

# Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*

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**An *Arabidopsis thaliana* line that is mutant for the R2R3 MYB gene, AtMYB4, shows enhanced levels of sinapate esters in its leaves. The mutant line is more tolerant of UV-B irradiation than wild type. The increase in sinapate ester accumulation in the mutant is associated with an enhanced expression of the gene encoding cinnamate 4-hydroxylase, which appears to be the principal target of AtMYB4 and an effective rate limiting step in the synthesis of sinapate ester sunscreens. AtMYB4 expression is downregulated by exposure to UV-B light, indicating that derepression is an important mechanism for acclimation to UV-B in *A.thaliana*. The response of target genes to AtMYB4 repression is dose dependent, a feature that operates under physiological conditions to reinforce the silencing effect of AtMYB4 at high activity. AtMYB4 works as a repressor of target gene expression and includes a repression domain. It belongs to a novel group of plant R2R3 MYB proteins involved in transcriptional silencing. The balance between MYB activators and repressors on common target promoters may provide extra flexibility in transcriptional control.**

**Keywords:** dose dependence/plants/repressor/R2R3 MYB transcription factor/UV-B tolerance

## Introduction

Because plants require sunlight for photosynthesis they are inevitably exposed to ultraviolet light, including that in the wavelength range of 280–320 nm (ultraviolet-B, UV-B). UV-B light is potentially very damaging to DNA and proteins, and increases free radical production. To provide a sunscreen, most plants synthesize secondary metabolites that can absorb UV-B and also act as free radical scavengers to counteract the damaging consequences of irradiation. Prominent amongst these sunscreens are the

phenylpropanoids, and from these compounds some plants produce flavonoids, others produce hydroxycinnamic acid derivatives and yet others, such as *Arabidopsis thaliana*, produce sinapate esters (hydroxycinnamic acid derivatives) in their leaves, but augment this protection with flavonoid production (Li *et al.*, 1993).

The control of production of UV-B-protecting metabolites has been intensively studied in several plant species and has been shown to be predominantly transcriptional (Chappell and Hahlbrock, 1984; Douglas *et al.*, 1987; Schulze-Lefert *et al.*, 1989; Li *et al.*, 1993; Hartmann *et al.*, 1998). The most detailed analysis of transcriptional regulation has been undertaken for the promoter of the chalcone synthase gene (*CHS*) where light responsive units (LRUs), consisting of an ACGT-containing element (ACE) and a MYB recognition element (MRE), confer responsiveness to UV-containing white light. Two bZIP transcription factors bind the ACE and are believed to be involved in transcriptional activation in response to UV-containing light (Feldbrugge *et al.*, 1994; Sprenger-Haussels and Weisshaar, 2000). Two MYB-related proteins have also been shown to interact with the MRE (Sablowski *et al.*, 1994; Feldbrugge *et al.*, 1997). Other members of the R2R3 MYB gene family have been implicated in regulation of other branches of phenylpropanoid metabolism, including phlobaphene biosynthesis (*P* in maize; Grotewold *et al.*, 1994), flavonol synthesis (*AmMYB305* and *AmMYB340*) in *Antirrhinum majus* (Moyano *et al.*, 1996) and hydroxycinnamic acid and monolignol biosynthesis (*AmMYB308* and *AmMYB330*) in tobacco (Tamagnone *et al.*, 1998a). Of these, the action of *AmMYB308* in regulating flux to soluble hydroxycinnamic acid derivatives could represent an important control point in regulating the synthesis of UV-protecting compounds, and in the acclimation to UV-B stress of those plants that use these particular phenylpropanoids as sunscreens.

*AmMYB308* was isolated from *A.majus* and overexpression in tobacco caused an inhibition of hydroxycinnamic acid and monolignol biosynthesis, implying that it could repress transcription of the biosynthetic genes. However, MYB-related proteins have generally been shown to operate as transcriptional activators (Weston and Bishop, 1989; Foos *et al.*, 1994). c-MYB has been shown to contain an activation domain, although negative regulatory domains within the C-terminus of the protein may limit the activation by the protein through interaction with negative regulatory proteins (Dubendorff *et al.*, 1992; Wang *et al.*, 1999). B-MYB, although reported to act as a repressor (Foos *et al.*, 1992; Watson *et al.*, 1993), also contains an activation domain (Ansieau *et al.*, 1997). In plants, the view that MYB-related transcription factors function predominantly as transcriptional activators follows from the demonstration of activation domains in the

**Table I.** Biosynthetic enzymes of phenylpropanoid metabolism

Enzyme	Abbreviation	Isoform used	Accession No.
Phenylalanine ammonia lyase	PAL	PAL2	L33678
Cinnamate 4-hydroxylase	C4H		D78596
<i>p</i> -coumaroyl 4-CoA ligase	4CL	4CL1 4CL3	U18675 AF106088
<i>p</i> -coumarate 3-hydroxylase	C3H		
Caffeic acid <i>o</i> -methyl transferase	OMT		U70424
Caffeoyl CoA <i>o</i> -methyl transferase	CCoAOMT		L40031
Ferulate 5-hydroxylase	F5H		AF068574
Caffeoyl CoA reductase	CCR		
Cinnamoyl alcohol dehydrogenase	CAD	CAD1	L37883
Sinapic acid UDPG:sinapoyl transferase	SGT		
Sinapoylglucose malate:sinapoyl transferase	SMT		
Chalcone synthase	CHS		AF012810

maize MYB-related proteins C1 and P (Sainz *et al.*, 1997), and the presence of regions with a high probability of forming amphipathic  $\alpha$ -helix within the predicted C-termini of many other R2R3 MYB-related proteins from plants (Martin and Paz-Ares, 1997; Kranz *et al.*, 1998). Given the evidence for R2R3 MYB-related proteins activating transcription of structural genes in different branches of phenylpropanoid metabolism (Martin and Paz-Ares, 1997), the action of AmMYB308 in repressing expression of some hydroxycinnamic acid synthesizing genes is unusual. Its effect in tobacco might reflect that it can bind to the same target sites as endogenous MYB-related activators, but not activate from these sites in a heterologous host.

To determine the biological role of MYB-related transcription factors in production of hydroxycinnamic acid derivatives, we have characterized a gene from *A.thaliana*, *AtMYB4*, orthologous to *AmMYB308*, and we have identified a knockout mutant. The mutant demonstrates that *AtMYB4* plays a significant role in controlling sinapate ester formation and in the development of sunscreens offering protection to UV-B light. *AtMYB4* appears to work as a repressor, particularly of one key target gene—that encoding cinnamate 4-hydroxylase. Through regulation of the expression of this gene, *AtMYB4* negatively modulates sinapate ester formation in the absence of UV-B light. Expression of *AtMYB4* is downregulated in response to UV-B, resulting in increased sinapate ester production.

## Results

### Identification of *AtMYB4* and a knockout mutant from *A.thaliana*

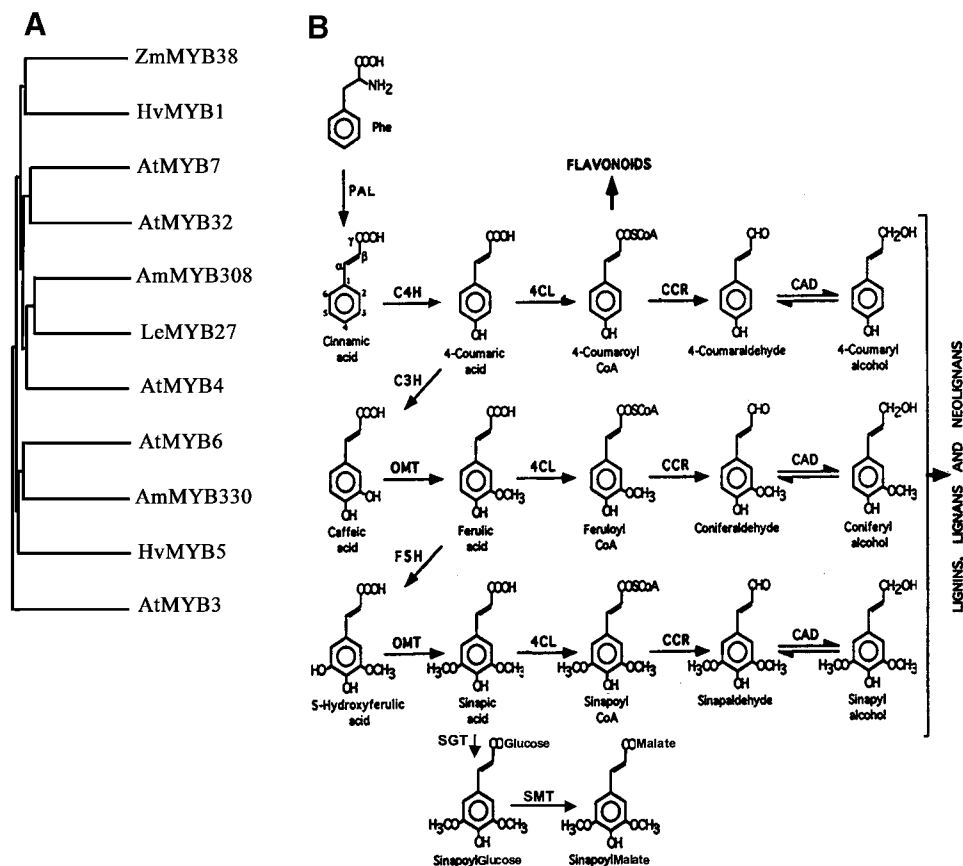
The R2R3-related transcription factor AmMYB308 had been shown to downregulate the *C4H*, *4CL* and *CAD* genes (Table I; Figure 1B) of hydroxycinnamic acid/monolignol biosynthesis when overexpressed in transgenic tobacco (Tamagnone *et al.*, 1998a). To determine the normal physiological role of *AmMYB308* we needed to find a mutation of the gene; the most appropriate strategy was to identify the orthologous gene from *A.thaliana* and screen the numerous insertion-mutagenized populations for knockout mutations. This task was facilitated by the near-complete cloning of R2R3 MYB genes from

*A.thaliana* (Kranz *et al.*, 1998; Romero *et al.*, 1998). Comparison of sequences encoded by these genes to that of *AmMYB308* revealed *AtMYB4* to encode the most similar protein (Figure 1A). Both proteins belong to R2R3 subgroup 4 proteins, which share both a conserved motif (pdLNL<sup>D</sup><sub>E</sub>Lxi<sup>G</sup>/S) and a region potentially forming a zinc finger domain at their C-termini (Kranz *et al.*, 1998). Given the near completion of the *A.thaliana* genome sequence and the identification of 111 R2R3 MYB genes from a total predicted number of ~125, it is highly likely that *AtMYB4* is the orthologous gene in *A.thaliana* to *AmMYB308* in *A.majus*.

A mutant of *AtMYB4* was sought by PCR-mediated reverse genetic screening of several insertion mutagenized populations, and a stable dSpm insertion was identified (ecotype Colombia; Meissner *et al.*, 1999). The insertion was in the first exon, 92 bp after the initiating ATG. It disrupted the sequence encoding the first repeat (R2) of the DNA binding domain of *AtMYB4*. Heterozygous plants were identified from pooled seed, and plants segregating as homozygous wild type or homozygous mutants were selected for phenotypic analysis. Two individuals, homozygous for the dSpm insertion, were propagated by selfing (*atmyb4*<sup>-</sup> A and B) as examples of the mutant, and one homozygous wild-type sibling line (WT) was similarly propagated. The effect of the dSpm insertion on the expression of *AtMYB4* was established by northern blots of RNA from leaves, where no transcript was visible in the mutant compared with a transcript of 1.2 kb in leaves from the wild-type plant. This was confirmed by RT-PCR (Figure 2A) of cDNA made from RNA isolated from leaves.

### Phenotype of *atmyb4*<sup>-</sup> mutant plants

Visual inspection of *atmyb4*<sup>-</sup> plants showed no obvious differences in growth rate or appearance in comparison to wild-type plants. Wild-type and mutant lines were grown on soil in the greenhouse for 12 days and methanolic extracts of leaves were analysed by HPLC. A single major peak corresponding to sinapoyl malate was apparent from wild-type leaves (Figure 2B). This is the major soluble phenolic in *A.thaliana* leaves and is synthesized via the hydroxycinnamic acid pathway (Figure 1B; Chapple *et al.*, 1992; Li *et al.*, 1993; Landry *et al.*, 1995; Lorenzen *et al.*, 1996). In the mutant seedlings this peak was ~6-fold



**Fig. 1.** (A) Dendrogram showing structural relationships between members of R2R3 MYB subgroup 4 from different plant species. Zm signifies *Zea mays*; Hv, *Hordeum vulgare*; Le, *Lycopersicon esculentum*; At, *Arabidopsis thaliana*; Am, *Antirrhinum majus*. For gene references see Kranz *et al.* (1998). (B) Pathway of hydroxycinnamic acid metabolism in *A.thaliana* leaves. Interconversions producing the precursors for flavonoid biosynthesis, monolignols and sinapate esters are indicated (for key to enzymes see Table I). CCoAOMT can use caffeoyl CoA and 5-hydroxyferuloyl CoA as substrates to produce feruloyl CoA and sinapoyl CoA in an alternative methylation pathway for sinapoyl ester production.

greater, indicating a significant increase in the production of sinapoyl malate in *atmyb4<sup>-</sup>* mutant plants. These experiments were repeated with leaf material from seedlings grown on agar, and consistently showed significantly higher levels of sinapate esters in the mutant lines compared with wild type, although the differences were not as great as the difference observed in the first analysis (Table II).

The enhanced levels of sinapoyl malate in the *atmyb4<sup>-</sup>* mutant lines suggested that these plants might be more tolerant of UV-B (Li *et al.*, 1993; Landry *et al.*, 1995). A dose–response curve showed that a 20 min exposure to UV-B light (3.2 mW/cm<sup>2</sup>) caused >80% death of wild-type seedlings. Treatment of seedlings homozygous for the *atmyb4<sup>-</sup>* mutation for the same length of time gave significantly reduced plant death (36%, 21%), establishing that loss of AtMYB4 activity increased sinapoyl malate levels, affording greater tolerance to UV-B (Figure 3A).

#### Expression of AtMYB4 in response to light and wounding

Given that AtMYB4 represses sinapoyl malate production, and that mutation of this gene will make *A.thaliana* seedlings more tolerant of UV-B, it was of interest to determine the normal response of AtMYB4 expression to

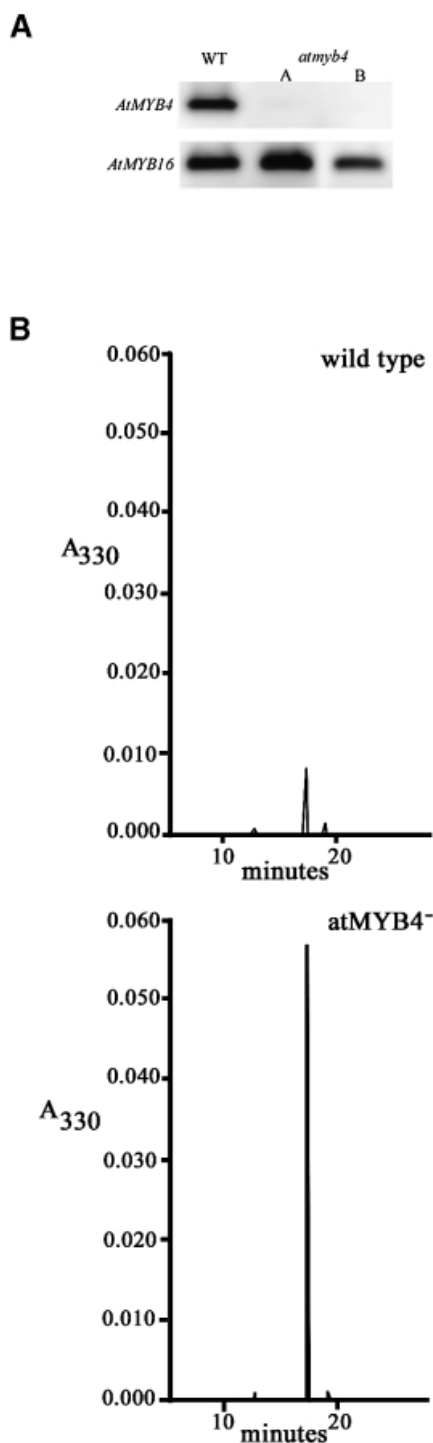
varying light stimuli. Generally, AtMYB4 expression was induced by all types of light (Figure 4). However, while prolonged (24 h) exposure to white light left AtMYB4 expression more or less the same as after 1 h, prolonged exposure to blue or UV light gave reduced levels of AtMYB4 expression. This was true for UV-A, UV-B and UV-C light, but the most dramatic change occurred in response to UV-B. Twenty-four hour exposure to relatively low levels of UV-B (0.2 mW per cm<sup>2</sup>) gave complete loss of AtMYB4 expression. This response was reproducible in independent extracts from seedlings.

In addition, the response of AtMYB4 expression to wounding was analysed (Figure 4). Transcript levels of AtMYB4 decreased in leaves 4–6 h after wounding, both in wounded leaves and systemically in non-wounded leaves from the same rosette. AtMYB4 transcript levels recovered after ~24 h.

#### Identification of genes regulated by AtMYB4

To analyse which genes AtMYB4 regulates, RNA was extracted from leaves of wild-type and mutant, soil-grown plants. First strand cDNA was synthesized and primer pairs were designed for each of the genes encoding enzymes in hydroxycinnamic acid and monolignol biosynthesis, where the gene sequence had been published.

These were used initially to amplify the relevant transcripts from wild-type RNA (Table I). cDNAs were subcloned and their identity was confirmed by sequencing.



**Fig. 2.** (A) Expression of *AtMYB4* in leaves of wild-type (WT) and *atmyb4*<sup>-</sup> mutant plants. Expression was monitored by RT-PCR amplification of first strand cDNA. The transcript of another *MYB* gene expressed in leaves, *AtMYB16*, was amplified from the same cDNA as a control. (B) HPLC traces of soluble phenolics in methanolic extracts from leaves of wild-type and *atmyb4*<sup>-</sup> mutant plants. A single major peak absorbing at A<sub>330</sub> was confirmed to be sinapoyl malate by liquid chromatography mass spectrometry (LCMS). In the mutant the levels of sinapoyl malate were 6- to 7-fold higher than in wild type.

The same primers used to amplify the biosynthetic gene transcripts were used for quantitative RT-PCR of cDNA from wild type and mutant. Compared with wild-type RNA, only one gene showed higher transcript levels in the two mutant lines—that encoding *C4H* (Table I; Figure 5). Given that *atmyb4*<sup>-</sup> mutant plants routinely showed higher levels of sinapate esters, this suggested that the expression of the *C4H* gene can significantly affect flux along this branch of phenylpropanoid metabolism. In other words, *C4H* represents an important control point in the synthesis of sinapate esters with an implied high flux-control coefficient.

Only one other gene showed a difference in expression between the *atmyb4*<sup>-</sup> mutants and the wild type, that encoding *CCoAOMT* (Figure 5). Interestingly, this showed reduced transcript levels in the mutant compared with the wild type, implying that its expression was upregulated in response to *AtMYB4* activity.

We examined expression of the gene encoding *C4H* in relation to expression of *AtMYB4*. Under blue or UV-B light there was a negative correlation between *AtMYB4* expression and *C4H* expression (Figure 4). This suggested that under these conditions *AtMYB4* activity is the principal factor influencing expression of the *C4H* gene, but under other light conditions other factors are probably more influential.

#### Overexpression of *AtMYB4* in tobacco and *A.thaliana*

Overexpression of *AtMYB4* gave rise to the same phenotype in transgenic tobacco that had been observed for *AmMYB308* (Figure 6): slow-growing plants with pale leaves that showed premature white lesions on the mature leaves indicative of reduced levels of hydroxycinnamic acid derivatives (Tamagnone *et al.*, 1998b). *AtMYB4* overexpression reduced the steady state transcript levels of the *C4H*, *4CL1* and *CAD* genes in tobacco in the same way as *AmMYB308* (data not shown; Tamagnone *et al.*, 1998a).

In *A.thaliana* plants overexpressing *AtMYB4*, white lesions were apparent in older leaves (Figure 6). The *atMYB4*<sup>-</sup> mutant line was also transformed with the construct giving high levels of *AtMYB4* expression. This line showed no phenotype compared with wild type.

Plants overexpressing *AtMYB4* were more sensitive to UV-B. Exposure to a transilluminator for 10 min, which caused no death in wild-type plants, resulted in 60 and 37% plant death in two independent lines overexpressing *AtMYB4* (Figure 3B). Analysis of the phenylpropanoids in these overexpressing lines confirmed that they had reduced levels of sinapate esters (Table II), but their flavonoid composition was not affected (data not shown).

#### Identification of genes regulated by *AtMYB4* in overexpressing *A.thaliana* lines

Comparison of transcript levels for the biosynthetic genes in two independent overexpressing lines with wild-type plants showed that expression of the *C4H* gene was considerably reduced by *AtMYB4* overexpression (Figure 5). Interestingly, the *CCoAOMT* gene showed increased transcript levels in both overexpressing lines, and increased expression in the mutant line overexpressing *AtMYB4*. The transcript levels of *PAL2*, *F5H*, *OMT* and *CAD1* were unaffected by overexpression of *AtMYB4*, in

contrast to the effects of overexpression of the gene in tobacco where *CAD* was downregulated. This result suggests that regulation of the *CAD* genes from tobacco

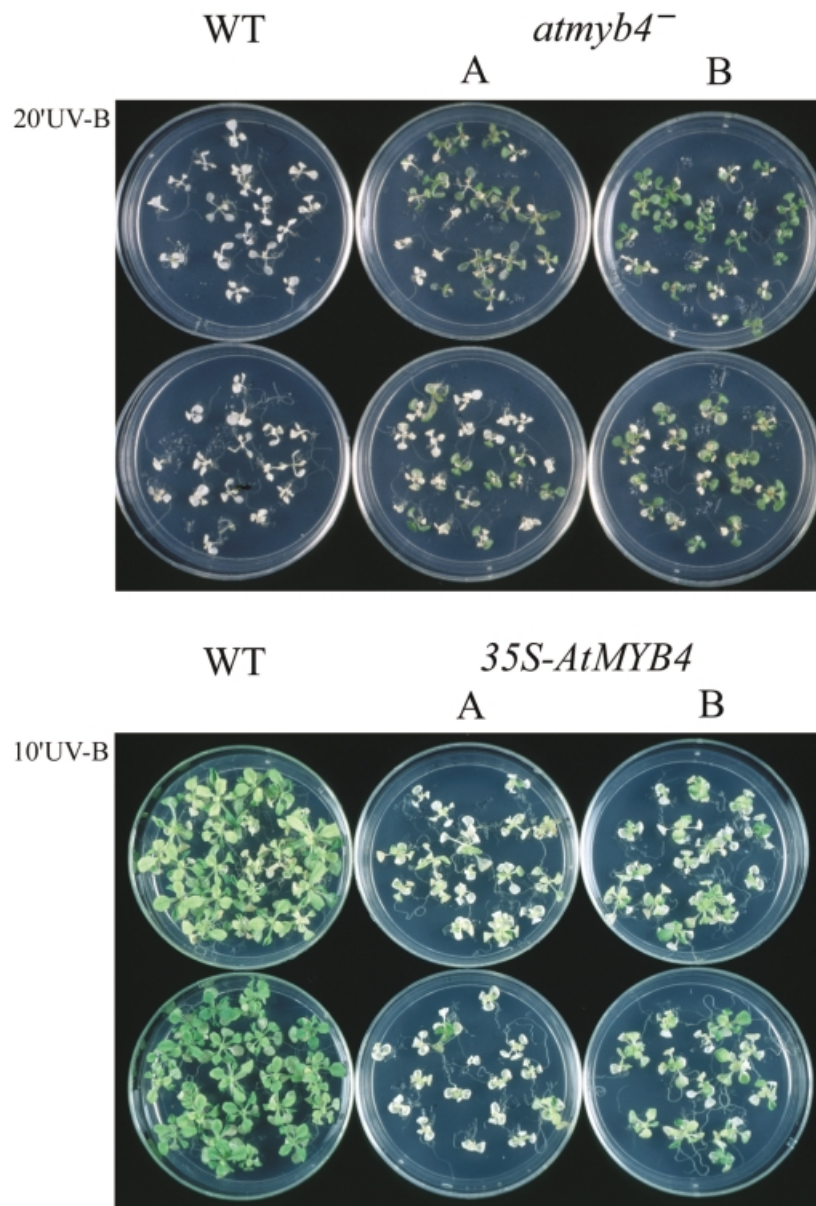
**Table II.** Sinapoyl malate content ( $\mu\text{g/g}$  fresh weight leaves) of *A.thaliana* lines

Line	Plants grown under	
	Short days	Long days
Wild type	458.9	295.7
atmyb4 <sup>-</sup> A	524.4	389.8
atmyb4 <sup>-</sup> B	557.7	352.8
35S-AtMYB4A	372.9	252.4
35S-AtMYB4B	258.2	238.7

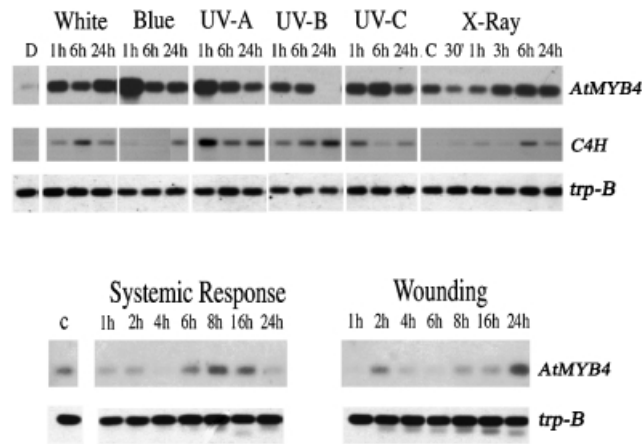
is distinct from regulation of the *CAD1* gene in *A.thaliana*. Interestingly, overexpression of *AtMYB4* also affected three genes that were unaffected in the atmyb4<sup>-</sup> mutant, *CHS*, *4CL1* and *4CL3* (Figure 5), which all showed reduced transcripts in overexpressing lines, while the atmyb4<sup>-</sup> mutant overexpressing *AtMYB4* showed an intermediate effect.

#### **Dose-dependent selection of target genes by AtMYB4**

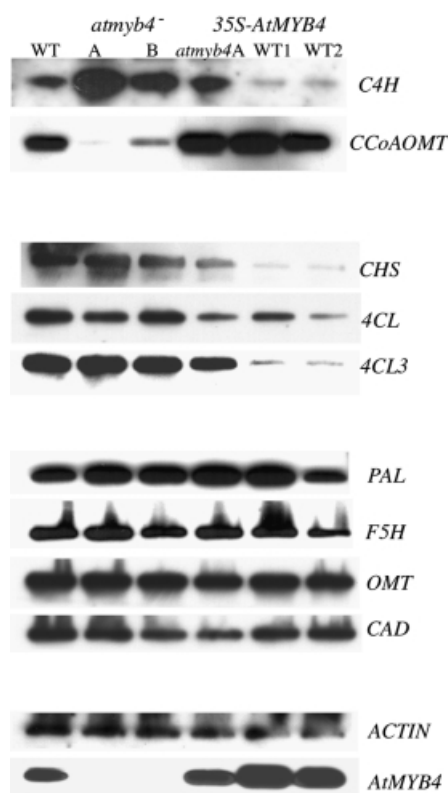
One of the most significant aspects of our data on AtMYB4 action was that it appeared to regulate new genes when present in high concentrations. Comparison of the transcript levels in wild-type and atmyb4<sup>-</sup> knockout plants suggested that only the *C4H* gene was negatively regulated by AtMYB4, and that the *CCoAOMT* gene was



**Fig. 3.** Effect of mutation of *AtMYB4* on the tolerance of *A.thaliana* seedlings to UV-B. The upper panel shows seedlings exposed to 20 min UV-B (3.2 mW/cm<sup>2</sup>). More seedlings survived in duplicate plates of atMYB4<sup>-</sup> mutants than did from the wild type. The lower panel shows the enhanced sensitivity of *AtMYB4*-overexpressing plants to UV-B. Plants were exposed to 10 min UV-B (3.2 mW/cm<sup>2</sup>). Two independent transgenic lines showed enhanced seedling death on duplicate plates compared with wild type.

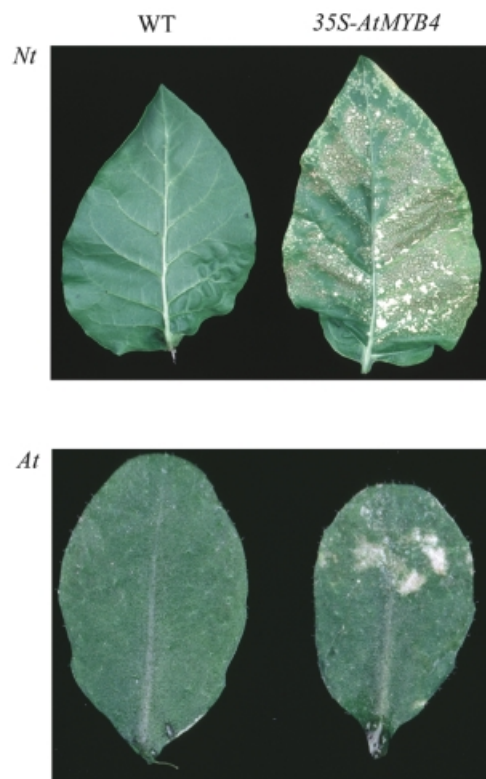


**Fig. 4.** Expression of *AtMYB4* in seedlings grown under different light conditions. Transcript levels were determined from total RNA from leaves. The expression of the *C4H* gene was determined for the same samples. As a control, the  $\beta$  subunit of tryptophan synthase (*trp-B*) was amplified. The expression of *AtMYB4* was also determined for wounded leaves and for leaves from wounded plants (systemic response).



**Fig. 5.** Effect of mutation of *AtMYB4* and overexpression of the gene on transcript levels of the genes of hydroxycinnamic acid and flavonoid metabolism. Transcript levels were determined by quantitative RT-PCR. Expression of *C4H* increased in the mutant and was decreased in overexpressing lines. The overexpression construct in the *atmyb4<sup>-</sup>* mutant background gave an intermediate transcript level. The *CCoAOMT* gene showed complementary responses; its transcript levels were decreased in the mutant and enhanced in the overexpressing lines. *CHS*, *4CL1* and *4CL3* genes were downregulated by overexpression of *AtMYB4*.

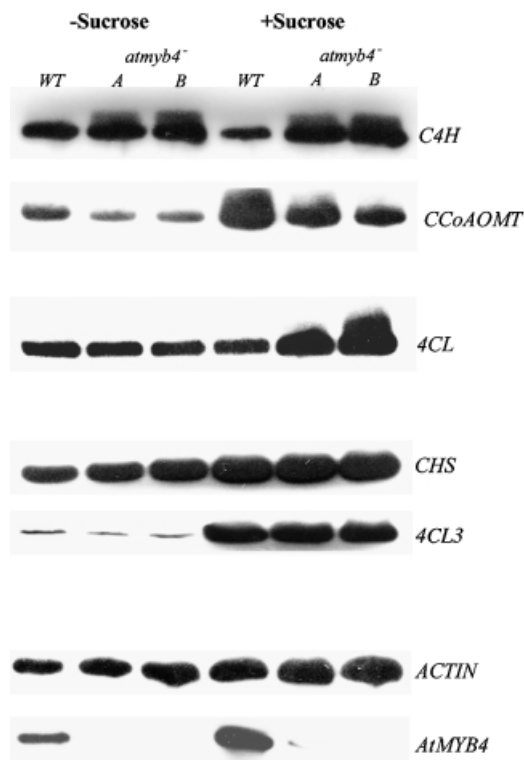
positively regulated by the transcription factor. However, in plants with higher activity of *AtMYB4*, as achieved by expression of the cDNA under the control of the double CaMV 35S promoter, the *CHS*, *4CL1* and *4CL3* genes were also negatively regulated by *AtMYB4*. Additional experiments were undertaken to determine whether dose-



**Fig. 6.** Phenotypic effects of *AtMYB4* overexpression in tobacco (*Nt*) and *A.thaliana* (*At*). In both species overexpression caused the appearance of white lesions on older leaves very similar to those observed for *AmMYB308* overexpression in tobacco (Tamagnone *et al.*, 1998b).

dependent selection of target genes by *AtMYB4* occurred under physiological conditions.

The steady state transcript levels of *AtMYB4* increased in plants grown on agar containing sucrose compared with plants grown on agar alone (Kranz *et al.*, 1998). Consequently, wild-type and *atmyb4<sup>-</sup>* mutant plants were grown on agar alone and on agar supplemented with 100 mM sucrose. This resulted in an induction of *AtMYB4* transcript levels in the wild-type plants grown on agar plus sucrose, confirming the previous observations (Figure 7).



**Fig. 7.** Dose dependence of target gene repression by AtMYB4. Transcript levels were analysed by quantitative RT-PCR. The effect of AtMYB4 was monitored by comparing transcript levels in wild type and mutants (*atmyb4*<sup>-</sup> A and B). The *C4H* gene was downregulated by AtMYB4 in plants grown with or without sucrose. The *CCoAOMT* gene was upregulated by AtMYB4 in plants grown with or without sucrose. On high sucrose, when AtMYB4 expression was elevated, the *4CL1* gene was negatively regulated by AtMYB4. There was no effect of AtMYB4 activity on transcript levels for *CHS* or *4CL3* genes.

Comparison of transcript levels for the *C4H* gene in wild-type and mutant plants showed reduced transcripts in wild-type plants grown on sucrose compared with those without sucrose, and higher levels of transcripts in the mutant lines compared with the wild type under both conditions, as expected. Also, as predicted, the *CCoAOMT* gene was upregulated in plants grown on sucrose, but *atmyb4*<sup>-</sup> mutant plants showed lower transcript levels than wild type when grown on agar with or without sucrose. Interestingly, the *4CL1* gene (Ehlting *et al.*, 1999) showed no difference in transcript levels between wild type and mutants on agar without sucrose, but a higher level of transcript was observed in mutant lines compared with wild type where plants were grown on agar plus sucrose. These data suggested that the induction of AtMYB4 expression by sucrose results in elevation of AtMYB4 activity sufficient to bring the *4CL1* gene under control of the repressor, and that the dose-dependent changes in the target specificity of AtMYB4 can play a physiological role in regulating target gene expression *in vivo*.

#### **Analysis of AtMYB4 regulation using transfection assays**

To determine whether the effects of AtMYB4 on target gene expression involved direct interaction of the encoded transcription factor with the target gene promoters,

transfection assays were undertaken in *A.thaliana* protoplasts. The promoters of the *C4H*, *CHS*, *4CL3* and *CCoAOMT* genes were fused to the *GUS* reporter gene. Each reporter gene construct was assayed with and without AtMYB4 in co-transfection assays. Expression from the *C4H* promoter was reduced 26-fold by co-transfection with AtMYB4, establishing that the effect of the transcription factor is to repress expression from the *C4H* promoter (Figure 8A). This relationship was confirmed by transforming wild-type and *atmyb4*<sup>-</sup> mutant lines with a *C4H* promoter–*GUS* reporter construct. *GUS* assays (three replicates each) from 10 independent transformants of the wild-type line gave  $5.7 \pm 1.2$  nmol 4-methylumbelliferone (MU) mg/protein/min, whereas assays from 10 independent transformants of the mutant line gave  $10.8 \pm 1.0$  nmol MU mg/protein/min, confirming the higher expression from the *C4H* promoter in the absence of AtMYB4 activity. Histochemical staining revealed that the *C4H* promoter gave *GUS* activity all over the leaf but particularly in the vascular tissue in the mutant, indicating that AtMYB4 is repressing expression of this gene all over the leaf (Figure 8B).

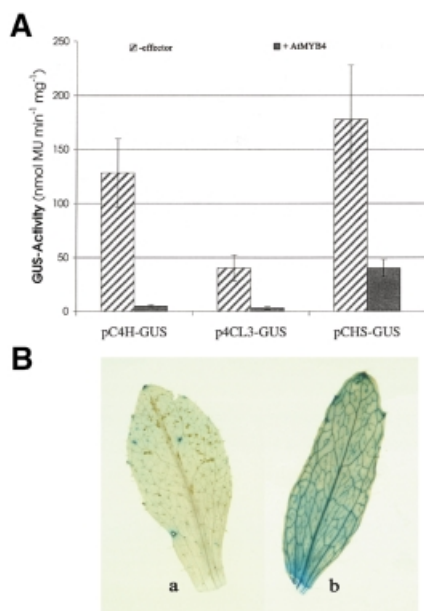
The transfection assays also revealed an inhibition by AtMYB4 of expression from the *4CL3* and the *CHS* promoters, although the downregulation of these reporter genes was not as great as for the *C4H* promoter (13- and 4-fold, respectively). These data confirmed that, at high concentrations, AtMYB4 can repress these genes, and that repression involves a direct interaction of the transcription factor with the promoters of its target genes. Interestingly, no activation or repression of the *CCoAOMT* promoter by AtMYB4 was observed in the transfection assays (data not shown), indicating that AtMYB4 does not act as a direct activator of the *CCoAOMT* gene.

#### **Mutational analysis of AtMYB4**

To investigate the mechanism of AtMYB4 repression, a series of mutations in the AtMYB4 gene construct were made and the effect of the altered proteins on the repression of the *C4H* promoter was monitored using transfection assays (Figure 9A). Mutagenesis of the tryptophan residue in the recognition helix of R3 should abolish DNA binding. This mutant (JAtMYB4Wmu) lost the ability to repress the *C4H* promoter, establishing that binding of DNA by AtMYB4 is essential for its function as a transcriptional repressor.

Deletion of the entire C-terminal domain reduced the repression, although it was not lost entirely at the concentration of effectors tested. Mutation of the cysteine residues within the region of the C-terminal domain suggested to form a zinc finger (Kranz *et al.*, 1998) had no effect on the repression by AtMYB4. Deletion of a conserved motif found in AtMYB4 (JAtMYB4cd) did reduce repression to a similar extent to complete removal of the C-terminal domain (Figure 9A). These results were confirmed by multiple replicates and by assays conducted with varying amounts of effector DNA. In each case repression by AtMYB4 and mutated versions of the protein was reduced when lower concentrations of effector were used.

The results suggested that part of the repression effect could be attributed to the C-terminal domain of AtMYB4, specifically the conserved region found in subgroup 4

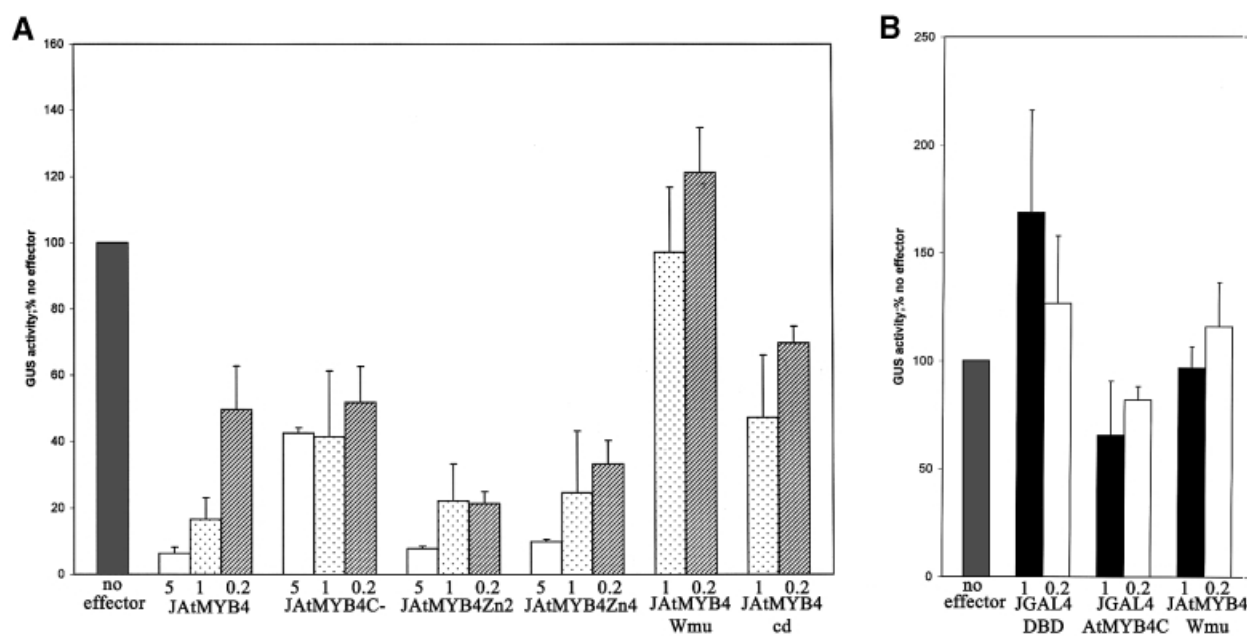


**Fig. 8.** (A) Transfection assays illustrating the effect of AtMYB4 on GUS expression driven by the *C4H* gene promoter, the *CHS* gene promoter and the *4CL3* gene promoter. (B) GUS staining of wild-type (a) and *atmyb4* mutant plants (b) following transformation with the *C4H* promoter–GUS fusion. The activity driven by the *C4H* promoter was significantly enhanced in the mutant line, in the general tissue of the leaf and particularly in the vascular tissue, indicating, by comparison, the regions where AtMYB4 repressed *C4H* expression most strongly.

(Kranz *et al.*, 1998). This motif is present in AmMYB308 and in AmMYB330, another MYB protein from *A. majus* that can repress hydroxycinnamic acid biosynthesis when overexpressed in tobacco (Tamagnone *et al.*, 1998a).

It seemed likely that part of the repressing action of AtMYB4 stems directly from its recognition and binding of DNA. It is very likely that there is transcriptional activation of the *C4H* promoter during the preparation of protoplasts for the transfection assays. All the genes of phenylpropanoid metabolism are induced by wounding stimuli of this type (Bell-Lelong *et al.*, 1997; Mizutani *et al.*, 1997; Ehling *et al.*, 1999; Figure 4). Indeed, empirical evidence for such activation is provided by the background activity of the *C4H* promoter used to determine repression by AtMYB4 in the transfection assays. It is also likely that such activation results, in part, from the action of MYB-related transcriptional activators (Sablowski *et al.*, 1994; Feldbrugge *et al.*, 1997). Therefore, part of the repressing effect of AtMYB4 is likely to be due to competition with endogenous MYB activators for a common binding site in the *C4H* promoter.

Although part of the repressing activity of AtMYB4 may work through competition with activators, our mutational analysis showed that part of the repression exercised by AtMYB4 operates through its C-terminal domain. To determine whether repression by this domain operates indirectly on specific transcription factors associated with the *C4H* promoter, or whether repression operates directly on the basal transcriptional machinery, a construct encoding the C-terminal domain of AtMYB4



**Fig. 9.** (A) Analysis of the ability of AtMYB4 mutants to inhibit transcription from the *C4H* promoter in transfection assays. AtMYB4 repressed basal expression of GUS from the *C4H* promoter between 20- and 40-fold depending on the concentration of effector supplied (5, 1 and 0.2 = 5, 1 and 0.2 μg of effector plasmid, respectively). The repression exercised by AtMYB4 (JAAtMYB4) was lifted by ~50% by removal of the C-terminal domain of the protein (JAAtMYB4C-). Mutation of either or both of the potential zinc finger domains had no effect on repression (JAAtMYB4Zn2 and JAAtMYB4Zn4). Mutation of the DNA binding domain of AtMYB4 (JAAtMYB4Wmu) caused complete loss of all repression. Removal of the conserved domain (JAAtMYB4cd) in the C-terminus of subgroup 4 family members reduced the ability of AtMYB4 to repress transcription, to a similar extent to complete removal of the C-terminus. Results are averages from at least four experiments. Error bars indicate standard deviations. (B) Analysis of the ability of the C-terminal domain of AtMYB4 to inhibit repression when linked to another DNA binding domain, using transfection assays. The GAL4 DNA binding domain slightly activated from the GAL4-90 promoter (JGAL4-DBD). Addition of the C-terminus of AtMYB4 to the GAL4 DNA binding domain (JGAL4AtMYB4C) reduced expression from the GAL4-90 promoter. This was not due to ‘squenching’ because the mutant of AtMYB4 with a defective DNA binding domain (JAAtMYB4Wmu) had no effect on expression from the GAL4-90 promoter.



fused to the DNA binding domain of GAL4 was made. This was tested in transfection assays on a multimerized GAL4 motif fused upstream of the -90 sequence of the CaMV35S promoter, fused in turn to the GUS coding sequence. This reporter had appreciable activity in *A.thaliana* protoplasts in the absence of any effectors. This reporter also responded strongly to a GAL4-VP16 fusion (10- to 25-fold activation depending on the concentration of effector supplied). A construct expressing the GAL4 DNA binding domain alone showed a small stimulation of reporter activity in agreement with previous reports in plants (Schwechheimer *et al.*, 1998). The GAL4 DNA binding domain-C-terminal AtMYB4 fusion reduced expression from the reporter gene by ~50%. This was not the result of 'quelching', since inclusion of the version of AtMYB4 with the mutated DNA binding domain had no effect on expression of the reporter. The repression of transcription exercised by the C-terminal domain of AtMYB4 is, therefore, independent of the promoter on which it is working and likely to be a direct effect on the basal machinery.

## Discussion

Analysis of *A.thaliana* lines mutant for the *AtMYB4* gene has demonstrated that AtMYB4 acts as a negative regulator of hydroxycinnamic acid metabolism, principally through regulating the expression of the gene encoding cinnamate 4-hydroxylase. Expression of AtMYB4 is reduced by different environmental conditions including wounding and exposure to UV-B light. In this way it brings about a derepression of *C4H* gene expression, resulting in higher synthesis of protecting sinapate esters. A mutant of *AtMYB4* that produces significantly higher levels of sinapate esters in its leaves also shows improved tolerance to UV-B treatment. It is interesting that the transcriptional response to UV-B that operates through AtMYB4 is focused on the regulation of expression of the *C4H* gene. There has been considerable debate as to which phenylpropanoids are involved in the response to UV-B and which provide the most effective sunscreens, and *C4H* is active in the synthesis of both sinapate esters and flavonoids. Recent evidence favours sinapate esters as being the primary source of protectant sunscreens in *A.thaliana* (Landry *et al.*, 1995; Booi-James *et al.*, 2000) and we observed a significant effect of the *atmyb4*<sup>-</sup> mutation on sinapoyl malate levels. Hydroxylation catalysed by *C4H* shifts the UV-absorbance spectrum of the hydroxycinnamic acid intermediates towards longer wavelengths to provide compounds that are effective in absorbing biologically relevant wavelengths of UV light (Landry *et al.*, 1995; Bell-Lelong *et al.*, 1997). Therefore, regulation of sinapate ester formation through control of *C4H* provides a biochemically effective strategy for plants to adopt in their acclimation to UV-B. *C4H* provides an effective rate-limiting step in the synthesis of sinapate esters because levels of sinapate esters are enhanced in the *atmyb4*<sup>-</sup> mutant, but, of the genes assayed, only the transcript levels of *C4H* also increase. Although it is possible that the control of flux along the hydroxycinnamic acid/monolignol pathway is vested in one of the genes not measured in our analysis, expression profiling of wild-type and mutant lines for 7000 expressed sequence tags on

microarrays has not revealed additional responding genes (data not shown). The strong correlation between sinapate ester accumulation and *C4H* transcript levels in both mutant and *AtMYB4*-overexpressing lines supports the view that *C4H* is the principal rate-limiting step, at least in leaves of *A.thaliana* plants.

Analysis of *atmyb4*<sup>-</sup> mutants showed that they were also downregulated in *CCoAOMT* transcripts compared with wild type. However, transfection assays showed no evidence for direct activation of the *CCoAOMT* promoter by AtMYB4. One explanation for our results is that AtMYB4 represses a repressor of *CCoAOMT*. A simpler explanation is that expression of the *CCoAOMT* gene responds to feedback regulation by hydroxycinnamic acid derivatives. This could explain the elevated levels of *CCoAOMT* transcripts in lines overexpressing *AtMYB4* (which have reduced hydroxycinnamic acid levels) and the lowered expression of the *CCoAOMT* gene in the *atmyb4*<sup>-</sup> mutant (where hydroxycinnamic acid intermediates are elevated). A similar type of metabolic regulation by transcinamic acid has been reported for *PAL* in tobacco (Blount *et al.*, 2000).

Our data reveal an important role for negative regulation in the synthesis of protecting sunscreens in plants. Such control has not been suggested by previous promoter analysis, although most of the detailed analysis of activation and silencing elements involved in UV response has been performed on the *CHS* gene promoter, for which negative regulation may not normally be operating. Activation of structural genes producing phenylpropanoid sunscreens by derepression is, however, unexpected. Our data suggest that AtMYB4 acts as both a direct repressor and as a competitive repressor through displacing activators binding to the MYB motifs common to the promoters of many genes in phenylpropanoid metabolism (P and L boxes: Sablowski *et al.*, 1994). Such MYB-related activators of the *C4H* gene remain to be identified. However, there is evidence from tobacco to suggest that the direct repression exercised by the orthologue of AtMYB4, AmMYB308, is of primary significance *in vivo*, because overexpression of a truncated version of AmMYB308, comprising just the DNA binding domain, failed to give any phenotypic effects in transgenic plants (Tamagnone *et al.*, 1998a).

Mutational analysis of AtMYB4 has shown the C-terminal region of the protein to be required for full repression. This domain can be transferred to a different DNA binding domain (GAL4) and confers silencing activity on the fusion protein. Although the direct silencing by the C-terminal domain was not strong, it was reproducible and was obtained with relatively low concentrations of effector plasmid. In fact, the GAL4 DNA binding domain retains some properties of a weak activator, as shown by our assays and those of others using this truncated protein (Schwechheimer *et al.*, 1998). The silencing effect of the AtMYB4 C-terminal domain, therefore, may be somewhat greater than apparent from the transfection assays because it has to operate against a background of residual activation. Deletion and mutational analysis of AtMYB4 showed that the region involved in repression included the motif NLELRISLPDDV, which is also conserved in a number of R2R3 MYB proteins belonging to subgroup 4 (pdLNL<sup>D</sup>/<sub>E</sub>Lxi<sup>G</sup>/<sub>S</sub>; Kranz *et al.*,

1998). This subgroup includes AmMYB308 and AmMYB330, which have very similar effects to AtMYB4 when overexpressed in tobacco (Tamagnone *et al.*, 1998a). Repressor functions have been described for MYB-related proteins before (Foos *et al.*, 1992; Marhamati and Sonenshein, 1996), although often operating through interaction with negative regulatory proteins (Kaspar *et al.*, 1999). No examples of R2R3 MYB-related repressors have previously been reported for plants.

The subgroup 4 MYB proteins include the products of four genes from *A.thaliana*, *AtMYB4*, *AtMYB6*, *AtMYB7* and *AtMYB32*, which may be functionally redundant. It is likely that they serve similar roles in negatively regulating hydroxycinnamic acid metabolism. We have analysed expression of *AtMYB32* and found transcripts to be significantly induced in the dark and in roots, unlike *AtMYB4*, which is constitutively expressed in most tissues. Therefore, *AtMYB32* may play a particular role in negatively regulating hydroxycinnamic acid metabolism in roots, and in reducing expression in dark-grown plants, while *AtMYB4* is particularly involved in the response to UV-B.

*AtMYB4* appears to function rather specifically in the regulation of the *C4H* gene to control hydroxycinnamic acid metabolism. However, at high levels of *AtMYB4* expression, additional genes fall under its negative transcriptional regulation. A similar reduction in target site selectivity has been reported for c-MYB activity *in vivo* (Andersson *et al.*, 1999). The additional genes regulated by *AtMYB4* are those encoding other steps in hydroxycinnamic acid metabolism and flavonoid biosynthesis. Indeed, the effect of high *AtMYB4* activity can be seen as reinforcing the silencing of its primary target (*C4H*) by silencing other structural genes in the same pathway to ensure downregulation of the specific metabolic pathway.

These results have some important implications for experimental analysis of transcription factor action, suggesting caution in evaluating target genes by activation tagging or any other method involving high levels of transcription factor activity. Results based on inducible activation of MYB proteins may also be subject to reduced selectivity of target genes as a result of high cellular concentrations of transcription factor, which could explain reports of more diverse effects of the *P* and *Cl* genes from maize than originally expected from mutant analysis (Bruce *et al.*, 2000).

Although our results suggest caution in deducing targets of MYB-transcription factors from overexpression analyses, they do provide some encouragement for the use of overexpression of regulatory genes to engineer metabolism. If high levels of specific regulatory genes are used, structural genes with similar regulatory motifs may fall under coordinate regulation. This reinforcement mechanism may be used normally by the plant, but could also be developed for biotechnological purposes to ensure effective metabolic regulation through coordinate control of several structural genes in a metabolic pathway.

## Materials and methods

### Screening for an insertion mutation of the *AtMYB4* locus

A dSpm insertion was found amongst the Sainsbury Laboratory *Arabidopsis thaliana* population using gene-specific primers to amplify the gene (Meissner *et al.*, 1999).

### Constructs for plant transformation

To produce a construct for constitutive high level expression of *AtMYB4*, the full-length cDNA was cloned into pJIT60 (Guerineau and Mullineaux, 1993) between the double CaMV35S enhancer and the CaMV Terminator to create pJAtMYB4. The entire expression cassette was cloned into the *Hind*III site of pSMAK251. To produce a reporter construct for the promoter of the *C4H* gene of *A.thaliana*, two oligonucleotides, one containing the sequence from 942 bp upstream of the initiating ATG and the other the region just (39 bp) upstream of the initiating ATG were used. The promoter was amplified by PCR and cloned into pSMAK251 replacing the CaMV35S promoter, to drive expression of the downstream *GUS* gene.

### Constructs for transfection assays

The reporter construct used for measuring expression from the *C4H* promoter consisted of a 1024 bp fragment upstream of the initiating ATG of the *C4H* gene, amplified by PCR and cloned *Xba*I-*Xho*I into pBT10GUS (Sprenger-Haussels and Weisshaar, 2000). The reporter for the *CCoAOMT* gene consisted of a 900 bp fragment upstream of the initiating ATG, amplified by PCR and cloned *Xba*I-*Xho*I into pBT10GUS. Reporter constructs for the *4CL3* and *CHS* genes of *A.thaliana* have been described by Ehling *et al.* (1999) and Hartmann *et al.* (1998). The reporter construct used for assaying regulation specified by the GAL4 DNA binding domain involved four copies of the GAL4 binding site cloned immediately upstream of the -90 CaMV 35S promoter in pBT10GUS.

Effector constructs consisted of *AtMYB4* in pJIT60 (pJAtMYB4) as described above, or modifications of the construct to change the amino acid sequence of the encoded protein. To produce pJAtMYB4C-, pJAtMYB4 was cut with *Bsa*BI, which cuts at +593 bp in the cDNA coding sequence, and *Sma*I, which cuts in the polylinker at the 3' end of the cDNA. Religation removed the intervening sequence to leave that encoding only the N-terminal 198 amino acids. The equivalent truncated version of AmMYB308 has been shown to form a stable protein in *Escherichia coli* (Tamagnone *et al.*, 1998a).

A C-terminal region predicted to form a zinc finger motif was also mutated (Kranz *et al.*, 1998), first by changing the sequence encoding amino acid 224 in pJAtMYB4 from TGT (cysteine) to TCT (serine) and that encoding amino acid 227 from TGC (cysteine) to TCC (serine) to form pJAtMYB4Zn2. pJAtMYB4Zn2 was mutagenized further, the sequence encoding amino acid 237 was changed from TGC (cysteine) to TCC (serine) and that encoding amino acid 239 was changed from TGC (cysteine) to TCC (serine) to form pJAtMYB4Zn4.

Digesting pJAtMYB4 with *Bsa*BI, which cuts twice at +513 bp and +629 bp in the *AtMYB4* cDNA coding sequence, created a construct expressing *AtMYB4* with a deletion of the region encoding the conserved domain. The cut plasmid was religated to lose the sequence encoding NLELRISLPDDV (amino acids 199–210), and amino acid 198 was changed from leucine to phenylalanine. The ligation was in-frame so that the rest of the C-terminus of *AtMYB4* was included in the protein expressed from this construct (pJAtMYB4cd).

A construct with a mutation in the *AtMYB4* DNA binding domain was created by mutagenizing the sequence around the conserved tryptophan of the recognition helix of R3, changing amino acids 108–110 from NYW to IDP to form pJMYB4Wmu.

To fuse the sequence encoding the C-terminal domain of *AtMYB4* to the DNA binding domain of GAL4, the sequences encoding amino acids 163–282 were amplified by PCR and cloned in-frame, *Bam*HI-*Eco*RI, behind the sequences encoding the GAL4 DNA binding domain in pJIT60 (pGAL4; Schwechheimer *et al.*, 1998). This plasmid was called pJGAL4AtMYB4C.

### Co-transfection assays

The At7 cell culture from *A.thaliana* ecotype Columbia was used for the co-transfection assays as described by Hartmann *et al.* (1998). Following co-transfection, protoplasts were incubated for 20 h prior to harvest. GUS activity was measured in the extracts and normalized to luciferase (LUC) activity formed by co-transfection of a 35S LUC construct in each assay.

### Stable transformation of *Arabidopsis* and tobacco

Tobacco (*Nicotiana tabacum* cv. Samsun NN) leaf discs were transformed using *Agrobacterium tumefaciens* strain LBA4404. Transformed plantlets were selected on Murashige and Skoog (MS) media containing 100 mg/ml kanamycin sulfate. More than 20 independent transformants were generated and analysed. *Arabidopsis thaliana* (ecotype Columbia) was transformed by vacuum infiltration with *A.tumefaciens* strain

GV3101. T<sub>0</sub> seeds were sown on germination medium (GM) containing 50 µg/ml kanamycin sulfate. At least 30 transformants carrying each construct were analysed.

#### UV treatment

*Arabidopsis thaliana* seedlings were grown on GM medium for 10 days (20°C, 10 h light/14 h dark). The lids of the Petri dishes were removed and the dishes were irradiated by a short wave transilluminator (UV-P 34003901 fitted with UV-B tubes F15T8 emitting 3.2 mW/cm<sup>2</sup>) for 10, 15, 20 or 25 min. After exposure the lids were replaced and the seedlings were grown for one week in the growth cabinet prior to scoring and photography.

#### Measurement of phenolics

*Arabidopsis thaliana* plants were grown for 12 days on MS agar or in soil. Leaves were frozen in liquid nitrogen and then phenolics were extracted in dry ice-cold methanol. Phenylpropanoids were analysed by HPLC as described in Tamagnone *et al.* (1998b). The identity of the major peaks was confirmed by LCMS.

#### RNA isolation and quantitative RT-PCR analysis

Plants were grown on agar for 12 days and total RNA was extracted from leaves according to Tamagnone *et al.* (1998a). RNA (5 µg) was treated with RNase-free DNase I to remove residual DNA. First strand cDNA was synthesized from 5 µg of RNA and amplified according to Frohman *et al.* (1988) using the dT<sub>17</sub> adaptor as a primer and Superscript II RNase-H reverse transcriptase at 42°C for 1 h. In preliminary tests, samples were taken after 15, 20, 25 and 30 cycles, the products were separated by gel electrophoresis and then blotted onto nitrocellulose. These filters were probed with the appropriate labelled cDNA probes to confirm that amplification was linear and to establish the number of amplification cycles for cDNA detection within the linear range. Genes (see Table I) encoding actin (McDowell *et al.*, 1996), C4H, 4CL, 4CL3, CHS, CAD1, F5H, OMT, PAL2, AtMYB4 (AF062860) and trpB (Berlyn *et al.*, 1989) were amplified for between 20 and 25 cycles. *CCoAOMT* was amplified for 30 cycles. Samples were loaded onto a gel and separated by electrophoresis. Staining with ethidium bromide confirmed that no products could be visualized in the samples. The gels were blotted onto nitrocellulose and hybridized to gene-specific probes.

#### Light treatment and wounding expression analysis

Wild-type (ecotype Columbia) plants were grown on soil for 6 weeks (16 h light/8 h dark) and then dark adapted for 2 days. Plants were then transferred to continuous white, blue, UV-A or UV-B light, and RNA was extracted from rosette leaves. Light treatments were: white light, Sylvania cool white F48T12/CW/HO 60 48 µE/m<sup>2</sup>/s; blue light, GTE Sylvania fluorescent F48T12/246/HO 5R5459/ Blue filter 63 µE/m<sup>2</sup>/s; UV-A, Philips TLK40W/10R 0.7 mW/cm<sup>2</sup>; UV-B, Philips TLK20W/12RS 0.2 mW/cm<sup>2</sup>. For UV-C treatment no dark adaptation was given; plants were given a single dose of UV-C light (600 J/m<sup>2</sup> from Philips TUV15W 2–75 mW/cm<sup>2</sup>).

Six-week-old plants were also used for wounding. Small cuts were made on half the rosette leaves and the other half was left intact. Wounded leaves were pooled separately from the non-wounded leaves that formed the sample exhibiting any systemic response. Control samples were from untreated plants of the same age.

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## Note added in proof

During the review of this paper it was noticed that CAD1 (accession No. L37883) shows low similarity to CAD genes from other plant species. RT-PCR analysis of mutant and *AtMYB4* over-expressing lines was repeated with another CAD gene from *A.thaliana* (accession No. Z31715), which is more similar to the CAD genes from other species. Exactly the same results were obtained for this gene as for CAD1 (Figure 5).