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Myb transcription factors have been implicated in a wide variety of plant-specific processes, including secondary metabolism, cell shape determination, cell differentiation, and stress responses. Very recently, AtMyb41 from Arabidopsis (Arabidopsis thaliana) was described as a gene transcriptionally regulated in response to salinity, desiccation, cold, and abscisic acid. The corresponding transcription factor was suggested to control stress responses linked to cell wall modifications. In this work, we have characterized AtMyb41 further by subjecting independent AtMyb41-overexpressing lines to detailed transcriptome and metabolome analysis. Our molecular data indicate that AtMyb41 is involved in distinct cellular processes, including control of primary metabolism and negative regulation of short-term transcriptional responses to osmotic stress.

Myb transcription factors (TFs) are widespread in animals, plants, and fungi. They were first identified as oncogenes in animals, where their function was linked to control of the cell cycle (Ito et al., 2001). Most animal Myb TFs are characterized by three repeats of the Myb domain, R1R2R3, which is an imperfect helix-turn-helix repeat of 50 to 53 amino acids. In contrast, most plant Myb TFs are of the R2R3 type that features only two repeats of the Myb domain. This basic structural difference between kingdoms raised questions about specific roles of Myb TFs in plants. Over the past decade, characterization of plant Myb TFs has implicated them in a wide variety of plant-specific processes, including secondary metabolism, cell shape determination, cell differentiation, and stress responses. For example, overexpression of AtMyb75 (AtPAP1) and AtMyb90 (AtPAP2) resulted in the accumulation of anthocyanin (Borevitz et al., 2000; Xie et al., 2006), while overexpression of AtMyb28 enhanced Met-derived glucosinolate content of Arabidopsis (Arabidopsis thaliana; Gigolashvili et al., 2007). AtMyb66 (WEREWOLF) promotes cell differentiation to the nonhair fate (Lee and Schiefelbein, 1999). AtMyb23 and AtGl1 both promote initiation of leaf edges, and AtMyb23 also controls trichome branching

(Kirik et al., 2005). A mutation in AtMyb26 impairs secondary wall thickening of endothecial cells in the anthers and leads to male sterility (Yang et al., 2007), and AtMyb46 has been linked to secondary cell wall biosynthesis (Zhong et al., 2007). Myb TFs have also been implicated in abiotic stress signaling pathways (Yamaguchi-Shinozaki and Shinozaki, 2005). For instance, AtPHR1 is a critical regulator of phosphorus stress responses (Rubio et al., 2001). AtMyb15 interacts with AtICE1 and negatively regulates the induction of cold-responsive genes (CBF regulon) independently of abscisic acid (ABA; Agarwal et al., 2006). AtMyb8 is required for basal freezing tolerance. Loss of AtMyb8 function also results in hypersensitivity to NaCl and increased expression of stress genes (Zhu et al., 2005). AtMyb2 mediates the ABA-dependent expression of salt- and dehydration-responsive genes and interacts with a calmodulin protein to enhance salt tolerance (Hoeren et al., 1998; Yoo et al., 2005). AtMyb60, AtMyb61, and AtMyb44 regulate stomatal aperture in response to drought stress (Cominelli et al., 2005; Liang et al., 2005; Jung et al., 2008).

Given the established stress elicitation of many Myb TFs (Yanhui et al., 2006), it is likely that other uncharacterized Myb TFs are also involved in plant responses to adverse environmental conditions. Very recently, the AtMyb41 gene was found to be transcriptionally regulated in response to salinity, desiccation, and cold as well as the endogenous plant hormone ABA (Cominelli et al., 2008). Overexpression of AtMyb41 in transgenic plants resulted in a dwarf phenotype due to alterations of cell expansion and cuticle integrity and increased their sensitivity to desiccation, suggesting that AtMyb41 may control stress responses linked to cell wall modifications (Cominelli et al., 2008). Here, we have characterized AtMyb41 further by subjecting independent AtMyb41-overexpressing lines to de-
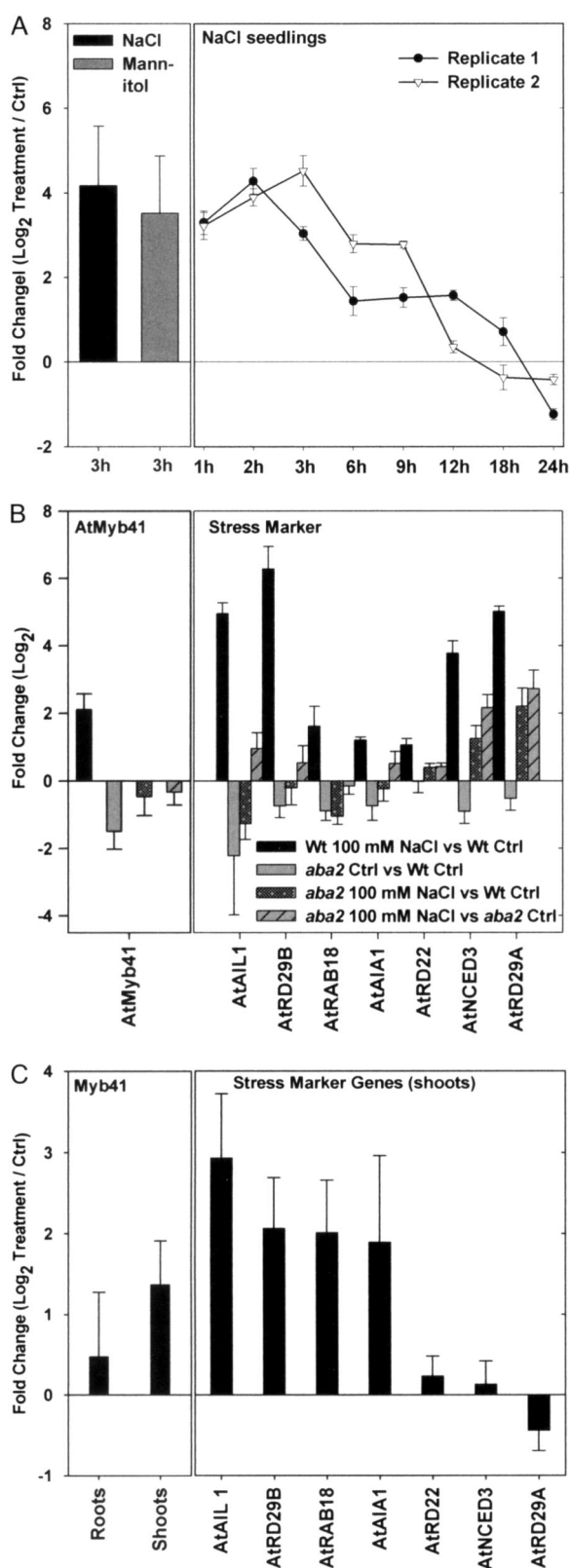

Figure 1. A, Expression of *AtMyb41* in 9-d-old seedlings after 3 h of treatment with 100 mM NaCl or 200 mM mannitol (left panel; data represent means ± SD of three independent replicates) and after 24 h of treatment with 100 mM NaCl (right panel; data from two independent experiments are shown separately, with error bars representing SEM from three biological replicates). Ctrl, Control. B, Expression of *AtMyb41* (left) and stress marker genes (right) after 3 h of 100 mM NaCl treatment in 9-d-old *aba2* mutant and wild-type seedlings. Data represent means ± SD of three independent replicates. Black bars represent salt-stressed wild type (Wt) versus control wild type (Wt Ctrl); gray bars represent control *aba2* versus control wild type; dotted black bars represent salt-stressed *aba2* versus control wild type; striped gray bars represent salt-stressed *aba2* versus control *aba2*. C, Expression of *AtMyb41* (left) and stress marker genes (right) in hydroponically grown 4-week-old plants. Plants were treated with increasing salt concentrations, reaching 50 mM NaCl in the final week. Data represent means ± SD of three independent replicates. The Arabidopsis Genome Initiative codes of the genes are provided in Supplemental Table S4.

tailed transcriptome and metabolome analysis. Our molecular data indicate that AtMyb41 is involved in distinct cellular processes, including control of primary metabolism and negative regulation of short-term transcriptional responses to osmotic stress.

RESULTS

AtMyb41 Expression Is Induced by Salt and Osmotic Stress in an ABA-Dependent Manner

We identified *AtMyb41* in a screen for salt shock- and osmotic shock-inducible TFs in 9-d-old Arabidopsis seedlings grown in sterile liquid culture using a high-throughput quantitative real-time reverse transcription (RT)-PCR resource for almost all known Arabidopsis TFs (Czechowski et al., 2004). *AtMyb41* transcript levels in seedlings increased reproducibly after 3 h of 100 mM NaCl or 200 mM mannitol treatment (Fig. 1A). Subsequent analysis of the kinetics of *AtMyb41* induction revealed a rapid increase in transcript levels within 1 h of NaCl addition, as described previously (Cominelli et al., 2008), but under our experimental conditions *AtMyb41* peaked between 2 and 3 h and subsequently declined to basal, non-stressed control levels (Fig. 1A). Induction of *AtMyb41* transcription was partially blocked in the *aba2* mutant with reduced capacity to synthesize ABA (Fig. 1B). Similar results were obtained for a set of known stress marker genes, including *AtRd29b* and *AtRab18*, confirming an ABA-dependent regulation (Fig. 1B). NaCl induction of *AtMyb41* expression was also observed in 4-week-old hydroponically grown plants acclimated to 50 mM NaCl (Fig. 1C), implicating *AtMyb41* in long-term adjustment of plant cells to moderate osmotic stress in addition to more dramatic, short-term responses to osmotic shock (Fig. 1A). In line with a previous report (Cominelli et al., 2008), we observed that *AtMyb41* expression was up-regulated by drought, cold, and ABA treatments (data not shown).

Ectopic Overexpression of *AtMyb41* Retards Arabidopsis Growth

To investigate the role of *AtMyb41* in plant response to stress, transgenic Arabidopsis lines were created using the constitutive promoter *Cauliflower mosaic*

virus-35S to drive *AtMyb41* expression. In the T3 generation, eight kanamycin-resistant lines were identified and *AtMyb41* overexpression was confirmed by quantitative real-time RT-PCR in four of five putative single-insertion lines (Fig. 2A). Growth was not affected significantly in seedlings of *AtMyb41* overexpressors (data not shown). However, prolonged overexpression of *AtMyb41* inhibited growth (Fig. 2, B and C), albeit to a much lower extent than described previously for independent *AtMyb41* transgenic lines (Cominelli et al., 2008). Despite the mild dwarf phenotype, no significant differences in relative growth rate were observed between *AtMyb41* overexpressors and wild-type plants in vitro on salt-containing plates, in long-term salt acclimation experiments performed in hydroponic culture, or in soil-grown salt- or drought-stressed plants (data not shown).

AtMyb41 Overexpression Deregulates a Subset of Salt-Induced Genes

Rapid induction of *AtMyb41* expression in response to osmotic and salt stress indicated a possible role for AtMyb41 in immediate/early responses of seedlings to osmotic stress. To identify potential targets of
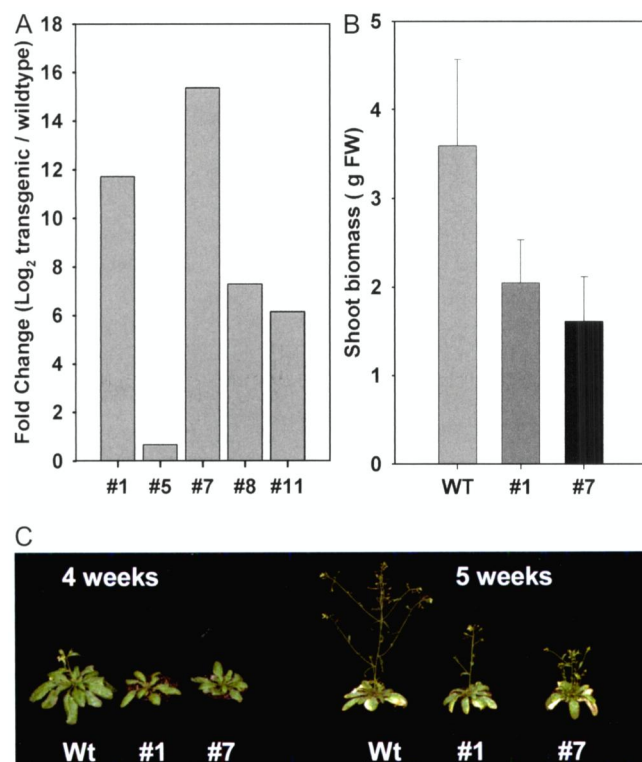

Figure 2. A, Analysis of transgene expression in *AtMyb41*-overexpressing lines using quantitative real-time RT-PCR. B, Growth of *AtMyb41*-overexpressing plants compared with wild-type plants, as estimated by shoot biomass. Data represent means ± SD of 45 plants from eight independent replicates, harvested before bolting. FW, Fresh weight; WT, wild type. C, *AtMyb41*-overexpressing plants from lines 1 and 7 compared with wild-type (Wt) plants at 4 and 5 weeks after sowing.

AtMyb41 action as a step toward defining its physiological role, transcriptome analysis of the two most highly overexpressing *AtMyb41* lines, lines 1 and 7, was performed using the Affymetrix GeneChip Arabidopsis ATH1 Genome Array. Three independent experiments were performed with seedlings of the transgenic lines and wild-type plants grown in vitro under control conditions and after treatment with 100 mM NaCl for 3 h. For a global overview, gene expression data were analyzed first via unsupervised principal component analysis, which demonstrated good reproducibility between independent experiments (Fig. 3A). Principal component 1 (PC1), representing 37% of the variance within the data set, separated the transgenic lines from wild-type seedlings, while PC2, encompassing 19% of the variance, separated stressed plants from controls (Fig. 3A). To assess differential expression among the genotypes and the treatments, data were further analyzed by a significance-based comparison applying a false discovery rate (FDR)-corrected false positive rate (type I error) of 5% (FDR $P < 0.05$; Benjamini and Hochberg, 1995) and a threshold >1.5-fold change ($\log_2$ scale). Comparisons were made between genotypes for a given treatment or between treatments for a given genotype (Supplemental Table S1). Genes differentially expressed between genotypes and/or treatments were clustered with the K-means method, revealing five groups with broad differential patterns of expression (Fig. 3B). Clusters A and B represented salt-induced and salt-repressed genes, respectively, regardless of the genotype, many of which are known to be stress responsive like *AtRd29a*, *AtCor15a*, and *AtKin1* (Supplemental Table S1). The other three clusters included genotype-specific genes. We found transcripts induced (cluster C) or repressed (cluster D) in the transgenics that did not respond to salt treatment, indicating stress-unrelated roles for AtMyb41 or reflecting pleiotropic effects of *AtMyb41* overexpression. Most interesting, cluster E contained genes that were salt induced in the wild type but not in *AtMyb41*-overexpressing plants, indicating a possible role for AtMyb41 in feedback repression of these genes. This cluster contained a number of known stress-responsive genes, including some late embryogenesis abundant genes, *AtDreb2a* and *AtNced3*.

To determine the broad functions of genes that may be controlled by AtMyb41, transcripts that were differentially expressed between *AtMyb41* overexpressors and the wild type under both stress and nonstress conditions (FDR $P < 0.05$, >1.5-fold change on a $\log_2$ scale) were collectively subjected to statistical overrepresentation analysis using PageMan software (Usadel et al., 2006). Overrepresented functional groups included large enzyme families, stress responses, hormone metabolism, secondary metabolism, Myb TFs, transport, and metal handling (Fig. 3C). This analysis indicated that AtMyb41 controls, directly or indirectly, a panoply of genes involved in multiple cellular processes.
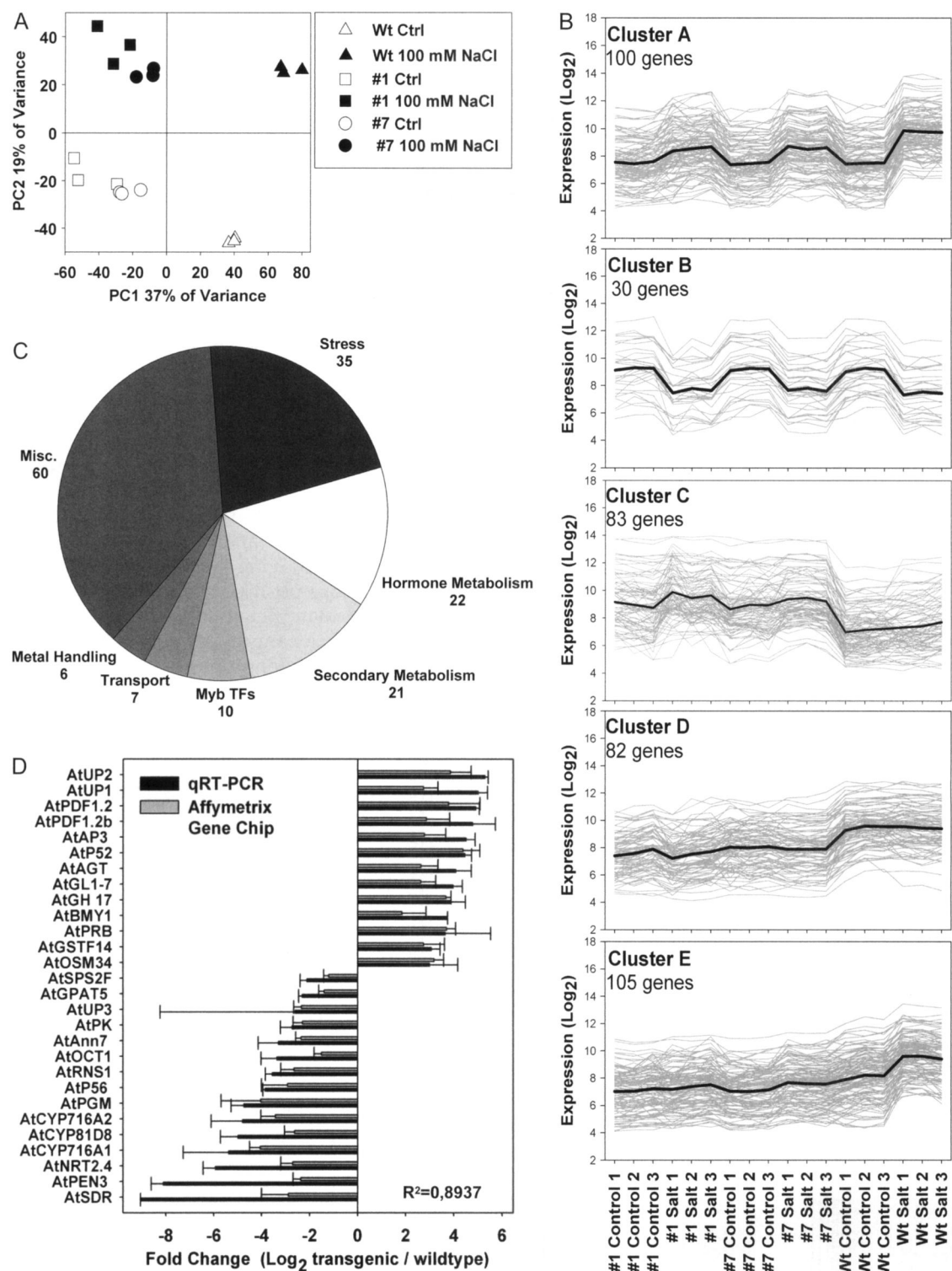
Figure 3. A, Principal component analysis of the transcriptomic profiles obtained from three independent biological replicates of each genotype and treatment. The first two principal components are shown, which constitute the major variation of the data set. Ctrl, Control; Wt, wild type. B, K-means clustering of the genes declared to be changed (FDR $P < 0.05$, >1.5-fold changed in $\log_2$ scale) in *AtMyb41* overexpressors versus the wild type and treatment versus control. Cluster A, Salt-induced genes; cluster B, salt-repressed genes; cluster C, genes up-regulated in the transgenic lines; cluster D, genes down-regulated in the transgenic lines; cluster E, salt-induced genes in the wild type but not in the transgenic lines. C, PageMan statistical overrepresentation analysis of genes significantly changed in expression due to *AtMyb41* overexpression (genes only responding to salt stress were excluded). D, Comparison of microarray and quantitative real-time RT-PCR (qRT-PCR) data (data represent means ± sᴅ of two overexpressing lines under control conditions in three independent experiments) of selected genes found significantly changed in the transcriptomic profiles. The Arabidopsis Genome Initiative codes of the genes are provided in Supplemental Table S4.

Quantitative real-time RT-PCR was used to confirm the results of the microarray studies for the most strongly up- and down-regulated genes (Fig. 3D). All of the genes tested were confirmed to be either induced or repressed in *AtMyb41* overexpressors compared with the wild type. In many cases, the magnitude of changes calculated from quantitative real-time RT-PCR data were greater than from array data, as expected, but the correlation between technologies was remarkably good (linear regression $r^2 = 0.8937$).

AtMyb41 Overexpression Alters Primary Metabolism in Seedlings

Metabolite profiling of seedlings, using gas chromatography coupled to electron impact ionization-time of flight-mass spectrometry (GC/EI-TOF-MS), was carried out to determine the extent to which AtMyb41-induced changes in gene expression altered plant metabolism. A total of 92 mass spectral tags (MSTs; Desbrosses et al., 2005) were recognized, and 60 of these were ascribed to known chemical compounds (Supplemental Table S2). Data were analyzed first using unsupervised independent component analysis (ICA), which revealed marked differences between genotypes (transgenic versus wild type) but not between the treatments (control versus NaCl; Fig. 4A). Further statistical analysis using ANOVA also demonstrated significant changes in metabolite pools between genotypes but only minor changes between treatments. Sugars, amino acids, and some organic acids were changed in the *AtMyb41* overexpressors relative to wild-type seedlings (Table I). Differences due to NaCl treatment were small, and only six metabolites, mostly sugars, accumulated in wild-type and transgenic lines in response to salt shock (Table II). Similar results in other plant species implicate glycolysis and Suc metabolism in evolutionarily conserved responses to short-term osmotic stress (Sanchez et al., 2008b). Only Glc and Gal accumulated under short-term osmotic stress in the wild type and constitutively in *AtMyb41* overexpressors (Tables I and II). Taken together, these results point to a role of AtMyb41 in the control of primary metabolism.

Long-Term Effects of *Myb41* Overexpression in Plants

Given the effects of *AtMyb41* overexpression on long-term plant growth (Fig. 2, B and C), it was of interest to us to investigate the molecular consequences of *AtMyb41* overexpression in older plants. Rosettes from *AtMyb41* transgenics and wild-type plants, subjected to control and various stress treatments, were profiled at the metabolome level. A total of 91 MSTs were recognized, but only 54 were found in both seedlings and mature plants. Seventy MSTs of rosettes of mature plants were ascribed to known chemical compounds (Supplemental Table S3). Changes in metabolite profiles following salt, drought, cold, and ABA treatments were revealed by unsuper-
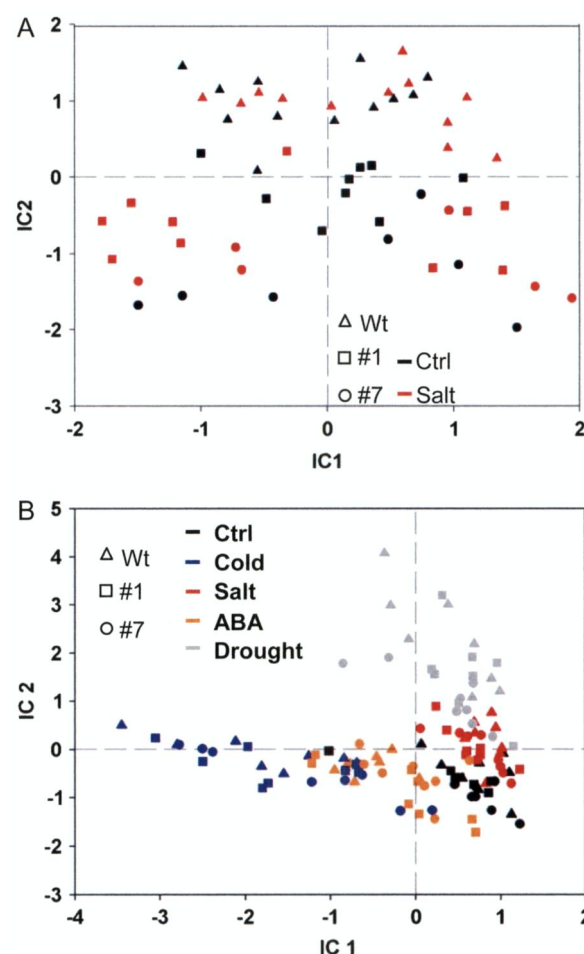

Figure 4. Unsupervised ICA of metabolomic profiles. A, Wild-type (Wt) and *AtMyb41*-overexpressing seedlings under control conditions (Ctrl; black symbols) and after 3 h of 100 mM NaCl treatment (red symbols). Triangles represent the wild type; squares represent line 1; and circles represent line 7. B, Wild-type and *AtMyb41*-overexpressing adult soil-grown plants under different stress conditions. Triangles represent the wild type; squares represent line 1; circles represent line 7; black represents control; red represents NaCl; orange represents ABA; blue represents cold; and gray represents drought.

vised ICA, but surprisingly, no clear separation was observed between genotypes for these treatments (Fig. 4B). A significance-based analysis (ANOVA) demonstrated that the pool sizes of only two metabolites were statistically different in the shoots of transgenic compared with wild-type plants: myoinositol and galactinol (Fig. 5). Myoinositol levels were significantly lower in control and cold-treated *AtMyb41* overexpressors than in wild-type plants, and galactinol was lower in the transgenic lines not only under these two conditions but also under salt-stress conditions. Raffinose levels also tended to be lower in the transgenic lines than in the wild type under control and stress conditions, although differences were not significant at $P < 0.01$ (Fig. 5). Note that myoinositol levels were also lower in seedlings of *AtMyb41* overexpressors than in wild-type controls (Table I).

Table I. *Significantly changed metabolites due to AtMyb41 overexpression in lines 1 and 7 (ANOVA P < 0.01)*

Data represent means from three independent experiments. To aid comprehension, the metabolite pool sizes in the overexpressors were averaged and expressed as ratios of wild-type seedlings under control or 100 mm NaCl conditions. The chemical identities of metabolites shown in brackets could not be accurately ascertained. The unknowns are unidentified metabolites that have been detected before and are denoted by Golm Metabolite Database code (Kopka et al., 2005).

Type	Metabolite	Lines 1 and 7 Control/Wild-Type Control	Lines 1 and 7 NaCl/Wild-Type NaCl
Amino acids	Pyroglutamic acid	5.40	5.68
	Ala	5.16	5.40
	Glu	3.87	4.16
	Asp	2.97	3.06
	Phe	2.78	2.50
	Thr	2.22	2.53
	Gly	2.08	2.58
	Se	1.93	2.18
	[Orn lactam]	0.57	0.75
Sugars	Maltose	1.98	2.92
	Xyl	1.97	2.12
	Rha	1.81	3.68
	1,6-Anhydro-β-D-Glc	1.57	2.47
	Glc-6-P	1.46	1.77
	Gal	1.40	1.83
	Glc	1.32	1.77
	Myoinositol	0.63	0.84
	[2-NH 2-deoxy Glc]	0.48	0.74
Organic acids	Gluconic acid	3.79	2.32
	Citric acid	2.15	2.41
	Sinapic acid	1.65	1.86
	Fumaric acid	1.56	2.94
	Malic acid	0.57	0.85
	Glyceric acid	0.09	0.09
Miscellaneous	Putrescine	9.17	7.81
	[Glycerophosphoglycerol]	1.67	1.84
	[Glycerol-3-P]	1.25	1.51
Unknown	A139006	10.12	7.01
	A151009	5.94	5.53
	A144007	3.09	3.05
	A302001	2.39	2.17
	A199004	1.76	1.92
	A203003	1.50	1.62
	A147001	0.64	0.83
	A1990904	0.48	0.75
	A126003	0.19	0.23
	A237006	0.13	0.31

To test if the genes that were differentially expressed in *Myb41* overexpressor compared with wild-type seedlings were also differentially expressed in adult plants of these genetic backgrounds, we measured expression of AtMyb41-responsive genes (Fig. 3D) in greenhouse-grown plants. A comparison between seedlings and adult plants grown under control, non-stress conditions revealed that only *AtAp3* was differentially expressed between rosettes of *AtMyb41* overexpressors and those of the wild type (Fig. 6A). Transcripts of several genes were barely detected, and subsequent analysis of seedlings revealed that some of these genes were predominantly expressed in roots, explaining the lack of expression in mature rosettes

(Supplemental Fig. S1). However, differences between transgenic and wild-type plants in the expression of some of these genes were observed when adult plants were exposed to abiotic stress, suggesting that *AtMyb41* overexpression alters stress signaling pathways not only in seedlings but also in mature rosettes (Fig. 6, B–E).

DISCUSSION

Although many TFs of the Myb family are expected to be involved in stress responses, few have been characterized functionally. Recently, AtMyb41 was implicated in stress-induced cell wall expansion and

Table II. *Significantly changed metabolites in seedlings under salt shock, regardless of the genotype (ANOVA P < 0.01)*

To aid comprehension, metabolite pool sizes were averaged for both overexpressors. Data represent means of three independent experiments and are expressed as ratios of the 100 mM NaCl treatment to controls. The unknown is an unidentified metabolite that has been detected before and is denoted by Golm Metabolite Database code (Kopka et al., 2005).

Type	Metabolite	Wild-Type NaCl/Control	Lines 1 and 7 NaCl/Control
Sugars	Suc	1.76	1.70
	Glc	1.37	1.87
	Gal	1.40	1.87
	Fru	1.39	1.62
Unknown	A1990904	1.19	1.89

modification in Arabidopsis, based on the dual observations that *AtMyb41* transcript levels could not be detected under normal growth conditions but accumulated under stress and that overexpression of *AtMyb41* resulted in abnormal morphology with severely inhibited cell and whole plant growth (Cominelli et al., 2008). Using quantitative real-time RT-PCR, we were able to detect *AtMyb41* transcripts under nonstress conditions not only in seedlings but also in shoots and roots of mature plants. We showed that *AtMyb41* was induced by osmotic stress in an ABA-dependent manner, similar to known ABA-responsive genes like *AtAil1*, *AtRab18*, *AtRd29b*, and *AtAia1*, suggesting that *AtMyb41* may be part of the AREB regulon (Fujita et al., 2005). Consistent with this idea, putative ABRE cis elements are present in the promoter region of *AtMyb41*, together with myc recognition sites (Supplemental Fig. S2). A gain-of-function approach was used to shed light on possible molecular and physiological roles of AtMyb41. Constitutive overexpression of *AtMyb41* in Arabidopsis inhibited plant growth, but to a much lower extent than described previously for similar transgenic plants (compare figure 2C with figure 2F in Cominelli et al., 2008).

Transcriptome analysis revealed a subset of genes that were induced by salt treatment in wild-type seedlings that failed to be induced in *AtMyb41* overexpressors (Fig. 3B). Clearly, *AtMyb41* overexpression interfered with the response of these genes, suggesting that this Myb TF may act as a transcriptional repressor. Among these transcripts were a number of known stress-responsive genes, including six late embryogenesis abundant-like proteins that are crucial in seed development and stress responses (Hundertmark and Hincha, 2008), *AtDreb2a* encoding a key TF involved in stress signaling (Sakuma et al., 2006), and *AtNced3* encoding a key enzyme of ABA biosynthesis with a major role under stress (Iuchi et al., 2001). Noteworthy, *AtBgl1*, which encodes a β-glucosidase involved in the activation of conjugated ABA pools (Lee et al., 2006), was more expressed in the *AtMyb41* overexpressors than in the wild type under both stress and nonstress conditions (Supplemental Table S1). Transcriptional

changes in *AtNced3* and *AtBgl1* indicate that a disturbance in ABA metabolism may have been responsible for the deregulation of stress-related genes in the *AtMyb41* overexpressors, but further work will be required to test this hypothesis.

Interestingly, 10 Myb TFs were differentially expressed in *AtMyb41* overexpressors compared with the wild type (Fig. 3C). Eight of these, *AtMyb71*, *AtMyb121*, *AtMyb9*, *AtMyb52*, *AtMyb49*, *AtMyb64*, *AtMyb102*, and *AtMyb93*, were responsive to salt treatment in the wild type but not in the transgenic lines, while expression of *AtMyb40* and *AtMyb74* was repressed in the *AtMyb41* overexpressors under nonstress conditions (Supplemental Table S1). Phylogenetic analysis of the protein sequences revealed that the majority of these TFs are closely related (Kranz et al., 1998), and a survey of publicly available transcriptomic data suggested that they are similarly transcriptionally regulated, particularly in the case of osmotic and salt stresses (Supplemental Fig. S3). Of the 10 Myb TFs, only *AtMyb102* has been characterized in any detail: *AtMyb102* transcripts accumulated rapidly following osmotic stress and wounding and contain a 5′ untranslated region with a crucial role in posttranscriptional regulation (Denekamp and Smeekens, 2003). Taken together, these results point to the existence of a network of interregulated Myb TFs that orchestrates transcriptional reprogramming in Arabidopsis to mount appropriate responses to abiotic stress.

Transcriptomic analysis identified a set of well-known stress-responsive genes that are part of the overall response to stress, such as *AtRd29a*, *AtCor15a*, and *AtKin1* (Ma et al., 2006), which were apparently not deregulated by AtMyb41 overexpression. Thus, AtMyb41 controls some but not all of the signaling pathways responding to salt shock in seedlings. In addition, transcriptome analysis also revealed a subset of transcripts that were either up- or down-regulated in *AtMyb41* overexpressors relative to the wild type, which were not responsive to salt treatment in any genotype (Fig. 3B; Supplemental Table S1). Altered expression of these genes in transgenic lines may have been a pleiotropic effect of prolonged, high-level *AtMyb41* expression. However, it may also reflect physiological roles of AtMyb41 not necessarily linked to osmotic stress. For example, AtMyb41 may be involved in sulfur homeostasis, because its expression increased under low sulfur and in the sulfate transporter *sultr1;2* mutant (*sel1-10*; Maruyama-Nakashita et al., 2003). In our data set, this transporter was up-regulated in the *AtMyb41*-overexpressing seedlings, while *AtSultr3;4* was down-regulated under salt treatment in the transgenic lines but not in the wild type (Supplemental Table S1). These results support the hypothesis that AtMyb41 may be involved in the regulation of nutrient homeostasis.

Metabolomic analysis revealed strong differences between seedlings of *AtMyb41* overexpressors and the wild type, indicated by constitutive changes in the level of primary metabolites including amino acids,
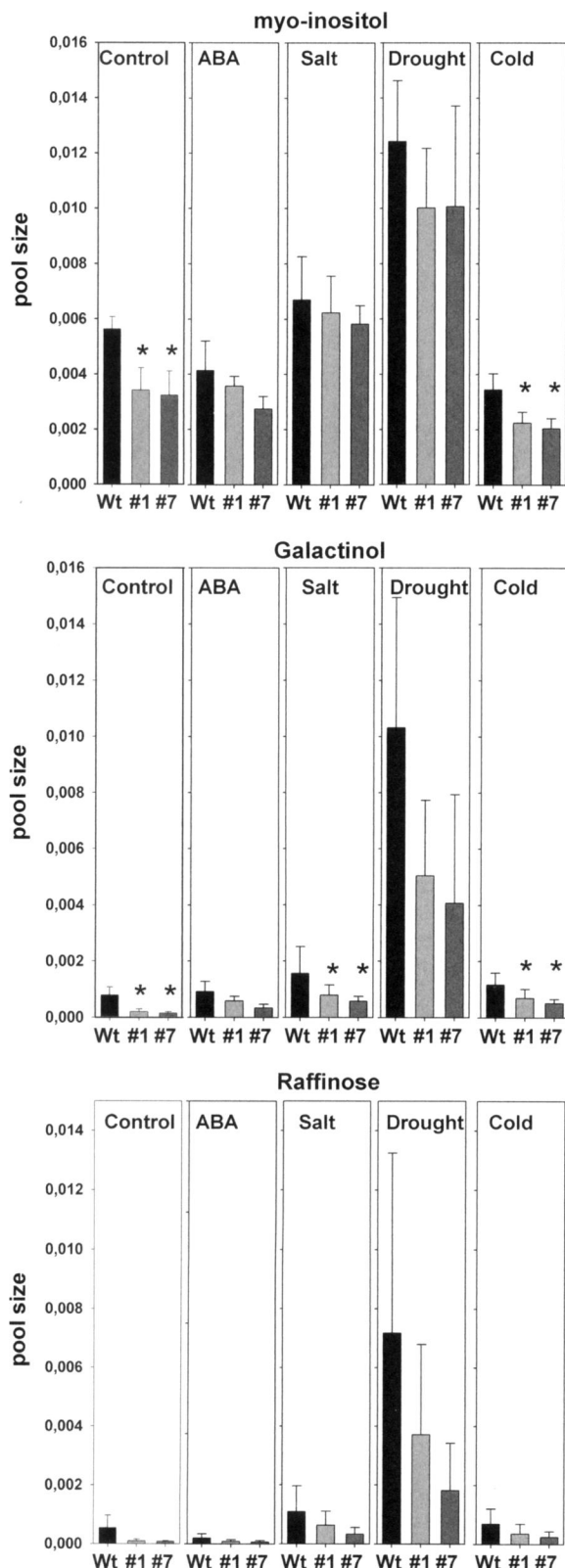
myo-inositol

Galactinol

Raffinose

organic acids, and sugars (Tables I and II). Some of the AtMyb41-induced metabolic changes, such as the increase in amino acids and organic acids, have been observed under various abiotic stress conditions (Guy et al., 2008; Sanchez et al., 2008b). It is unlikely that the altered metabolic phenotype of *AtMyb41* overexpressor seedlings was a result of changes in growth rate, as reduced plant size was evident only later in plant development. Almost all of the major metabolic changes were observed in seedlings but not in adult plants of the *AtMyb41* transgenic lines, which indicates some form of developmental control of AtMyb41 function. The potential involvement of Myb TFs in the control of primary metabolism is an interesting area for further research, since, to the best of our knowledge, this family of TFs has so far only been shown to be involved in the control of secondary metabolism (Borevitz et al., 2000; Xie et al., 2006). Consistent with the changes in metabolite levels, transcript levels of genes encoding a variety of enzymes involved in central metabolism were affected in the *AtMyb41* overexpressors, including β-amylase (*AtBmy1*), pyruvate kinase (*AtPk*), phosphoglycerate/biphosphoglycerate mutase (*AtBpm*), Suc-P synthase (*AtSps2f*), Ala-glyoxylate aminotransferase (*AtAgt*), Gln synthetase (*AtGln-1;4*), and Tyr aminotransferase (*AtTat3*). Furthermore, transcripts for several transporters, including sulfate (*AtSultr1;2* and *AtSultr3;4*), nitrate (*AtNrt2;4*), sugar, and amino acid transporters were differentially regulated (Supplemental Table S1).

Despite the negative impact of *AtMyb41* overexpression on the growth of adult plants (Fig. 2), these lines showed few differences in metabolite levels compared with wild-type controls under nonstressed or stressed conditions at this developmental stage. Only myoinositol and galactinol levels were significantly lower in adult *AtMyb41* overexpressors compared with wild-type controls, and raffinose levels showed a similar trend (Fig. 5). Note that the raffinose biosynthetic pathway, which includes all of these three compounds, has been linked to osmotic and cold stresses in Arabidopsis (Taji et al., 2002; Zuther et al., 2004). Two galactinol synthase genes (*AtGols2* and *AtGols4*) were deregulated in the *AtMyb41*-overexpressing seedlings (Supplemental Table S1), suggesting the involvement of AtMyb41 in transcriptional control of this metabolic pathway. Taken together, this indicates that at least some of the stress-related roles of AtMyb41 in seedlings are conserved at later stages of plant development. Consistent with this idea, some of the transcripts that were differentially expressed between wild-type and transgenic seedlings were also differentially regulated under stress conditions in adult plants (Fig. 6).

Figure 5. Metabolites significantly changed in shoots of mature *AtMyb41*-overexpressing plants grown in soil under different stress conditions. Data represent means ± SD of eight independent biological replicates. Asterisks highlight statistically significant difference between the wild type (Wt) and transgenics within each treatment at *P* < 0.01 (ANOVA).
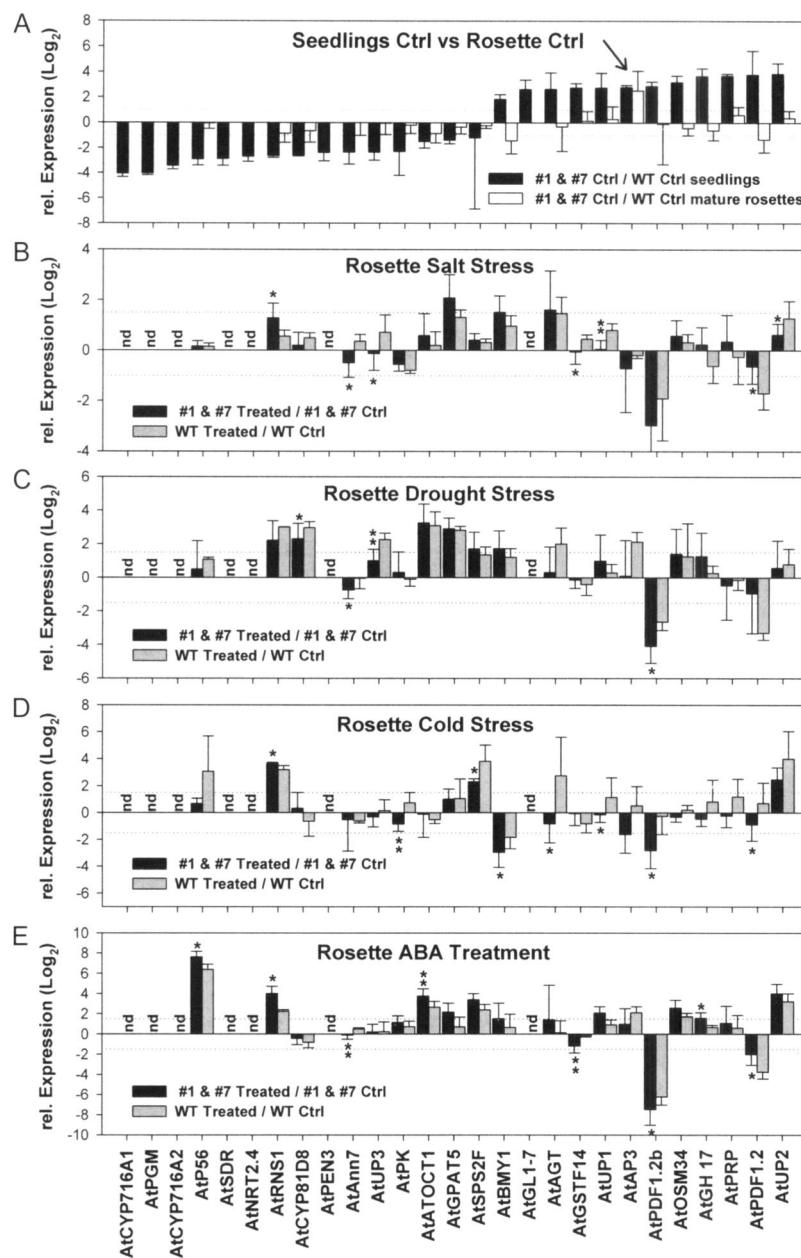
Figure 6. Expression analysis of the most highly regulated transcripts in *AtMyb41*-overexpressing rosettes. A, Expression comparison of *AtMyb41*-overexpressing plants grown under greenhouse control conditions with in vitro-grown seedlings. Ctrl, Control; WT, wild type. B to E, The expression of these genes under salt (B), drought (C), or cold (D) stress and after ABA treatment (E) in rosettes from greenhouse-grown plants. Data represent means ± SD of four biological replicates, each a pool of eight plants. The arrow in A highlights the similar expression pattern of *AtAp3* between rosettes and seedlings, while the asterisks in B to E indicate differentially expressed genes between adult wild-type and overexpressor plants, according to *t* test (* $P < 0.05$, ** $P < 0.01$). nd, Not detected in rosettes. The Arabidopsis Genome Initiative codes of the genes are provided in Supplemental Table S4.

Cominelli et al. (2008) identified numerous genes that were either up- or down-regulated in *AtMyb41* overexpressors compared with the wild type under nonstress conditions. We retested 20 of these by real-time quantitative RT-PCR but found few that were regulated in the same way in our *AtMyb41* overexpressors (Supplemental Fig. S4A). Although some of these genes were regulated in the wild type by abiotic stress (salt, drought, cold) or ABA, most were regulated in the same way in our *AtMyb41* overexpressors (Supplemental Fig. S4, B–E). Therefore, few if any of these genes appear to be controlled by AtMyb41. The discrepancies between our results and those of Cominelli et al. (2008) may be explained, in part, by differences in the magnitude of the effects of *AtMyb41* overexpression in the

different transgenic lines. The *AtMyb41* overexpressors of Cominelli et al. (2008) were highly stunted, while those in this study were only moderately affected in growth, and only at later stages of development. Differences in plant culture and treatment conditions may also account for some of the differences observed between the two studies. It should also be pointed out that the results of Cominelli et al. (2008) lack internal consistency: some of their results on cell wall-related gene transcripts obtained by RT-PCR were not confirmed by their microarray experiments, undermining their conclusion that changes in the expression of such genes in the *AtMyb41* overexpressors may account for the growth defects of their transgenic lines. In fact, no causal relationship between cell wall-related genes and

the dwarf phenotype of their *AtMyb41* overexpressors was proven (Cominelli et al., 2008).

Whatever the explanation for the growth defect in *AtMyb41* overexpressors, it seems unlikely that it was caused by constitutive activation of stress pathways by AtMyb41, as none of the stress-related metabolites identified in previous studies, including amino acids, organic acids, sugars, and polyols (Sanchez et al., 2008b), were altered in mature, nonstressed transgenic plants compared with the wild-type controls (Fig. 4). In contrast to previous results (Cominelli et al., 2008), we observed no increase in the sensitivity of growth to salt and drought stresses of *AtMyb41*-overexpressing plants compared with wild-type controls, which is perhaps not surprising given the multigenic nature of abiotic stress tolerance. Again, differences between these two studies in this regard may be related to differences in the severity of the growth defects exhibited by the two sets of *AtMyb41* overexpressors, with the dramatic dwarf phenotype observed by Cominelli et al. (2008) reflecting marked pleiotropic effects that would confound any interpretation of the physiological roles of AtMyb41.

In summary, we demonstrated the involvement of the ABA-regulated AtMyb41 TF in the control of distinct cellular processes in Arabidopsis seedlings, including negative regulation of short-term transcriptional responses to osmotic stress and associated changes in primary metabolism.

MATERIALS AND METHODS

Plant Material, Growth, and Experimental Conditions

Arabidopsis (*Arabidopsis thaliana*) lines overexpressing *AtMyb41* were obtained using the PyAt4g28110 cDNA clone from the ORFeome collection (Gong et al., 2004). Through the Gateway system (Invitrogen), the At4g28110 open reading frame was cloned into PMDC 32 (Curtis and Grossniklaus, 2003), where the expression is driven by the constitutive *Cauliflower mosaic virus*-35S promoter. Plants were transformed and selected with kanamycin for at least three generations.

For in vitro culture, approximately 100 seedlings were grown at 22°C in sterile liquid medium in Erlenmeyer flasks on a shaker under constant fluorescent light (approximately 50 μE m^{-2} s^{-1} in the flask; Scheible et al., 2004). Seven days after sowing, the medium was changed, and on day 9, NaCl or mannitol was added at a final concentration of 100 or 200 mM, respectively. Whole seedlings were harvested after 1, 2, 3, 6, 12, 18, and 24 h. Each experiment consisted of sample sets with at least three biological replicates (i.e. independent flasks). For hydroponic culture, seeds were sown in 0.5-mL plastic tubes filled with half-strength Hoagland medium (Hoagland and Arnon, 1938) including 0.8% agar. After 1 week, tubes were cut open at the bottom and transferred to a 2-L box filled with nutrient solution without agar and grown at 20°C and 12 h of light/12 h of darkness. The salt acclimation experiment was performed by increasing NaCl concentration weekly in three steps, from 0 to 10 mM, 25 mM, and 50 mM NaCl, with fresh nutrient solution each week. Medium was also changed every week for control plants. For greenhouse culture, plants were grown in soil (type GS90 + vermiculite; Einheitserde) using 10-cm pots under 20°C/18°C day/night temperatures with a long-day (16 h) photoperiod (minimum 200 μE m^{-2} s^{-1}). Salt and drought stress treatments were performed on 1-week-old plants, watering with a 50 mM NaCl solution or withholding irrigation for 2 weeks, respectively. For cold stress, 3-week-old plants were transferred to a 4°C growth chamber with a 16-h photoperiod at 90 μE m^{-2} s^{-1} for 24 h. ABA-treated plants were sprayed with 100 μM ABA solution 24 h before harvest. For each condition, rosette leaves were harvested before bolting in eight biological replicates consisting of pools of five plants each.

Gene Expression Analysis

For the Affymetrix GeneChip Arabidopsis ATH1 Genome Array experiments, RNA extraction was performed from seedling material grown in vitro from three independent experiments in pools of three biological replicates each (i.e. independent flasks). Total RNA was isolated using the hot borate method (Wan and Wilkins, 1994), and quality and quantity were assessed with a Bioanalyzer- 2100 using RNA 6000 NanoChips (Agilent Technologies) and a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), respectively. RNA was labeled with the Message AmpII-Biotin Enhanced kit following the manufacturer's instructions (Ambion). Hybridization and scanning were performed at ATLAS Biolabs. For quantitative real-time RT-PCR analysis, total RNA was treated with TURBO DNAseI (Ambion) and first-strand cDNA was synthesized with an oligo(dT) primer and SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was performed using the 2xSYBR GreenI PCR Mastermix and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Primer design, reaction conditions, cycling and dissociation curve parameters, DNA contamination, and 3' to 5' ratio checks were performed as described by Czechowski et al. (2005) without spike-in controls in a reaction volume of 10 μL. Amplification efficiency was assessed with the LinRegPCR program (Ramakers et al., 2003). Analysis of expression data was performed as described previously (Czechowski et al., 2004, 2005) using the geometric mean of three housekeeping genes for normalization (Vandesompele et al., 2002). The housekeeping genes were *AtSand* (At2g28390), *AtGapdh* (At1g13440), and *AtPdf2* (At1g13320), selected among the most stably expressed genes in Arabidopsis (Czechowski et al., 2005). A list of the primers used is available in Supplemental Table S5.

Metabolite Analysis

Metabolomic analysis was performed essentially as described by Sanchez et al. (2008a). In brief, plant material (60 mg) was extracted with chloroform/methanol, and the polar fraction was prepared by liquid partitioning into water, derivatized, and injected in a GC/EI-TOF-MS system (Wagner et al., 2003). Peak height representing arbitrary mass spectral ion currents was normalized with the sample fresh weight and ribitol content for internal standardization, and the resulting metabolomic profiles were analyzed using the TagFinder software (Luedemann et al., 2008). A series of discrete metabolic features were obtained after filtering for those represented by three or more intercorrelated mass fragments within the whole data set. Metabolites were identified using the NIST05 software (www.nist.gov/srd/mslist.htm) and the mass spectral and retention time index collection of the Golm Metabolome Database (Kopka et al., 2005). Mass spectral matching was manually supervised and accepted with thresholds of match > 650 (maximum 1,000) and retention time index < 1.0%.

Data Analysis and Statistics

K-means clustering, principal component analysis, and ICA were used as clustering algorithms to analyze profiling data in a nonsupervised approach, using The Institute for Genomic Research multiple experiment viewer software (TMEV_3.1), the pcaMethods package for the R programming language (www.bioconductor.org/packages/2.1/bioc/html/pcaMethods.html), and the MetaGeneAlyse Web page (http://metagenealyse.mpimp-golm.mpg.de).

Microarray results were analyzed using the bioconductor software package for R (Gentleman et al., 2004). Data quality was assessed with the affy (Gautier et al., 2004) and AffyPLM packages, and expression estimates were obtained with the Robust Multi-Array algorithm (Irizarry et al., 2003). Genes assigned as present ($P < 0.05$) using the MAS5 present/absent algorithm were statistically tested for differential expression using mixed models with the LIMMA bioconductor package (Smyth, 2004). P values describing control versus treatment and genotype comparisons were corrected for multiple testing using the FDR correction (Benjamini and Hochberg, 1995). Metabolomic data were log$_{10}$ transformed prior to statistical analysis, performed with ANOVA using the TMEV_3.1 software.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Root-shoot expression ratio of selected genes identified in the microarray analysis as differentially expressed in *AtMyb41*-overexpressing seedlings.

Supplemental Figure S2. cis-Regulatory elements in the putative promoter region of *AtMyb41* identified from the PLACE database (www. dna.affrc.go.jp/PLACE).

Supplemental Figure S3. Coexpression analysis of the 10 Myb TFs using the Genevestigator database (www.genevestigator.ethz.ch).

Supplemental Figure S4. Comparison of the expression of the genes most highly regulated by *AtMyb41* overexpression in the study of Cominelli et al. (2008) with the expression in the independent transgenic lines (lines 1 and 7) described in our study.

Supplemental Table S1. Transcriptomic profile data from seedlings.

Supplemental Table S2. Metabolic profile data from seedlings.

Supplemental Table S3. Metabolic profile data from rosettes.

Supplemental Table S4. Arabidopsis Genome Initiative codes of all of the genes described.

Supplemental Table S5. List of primers used for quantitative real-time RT-PCR.

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