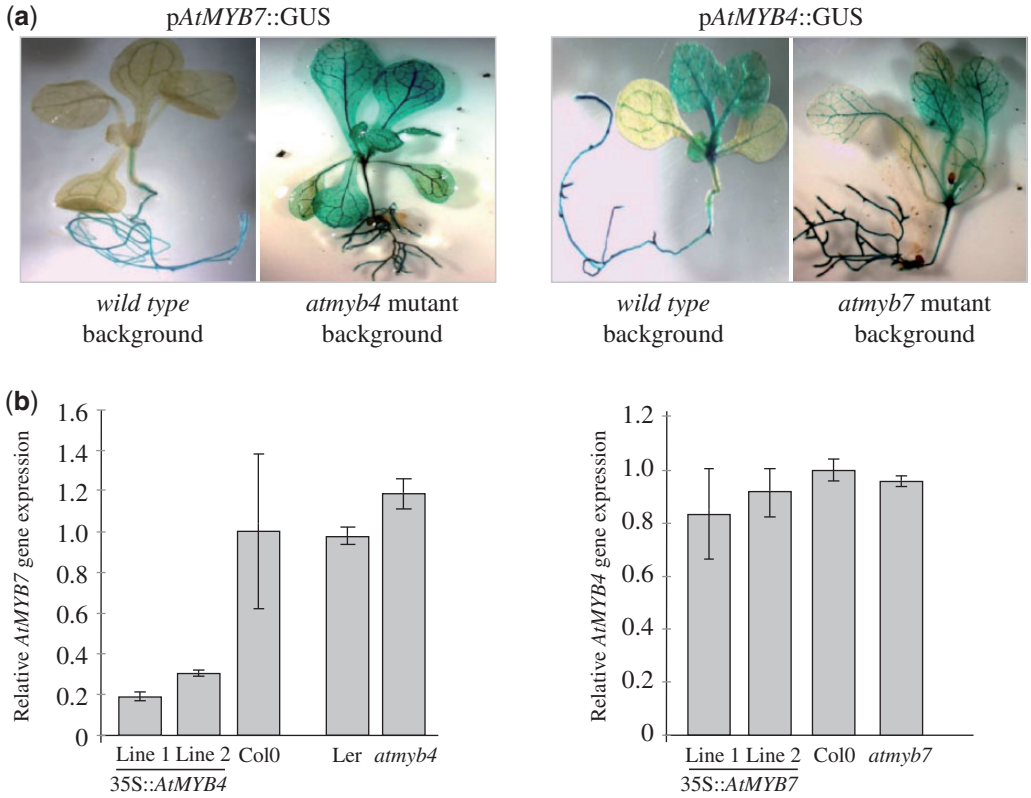

Fig. 2 Expression pattern of AtMYB7 and AtMYB4 in *atmyb4* and *atmyb7* mutant backgrounds. (a) Arabidopsis plants expressing GUS under the control of the *AtMYB7* gene promoter in the *atmyb4* mutant background (left panel) and Arabidopsis plants expressing GUS under the control of the *AtMYB4* gene promoter in the *atmyb7* mutant background (right panel). (b) Relative expression levels of *AtMYB7* and *AtMYB4* in the wild type, 35S::*AtMYB7* and 35S::*AtMYB4* and *atmyb7* and *atmyb4* mutant plants.

showed that AtMYB32 regulates the synthesis of lignin in pollen (Preston et al. 2004).

Thus, to study whether AtMYB7 regulates the phenylpropanoid pathway, we characterized an *atmyb7* T-DNA insertion mutant and we studied the effect on the synthesis and accumulation of lignin and flavonoids, the two main end-products of this metabolic route.

As in the case of *atmyb4* and *atmyb32* plants, no obvious changes were observed in the phenotype of the single *atmyb7* or double *atmyb7 atmyb4* plants (**Supplementary Fig. S1**), and the expression analysis of the main lignin genes did not show changes compared with wild-type stems (**Supplementary Fig. S2**). In agreement with these data, the distribution of lignin was not significantly altered in mutant stems (**Supplementary Fig. S2**) and the determination of lignin content and of its composition (S/G ratio) did not indicate changes with respect to control plants (**Supplementary Fig. S2**).

The expression analysis of the main flavonoid biosynthetic genes in rosette leaves from *atmyb7* and wild-type plants indicated that the lack of AtMYB7 produces an increase in the expression of the early phenylpropanoid genes, *PAL* (*phenylalanine ammonia-lyase*), *C4H* (*cinnamate 4-hydroxylase*) and *4CL* (*4-coumarate-CoA ligase*), of two flavonoid-specific genes, *F3′H* (*flavonoid 3′-hydroxylase*) and *DFR* (*dihydroflavonol reductase*), and *UGT* (*UDP sugar glycosyltransferase*) (**Fig. 3**). These changes are in line with the 2-fold increase in flavonols that can be detected in *atmyb7* rosettes (**Fig. 4**) and suggest that AtMYB7 acts as a repressor of flavonols. On the other hand, a 30% decrease of anthocyanins is observed in mutant plants, while the amount of soluble phenolics does not change significantly compared with control plants (**Fig. 4**). In accordance with this finding, the overexpression of AtMYB7 reduces exclusively the accumulation of flavonols and leaves unaffected the endogenous levels of anthocyanins (**Fig. 5**).

In *A. thaliana*, flavonols acts as UV-sunscreens together with sinapate esters, and both class of compounds are synthesized through the phenylpropanoid pathway. Thus, to study if the higher flavonoid content of *atmyb7* plants influences the synthesis of sinapate esters, we also analyzed the expression of their main biosynthetic genes. The results indicate that *SMT* (*sinapoylglucose malate sinapoyltransferase*) and *ALDH* (*aldehyde dehydrogenase*) are repressed in *atmyb7* rosettes, while the expression of *SGT* (*sinapate UDP-glucose sinapoyltransferase*) is induced (**Fig. 6**).

To better characterize the function of AtMYB7 in the regulation of flavonoid synthesis, we produced transgenic plants in which the AtMYB7 coding sequence was fused to that of the glucocorticoid receptor (GR) and placed under the control of

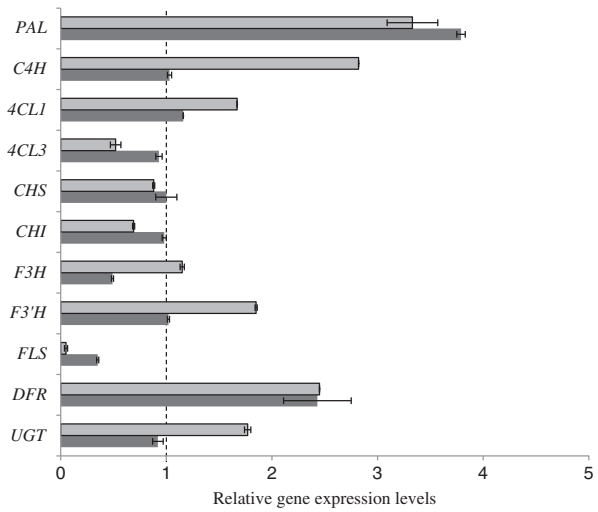

Fig. 3 AtMYB7 regulates genes involved in the biosynthesis of flavonoids. Gene expression analysis of genes involved in flavonoid biosynthesis in *atmyb7* (gray bar) and *atmyb7 atmyb4* (black bars) rosette leaves. The flavonoid biosynthetic pathway. *CHS*, *chalcone synthase*; *CHI*, *chalcone isomerase*; *F3H*, *flavanone 3-hydroxylase*; *F3′H*, *flavonoid 3′-hydroxylase*; *FLS*, *flavonol synthase*; *UGTs*, *UDP sugar glycosyltransferases*; *DFR*, *dihydroflavonol reductase*. The broken line represents the relative gene expression level in wild-type plants fixed as one.

the 35S *Cauliflower mosaic virus* (CaMV) promoter. After a 3 h treatment with dexamethasone (DEX), we analyzed the expression of the main genes that participate in the synthesis of flavonoids. The activation of AtMYB7 transcriptional activity resulted in a general repression of the whole flavonoid biosynthetic pathway, and particularly of *CHI* (*chalcone isomerase*), *DFR* and *FLS* (*flavonol synthase*) (**Fig. 7**). We also performed the assay in the presence of the protein synthesis inhibitor cycloheximide (CHX) to identify AtMYB7 early target genes. The addition of CHX restored the wild-type gene expression level of the majority of the genes, except that of *DFR* and *UGT* genes, indicating that these two genes are early targets of AtMYB7 (**Fig. 7**).

The function of AtMYB4 and AtMYB7 is not redundant

AtMYB4 acts as a negative regulator of sinapate ester synthesis (Jin et al. 2000) and possibly of AtMYB7, which in turn seems to repress the synthesis of flavonols. To better define the interactions between these two factors, we generated *atmyb7 atmyb4* double mutant plants and we studied the effects on the biosynthesis of flavonoids and sinapate esters. These double mutant plants do not show phenotypic alterations

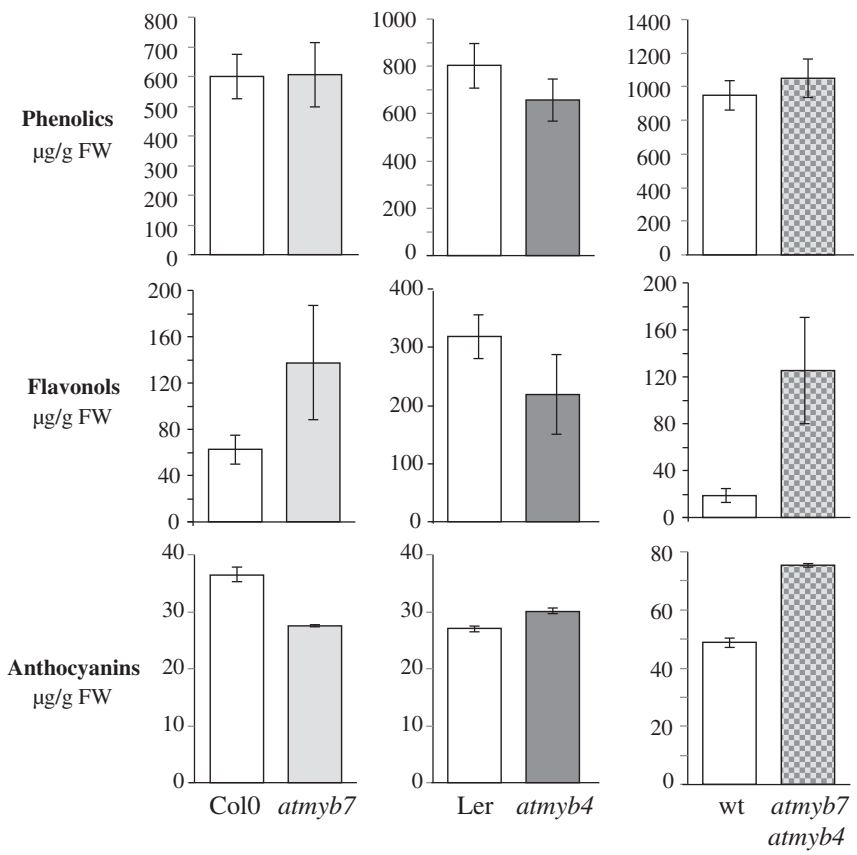

Fig. 4 Quantification of soluble phenolics, flavonoids and anthocyanins in wild-type, *atmyb7*, *atmyb4* and *atmyb7 atmyb4* plants. For the double mutant plant assays, the wild type (wt) plants are the cross between Col-0 and Ler ecotypes. Soluble phenolics, flavonols and anthocyanins are expressed as equivalents of gallic acid, rutin and cyanidine chloride, respectively. Data are the mean of three replicates ± SD.

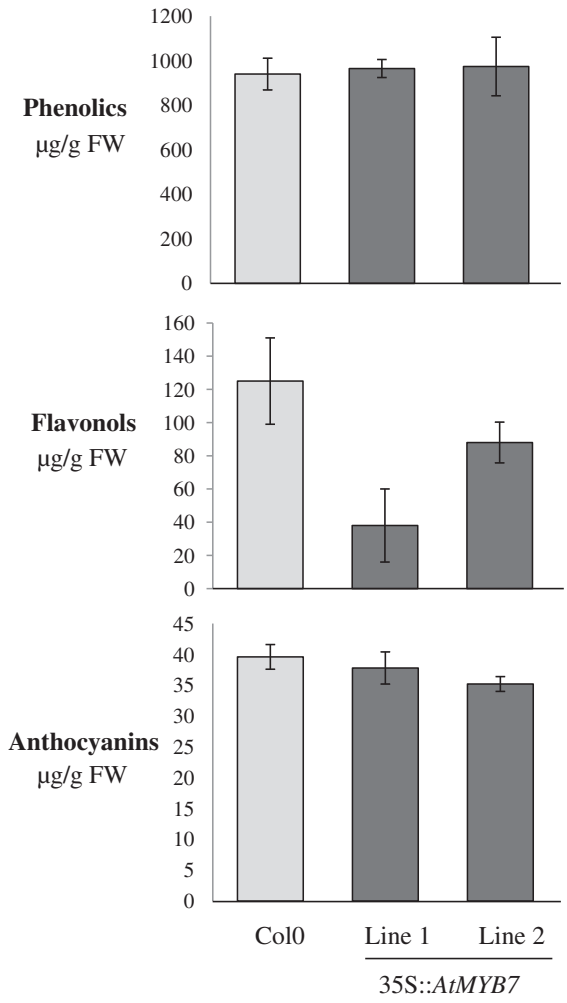

Fig. 5 Quantification of soluble phenolics, flavonoids and anthocyanins in wild-type and 35S::*AtMYB7* plants. Soluble phenolics, flavonols and anthocyanins are expressed as equivalents of gallic acid, rutin and cyanidine chloride, respectively. Data are the mean of three replicates ± SD.

(**Supplementary Fig. S1**), and our results indicate that the lack of both MYB factors induces the expression of all the genes whose expression is induced in *atmyb7* rosettes (**Fig. 3**). In addition, *atmyb7 atmyb4* rosettes display an increase in flavonols similar to that of *atmyb7* plants (**Fig. 4**), making the occurrence of a functional redundancy between AtMYB7 and AtMYB4 unlikely and confirming the specific role of AtMYB7 as a repressor of flavonol synthesis. The quantification of soluble phenolics in *atmyb7 atmyb4* rosette leaves showed no difference compared with wild-type plants, while anthocyanin levels are higher in the absence of both MYB factors (**Fig. 4**).

The lack of AtMYB4 increases the levels of sinapate esters (Jin et al. 2000), and our results indicate that this also led to the up-regulation of *AtMYB7* (**Fig. 2**). To study the effect on the accumulation of flavonoids, we also determined their levels in *atmyb4* and wild-type rosette leaves (**Fig. 4**). The reduced levels of flavonols in *atmyb4* mutant plants indicate once more that AtMYB7 acts a transcriptional repressor of these compounds. On the other hand, anthocyanins are slightly increased in *atmyb4* leaves, while the total content of soluble phenolics is not significantly altered (**Fig. 4**).

To study the interactions between AtMYB7 and AtMYB4 in more depth, we also analyzed the expression of the main genes involved in the synthesis of sinapate esters in both *atmyb7* and *atmyb7 atmyb4* leaves. The lack of *AtMYB7* gene expression reduces the transcript levels of the *ALDH* gene but its expression is restored to wild-type levels in the double *atmyb7 atmyb4* mutant plants (**Fig. 6**), in accordance with the specific function of AtMYB4 as a repressor of the synthesis of sinapate esters.

Finally, as *atmyb7* plants do not display changes in the synthesis of lignin (**Supplementary Fig. S2**) we analyzed stems from *atmyb7 atmyb4* plants to study if AtMYB4 plays a role in the regulation of this branch of the phenylpropanoid pathway (**Supplementary Fig. S2**). The lack of alterations in both lignin content and composition indicates that AtMYB4 is not directly involved in the regulation of lignin synthesis.

AtMYB7 is induced by salt stress conditions

The data obtained indicate that AtMYB7 acts as a repressor of flavonol synthesis and that its function is at least partially controlled by AtMYB4. To better define the physiological role of AtMYB7, additional experiments were undertaken to identify additional factors that influences its gene expression. The in silico analysis of the *AtMYB7* gene expression pattern (Arabidopsis eFP Browser, http://bar.utoronto.ca) revealed that it can be induced under abiotic stress conditions such as salt stress. Thus we supplied wild-type plants with NaCl during 4 h and we studied the response of these two factors to this treatment. Under these experimental conditions, we observed a clear induction of *AtMYB7*, while *AtMYB4* gene expression remains unchanged, indicating that the salt response of *AtMYB7* is independent of AtMYB4 (**Fig. 8**).

Discussion

Plant R2R3-MYB proteins are major regulators of phenylpropanoid metabolism, and their classification according to the presence of conserved domains allowed the identification of 22 subgroups in *A. thaliana* (Kranz et al. 1998, Stracke et al. 2001). An increasing amount of data indicates that MYB factors belonging to the same subgroup share a degree of functional conservation. This is the case for subgroup 4, which comprises AtMYB4 and AtMYB32 that act as transcriptional repressors (Jin et al. 2000, Preston et al. 2004). Thus, AtMYB4 was shown to control the synthesis of sinapate esters in a UV-dependent fashion by repressing the gene encoding C4H (Jin et al. 2000), while AtMYB32 regulates the synthesis of lignin in the pollen (Preston et al. 2004). Two other MYB factors are clustered within this subgroup, AtMYB3 and AtMYB7, and, to date, no functional data are available on the physiological role of these regulators.

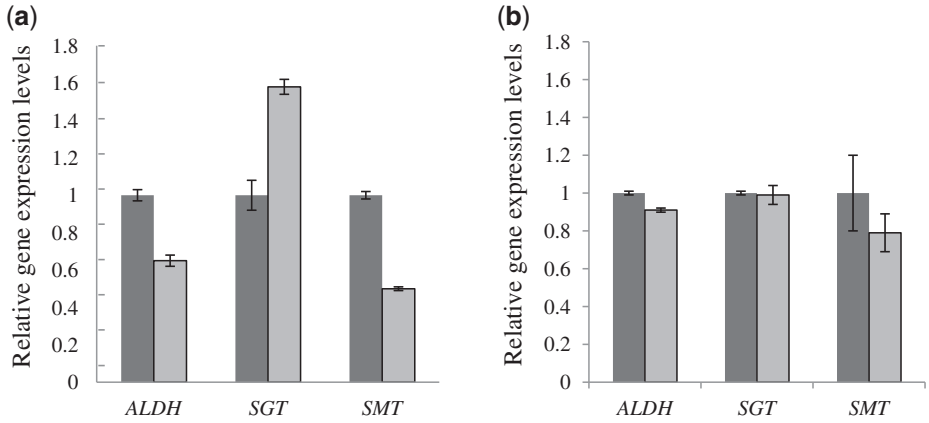

Fig. 6 Sinapate ester biosynthetic genes are not regulated by AtMYB7. Gene expression analyses of sinapate ester biosynthetic genes in (a) *atmyb7* and (b) *atmyb7 atmyb4* plants. Sinapate ester biosynthesis: *ALDH*, *aldehyde dehydrogenase*; *SGT*, *sinapate UDP-glucose sinapoyltransferase*; *SMT*, *sinapoylglucose malate sinapoyltransferase*. Black bars represent wild-type plants and gray bars the mutant plants.

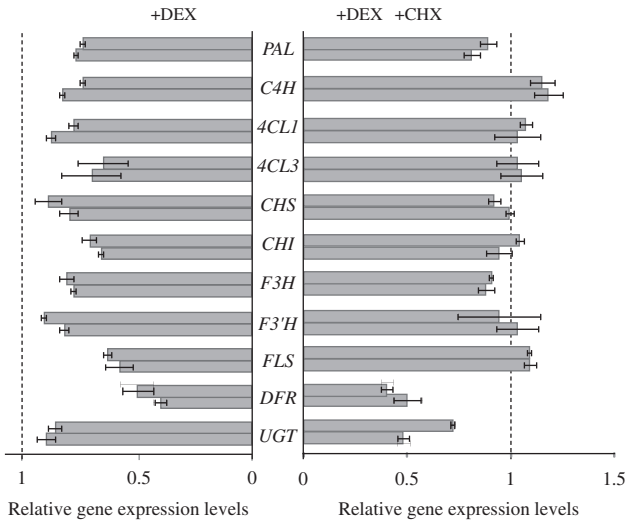

Fig. 7 Identification of AtMYB7 early target genes involved in the biosynthesis of flavonoids. Relative expression levels of flavonoids genes in 35S::*AtMYB7*:GR plants. The left panel represents expression analyses of flavonoid genes in 35S::*AtMYB7*:GR plants induced with dexamethasone (+DEX). The right panel represents expression analyses of flavonoid genes in 35S::*AtMYB7*:GR plants induced with DEX (+DEX) in the presence of cycloheximide (+CHX). Two biological replicates (with three technical replicates) are shown. Broken lines represent the relative gene expression level of untreated plants fixed as one.

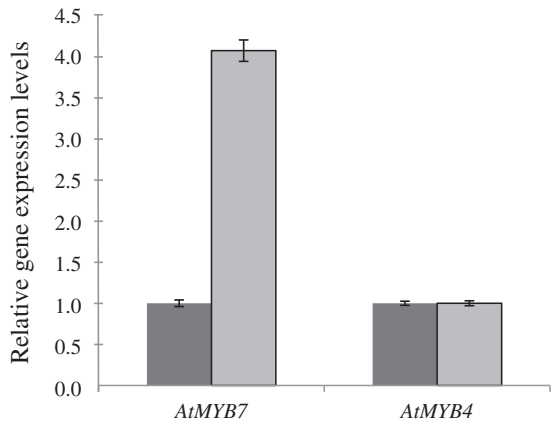

Fig. 8 AtMYB7 is induced by salt. Relative expression levels of *AtMYB7* and *AtMYB4* in wild-type and *atmyb7* and *atmyb4* mutant plants treated with salt. Black bars represent untreated plants and gray bars represent salt-stressed plants.

The phylogenetic analysis of the subgroup 4 R2R3-MYB proteins revealed that AtMYB7 is the closest one to the already characterized AtMYB4 and AtMYB32 (Dubos et al. 2010) and in this work we undertook its characterization to unravel its function in *A. thaliana*.

The GUS reporter gene analysis indicated that *AtMYB7* is mainly expressed in roots and in anthers, while in rosettes it is mainly detected in the leaf vasculature. This expression pattern is similar to that of *AtMYB4* but comparatively weaker. Previous studies showed that *AtMYB32* is strongly expressed in root primordia (Preston et al. 2004), but this is not the case for either *AtMYB7* or *AtMYB4*.

The lack of AtMYB4 induces the expression of *AtMYB7* in tissues where normally it is not detected. In addition, overexpression of AtMYB4 represses *AtMYB7* gene expression in transgenic plants. Thus, it is likely that AtMYB4 limits (direct or indirectly) the expression pattern of *AtMYB7*. A direct interaction of AtMYB4 with the *AtMYB7* gene promoter is not excluded due to the presence of several *cis*-elements typically recognized by MYB factors (data not shown) in the *AtMYB7* gene promoter.

The characterization of *atmyb7* and *atmyb7 atmyb4* mutant plants makes a role for both AtMYB7 and AtMYB4 in the synthesis of lignin unlikely. The higher flavonol levels of *atmyb7* plants indicate a function for AtMYB7 in the regulation of this branch of the phenylpropanoid pathway. In addition, the comparison of the flavonoid profiles of *atmyb7*, *atmyb4* and *atmyb7 atmyb4* mutants suggests that the function of AtMYB7 in

flavonol synthesis is specific, as the lack of AtMYB4 in the double *atmyb7 atmyb4* mutant does not modify the higher levels detected in the *atmyb7* single mutant. In accordance with this, the overexpression of *AtMYB7* reduces flavonol accumulation. The increase in flavonol content is accompanied by a reduction of the anthocyanin levels in the *atmyb7* mutant. This could be the effect of a homeostatic response leading to the redistribution of the common flavonoid precursors. On the other hand, the reduced flavonol levels of the *atmyb4* mutant can be caused by an enhanced activity of AtMYB7, whose expression is induced in the absence of AtMYB4. When both factors are absent, an increased level of both anthocyanins and flavonols is observed. This effect could be due to a more extended misregulation of the general phenylpropanoid pathway as AtMYB4 regulates one of the early phenylpropanoid genes (Jin et al. 2000).

At the molecular level, the lack of *atmyb7* induces the expression of several genes involved in the synthesis of flavonoids such as the early phenylpropanoid genes *C4H* and *4CL1* and the specific flavonoid genes *F3′H*, *DFR* and *UGT*. The same pattern of up-regulation is found in *atmyb7 atmyb4* plants and this reinforces the idea that the role of AtMYB7 on this branch of the phenylpropanoid pathway is not redundant with that of AtMYB4. In addition, inducible AtMYB7 assays showed that some genes of the flavonoid pathway are early targets of this factor, in agreement with a direct role for AtMYB7 in the repression of this branch of the phenylpropanoid pathway.

The *atmyb4* mutants accumulate sinapate esters (Jin et al. 2000) and in this work we showed that in these plants, flavonols are reduced. This effect is in line with the induction of *AtMYB7* expression that takes place in the absence of AtMYB4 and leads us to hypothesize that this metabolic balance between the two main *A. thaliana* UV-protecting sunscreens would be regulated by AtMYB4 and AtMYB7.

Our results show that *AtMYB7* gene expression is induced by salt treatment. This is in agreement with previous results showing that R2R3-MYB factors can play a role in the synthesis of flavonols that takes place in response to this abiotic stress (Fini et al. 2011). Flavonols act as antioxidants against the reactive oxygen species generated by salt stress (Hong et al. 2009). Therefore, AtMYB7 can be proposed as a component of the regulatory network that controls the response to salt stress. In contrast, the gene expression of *AtMYB4* is not altered by salt stress, suggesting once more that the main function of this factor is the regulation of sinapate esters.

The role of AtMYB7 as a repressor of flavonol biosynthesis provides an example of functional divergence within the members of the same subgroup 4 of the R2R3-MYB family. The duplication of these factors during evolution led to a divergence of their function in the regulation of the different branches of the phenylpropanoid pathway (Feller et al. 2011). Thus, while AtMYB4 evolved to regulate sinapate ester biosynthesis and AtMYB32 to control lignin biosynthesis in pollen, in this work we have shown that AtMYB7 has evolved to repress flavonol biosynthesis. In addition, the regulation of AtMYB7 gene expression by AtMYB4 led us to propose the existence of a functional interplay between these two closely related factors to control the balance of the main *A. thaliana* UV-sunscreens.

Materials and Methods

Plant material

Arabidopsis thaliana atmyb7 (ecotype Columbia) T-DNA mutant seeds were obtained from the SALK collection (http://signal.salk.edu/cgi-bin/tdnaexpress) and *atmyb4* (ecotype Landsberg erecta) mutant seeds from the transposon GENETRAP collection (http://genetrap.cshl.edu). The double mutant *atmyb7 atmyb4* was obtained by cross-pollination of the homozygous single mutants. The segregation of the mutant alleles was followed by genotyping the offspring until the double homozygous plant was obtained. The primers used for the screening of single and double mutants are listed in **Supplementary Table S1**.

Approximately 2 kb of the *AtMYB7* and *AtMYB4* gene promoters (p*ATMYB7* and p*ATMYB4*) were cloned (**Supplementary Table S1**) in a pDONOR221 vector (Invitrogen) and then fused to the *GUS* coding sequence using the pHGWFS7 vector (Karimi et al. 2002). The resulting p*AtMYB7*::*GUS* and p*AtMYB4*::*GUS* constructs were then transferred by floral dip (Clough and Bent 1998) to *A. thaliana* wild-type, *atmyb7* and *atmyb4* mutant plants. The primer sequences used to clone both promoters are shown in **Supplementary Table S1**. For the production of the overexpressing plants, the cDNA sequence of AtMYB7 and AtMYB4 was placed under the control of the 35S CaMV promoter and the pA35S transcription terminator, and the construct was transferred to the pCAMBIA1300 vector as described elsewhere (Fornalé et al. 2006).

The cDNA sequence of *AtMYB7* was amplified by PCR (**Supplementary Table S1**) and cloned into the pDONOR221 vector (Invitrogen) and fused through LR recombination (Invitrogen) in the multisite vector pH7m34GW (Karimi et al., 2002) with the 35S CaMV promoter (Karimi et al., 2002) and the glucocorticoid receptor (GR). *Arabidopsis thaliana* plants were transformed by floral dip using *Agrobacterium tumefaciens*. (Clough and Bent 1998).

For the in vitro culture, surface-sterilized *A. thaliana* wild-type and mutant seeds were sown on MS solid medium (Murashige and Skoog 1962), kept for 2 d at 4°C in the dark and then transferred to a growth chamber with a 16 h light period at 22°C. For the greenhouse culture, seeds were grown under standard condition (25°C day and 22°C night with 50% humidity) with a 16/8 h photoperiod of natural and supplemented light.

Once harvested, plant material was immediately frozen with liquid nitrogen and stored at −80°C for the following biochemical and molecular analyses, or immediately used as fresh material for the histochemical studies.

Cycloheximide and dexamethasone treatments

CHX (Sigma-Aldrich) was dissolved in 50% (v/v) ethanol at 50 mM and DEX (Sigma-Aldrich) was dissolved in 100% ethanol (v/v) at 5 mM. Both solutions were kept at −20°C until use. 35S::*AtMYB7*::GR seedlings were grown on filter paper circles for 14 d and then transferred to new plates containing 50 μM DEX or 50 μM DEX plus 5 μM CHX. Controls were prepared using water containing the same amount of ethanol (Roig-Villanova et al. 2006). Treatments were performed for 3 h and replicates consisting of pools of 10 plants were sampled and immediately frozen in liquid nitrogen for the quantitative reverse transcription–PCR (RT–PCR) assays.

Histology and lignin analysis

Handmade cross-sections were prepared from the basal part of inflorescence stems, stained with Wiesner solution (1% phloroglucinol in ethanol : 37% HCl, 1 : 1) and immediately observed under light microscopy.

The lignin content and composition were determined in mature stems of mutant and wild-type plants. Thioacidolysis and gas chromatography were used to determine the lignin composition according to the method published by Lapierre et al. (1986), and the lignin content was determined by derivatization with thioglycolic acid (Withmore, 1978).

Constructions and RT–PCR

Total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen). Approximately 1.5 μg of total RNA were reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was generated using an oligo$(dT)_{15}$ primer, and 2 μl of the first-strand cDNA was used as a template in subsequent PCRs. Quantitative RT–PCR assays were run using the Light Cycler 480 (Roche) and LC480 SYBR Green I Master (Roche). Gene-specific primers were used to amplify *AtMYB7*, *AtMYB4* and all the main phenylpropanoid biosynthetic genes (**Supplementary Table S2**), and actin was used for data normalization.

GUS staining

Histochemical staining for GUS activity in transgenic plants was performed as described previously (Jefferson et al. 1987) with minor modifications. Seedlings grown in vitro on MS plates for 10 d, rosettes or flowering plants were harvested and immediately immersed in the reaction solution composed of 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 50 mM sodium phosphate, 2 mM ferricyanide, 2 mM ferrocyanide and 0.2% Triton X-100, pH 7.0. After 5 min of vacuum infiltration, samples were incubated overnight at 37°C. Serial washes of 30 min each were then performed with increasing concentrations of ethanol (from 20% to 70%) and pictures of the stained tissues were taken with a stereomicroscope.

Salt treatments

Wild-type, *atmyb7* and *atmyb4* mutant plants grown in vitro for 2 weeks were transferred to a liquid MS medium containing 150 mM NaCl for 4 h. For each treatment, the corresponding control was prepared by incubating seedlings in MS liquid medium for the same time. After this time period, whole plants were collected and immediately frozen with liquid nitrogen.

Quantification of flavonoids and soluble phenolics

Total flavonols were determined according to Chang et al. (2002). Leaf tissues were extracted in 80% methanol at 4°C for 2 h. After centrifugation, aliquots of supernatant were taken to 2 ml with methanol and sequentially mixed with 0.1 ml of aluminum chloride (10% water solution), 0.1 ml of 1 M K-acetate and 2.8 ml of distilled water. After 30 min incubation at room temperature, absorbance at 415 nm was recorded. Flavonol content was quantified as equivalents of rutin used as standard.

Total anthocyanins were determined according to Laitinen et al. (2008). Leaf tissues (100 mg) were extracted with 1 ml of extraction solvent (methanol, water, hydrochloric acid, 7 : 2 : 1) at 4°C for 20 h and centrifuged (20 min, 10,000 r.p.m., 4°C). The absorbance of the supernatants was measured at 530 nm and the anthocyanin content was quantified as equivalents of cyanidine chloride used as standard.

Total phenolics were determined using a modified Folin–Ciocalteu colorimetric method (Singleton and Rossi 1965). Fresh leaf tissue (100 mg) was extracted in 1 ml of ethanol (80%), incubated for 2 h at 4°C in the dark and then centrifuged to remove cell debris. Aliquots of supernatant were made up to a volume of 3 ml with distilled water. Then 0.5 ml of Folin–Ciocalteau reagent (1 : 1 with water) and 2 ml of Na_2CO_3 (20%) were added. The solution was warmed for 15 min at 45°C, cooled to room temperature and the absorbance was measured at 650 nm. Phenolics were quantified as equivalents of gallic acid used as standard.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Spanish 'Ministerio de Economía y Competitividad' [AGL2011-30545-C02-01 to D.C.-R.]; the Spanish Ministerio de Ciencia e Innovación [CONSOLIDER-INGENIO program (CSD2007-00036)]; Autonomous Government of Catalonia [grant AGAUR (2008FI-B 00399) to J.E.S.-H.]; CRAG [a contract to J.E.S.-H.]. This work was carried out within the framework of the 'Xarxa de Referència de Biotecnologia' (XarBa) from the Autonomous Government of Catalonia

Acknowledgments

We are indebted to Dr. Matus for enriching discussions during the elaboration of this work, and to Nuria Mir, Dr. Capellades and Yasin Torres for technical support and helpful comments. We also thank Dr. Miguel Moreno-Risueno and Dr. Rossangela Sozzani for kindly providing the p2P3R entry vector with the GR tag.

Disclosures

The authors have no conflicts of interest to declare.

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