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

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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; 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 truncatula (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 et al., 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 3SS 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](image-url)
analysed. However, \textit{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. \textit{AtMYB41} transcript accumulated in response to these treatments at similar levels both in rosette leaves and in roots (data not shown). \textit{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 \textit{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 \textit{AtMYB41} by RNA interference approaches (data not shown), we decided to characterize this transcription factor using an over-expression strategy. We generated 35S::\textit{AtMYB41} transgenic Arabidopsis plants in which the \textit{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 \textit{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 \textit{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 \textit{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::\textit{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::\textit{AtMYB41} plants were 25% of the values for wild-type leaves (Table 1).

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::\textit{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::\textit{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 \textit{AtMYB41} over-expression inhibits plant cell expansion.

**Table 1 Phenotypic analysis of transgenic 35S::\textit{AtMYB41} plants**

<table>
<thead>
<tr>
<th>Plant height (cm)</th>
<th>Rosette leaf length (cm)</th>
<th>Rosette leaf width (cm)</th>
<th>Number of siliques per plant</th>
<th>Number of seeds per silique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>20.2 ± 2.4</td>
<td>3.7 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>35S::\textit{AtMYB41}-2</td>
<td>1.7 ± 0.4</td>
<td>0.8 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>35S::\textit{AtMYB41}-7</td>
<td>1.4 ± 0.5</td>
<td>0.7 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>35S::\textit{AtMYB41}-9</td>
<td>1.4 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>
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 over-expression 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 60% 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 3b, 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
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 silique fragments 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.

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 AtEXP10, 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)

<table>
<thead>
<tr>
<th>Description</th>
<th>Affymetrix IDa</th>
<th>AGIb</th>
<th>FCc</th>
<th>Odf</th>
<th>Ae</th>
<th>Nf</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integral membrane family protein</td>
<td>258905_at</td>
<td>At3g06390</td>
<td>242.6</td>
<td>+</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Lipid transfer protein family protein</td>
<td>250230_at</td>
<td>At5g13900</td>
<td>88.5</td>
<td>+</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>263005_at</td>
<td>At1g54540</td>
<td>83.3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>myb family transcription factor (MYB41)</td>
<td>253851_at</td>
<td>At4g28110</td>
<td>83.0</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>GDSL motif lipase/hydrolase family protein</td>
<td>260234_at</td>
<td>At1g74460</td>
<td>79.8</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydroxyproline-rich glycoprotein family protein</td>
<td>245899_at</td>
<td>At5g09480</td>
<td>73.8</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peroxidase, putative</td>
<td>247857_at</td>
<td>At5g58400</td>
<td>64.3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>GDSL motif lipase/hydrolase family protein</td>
<td>267121_at</td>
<td>At2g23540</td>
<td>58.3</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Lipid transfer protein family protein</td>
<td>262317_at</td>
<td>At2g48140</td>
<td>52.8</td>
<td>+</td>
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<tr>
<td>Transferase family protein</td>
<td>249289_at</td>
<td>At5g10400</td>
<td>39.8</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Endonuclease/exonuclease/phosphatase family protein</td>
<td>266011_at</td>
<td>At2g37440</td>
<td>38.8</td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<tr>
<td>Protein kinase, putative</td>
<td>261966_at</td>
<td>At2g39110</td>
<td>37.6</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pectinesterase family protein</td>
<td>267464_at</td>
<td>At1g06590</td>
<td>36.8</td>
<td>–</td>
<td>NA</td>
<td>–</td>
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<tr>
<td>Glycine-rich protein</td>
<td>262097_at</td>
<td>At1g55990</td>
<td>34.2</td>
<td>+</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>Auxin-responsive family protein</td>
<td>245412_at</td>
<td>At4g17280</td>
<td>33.2</td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<td>Lipid transfer protein family protein</td>
<td>256937_at</td>
<td>At3g22620</td>
<td>32.9</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Glutaredoxin family protein</td>
<td>260831_at</td>
<td>At1g06830</td>
<td>7.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Xyloglucan:xyloglucosyl transferase, putative (XTH7)</td>
<td>253040_at</td>
<td>At4g37800</td>
<td>5.7</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Copia-like retrotransposon family</td>
<td>258003_at</td>
<td>At2g29030</td>
<td>5.2</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Expansin, putative (EXPS)</td>
<td>249367_at</td>
<td>At5g06530</td>
<td>4.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Trehalose-6-phosphate phosphatase, putative</td>
<td>263452_at</td>
<td>At2g22190</td>
<td>2.8</td>
<td>NA</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Peptidase M20/M25/M40 family protein</td>
<td>254496_at</td>
<td>At4g20070</td>
<td>2.6</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Non-specific lipid transfer protein 5</td>
<td>252115_at</td>
<td>At3g51600</td>
<td>2.6</td>
<td>–</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Cytochrome P450, putative</td>
<td>246380_at</td>
<td>At1g57750</td>
<td>2.5</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Plastocyanin-like domain-containing protein</td>
<td>252638_at</td>
<td>At1g64640</td>
<td>2.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Shikimate kinase-related</td>
<td>266608_at</td>
<td>At2g35500</td>
<td>2.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AP2 domain-containing transcription factor TINY, putative</td>
<td>252638_at</td>
<td>At1g64640</td>
<td>2.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td><em>Expressed protein</em></td>
<td>263287_at</td>
<td>At2g36145</td>
<td>2.4</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Sulfate adenylyltransferase 3/ATP-sulfurylase 3 (APS3)</td>
<td>245254_at</td>
<td>At4g14680</td>
<td>2.3</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Inorganic carbon transport protein-related</td>
<td>262828_at</td>
<td>At1g17070</td>
<td>2.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Photosystem II reaction centre W (PsbW) family protein</td>
<td>253790_at</td>
<td>At4g28660</td>
<td>2.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Immunophilin</td>
<td>256130_at</td>
<td>At1g18170</td>
<td>2.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Haloacid dehalogenase-like hydrolase family protein</td>
<td>259603_at</td>
<td>At1g56500</td>
<td>2.2</td>
<td>–</td>
<td>NA</td>
<td>–</td>
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<tr>
<td>Chlorophyll a/b binding protein, putative/LHCl type II, putative</td>
<td>258015_at</td>
<td>At1g19150</td>
<td>2.2</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Membrane protein, putative</td>
<td>255719_at</td>
<td>At1g32060</td>
<td>2.1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Photosystem II reaction center PsbP family protein</td>
<td>245368_at</td>
<td>At4g15510</td>
<td>2.1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>At1g18730</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>262785_at</td>
<td>At1g10750</td>
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<td>–</td>
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<tr>
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<td>At5g43750</td>
<td>2.1</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>

aIdentification number on the Affymetrix Arabidopsis GENECHIP (ATH1).
AbAbiordopsis gene index number.
CcFold 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,fResponse 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.
(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 synthetases, 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 ω/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., 2005; 2006). 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, 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 BamHI and XbaI and cloned in the corresponding sites of pRT-β/Not/AscI under the control of the CamV 35S promoter and the ß untranslated sequence of TMV (Overlacker and Werr, 1996). The chimeric expression cassette was then transferred into the AscI site of the binary vector pGPTV-KAN-Asc (Overlacker 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 H2O),
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 silicates 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).

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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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