The Plant Journal (2008) **53**, 53–64 doi: 10.1111/j.1365-313X.2007.03310.x

Over-expression of the Arabidopsis *AtMYB41* gene alters cell expansion and leaf surface permeability

Eleonora Cominelli, Tea Sala, Daniele Calvi, Giuliana Gusmaroli[†] and Chiara Tonelli[*]
Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, 20133 Milan, Italy

Received 26 July 2007; accepted 23 August 2007.
[*]For correspondence (fax +39 0250315044; e-mail chiara.tonelli@unimi.it).
[†]Present address: Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520-8104, USA.

Summary

The Arabidopsis *AtMYB41* gene encodes an R2R3-MYB transcription factor whose expression is not detectable under normal growth conditions in any organ or at any developmental stage analysed. It is expressed at high levels in response to drought, ABA and salt treatments, suggesting a possible role in stress responses. Transgenic lines over-expressing this transcription factor showed a pleiotropic phenotype similar to that exhibited by some mutants that affect cuticle biosynthesis. This includes a dwarf appearance, dependent on smaller cells with abnormal morphology, enhanced sensitivity to desiccation, and enhanced permeability of leaf surfaces, suggesting discontinuity in the cuticle. The expression of genes involved in lipid metabolism and transport, in cell-wall modifications and cell expansion, genes coding for membrane-associated proteins and genes specifically involved in cuticle metabolism was differentially modulated between wild-type and transgenic plants, suggesting a direct or indirect role of AtMYB41 in the regulation of their transcription. Taken together, our results suggest that AtMYB41 is part of a complex network of transcription factors controlling cell expansion and cuticle deposition in response to abiotic stress.

Keywords: *MYB*, over-expression, cuticle, cell expansion, *Arabidopsis thaliana*.

Introduction

The cuticle is one of the major barriers in plants that protects aerial organs from damage caused by abiotic and biotic stresses. It is a complex structure, usually composed of several layers: the outermost is formed by crystals of epicuticular waxes and overlies the cuticle membrane layer, which is formed by an outer translucent cuticle proper and an inner opaque cuticular layer, composed primarily of insoluble cutin polyesters (Jeffree, 1996).

Several loss-of-function mutants affecting wax biosynthesis and deposition have been identified in various species (Nawrath, 2006). The most obvious change in many of these mutants is the presence of a shiny, glossy stem instead of a glaucous one, but in some cases these mutants also display pleiotropic phenotypes involving stunted growth, elevated transpiration rate, reduced fertility, increased sensitivity to chemical exposure and to pathogens, organ fusion, morphological irregularities in cell shape, and cell death (Jenks *et al.*, 2002; Nawrath, 2006; Yephremov and Schreiber, 2005). Arabidopsis genes involved in the regulation or synthesis of cutin components, and, in some cases, also synthesis of wax include *LCR*, *WIN1*, *LACS2*, *ATT1*, *WAX2*, *ACE/HTH* and *BDG* (Chen *et al.*, 2003; Kannangara *et al.*, 2007; Kurdyukov *et al.*, 2006a,b; Schnurr *et al.*, 2004; Wellesen *et al.*, 2001; Xiao *et al.*, 2004). These mutants show phenotypic alterations similar to those described for the most severe wax synthesis mutants and very similar to that obtained in Arabidopsis lines over-expressing a fungal cutinase (Sieber *et al.*, 2000). Recently, enhanced resistance to *Botrytis cinerea* has been described for some of these mutants and for the lines over-expressing a fungal cutinase (Bessire *et al.*, 2007; Chassot *et al.*, 2007; Tang *et al.*, 2007).

Many of the mutants described affect genes coding for enzymes involved in cuticle biosynthesis, but very few regulatory or putative regulatory genes controlling these pathways are known (Yephremov and Schreiber, 2005). In some cases, the genes involved have been isolated by genetic approaches through study of the corresponding loss-of-function mutants; in other cases, gain-of-function mutants or over-expression lines have been analysed.

Some members of the AP2/EREBP family of transcription factors have been characterized for their possible role in this process, such as WIN1/SHN in Arabidopsis (Aharoni *et al.*, 2004; Broun *et al.*, 2004; Kannangara *et al.*, 2007), WXP1 and WXP2 in *Medicago trunculata* (Zhang *et al.*, 2005, 2007), and GL15 in maize, which is highly similar to the Arabidopsis transcription factor AINTEGUMENTA (Hannoufa *et al.*, 1996; Moose and Sisco, 1996).

In Arabidopsis, the *WIN1/SHN* gene is involved in the regulation of wax and cutin production, as demonstrated by the study of lines in which its expression is up or downregulated (Aharoni *et al.*, 2004; Broun *et al.*, 2004; Kannangara *et al.*, 2007). The maize *gl15* mutation shortens the duration of expression of juvenile epidermal cell traits, among them the transition between the expression of juvenile and adult waxes, which occurs earlier than in wild-type plants (Moose and Sisco, 1996). Moreover, maize lines over-expressing the *GL15* gene show an increased number of leaves expressing juvenile waxes, indicating that *GL15* is involved in the promotion of the juvenile phase (Lauter *et al.*, 2005). For comparison, in Arabidopsis, the *GIS* gene encodes a putative C2H2 transcription factor that plays a role in the juvenile–adult transition of epidermal differentiation (Gan *et al.*, 2006).

WXP1 and WXP2 of the model legume *Medicago truncatula* also belong to the AP2/EREBP transcription factor family. Over-expression of the *WXP1* gene in transgenic alfalfa (*Medicago sativa*) increases cuticular wax accumulation and enhances drought tolerance (Zhang *et al.*, 2005). Transgenic expression of *WXP1* or of its paralog *WXP2* in Arabidopsis also leads to increased wax deposition and enhanced drought tolerance (Zhang *et al.*, 2007).

MYB proteins are a class of transcription factors that are present in all eukaryotes, and share a common DNA-binding domain. In plants, the most highly represented MYB protein group is the R2R3 subfamily, members of which contain two MYB repeats in their DNA-binding domains (Martin and Paz-Ares, 1997). In *Arabidopsis thaliana*, 126 *R2R3-MYB* genes have been identified (Stracke *et al.*, 2001), and involvement in the regulation of plant-specific processes has been reported for some of them, such as the regulation of phenylpropanoid metabolism, the control of specialized cell morphology, and the regulation of plant responses to biotic and abiotic stresses, hormones and light (Martin and Paz-Ares, 1997; Petroni *et al.*, 2002; Stracke *et al.*, 2001). Arabidopsis *MYB* genes involved in the response to abiotic stresses have been described (Abe *et al.*, 1997, 2003; Cominelli *et al.*, 2005; Denekamp and Smeekens, 2003; Jin *et al.*, 2000; Urao *et al.*, 1993; Zhu *et al.*, 2005).

Here we report the characterization of transgenic Arabidopsis lines over-expressing *AtMYB41*, which encodes an R2R3-MYB transcription factor whose expression is specifically induced in response to abiotic stress in wild-type plants. Over-expression of *AtMYB41,* under the control of the CaMV 35S promoter, results in a pleiotropic phenotype resembling that of numerous cuticle mutants, suggesting a possible role for AtMYB41 in the regulation of cuticle biosynthesis. Consistently, at the molecular level, over-expression of *AtMYB41* is accompanied by changes in the level of expression of some genes involved in cuticle biosynthesis and cell expansion.

Results

The AtMYB41 *gene is induced in response to abiotic stresses*

The predicted protein encoded by the *AtMYB41* (At4g28110) gene belongs to subgroup 11 of the R2R3-MYB transcription factor family of Arabidopsis, together with AtMYB49, AtMYB74 and AtMYB102, as revealed by phylogenetic analysis (Kranz *et al.*, 1998; Stracke *et al.*, 2001). Of the members of this subgroup, only AtMYB102 has been characterized, and a role for it in integrating signals derived from wounding and osmotic stresses has been suggested (Denekamp and Smeekens, 2003). To gain insight into the regulation of the *AtMYB41* gene, quantitative RT-PCR analysis was performed on RNA obtained from several organs and at various stages of development of seedlings, rosette leaves, flowers and siliques. As shown in Figure 1, *AtMYB41* transcript was not detectable, under normal growth conditions in any organs or at any of the developmental stages

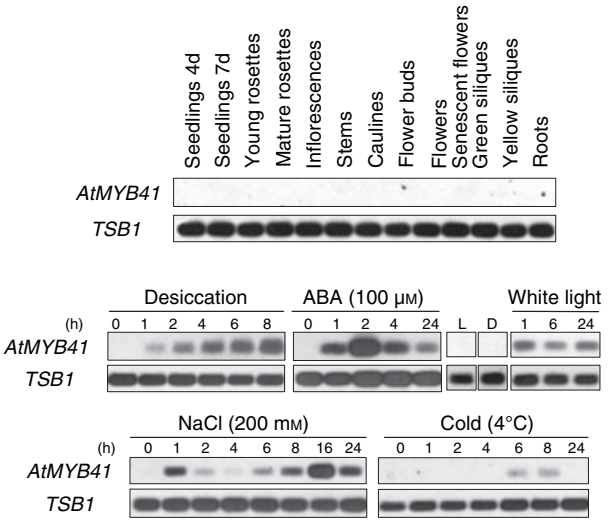

Figure 1. Analysis of *AtMYB41* gene expression.
Quantitative RT-PCR analysis of *AtMYB41* expression in various organs of wild-type plants grown under standard conditions and in response to various treatments. For the desiccation and cold treatments, 3-week-old wild-type plants, grown on soil, were dehydrated on Whatman 3 MM paper or transferred to 4°C; for ABA and NaCl treatments, 3-week-old wild-type plants grown in liquid MS medium were supplemented with 100 μM ABA or 200 mM NaCl. For light treatment, plants grown under a 16 h light/8 h dark cycle for 3 weeks (L sample) were dark-adapted for 2 days (D sample) and then transferred to continuous white light for up to 24 h. The *TSB1* gene was used as a control (Berlyn *et al.*, 1989).

analysed. However, *AtMYB41* expression was significantly induced in response to desiccation, ABA and salt treatments (Figure 1). Interestingly, in the case of salt treatment, we observed a two-step induction kinetic, with a first peak after 1 h of treatment and a second one after 16 h. *AtMYB41* transcript accumulated in response to these treatments at similar levels both in rosette leaves and in roots (data not shown). *AtMYB41* transcript also accumulated in response to cold (Figure 1), white light (Figure 1) and heat shock (data not shown) treatments, but not in response to wounding, either in terms of the local or the systemic response (data not shown).

Ectopic expression of AtMYB41 *confers a dwarf phenotype to plants*

Due to the absence of insertion mutants in the T-DNA and transposon databases/germplasm collections, and the inability to efficiently silence *AtMYB41* by RNA interference approaches (data not shown), we decided to characterize this transcription factor using an over-expression strategy. We generated 35S::*AtMYB41* transgenic Arabidopsis plants in which the *AtMYB41* cDNA was over-expressed under the control of the strong CaMV 35S promoter and the tobacco mosaic virus omega sequence, which has been shown to elevate the translation level of the transgene. We selected ten kanamycin-resistant lines. Eight of them showed very severe phenotypic alterations: plants had reduced stature, rosette and cauline leaves had reduced dimensions and were often wrinkled and in some cases had curled-up edges. As shown in Figure 2(a), the expression level of the *AtMYB41* gene was examined by quantitative RT-PCR analyses in three of the eight over-expressing lines described above. In all three transgenic lines molecularly analysed, very high *AtMYB41* expression levels were detected, and absence of the transcript in wild-type control plants was confirmed (Figure 2a). Comparison between transgenic and wild-type plants showed that over-expression of *AtMYB41* is accompanied by impressive phenotypic alterations in plants grown either on Petri dishes for 3 weeks (Figure 2b–e) or on soil under standard conditions for 5 weeks (Figure 2f). To quantify the differences between the 35S::*AtMYB41* and wild-type plants grown on soil for 5 weeks, we measured plant height and rosette leaf size: transgenic plants were less than 2 cm high, while wild-type plants reached 20 cm high (Table 1). The length and the width of the third rosette leaf of 35S::*AtMYB41* plants were 25% of the values for wild-type leaves (Table 1).

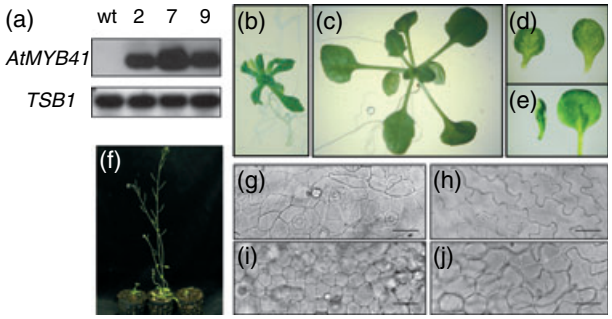

Figure 2. Molecular characterization and phenotype of 35S::*AtMYB41* transgenic Arabidopsis lines.
(a) Quantitative RT-PCR analysis of *AtMYB41* expression from wild-type and three transgenic lines (line number at the top), grown on soil for 3 weeks. The *TSB1* gene was used as a control.
(b, c) 35S::*AtMYB41*-2 and wild-type plants grown on solid MS for 2 weeks.
(d, e) Detail of the cotyledons and 3rd leaf from a 35S::*AtMYB41*-2 plant (left) and wild-type plant (right).
(f) 35S::*AtMYB41*-2 (left), 35S::*AtMYB41*-7 (right) and wild-type plants (middle) grown on soil for 5 weeks.
(g, h) Adaxial leaf epidermis of 35S::*AtMYB41*-2 (g) and wild-type plants (h) at the same magnification; bar = 40 μm.
(i, j) Adaxial leaf palisade parenchyma of 35S::*AtMYB41* (i) and wild-type (j) at the same magnification; bar = 40 μm.

More detailed microscopic investigation of this aspect of the phenotype of the over-expressing lines revealed that the cells in the leaf palisade parenchyma and in the leaf epidermis of the 35S::*AtMYB41* plants were much smaller than those of the wild-type leaves (Figure 2g–j). In addition to the differences in cell dimensions, the transgenic plants showed morphological alterations in the shape of their epidermal cells, which were polygonal instead of displaying the characteristic multi-lobed shape, reminiscent of a piece from a jigsaw puzzle (Telfer and Poethig, 1994) (Figure 2g–j). This result suggested that the decreased size of 35S::*AtMYB41* plants is probably the result of smaller cell size rather than decreased cell number. Therefore, whereas cell division does not seem to be compromised, it is possible that AtMYB41 over-expression inhibits plant cell expansion.

Table 1 Phenotypic analysis of transgenic 35S::AtMYB41 plants

	Plant height (cm)	Rosette leaf length (cm)	Rosette leaf width (cm)	Number of siliques per plant	Number of seeds per silique
Wild-type	20.2 ± 2.4	3.7 ± 0.3	1.2 ± 0.1	93 ± 9	46 ± 4
35S::AtMYB41-2	1.7 ± 0.4	0.8 ± 0.2	0.4 ± 0.1	19 ± 3	21 ± 1
35S::AtMYB41-7	1.4 ± 0.5	0.7 ± 0.2	0.4 ± 0.2	20 ± 2	22 ± 1
35S::AtMYB41-9	1.4 ± 0.3	0.8 ± 0.3	0.3 ± 0.1	18 ± 3	20 ± 2

Although developmental defects in 35S::*AtMYB41* plants were evident at both the vegetative and reproductive phases (Figure 2b–f), we did not observe any detectable alterations at the seedling stage (until the 6th day after germination), either in aerial tissues or in roots (data not shown). Moreover, we did not observe organ fusion or other abnormalities in flowers or trichomes of adult plants (data not shown). However, the transgenic plants showed a severe reduction in seed production, due to the reduced number and size of the siliques (Table 1). No differences were observed in the weight of the seeds between wild-type and transgenic lines (data not shown), even though 35S::*AtMYB41* seeds showed a reduced germination rate. To quantify this aspect of the phenotype, seeds were sown on soil and covered with plastic wrap to maintain high humidity. After 3 weeks, 90.4% of the wild-type seeds had germinated and 3% of the developing plants died, whereas only 39.2% of the transgenic seeds had germinated and 10% of the developing transgenic seedlings died.

All the experiments described were performed on transgenic lines 35S::*AtMYB41-2*, 35S::*AtMYB41-7* and 35S::*AtMYB41-9*, giving similar results.

*35S::*AtMYB41 *plants show higher transpiration rates*

As *AtMYB41* expression was upregulated in response to drought stress, we investigated the effects of *AtMYB41* overexpression on water loss and transpiration rate during drought. Water loss was measured from detached rosette leaves of wild-type and 35S::*AtMYB41* plants. During the first minutes following the start of the treatment, the transgenic plants showed a more rapid water loss than the wild-type plants, such that almost 80% of the FW of leaves was lost within 2 h following excision, whereas wild-type leaves lost only 35% of their FW in the same period of time (Figure 3a). Although in Figure 3, we only show data obtained from the 35S::*AtMYB41-2* transgenic line, we obtained very similar results for the 35S::*AtMYB41-7* and 35S::*AtMYB41-9* lines also (data not shown).

*35S::*AtMYB41 *plants have a discontinuous cuticle*

As the pleiotropic phenotype described for the 35S::*AtMYB41* plants was reminiscent of that of the most severe wax synthesis and cutin mutants (reviewed by Jenks *et al.*, 2002; Nawrath, 2006; Yephremov and Schreiber, 2005), we investigated the possibility that the cuticle had been altered in these plants. We performed a chlorophyll-leaching experiment in 80% ethanol. As shown in Figure 3(b), chlorophyll was extracted much faster from the rosette leaves of 35S::*AtMYB41* plants than from wild-type leaves. We then analysed the surfaces of leaves and siliques using the toluidine blue (TB) test (Figure 3c–g). Wild-type leaves did not show TB staining, as expected for plants with a complete

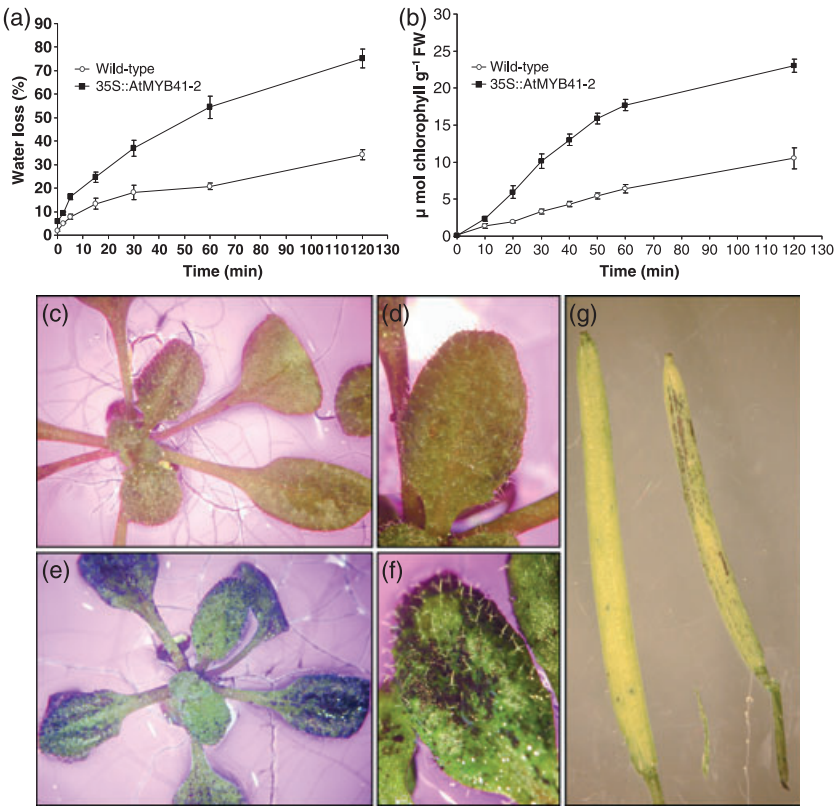

Figure 3. Surface permeability of leaves.
(a) Rate of water loss from 35S::*AtMYB41*-2 and wild-type plants. Detached rosettes were weighed at the time intervals shown. The results are derived from three independent experiments and are shown with SE for the mean for each time point.
(b) Chlorophyll-leaching assays with mature rosette leaves of 35S::*AtMYB41*-2 and wild-type immersed in 80% ethanol for various time intervals. The results are derived from three independent experiments and are shown with SE for the mean for each time point. FW, fresh weight.
(c–f) Two-week-old plants [Columbia wild-type (c, d) and 35S::*AtMYB41*-2 (e,f)] stained with TB.
(g) Siliques from wild-type (left) and 35S::*AtMYB41*-2 (right) stained with TB.

cuticle (Figure 3c,d), but the 35S::*AtMYB41* plants exhibited patchy and random staining (Figure 3e,f), as described for class II cutin mutants by Tanaka *et al.* (2004). Analysis of siliques gave similar results (Figure 3g).

Taken together, the TB test results (Figure 3c–g), the greater sensitivity to drought (Figure 3a) and the faster chlorophyll leaching in ethanol (Figure 3b) suggest that over-expression of *AtMYB41* causes a reduction in the insulating properties of the cuticle of 35S::*AtMYB41* plants. We obtained similar results for the three transgenic lines analysed (data not shown).

Effects of AtMYB41 *over-expression on gene expression*

As phenotypic analysis performed on three transgenic lines suggested the presence of a discontinuous cuticle in the 35S::*AtMYB41* plants, quantitative RT-PCR analysis was used to compare the expression level of genes involved in wax and cutin biosynthesis in the wild-type and in transgenic plants of the 35S::*AtMYB41-2* line (Figure 4). Total RNA was isolated from rosette leaves of wild-type and transgenic plants. We analysed the expression of *KCS1* (Todd *et al.*, 1999), *FDH* (Yephremov *et al.*, 1999) and *CER6* (Fiebig *et al.*, 2000), which are involved in elongation of fatty acyl chains, *ATT1* (Xiao *et al.*, 2004), *WAX2* (Chen *et al.*, 2003), *LACS2* (Schnurr *et al.*, 2004) and *LCR* (Wellesen *et al.*, 2001), which are involved in biosynthesis of cutin, *CER2* (Negruk *et al.*, 1996; St-Pierre *et al.*, 1998; Xia *et al.*, 1996), encoding a putative coenzyme A-dependent acyltransferase involved in cuticle biosynthesis, and *WIN1/SHN*, encoding an AP2/EREBP transcription activator of epidermal wax accumulation and cutin deposition (Aharoni *et al.*, 2004; Broun *et al.*, 2004; Kannangara *et al.*, 2007).

Of these genes, only *LACS2*, *ATT1* and *WIN1/SHN* showed a change in gene expression in response to *AtMYB41* over-expression. Specifically, whereas their transcripts were present in wild-type plants (as expected), *LACS2* mRNA was completely absent in transgenic 35S::*AtMYB41* plants, *ATT1* was expressed at a lower level, and *WIN1/SHN* showed increased expression.

As 35S::*AtMYB41* cells had very reduced dimensions, suggesting problems in cell expansion, we investigated a possible role of *AtEXP10*, the only Arabidopsis gene encoding an expansin that has been characterized in any detail. In fact, the corresponding antisense lines for *AtEXP10* were significantly smaller than wild-type (Cho and Cosgrove, 2000). Interestingly, as shown in Figure 4, we observed decreased expression levels of *AtEXP10* in 35S::*AtMYB41* lines compared to wild-type plants.

To identify other target genes of the AtMYB41 transcription factor, we used Affymetrix ATH1 GENECHIP arrays, representing approximately 24 000 genes. The expression profile in one 35S::*AtMYB41* line under unstressed conditions was compared with that of wild-type plants. The 25 most up- and downregulated genes are summarized in Table 2. The complete list of regulated genes detected by microarray analysis is provided in Table S1. Some genes were chosen and used to confirm the reliability of the microarray data using quantitative RT-PCR analysis. The results shown in Figure 5 support the reliability of the microarray data.

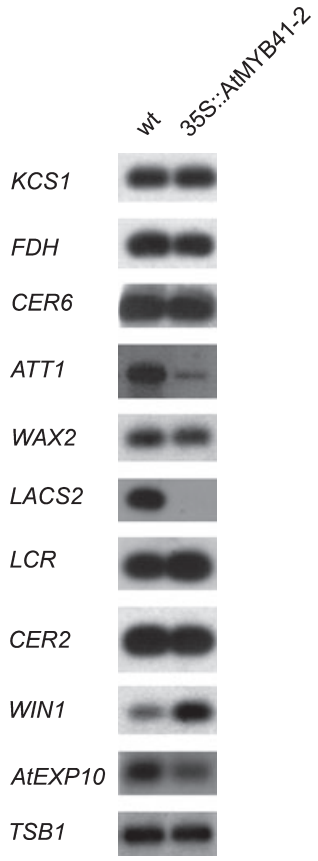

Figure 4. Expression of genes involved in wax and cutin biosynthesis and of *AtEXP10* in wild-type and the 35S::*AtMYB41*-2 transgenic line.
The *TSB1* gene was used as a control.

The transcript levels of 149 genes were induced, and those of 28 genes were suppressed in the 35S::*AtMYB41* plants, compared with wild-type, using a twofold change threshold (P value < 0.01, Table S1). The putative target genes of AtMYB41, involved in cuticle deposition and cell expansion, previously identified by the quantitative RT-PCR analysis, as described above, were not identified as significantly differentially regulated in the DNA microarray analysis of the 35S:*AtMYB41* plants. Among the 25 most upregulated genes shown in Table 2 (quantitative RT-PCR analysis is shown in Figure 5 for some of them), one is *AtMYB41*, as expected, and there are genes coding for proteins with a known or possible involvement in lipid biosynthesis or transport, such as the three genes coding for lipid transfer proteins

Table 2 Genes up- or downregulated in 35S::*AtMYB41* plants identified by GENECHIP analysis (list of the 25 most up- and downregulated genes)

Description	Affymetrix ID[a]	AGI[b]	FC[c]	O[d]	A[e]	N[f]
Upregulated genes						
Integral membrane family protein	258905_at	At3g06390	242.6	+	NA	–
Lipid transfer protein family protein	250230_at	At5g13900	88.5	+	+	NA
Hypothetical protein	263005_at	At1g54540	83.3	+	+	+
myb family transcription factor (MYB41)	253851_at	At4g28110	83.0	+	+	+
GDSL motif lipase/hydrolase family protein	260234_at	At1g74460	79.8	+	+	–
Hydroxyproline-rich glycoprotein family protein	245889_at	At5g09480	73.8	+	–	–
Peroxidase, putative	247857_at	At5g58400	64.3	NA	NA	NA
GDSL motif lipase/hydrolase family protein	267121_at	At2g23540	58.3	+	+	–
Lipid transfer protein family protein	262317_at	At2g48140	52.8	+	+	NA
Transferase family protein	249289_at	At5g41040	39.8	+	+	+
Endonuclease/exonuclease/phosphatase family protein	266011_at	At2g37440	38.8	+	+	NA
Protein kinase, putative	266196_at	At2g39110	37.6	+	+	+
Pectinesterase family protein	267464_at	At2g19150	35.6	–	NA	–
Glycine-rich protein	262097_at	At1g55990	34.2	+	+	NA
Auxin-responsive family protein	245412_at	At4g17280	33.2	+	+	NA
Lipid transfer protein family protein	256937_at	At3g22620	32.9	+	+	+
Glutamine amidotransferase-related	260741_at	At1g15040	32.1	+	+	+
Hydroxyproline-rich glycoprotein family protein	263998_at	At2g22510	25.5	+	+	+
Peroxidase, putative	260035_at	At1g68850	25.3	+	+	+
ABC transporter family protein	250239_at	At5g13580	24.4	+	+	+
Protein kinase, putative	267372_at	At2g26290	23.8	+	+	+
Hydrolase, α/β fold family protein	246203_at	At4g36610	19.4	+	+	NA
Acyl CoA reductase, putative	252638_at	At3g44540	18.8	+	+	+
Late-embryogenesis-abundant group 1 domain-containing protein	266544_at	At2g35300	18.1	+	+	+
Calcium-dependent protein kinase-related	266111_at	At2g02060	17.3	NA	+	+
Downregulated genes						
Glutaredoxin family protein	260831_at	At1g06830	–7.4	–	–	–
Xyloglucan:xyloglucosyl transferase, putative (XTH7)	253040_at	At4g37800	–5.7	–	NA	–
copia-like retrotransposon family	254542_s_at	At4g19790	–5.2	NF	NF	NF
Xyloglucan:xyloglucosyl transferase, putative (XTH8)	261825_at	At1g11545	–4.2	–	–	–
Expansin, putative (EXP5)	258003_at	At3g29030	–3.6	–	–	–
Ubiquitin family protein	249367_at	At5g40630	–2.9	–	–	–
Trehalose-6-phosphate phosphatase, putative	263452_at	At2g22190	–2.8	NA	+	+
Peptidase M20/M25/M40 family protein	254496_at	At4g20070	–2.6	+	+	–
Non-specific lipid transfer protein 5	252115_at	At3g51600	–2.6	–	NA	NA
Cytochrome P450, putative	246380_at	At1g57750	–2.5	–	–	+
Plastocyanin-like domain-containing protein	261975_at	At1g64640	–2.5	–	–	–
Shikimate kinase-related	266608_at	At2g35500	–2.5	–	–	–
AP2 domain-containing transcription factor TINY, putative	266820_at	At2g44940	–2.4	–	+	+
Expressed protein	263287_at	At2g36145	–2.3	–	NA	–
Sulfate adenylyltransferase 3/ATP-sulfurylase 3 (APS3)	245254_at	At4g14680	–2.3	–	–	+
Inorganic carbon transport protein-related	262288_at	At1g70760	–2.3	–	NA	–
Photosystem II reaction centre W (PsbW) family protein	253790_at	At4g28660	–2.3	–	–	–
Immunophilin	256130_at	At1g18170	–2.2	–	–	–
Haloacid dehalogenase-like hydrolase family protein	259603_at	At1g56500	–2.2	–	NA	–
Chlorophyll *a*/*b* binding protein, putative/LHCl type II, putative	256015_at	At1g19150	–2.2	–	NA	–
Membrane protein, putative	255719_at	At1g32080	–2.1	–	–	–
Photosystem II reaction center PsbP family protein	245368_at	At4g15510	–2.1	–	NA	–
Expressed protein	261422_at	At1g18730	–2.1	–	NA	–
Expressed protein	262785_at	At1g10750	–2.1	–	–	–
Expressed protein	249120_at	At5g43750	–2.1	–	NA	–

[a]Identification number on the Affymetrix Arabidopsis GENECHIP (ATH1).

[b]Arabidopsis gene index number.

[c]Fold change: genes from 35S::*AtMYB41* RNA samples that have normalized data values that are greater or less than those in wild-type samples by a factor of twofold were selected (*P* value < 0.01).

[d,e,f] Response to osmotic stress (O), ABA (A) and salt (N) treatments as collected from Genevestigator website using the Meta Analyzer tool. '+' corresponds to a positive response to the treatment (red colour in red/green coding in Genevestigator website); '–' corresponds to a negative response (green); 'NA', not affected by the treatment (black); 'NF', gene not found.

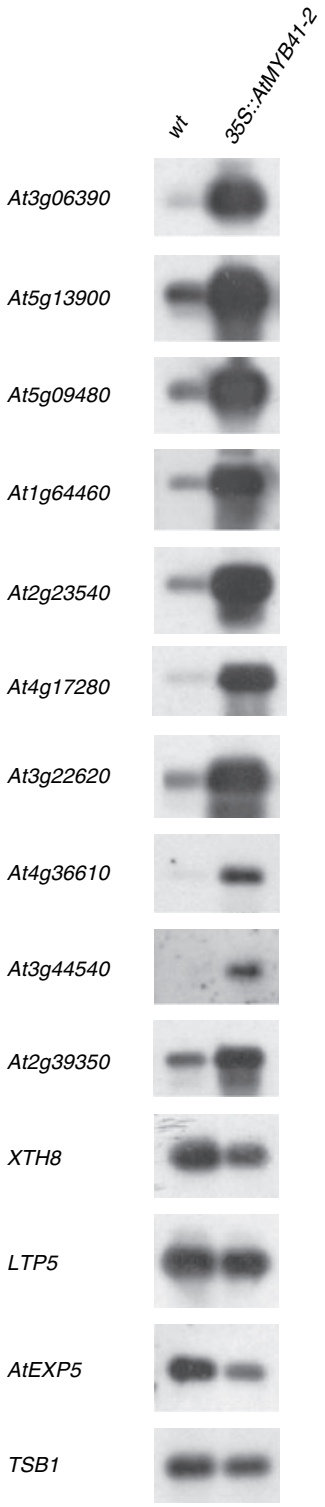

Figure 5. RT-PCR analysis of some genes identified as differentially modulated between 35S::*AtMYB41* and wild-type plants by microarray analysis.

(At5g13900, At2g48140 and At3g22620), two for GDSL motif lipase/hydrolase family proteins (At1g74460 and At2g23540), one for a putative acyl CoA reductase (At3g44540), and four for membrane proteins (At3g06390, At5g09480, At2g22510 and At5g13580) with unknown function, as predicted by the 'gene ontology cellular component' (data not shown) (one belongs to the ABC transporter family). Among the most downregulated genes in 35S::*AtMYB41* plants, there are three genes that have a possible role in cell expansion (At4g37800, At1g11545 and At3g29030), in lipid biosynthesis and transport, such as a gene encoding a cytochrome P450 (At1g57750), and one for a lipid transfer protein (At3g51600). If we consider all the genes that are either upregulated or downregulated in 35S::*AtMYB41* plants (Table S1), many encode for proteins belonging to the same families as encoded by the genes listed in Table 2. Among the genes not represented in Table 2 (but showing changes in expression in response to *AtMYB41* over-expression), there are 22 genes coding for transcription factors (belonging to the MYB, NAM, zinc finger, AP2, WRKY, HB and MADS families).

As *AtMYB41* expression is highly induced in response to desiccation, ABA and salt treatments, we used the Genevestigator website (http://www.genevestigator.ethz.ch; Zimmermann *et al.*, 2004) to obtain data on expression of all the genes listed in Table 2. Strikingly, we found that many of the genes upregulated in the 35S::*AtMYB41* line are also induced in response to these treatments, particularly in response to osmotic stress; conversely the expression of many genes downregulated in the transgenic line is also repressed or unaffected in response to these treatments.

Discussion

The complete absence of *AtMYB41* transcript under normal growth conditions, accompanied by its rapid induction soon after the onset of stress signals, suggests a role for this transcription factor in regulation of plant responses to these abiotic stresses.

Recent reports suggest that over-expression of some stress-inducible transcription factors belonging to different families can increase the tolerance of plants to drought, salinity or low temperature (reviewed by Umezawa *et al.*, 2006). However, over-expression of *AtMYB41* led to higher rates of water loss from leaves, although this gene is normally induced in response to desiccation stress. Moreover, when we monitored the expression of some genes specifically induced by various types of abiotic stress and commonly used as markers for the drought response, we did not observe any difference between lines over-expressing *AtMYB41* and wild-type plants (data not shown).

The pleiotropic phenotype of our over-expression lines is reminiscent of that of some cuticle mutants that have already been described (reviewed by Nawrath, 2006). However, it is important to note that 35S::*AtMYB41* plants did not show either the glossy phenotype or the organ fusion that are characteristic of many wax mutants (Nawrath, 2006).

These defects are also absent in some cutin mutants such as *lacs2* or *att1* (Xiao *et al.*, 2004).

So far, there has been no evidence of involvement of MYB proteins in the regulation of cuticle biosynthesis, although comparison of the promoter sequences of two genes encoding putative β-ketoacyl CoA synthases, *FDH* of Arabidopsis and *AFI* of *Antirrhinum*, involved in fatty acid metabolism, suggests a possible role of members of this family of transcription factors in the regulation of this process (Efremova *et al.*, 2004). In fact, analysis of defined portions of both promoters, which confer identical expression patterns to reporter genes in the heterologous species, revealed the presence of three conserved regions, two of which contain putative binding sites for MYB transcription factors (Efremova *et al.*, 2004).

The expression data that we obtained support our hypothesis of involvement of AtMYB41 in wax and cutin biosynthesis or deposition and in cell expansion, because many genes differentially modulated between 35S::*AtMYB41* and wild-type plants are directly involved or similar to other genes that have a role in these processes.

Because of the opposite effects of AtMYB41 on transcript levels of various genes, it is possible that this MYB protein might act as both a transcriptional activator and a repressor, depending on the context of the target sequence, which might influence its interaction with other regulatory proteins, as previously suggested for AtMYB15, for example (Agarwal *et al.*, 2006). Alternatively AtMYB41 may always act as a positive regulator, and the genes negatively regulated may be its indirect targets. Interestingly, expression data collected from the Genevestigator website (http://www.genevestigator.ethz.ch; Zimmermann *et al.*, 2004) for genes present in Table 2, in response to various abiotic stresses, clearly correlate in many cases with their expression levels in 35S::*AtMYB41* plants.

As previously mentioned, the putative targets of AtMYB41 are principally genes involved in the synthesis and transport of cuticle components and in cell-wall modification. With regard to the synthesis of cuticle components, through single gene expression analysis (Figure 4), we found that *LACS2*, which codes for a long-chain acyl CoA synthetase (Schnurr *et al.*, 2004), and *ATT1*, involved in cutin-related fatty acid oxidation (Xiao *et al.*, 2004), are downregulated in 35S::*AtMYB41* plants, while *WIN1/SHN*, a positive regulator of some wax and cutin biosynthetic genes (Aharoni *et al.*, 2004; Broun *et al.*, 2004; Kannangara *et al.*, 2007), was upregulated. The pleiotropic phenotype of 35S::*AtMYB41* lines is very similar to that previously described for the *lacs2* mutant (Schnurr *et al.*, 2004), and there was a good correlation between the phenotype of our transgenic lines and the lack of detectable expression of the *LACS2* gene in these plants. The *att1* mutant does not exhibit phenotypic alterations under normal growth conditions, but has a higher transpiration rate (Xiao *et al.*, 2004), similar to the *AtMYB41* over-expression lines, in which *ATT1* gene expression is reduced. It has been shown that WIN1/SHN regulates the expression of some wax and cutin genes including *KCS1*, *CER2* and *LACS2* (Broun *et al.*, 2004; Kannangara *et al.*, 2007). However, in the case of our transgenic line, despite higher *WIN1/SHN* transcript levels, we did not observe an increase in the expression of its putative targets, *KCS1*, *CER2* and *LACS2*. This apparent discrepancy might be explained by a complex regulatory network, in which *AtMYB41* over-expression might deregulate other factors required for the expression of these genes.

Through microarray analysis, we found that some genes with a demonstrated or putative role in cuticle component synthesis are differentially modulated in transgenic and wild-type plants in response to AtMYB41 expression. A gene coding for an alcohol-forming fatty acyl CoA reductase (FAR, At3g44540), which shows a high degree of homology with Arabidopsis *CER4*, which is involved in the acyl reduction pathway of wax biosynthesis (Rowland *et al.*, 2006), is upregulated in 35S::*AtMYB41* plants (Table 2 and Figure 5). We also found two genes coding for GDSL motif lipases that were upregulated to high levels in the transgenic line (At1g74460 and At2g23540, Table 2 and Figure 5). For this kind of enzyme, there is no precise information available about a possible role in wax or cutin biosynthesis, but one of these genes has been reported to be a target of WIN1/SHN transcription factors (Kannangara *et al.*, 2007). These authors made some interesting suggestions about its possible role in remodelling of monoacyl glycerol or transferring additional fatty acid moieties to the glycerol backbone. A similar role might be suggested for the two GDSL motif lipases that we identified as induced by *AtMYB41* expression. We also identified At4g36610 as positively regulated by AtMYB41 that codes for an α/β fold hydrolase, similar to BDG, which is involved in polymerization of carboxylic esters in the cuticular layer of the cell wall or the cuticle proper (Kurdyukov *et al.*, 2006a).

There is strong evidence supporting involvement of lipid transfer proteins (LTPs) and ABC transporters in cuticle deposition, even if, to date, it is not known exactly how this process takes place (Cameron *et al.*, 2006; Pighin *et al.*, 2004). In particular, the LTPs identified in our microarray analysis are of types 1 and 5 (Beisson *et al.*, 2003), the two groups that are considered the best candidates for a function in cuticle synthesis (Suh *et al.*, 2005). Furthermore, the ABC transporter belongs to the WBC sub-family and was suggested as a good candidate for wax export to the cuticle, because it is upregulated in the epidermis and belongs to the same group as CER5, a protein shown to have this function (Pighin *et al.*, 2004; Suh *et al.*, 2005). Furthermore, many *LTP* and *ABC* genes are induced by drought stress (Colmenero-Flores *et al.*, 1997; Jang *et al.*, 2004; Rea, 2007). In tobacco, it was recently shown that there is a strong induction of *LTP* gene expression and a concomitant increase in wax depo-

sition in response to drying events (Cameron *et al.*, 2006). Interestingly, among the four *LTP* genes, those that are upregulated (all belonging to the type 5 group) in the 35S::*AtMYB41* line (Table 2 and Figure 5) are also induced in response to osmotic stress and ABA (as shown by the Genevestigator website; Zimmermann *et al.*, 2004), as is the gene coding for the ABC transporter, which is also induced by salt stress (Table 2). Our data suggest that AtMYB41 regulates the expression of genes that may be involved in cuticle component transport in response to stress. On the other hand, *LTP5*, which encodes an LTP belonging to the type 1 group, is expressed at lower levels in the transgenic line than in wild-type (Table 2 and Figure 5), and is also downregulated in response to osmotic stress (Table 2). LTP5 might be involved in the transport of other lipids that are not required in response to stress, and AtMYB41 might negatively regulate its expression (either directly or indirectly).

In our expression analysis, we found that, in the transgenic line, the expression of genes coding for two expansins (AtEXP5 and AtEXP10) and for two xyloglucan:xyloglucosyl transferases (XTH7 and XTH8, Becnel *et al.*, 2006), all enzymes involved in cell wall modification, is downregulated (Figures 4 and 5, and Table 2). These data suggest a direct or indirect role for AtMYB41 in the negative regulation of *AtEXP5* and *AtEXP10*, and are completely consistent with the reduced dimensions and abnormal morphology of the cells observed in 35S::*AtMYB41* plants. Abnormalities in morphology of epidermal cells have also been shown for some cuticle mutants, such as *lcr*, *lacs2*, *pel1*, *pel3*, *cer10* and *ace/hth* (Kurdyukov *et al.*, 2006a; Schnurr *et al.*, 2004; Tanaka *et al.*, 2004, 2007; Yephremov *et al.*, 1999; Zheng *et al.*, 2005), but a possible link between expansins and cuticle synthesis or deposition has not been described previously.

The relationship between cuticle composition and structure and the tolerance to water stress is not very clear. In fact, there are some data suggesting that greater amounts of waxes enable plants to have lower transpiration rates, and there are many examples in which defects in the synthesis of cuticular components enhance plant transpiration (reviewed by Jenks *et al.*, 2002; Shepherd and Griffiths, 2006). On the other hand, in some cases, greater amounts of waxes do not improve transpiration rates (Jenks *et al.*, 2002; Shepherd and Griffiths, 2006).

A clear link between drought stress and inhibition of leaf growth is well established, and growth inhibition under these conditions generally results from decreases in cell-wall extensibility, a process mediated by expansins (Cosgrove *et al.*, 2002).

Genes described as differentially expressed between 35S::*AtMYB41* and wild-type plants may all be part of a molecular network that is important for cell-wall modification, cuticle synthesis and deposition, in response to osmotic stress, directed by the activity of AtMYB41.

Experimental procedures

Plant material

Seeds of wild-type *A. thaliana* ecotype Columbia were used in this study.

Seeds were incubated for 4 days at 4°C in the dark, to break seed dormancy, then transferred to 22°C with a 16 h light/8 h dark cycle, and plants were grown for the various periods indicated.

For *in vitro* experiments, seeds were surface-sterilized with ethanol for 2 min, then with a solution of sodium hypoclorite (0.5% v/v) for 5 min, rinsed three times with sterilized distilled water, and then seeds were sown on Petri dishes or on Phytatray II® (Sigma, http://www.sigmaaldrich.com/) with solid MS medium (Sigma M-5519) containing 1% w/v sucrose, 0.5 g l^{-1} MES (Sigma M-8652) and 0.8% w/v agar (Bactoagar, Difco; http://www.vgdusa.com).

Treatments and RT-PCR analysis

Samples were collected for expression analysis from various organs and at various developmental stages as previously described (Gusmaroli *et al.*, 2001).

Desiccation, ABA and white light treatments were performed as described by Cominelli *et al.* (2005). For cold treatment, seeds were sown on Einhietserde soil (Manna-Italia; http://www.manna.it), then plants were grown for 4 weeks and subsequently incubated at 4°C for up to 24 h in the dark. The entire aerial part of the plants was collected after 1, 2, 4, 6, 8 and 24 h. For NaCl treatments, plants were grown in liquid MS medium as previously described for ABA treatment (Cominelli *et al.*, 2005), then NaCl was added at a final concentration of 200 mM; samples were collected after 1, 2, 4, 6, 8, 16 and 24 h. All collected organs and treated plants were frozen in liquid nitrogen and stored at −80°C.

RNA extraction and RT-PCR analysis were performed as previously described (Cominelli *et al.*, 2005). The sequences of the primers used in this study are listed in Table S2. For each experiment, the RT-PCR analysis was repeated at least three times giving similar results.

Transgene construction and generation of transgenic plants

The *AtMYB41* cDNA was amplified from cDNA of drought-stressed plants, using MYB41F4 and MYB41R3 primers (see Table S2), and cloned in the pCR-Blunt II-TOPO vector (Invitrogen, http://www.invitrogen.com/). The fragment was then excised using *Bam*HI and *Xba*I and cloned in the corresponding sites of pRT-Ω/*Not*I/*Asc*I under the control of the CaMV 35S promoter and the Ω untranslated sequence of TMV (Überlacker and Werr, 1996). The chimeric expression cassette was then transferred into the *Asc*I site of the binary vector pGPTV-KAN-*Asc* (Überlacker and Werr, 1996). Arabidopsis plants were transfected with *Agrobacterium tumefaciens* strain GV3101 by the vacuum infiltration method (Bechtold and Pelletier, 1998), and transgenic plants were grown on agar plates containing kanamycin.

Microscopy

Adaxial surface shapes of the leaf epidermis and palisade parenchyma cells of fully expanded 3rd true leaves were examined. The samples, rendered transparent by incubation overnight in a chloral hydrate solution (200 g chloral hydrate, 20 g glycerol, 50 ml H_2O),

were then observed with a Zeiss Axioskop 20 microscope (Zeiss, http://www.zeiss.com/).

Transpirational water loss

For measurement of transpirational water loss, detached rosette leaves of 3-week-old plants grown on soil were placed on 3 mm filter papers set in 9 cm Petri dishes at 22°C for the indicated time periods. The degree of dehydration was measured by comparing the fresh weight (FW) of the leaves before and after the dehydration treatment. The assay was performed in triplicate. Ten plants were used for each time point in each assay.

Chlorophyll-leaching assay and staining with toluidine blue (TB test)

The chlorophyll-leaching assay was performed using rosettes of 3-week-old plants. For each experiment, three samples of four 35S::*AtMYB41* plants and four wild-type plants were prepared. Chlorophyll extraction and the determination of chlorophyll content were performed as previously described (Lolle *et al.*, 1997).

The TB test was performed using 2-week-old plants grown on plates solidified with 0.4% w/v gellan gum, as described by Tanaka *et al.* (2004). The same staining and wash were used for the analysis of green siliques of plants grown on soil.

Affymetrix ATH1 GENECHIP experiment

For total RNA isolation, wild-type and 35S::*AtMYB41* plants were grown for 21 days on soil under long-day conditions (16 h light/8 h dark). The plant samples (aerial parts) were pooled from several batches of plants to minimize variation in gene expression patterns caused by subtle changes in environmental conditions. For reproducibility, all samples were duplicated. Total RNA was extracted using TRIzol reagent (Invitrogen), followed by clean-up on RNeasy mini/midi kits (Qiagen, http://www.qiagen.com/). All methods for the preparation of cRNA, starting from 3 μg of total RNA, as well as the subsequent steps leading to hybridization and scanning of the ATH1 GENECHIP Arrays, were performed according to the methods supplied by Affymetrix (http://www.affymetrix.com). The average difference and expression call for each of the duplicated samples was computed using GENECHIP operating software, version 1.4 (GCOS1.4),using default parameters, scaling all images to a value of 500. Full details of microarray methods are available online (http://services.ifom-ieo-campus.it/).

For each of the two experimental conditions tested, two Arabidopsis ATH1 genome arrays were used, with a total of four GENECHIP arrays.

Data analysis was performed using the software GENESPRING GX version 7.3.1 (Agilent Technologies; http://www.agilent.com).

Acknowledgements

We thank Cathie Martin (John Innes Centre, Norwich, UK) for critically reviewing the manuscript. This work was supported by grants from the European Regulatory Gene Initiative in Arabidopsis (REGIA) project (QLRT-1999-00876) and the Italian 'Ministero dell'Istruzione, dell'Università e della Ricerca-Fondo per gli Investimenti della Ricerca di Base' (MIUR-FIRB) and 'Programmi di ricerca scientifica di Rilevante Interesse Nazionale' (PRIN) projects to C.T.

Supplementary Material

The following supplementary material is available for this article online:

Table S1. Genes up- or downregulated in 35S::AtMYB41 plants identified by GENECHIP analysis (complete list).

Table S2. Primers used in this study

This material is available as part of the online article from http://www.blackwell-synergy.com

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