Overexpression of *AtMYB44* Enhances Stomatal Closure to Confer Abiotic Stress Tolerance in Transgenic Arabidopsis[1][C][W][OA]

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AtMYB44 belongs to the R2R3 MYB subgroup 22 transcription factor family in Arabidopsis (*Arabidopsis thaliana*). Treatment with abscisic acid (ABA) induced *AtMYB44* transcript accumulation within 30 min. The gene was also activated under various abiotic stresses, such as dehydration, low temperature, and salinity. In transgenic Arabidopsis carrying an *AtMYB44* promoter-driven β-glucuronidase (GUS) construct, strong GUS activity was observed in the vasculature and leaf epidermal guard cells. Transgenic Arabidopsis overexpressing *AtMYB44* is more sensitive to ABA and has a more rapid ABA-induced stomatal closure response than wild-type and *atmyb44* knockout plants. Transgenic plants exhibited a reduced rate of water loss, as measured by the fresh-weight loss of detached shoots, and remarkably enhanced tolerance to drought and salt stress compared to wild-type plants. Microarray analysis and northern blots revealed that salt-induced activation of the genes that encode a group of serine/threonine protein phosphatases 2C (PP2Cs), such as ABI1, ABI2, AtPP2CA, HAB1, and HAB2, was diminished in transgenic plants overexpressing *AtMYB44*. By contrast, the *atmyb44* knockout mutant line exhibited enhanced salt-induced expression of PP2C-encoding genes and reduced drought/salt stress tolerance compared to wild-type plants. Therefore, enhanced abiotic stress tolerance of transgenic Arabidopsis overexpressing *AtMYB44* was conferred by reduced expression of genes encoding PP2Cs, which have been described as negative regulators of ABA signaling.

Transcription factors are critical regulators of the changes in gene expression that drive developmental processes and environmental stress responses. Over 1,600 transcription factors, representing approximately 6% of the total number of genes, have been identified in the Arabidopsis (*Arabidopsis thaliana*) genome (Arabidopsis Genome Initiative, 2000; Riechmann et al., 2000; Gong et al., 2004). These transcription factors can be classified into several families based on the structure of their DNA-binding domains. Members of the MYB, ERF, bZIP, and WRKY transcription factor families have been implicated in the regulation of stress responses (Schwechheimer et al., 1998; Singh et al., 2002). The *MYB* family comprises 163 genes, making it one of the largest transcription factor families in Arabidopsis (Yanhui et al., 2006).

The MYB domain consists of two or three 50- to 53-amino acid imperfect repeats that form the helix-turn-helix motifs R1, R2, and R3 (Rosinsky and Atchley, 1998). MYB proteins in animals generally contain three repeats having significant structural homology to cellular proto-oncogenes and play roles in cell cycle control (Lipsick, 1996). In contrast, two-repeat (R2R3) MYB family members predominate in plants. A total of 126 (77% of *MYB* genes) R2R3 MYB-encoding genes have been identified in the Arabidopsis genome (Yanhui et al., 2006).

Extensive functional analyses using large-scale insertional mutagenesis (Meissner et al., 1999) and expression profiling (Kranz et al., 1998; Yanhui et al., 2006) have been performed to examine R2R3 MYB proteins in Arabidopsis. In parallel, the roles of individual plant R2R3 MYB proteins in diverse plant processes have been explored, including hormonal signaling, cell cycle control, stress responses, secondary metabolism, cellular morphogenesis, and meristem formation (Martin and Paz-Ares, 1997; Jin and Martin, 1999).

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In particular, several R2R3 MYB genes play important roles in the responses to environmental stimuli in Arabidopsis. AtMYB2, in cooperation with AtMYC2, functions as a transcriptional activator in the dehydration- and abscisic acid (ABA)-inducible expression of *RD22* (for *RESPONSIVE TO DEHYDRATION22*; Urao et al., 1993; Abe et al., 2003). *AtMYB102* is a regulatory component that integrates dehydration, osmotic, or salinity stress, ABA application, and wound-signaling pathways (Denekamp and Smeekens, 2003). In addition, the Arabidopsis mutant *hos10-1* (conferring high expression of osmotically responsive genes) exhibits altered expression of ABA-responsive genes, showing dramatically reduced capacity for cold acclimation and hypersensitivity to dehydration and salinity (Zhu et al., 2005). As reported recently, *AtMYB60* is specifically expressed in guard cells and involved in light-induced opening of stomata (Cominelli et al., 2005), whereas *AtMYB61* is expressed under conditions necessary for dark-induced stomatal closure (Liang et al., 2005).

AtMYB44 (synonym AtMYBR1), together with AtMYB70, AtMYB73, and AtMYB77 (synonym AtMYBR2), belongs to R2R3 MYB subgroup 22. Members of this subgroup share two conserved motifs: TGLYMSPxSP and GxFMxVVQEMIxxEVRSYM (Kranz et al., 1998; Romero et al., 1998; Stracke et al., 2001). Genes encoding subgroup 22 proteins have similar expression patterns and are associated with stress responses. *AtMYB44*, *AtMYB73*, and *AtMYB77* are induced by wounding (Cheong et al., 2002) and white-light treatment (Ma et al., 2005) and are transiently up-regulated by cold stress (Fowler and Thomashow, 2002). Microarray analysis revealed that these genes are up-regulated together by salt stress in *sos2* (*salt overly sensitive2*) mutants (Kamei et al., 2005). In addition, *AtMYB44* and *AtMYB77* expression is reduced in *fus3* (for *fusca3*), *lec1* (for *leafy cotyledon1*), and *abi3* (for *ABA-insensitive3*) mutants that are defective in dormancy development and desiccation tolerance during late embryogenesis and seed maturation (Kirik et al., 1998). These observations suggest that subgroup 22 genes are involved in abiotic stress responses.

The *AtMYB44* (At5g67300) gene has an open reading frame of 918 bp encoding a putative 305-amino acid polypeptide with a predicted molecular mass of 33.3 kD. We characterized *AtMYB44* in more detail, examining its expression and the phenotype of transgenic plants with altered *AtMYB44* expression. Our data indicate that the AtMYB44 transcription factor plays a role in an ABA-mediated signaling pathway that confers abiotic stress tolerance via the enhancement of stomatal closure.

RESULTS

AtMYB44 Expression

Northern blots showed that *AtMYB44* transcript accumulation was induced within 30 min after the application of 100 μM ABA, 100 μM methyl jasmonate, or 50 μM ethylene to Arabidopsis rosette leaves (Fig. 1A). *AtMYB44* transcript levels also increased when Arabidopsis was exposed to dehydration, high salt levels, or cold (Fig. 1B). The increase in *AtMYB44* tran-

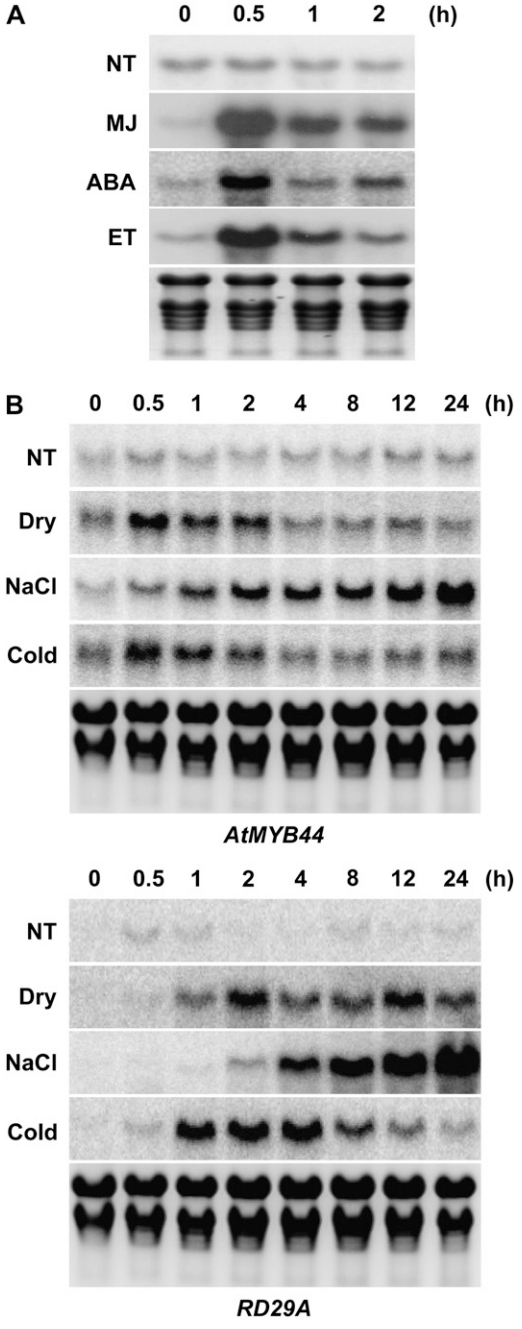

Figure 1. Northern blots of *AtMYB44* expression. A, Induction of *AtMYB44* by ABA. Sterilized water (nontreatment; NT), 100 μM methyl jasmonate (MJ), 100 μM ABA, or 50 μM ethephon (ET) was applied to the surface of solid Murashige and Skoog agar medium in which 2-week-old Arabidopsis seedlings were growing. Total RNA was extracted from plants harvested at the indicated times after each treatment. B, Induction of *AtMYB44* and *RD29A* by abiotic stresses. Two-week-old seedlings were dried on Whatman 3MM paper (Dry), treated with 250 mM NaCl (NaCl), or incubated at 4°C (Cold).

script levels occurred before the increase in *RD29A* (a marker gene for abiotic stress) transcripts, which was detected at least 1 h after hormone or stress treatment.

In transgenic Arabidopsis expressing the GUS reporter gene driven by the *AtMYB44* promoter (approximately 3.0 kb), GUS activity was observed in all tissues examined in transgenic plants, including the filament, stigma, pedicle, sepal, petal, and floral nectary (Fig. 2A). In most tissues, strong GUS expression was observed in the vasculature. In seedlings grown on Murashige and Skoog medium, the highest levels were observed in the veins and guard cells of the leaf epidermis (Fig. 2B).

For subcellular localization of the protein, *AtMYB44* cDNA was fused in frame to the N-terminal side of the GFP marker gene and expressed in transgenic Arabidopsis under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Confocal imaging of GFP revealed that the AtMYB44-GFP fusion protein accumulated in the nuclei (Fig. 2C).

35S:AtMYB44 Transgenic Arabidopsis

Transgenic Arabidopsis constitutively expressing *AtMYB44* cDNA (*35S:AtMYB44*) were also generated. Five independent T_3 or T_4 homozygote lines (denoted with numerals 10, 14, 17, 18, and 21) containing one (lines T-10 and T-21) or two (lines T-14, T-17, and T-18) copies of the transgene (Fig. 3A) and showing the highest levels of expression (Fig. 3B) were selected for further analyses. Western blots confirmed AtMYB44 protein (approximately 33 kD) accumulation in transgenic plants and the absence of protein in the *atmyb44* knockout plants (SALK_039074; Fig. 3C).

The *35S:AtMYB44* plants germinated uniformly, as measured 1 week after growing on Murashige and Skoog medium (Fig. 4A). In early stages of vegetative growth, however, rosette leaves of *35S:AtMYB44* plants were smaller, but became longer and wider than those of wild-type plants after flowering (Fig. 4B). Transgenic plants were dwarfed during the first 5 weeks of growth and were prostrate compared to wild-type plants (Fig. 4C). Extent of growth retardation was correlated with the expression level of the transgene in the transgenic plants.

Flowering time also differed between wild-type and *35S:AtMYB44* plants, as determined when the main florescence shoot elongated to 1 cm. Wild-type plants began to flower at 30 d after sowing (DAS), whereas *AtMYB44* transgenic plants took 36 to 37 DAS to reach the same stage (Fig. 4D). At this time point, all *35S:AtMYB44* plants had 16 to 18 leaves, whereas wild-type and *atmyb44* knockout plants had 13 leaves on average per rosette. Thus, the delay of flowering was not merely caused by a slower overall growth rate, but reflected developmental retardation in the flowering process.

When flowering (i.e. 6 weeks after sowing), heights of transgenic plants were comparable to that of wild-type plants. Adult *35:AtMYB44* plants had much shorter

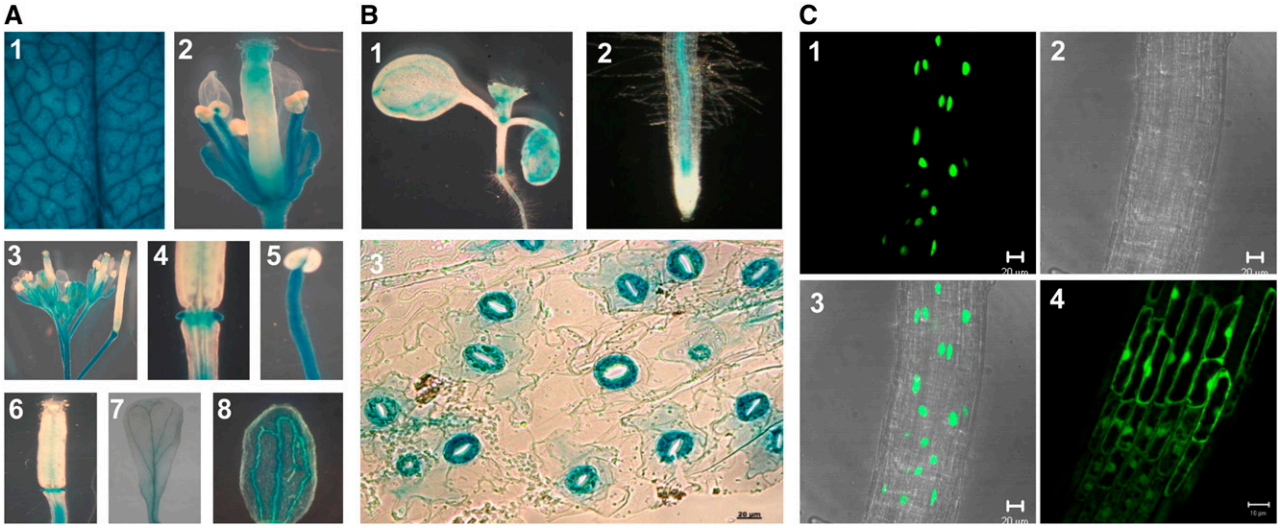

Figure 2. Localization of *AtMYB44* expression. A, Histochemical GUS assay. An approximately 3.0-kb fragment of the *AtMYB44* promoter was fused to the *GUS* gene and transformed into Arabidopsis. Histochemical assays for GUS activity in transgenic plants were performed as described by Jefferson et al. (1987). GUS staining patterns were confirmed by observing at least eight different transgenic lines. 1, Rosette leaf; 2, flower; 3, inflorescence; 4, floral nectar; 5, stamen; 6, carpel; 7, petal; 8, sepal. B, GUS activity in transgenic Arabidopsis seedlings grown on Murashige and Skoog medium. 1, One-week-old whole seedling; 2, root tip (1 week old); 3, paradermal section of the abaxial epidermis (200×) from 2-week-old plant. Scale bar = 20 μm. C, Subcellular localization of AtMYB44 protein. *AtMYB44* cDNA was fused to GFP and the construct was expressed in transgenic Arabidopsis under the control of the CaMV 35S promoter. GFP fluorescence patterns were confirmed by observing at least five different transgenic lines under a confocal laser-scanning microscope. 1, GFP fluorescence; 2, differential interference contrast (DIC; optical microscopic image); 3, merged image (GFP + DIC); 4, GFP from *35S:GFP* control plant. Scale bars = 20 μm for the images from the *35S:AtMYB44-GFP* plant (1, 2, and 3) and 10 μm for that from the *35S:GFP* plant (4), respectively.

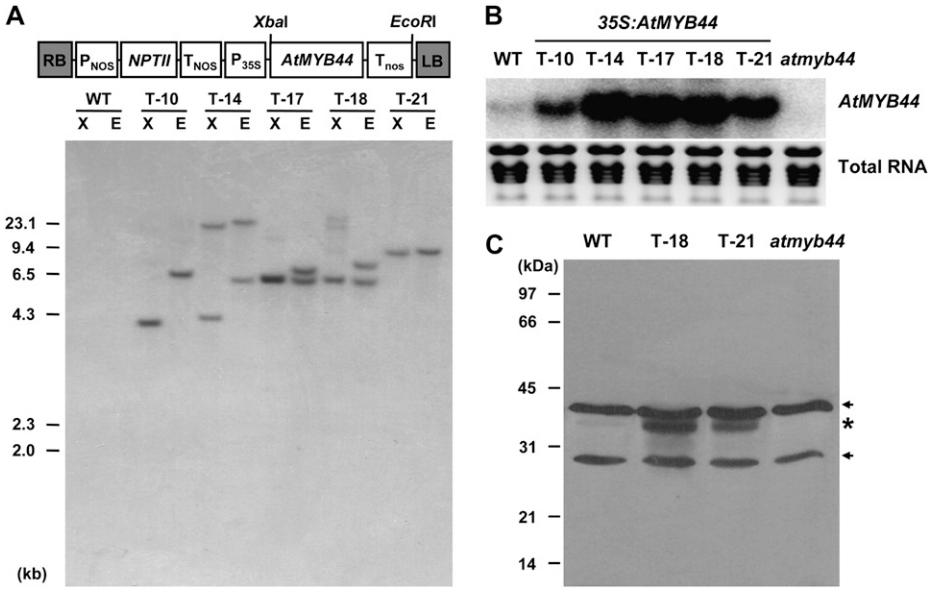

Figure 3. Blot analyses of transgenic Arabidopsis. *AtMYB44* cDNA was fused to the CaMV 35S promoter and transformed into Arabidopsis (*35S:AtMYB44*). T-10, T-14, T-17, T-18, and T-21 denote the transgenic line. The *atmyb44* knockout line (SALK_039074) was obtained from the SALK collection. A, Southern blot indicating copy numbers of the inserted T-DNA. Genomic DNA was digested with *Xba*I (X) and *Eco*RI (E), and the blot was hybridized with a *NEOMYCIN PHOSPHOTRANSFERASEII* (*NPTII*) probe. B, Northern blot demonstrating the constitutive expression of *AtMYB44* in transgenic plants. C, Western blot showing the AtMYB44 protein levels in transgenic plants. Asterisk, AtMYB44 protein band (approximately 33 kD); arrowheads, bands of two unknown cross-reacted proteins (approximately 40 and 29 kD, respectively).

petioles and smaller seeds than wild-type plants; *atmyb44* knockout plants exhibited no distinguishable phenotypes in terms of germination, growth, and flowering when compared to wild-type plants.

ABA Sensitivity of *35S:AtMYB44* Plants

Without treatment with ABA, the seed germination rate of *35S:AtMYB44* plants was comparable to that of wild-type plants (Fig. 5A). However, ABA inhibited germination of *35S:AtMYB44* plants more severely than that of wild-type plants, indicating ABA hypersensitivity of transgenic plants. Treatment with 3 μM ABA decreased the seed germination rate of *35S:AtMYB44* plants to approximately 20%, whereas wild-type seeds retained 70% germination under the same conditions. The *atmyb44* T-DNA insertion knockout line showed no difference from wild-type plants in the ABA germination experiment.

Stomata of *35S:AtMYB44* plants had smaller guard cells and apertures than did wild-type plants by approximately 80% (Fig. 5B). Density of guard cells (num-

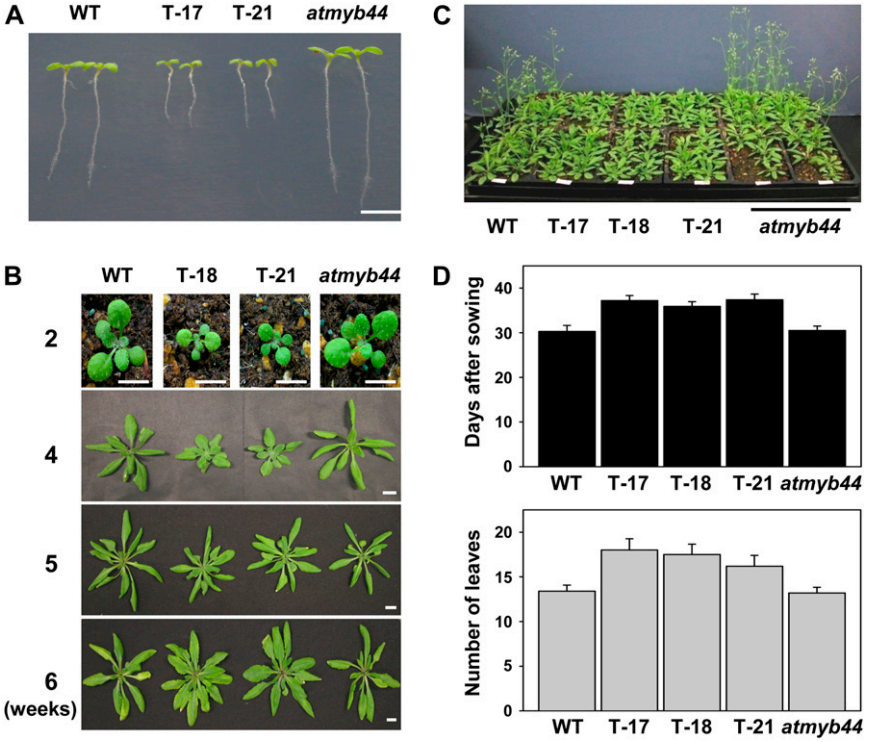

Figure 4. Growth of transgenic Arabidopsis overexpressing *AtMYB44*. A, One-week-old seedlings grown on Murashige and Skoog medium. Scale bar = 1 cm. B, Growth of rosette leaves after growing on soil. Scale bars = 1 cm for all the images. C, Appearance of transgenic plants 5 weeks after sowing. D, Flowering time of *35S:AtMYB44* plants. The time (DAS) at which the main inflorescence shoot had elongated to 1 was recorded. In addition, the number of rosette leaves when plants were flowering was counted. In all cases, 20 plants were counted to calculate the average ± SD. [See online article for color version of this figure.]

bers on unit area) was not differentiated in *35S*:*AtMYB44* when compared to wild-type plants (data not shown). ABA treatment resulted in a higher rate of stomatal closure in *35S*:*AtMYB44* plants than in wild-type plants. Treatment with 1 μM ABA reduced the stomatal apertures of wild-type plants to approximately 85% of those of nontreated plants. In *35S*:*AtMYB44* plants, the same treatment reduced stomatal apertures to 60% to 70% of those of nontreated plants. Therefore, transgenic plants overexpressing *AtMYB44* exhibited more rapid ABA-induced stomatal closure than did wild-type plants. Stomatal apertures of *atmyb44* knockout plants were slightly larger (approximately 105%) than those of wild-type plants and were reduced to 80% level in this experiment.

Stress Tolerance of *35S:AtMYB44* Plants

The rate of water loss from *35S*:*AtMYB44* plants was lower than that from wild-type plants, as measured by the fresh-weight loss of detached shoots (Fig. 6A). After dehydration for 3 h, the fresh weight of *35S*:*AtMYB44* plants was reduced to approximately 60%, whereas wild-type and *atmyb44* knockout plants retained 70% of their initial weight.

In addition, three *35S*:*AtMYB44* lines had higher survival rates than did wild-type plants on rewatering after 12 d of water deprivation (Fig. 6B). In 10 independent experiments, 231 of 282 *35S*:*AtMYB44* (T-21 line) plants survived this test, for a survival rate of 82%, whereas 70 of 411 (17%) wild-type plants and 11 of 134 (8%) *atmyb44* knockout plants survived. Two other *35S*:*AtMYB44* lines, T-17 (252 of 283) and T-18 (176 of 198), both had 89% survival rates.

The *35S*:*AtMYB44* plants also showed significantly enhanced salt stress tolerance. On watering with increasing concentrations of NaCl up to 300 mM, transgenic plants grew relatively well, whereas wild-type plants became wilted and chlorotic (Fig. 6C). In 10 independent experiments, 292 of 353 T-21 line plants survived the salt tolerance test, for a survival rate of 83%, whereas 40 of 229 (17%) wild-type plants and nine of 131 (7%) *atmyb44* knockout plants survived. Lines T-17 (243 of 278) and T-18 (209 of 235) had 87% and 89% survival rates, respectively.

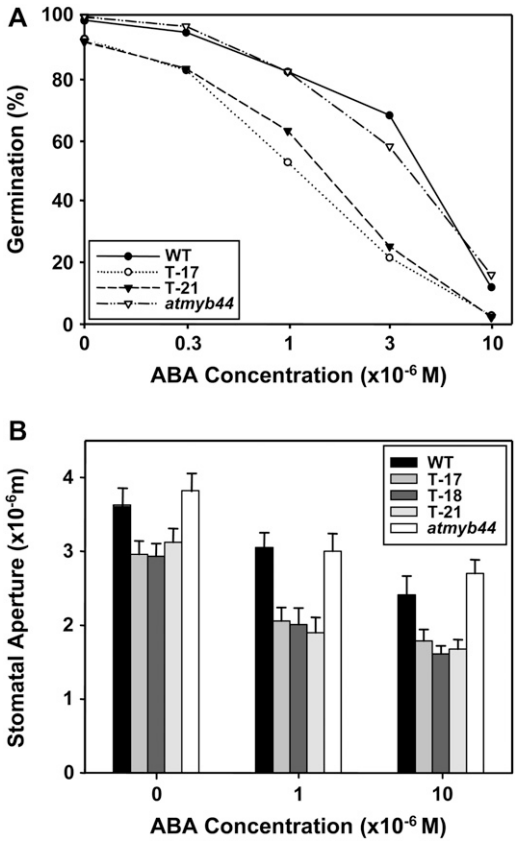

Figure 5. Responses of *35S*:*AtMYB44* and *atmyb44* knockout plants to ABA. A, Germination rate. Seeds were germinated and grown on Murashige and Skoog agar plates with or without ABA for 7 d. B, Size of stomatal apertures. Stomata were fully opened prior to ABA treatment. Rosette leaves of 5-week-old plants were detached and floated abaxial-side down on opening solution for 2 h prior to ABA treatment. Leaves were then treated with ABA for 2 h by adding it to the solution. Stomatal apertures in epidermal peels were observed under a microscope and measured. The sizes of at least 50 stomatal apertures were measured for each treatment.

Expression of Salt-Induced Genes in Transgenic Plants

Microarray experiments were performed twice using 10 μg of total RNA extracted from wild-type or transgenic Arabidopsis plants (line T-21) treated with or without 250 mM NaCl for 24 h. Hybridization was conducted using Affymetrix ATH1 genome arrays. Microarray experiments using the synthetic oligonucleotide chip demonstrated a high degree of reproducibility between the two sets of independent experiments. Transcript-level data were deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number E-ATMX-30.

Only probe sets that showed significant differences in the two experiments were selected for further analysis. Without salt treatment, *35S*:*AtMYB44* and *atmyb44* knockout Arabidopsis did not show significant alteration in overall expression patterns (Supplemental Tables S1 and S2). Based on the 2-fold criterion, 112 (0.5% of the total 22,500 probe sets) and 26 (0.1% of the total) genes, respectively, had altered transcription levels.

By contrast, on treatment with 250 mM NaCl for 24 h, *35S*:*AtMYB44* plants exhibited significantly altered gene expression patterns. Compared to wild-type plants, 816 genes (3.6% of the total) had transcription levels enhanced by more than 2-fold in *35S*:*AtMYB44* transgenic plants, whereas 496 genes (2.2% of the total) had transcription levels reduced by more than 2-fold (Supplemental Table S3). In *atmyb44* knockout plants, with the salt treatment, 102 genes (0.5% of the total) had transcription levels enhanced by more than 2-fold, whereas 38 genes (0.2% of the total) had 2-fold lower levels compared to wild-type plants (Supplemental Table S4).

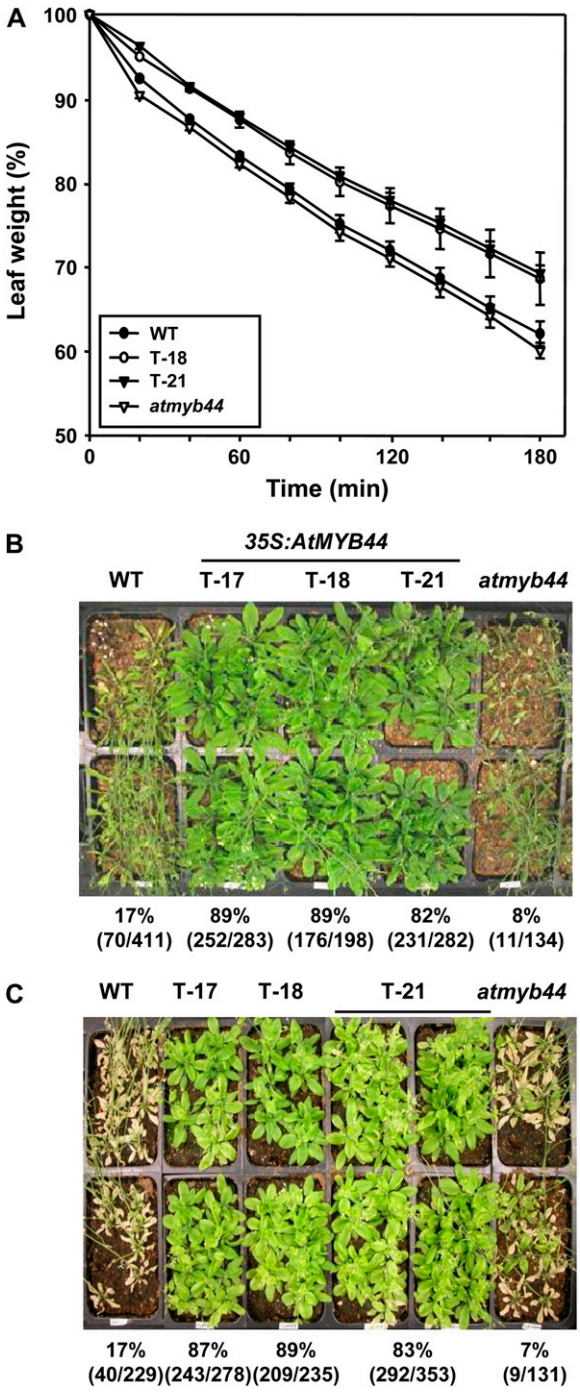

Figure 6. Abiotic stress tolerance tests of *35S:AtMYB44* plants. A, Transpiration rates. For water-loss measurements, the aerial part of 5-week-old plants was separated from the roots, placed on weighing dishes, and allowed to dry slowly on the laboratory bench (25°C, 60% relative humidity). Weights of the samples were recorded at regular intervals. B, Drought tolerance test. Watering of 4-week-old plants was stopped for 12 d and then resumed for 3 d. C, Salt tolerance test. Four-week-old plants were watered for 12 d at 4-d intervals with increasing concentrations of NaCl: 100 mM, 200 mM, and 300 mM. In B and C, survival rates (%) were calculated from the numbers of surviving plants per total plants tested in 10 independent experiments and are indicated under each of the plant lines. [See online article for color version of this figure.]

Genes showing enhanced salt-induced expression in *35S:AtMYB44* plants included those encoding aquaporins, arabinogalactan proteins (AGPs), auxin-induced proteins, cell wall biosynthetic or modifying enzymes, chlorophyll biosynthetic enzymes, and RNA-binding proteins (Supplemental Table S3). In addition, enhanced transcript levels of several types of protein kinase, xyloglucan endotransglucosylase/hydrolase, and calcium-binding proteins were observed in salt-treated *35S:AtMYB44* plants.

Microarray analysis revealed that transcript accumulation of well-studied ABA-dependent abiotic stress-inducible marker genes was not significantly enhanced in *35S:AtMYB44* transgenic plants on treatment with 250 mM NaCl, but was comparable to that in wild-type plants (Table I). In particular, the numbers of gene transcripts encoding DREB/CBF and AREB, which bind to the dehydration-responsive element (DRE/CRT) and the ABA-responsive element (ABRE), respectively, were not enriched or rather reduced in some cases.

Instead, salt-induced activation of the genes encoding Ser/Thr protein phosphatases 2C (PP2Cs) was suppressed in *35S:AtMYB44* transgenic plants (Table I). Salt induction of *AtHB-7* and *AtHB-12*, which are regulated by ABI1 in ABA signaling (Hoth et al., 2002), was also decreased (Supplemental Table S3). In addition, salt-induced expression of the genes encoding proteins involved in flavonoid biosynthesis, such as CHS, DFR, and F3H, was lower in *35S:AtMYB44* plants than in wild-type plants. Notably, the expression level of various cytochrome *P450* genes was also lower in *35S:AtMYB44* plants than in wild-type plants, supporting a previous observation that expression of these genes is related to abiotic stresses (Narusaka et al., 2004).

The result from microarray experiments on abiotic stress marker genes was confirmed by northern blots (Fig. 7). No increase in the well-known drought/salt stress marker genes *RD29A*, *RD22*, and *RAB18* was observed in transgenic plants, whereas the increase in the PP2C-encoding genes, such as *ABI1*, *ABI2*, *AtPP2CA*, *HAB1*, and *HAB2*, was diminished in *35S:AtMYB44* plants. The *atmyb44* knockout mutant line exhibited somewhat enhanced salt-induced expression of the PP2C-encoding genes.

DISCUSSION

AtMYB44 transcript accumulation was induced within 30 min after ABA, methyl jasmonate, or ethylene was applied to Arabidopsis rosette leaves (Fig. 1A). Expression of *AtMYB44* was also induced by dehydration, salt treatment, and low temperatures (Fig. 1). The increase in transcript accumulation occurred rapidly (i.e. within 30 min) and preceded the increase in transcripts of the ABA-inducible abiotic stress response marker gene *RD29A* (Fig. 1B). This is consistent with previous reports that *AtMYB44* tran-

Table I. *Comparison of the transcription levels of selected genes in 35:AtMYB44 plants treated with 250 mM NaCl*

Probe ID[a]	AGI No.[b]	Description	No Treatment				Salt Treatment			
			Experiment 1		Experiment 2		Experiment 1		Experiment 2	
			Fold[c]	*P* Value[d]	Fold	*P* Value	Fold	*P* Value	Fold	*P* Value
247025_at	At5g67030	Zeaxanthin epoxidase (ABA1)	NC[e]	0.91106	−1.1	0.99903	−1.1	0.99914	−1.8	0.99998
259669_at	At1g52340	Short-chain alcohol dehydrogenase (ABA2)	NC	0.04726	NC	0.01543	NC	0.09428	2.0	0.00004
246325_at	At1g16540	Aldehyde oxidase (ABA3)	NC	0.14568	NC	0.30741	NC	0.90572	NC	0.98692
256898_at	At3g24650	ABA-insensitive protein 3 (ABI3)	NC	0.50000	NC	0.93019	NC	0.24259	NC	0.50000
263377_at	At2g40220	AP2 domain transcription factor (ABI4)	NC	0.97330	NC	0.04415	NC	0.08383	NC	0.11801
263907_at	At2g36270	ABA-insensitive 5 (ABI5)	NC	0.60871	NC	0.64556	NC	0.26334	NC	0.50000
246314_at	At3g56850	ABRE-binding factor (AREB3, DPBF3)	NC	0.73666	NC	0.50000	NC	0.50000	NC	0.93443
253263_at	At4g34000	ABRE-binding factor 3 (ABF3)	NC	0.98457	−2.1	0.99961	NC	0.93848	−3.2	0.99987
258026_at	At3g19290	ABRE-binding factor 4 (ABF4, AREB2)	NC	0.73666	−1.5	0.99965	NC	0.50000	NC	0.75742
248487_at	At5g51070	Clp protease ATP-binding subunit (ERD1)	−1.7	0.99998	−1.5	0.99998	−2.8	0.99998	−3.4	0.99998
258258_at	At3g26790	B3 DNA-binding factor (FUS3)	NC	0.50000	NC	0.60871	NC	0.17741	NC	0.54523
260854_at	At1g21970	CCAAT box-binding factor HAP3 homolog (LEC1)	NC	0.49352	NC	0.50000	NC	0.81402	NC	0.50000
246481_s_at	At5g15960	Cold and ABA-inducible protein (KIN1)	−1.3	0.99979	−2.4	0.99998	NC	0.95880	−2.2	0.99998
263497_at	At2g42540	Cold-regulated protein (COR15a)	−1.7	0.99993	−3.7	0.99998	−1.4	0.99945	−9.1	0.99998
263495_at	At2g42530	Cold-regulated protein (COR15b)	−4.0	0.99998	−4.5	0.99998	−2.0	0.99995	−3.7	0.99994
245803_at	At1g47128	Cys proteinase (RD21A)	NC	0.51942	−1.2	0.99998	−1.1	0.99866	−1.8	0.99998
246908_at	At5g25610	Dehydration-induced protein (RD22)	NC	0.50000	−1.4	0.99997	NC	0.90015	−2.8	0.99998
259570_at	At1g20440	Dehydrin (COR47)	NC	0.50000	−2.6	0.99998	−1.6	0.99998	−2.8	0.99998
248352_at	At5g52300	Low temperature-induced protein 65 (RD29B)	NC	0.19480	−2.2	0.99994	−5.2	0.99998	−194	0.99998
248337_at	At5g52310	Low temperature-induced protein 78 (RD29A)	NC	0.10566	−2.1	0.99998	−1.4	0.99995	−8.5	0.99998
247095_at	At5g66400	Dehydrin (RAB18)	−1.8	0.99997	−2.6	0.99998	−3.0	0.99998	−39.3	0.99998
251775_s_at	At3g55610	δ-1-Pyrroline-5-carboxylate synthetase (P5CS)	NC	0.57086	−1.8	0.99998	NC	0.50000	−3.2	0.99998
254066_at	At4g25480	DRE/CRT-binding factor 1A (DREB1A, CBF3)	−1.7	0.99725	NC	0.98798	−5.6	0.99998	−8.5	0.99998
254074_at	At4g25490	DRE/CRT binding factor 1B (DREB1B, CBF1)	NC	0.87545	NC	0.79613	NC	0.50000	NC	0.74715
254075_at	At4g25470	DRE/CRT-binding factor 1C (DREB1C, CBF2)	NC	0.81402	NC	0.50000	NC	0.54523	NC	0.17741
248389_at	At5g51990	DRE/CRT-binding factor 1D (DREB1D, CBF4)	NC	0.46766	NC	0.50000	−9.1	0.99851	−6.4	0.99981
250781_at	At5g05410	DRE/CRT-binding factor 2A (DREB2A)	−2.1	0.99459	−3.0	0.99996	−1.6	0.99998	NC	0.91106
256430_at	At3g11020	DRE/CRT-binding factor 2B (DREB2B)	NC	0.50000	NC	0.00370	−1.2	0.99725	−1.8	0.99630
259831_at	At1g69600	Zinc finger homeodomain (ZFHD1)	NC	0.50000	NC	0.01543	NC	0.72595	NC	0.50648
261713_at	At1g32640	bHLH transcription factor (AtMYC2, JIN1)	NC	0.84677	−2.4	0.99998	−1.6	0.99996	−1.7	0.99997
258310_at	At3g26744	bHLH transcription factor (ICE1)	NC	0.45477	NC	0.54523	NC	0.23255	NC	0.51942
260581_at	At2g47190	R2R3 MYB transcription factor (AtMYB2)	NC	0.99817	−2.8	0.99981	−2.1	0.99998	−8.0	0.99998
264782_at	At1g08810	R2R3 MYB transcription factor (MYB60)	NC	0.01543	NC	0.44192	NC	0.11801	2.0	0.00002
264556_at	At1g09540	R2R3 MYB transcription factor (MYB61)	−1.6	0.99914	NC	0.02476	NC	0.06152	NC	0.04415
261648_at	At1g27730	Salt-tolerance zinc finger protein (STZ, ZAT10)	−2.8	0.99998	−2.6	0.99998	−1.7	0.99998	1.5	0.00002
245120_at	At2g39810	E3 ubiquitin ligase (HOS1)	NC	0.13134	NC	0.50000	NC	0.50000	NC	0.50000
247313_at	At5g63980	3(2),5-Bisphosphate nucleotidase (HOS2, FRY1)	1.1	0.00039	NC	0.57086	NC	0.81402	NC	0.74715
249405_at	At5g40280	β-Subunit of protein farnesyl transferase (ERA1)	NC	0.50000	NC	0.50000	NC	0.93019	NC	0.48058
260203_at	At1g52890	NAM-like protein (ANAC019)	−3.0	0.99997	−2.6	0.99997	−1.7	0.99998	−9.1	0.99998
258395_at	At3g15500	NAM-like protein (ANAC055)	−2.1	0.99938	−2.6	0.99973	NC	0.44192	NC	0.92106
253872_at	At4g27410	NAM-like protein (ANAC072)	−3.2	0.99998	−2.8	0.99998	−1.3	0.99998	−5.2	0.99998
254305_at	At4g22200	Potassium channel protein (AKT2/3)	NC	0.50000	−2.1	0.99998	−1.2	0.99552	−3.0	0.99998
248888_at	At5g46240	Potassium channel protein (KAT1)	NC	0.50000	−1.3	0.99976	−1.5	0.99508	−1.7	0.99976
267610_at	At2g26650	Potassium transporter (AKT1)	NC	0.68109	NC	0.30741	NC	0.48058	NC	0.00370
253264_at	At4g33950	ABA-activated protein kinase (OST1)	NC	0.62112	NC	0.50000	NC	0.96159	−2.4	0.99998
260633_at	At1g62400	Protein kinase (HT1)	NC	0.50000	NC	0.99508	NC	0.93848	NC	0.75742
265252_at	At2g01980	Na^+/H^+ antiporter (SOS1)	NC	0.30741	NC	0.81402	NC	0.99221	NC	0.93443
246614_at	At5g35410	Ser/Thr protein kinase (SOS2)	NC	0.50000	NC	0.55808	NC	0.21319	NC	0.87545
249783_at	At5g24270	Calcineurin B-like protein 4 (CBL4, SOS3)	NC	0.25285	NC	0.29609	NC	0.50000	NC	0.00120
255731_at	At1g25490	PP2A, subunit A (RCN1)	NC	0.50000	NC	0.50000	NC	0.41643	NC	0.50000
253994_at	At4g26080	PP2C (ABI1)	NC	0.88199	−2.0	0.99998	−1.4	0.99931	−3.2	0.99998
247957_at	At5g57050	PP2C (ABI2)	NC	0.66941	−1.6	0.99938	−2.0	0.99981	−6.9	0.99973
259231_at	At3g11410	PP2C (AtPP2CA)	−2.0	0.99998	−1.6	0.99998	−1.2	0.99979	−6.9	0.99998
259922_at	At1g72770	PP2C (HAB1)	NC	0.96903	−1.6	0.99997	−2.2	0.99998	−7.4	0.99998
260712_at	At1g17550	PP2C (HAB2)	NC	0.50000	−1.4	0.99593	−1.5	0.99973	−1.8	0.99797
247723_at	At5g59220	PP2C	−2.1	0.99956	−4.0	0.99998	−3.7	0.99998	−19.6	0.99998
261077_at	At1g07430	PP2C	−1.8	0.99923	−4.5	0.99998	−3.0	0.99998	−29.8	0.99998
266274_at	At2g29380	PP2C	NC	0.42914	NC	0.69259	NC	0.50000	NC	0.85432
248428_at	At5g51760	PP2C	NC	0.35444	NC	0.23255	NC	0.99350	NC	0.96422

[a]Describes name of probe set on Affymetrix GeneChip ATH1. [b]Arabidopsis Genome Initiative number. [c]Relative gene transcript level compared with the same gene in wild-type plants. [d]Change *P* value, which measures the probability that the expression levels of a probe in two different arrays are the same. [e]NC, No change.

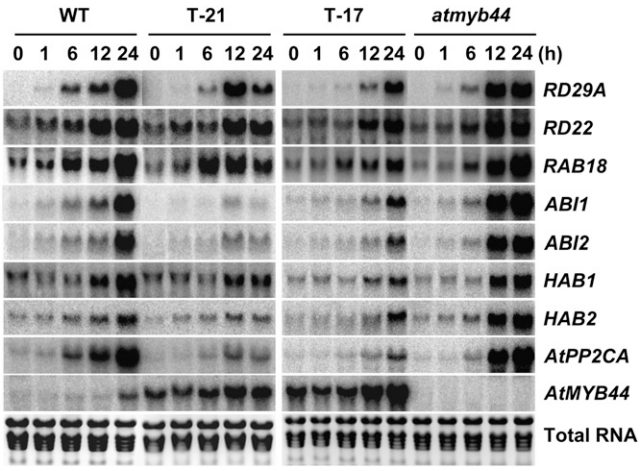

Figure 7. Northern blots of salt-inducible genes in *35S:AtMYB44* plants. Five-week-old plants were treated with 250 mM NaCl and harvested at the indicated times. cDNA probes used were EST clones obtained from TAIR.

scripts are induced in most tissues and by a variety of hormone treatments, environmental conditions, and microbial infections (Kranz et al., 1998; Yanhui et al., 2006). In our previous microarray experiment, *AtMYB44* was identified as a jasmonate-inducible gene (Jung et al., 2007). The signaling mechanism leading to the multihormonal activation of *AtMYB44* has not been investigated.

We found that six copies of the highly conserved RY motif CATGCA(TG), an essential target of FUS3 and ABI3 transcription factors (Mönke et al., 2004), are present in the *AtMYB44* promoter. ABI3 and FUS3 transcription factors are associated with ABA action (Nambara et al., 2000; Gazzarrini et al., 2004). This explains the lower levels of *AtMYB44* transcripts that were observed in the *fus3*, *lec1*, and *abi3* mutants (Kirik et al., 1998). These mutants are defective in dormancy development and desiccation tolerance during late embryogenesis and seed maturation (To et al., 2006).

Transgenic Arabidopsis overexpressing *AtMYB44* (*35S:AtMYB44*) was hypersensitive to ABA during seed germination, dwarfed in the early stages of growth, and delayed in flowering (Fig. 4). Similar phenotypes have been observed in Arabidopsis lines that overexpress well-known ABA-dependent, drought-response genes such as *DREB1A/CBF3* (Kasuga et al., 1999; Gilmour et al., 2000), *DREB2A* (Sakuma et al., 2006), *ABF3* (Kang et al., 2002), and *ABF4* (Kang et al., 2002). This suggests that AtMYB44 plays a role in ABA-mediated responses to abiotic stresses such as drought, high salinity, and low temperature.

In *AtMYB44* promoter-GUS expression assays, particularly high levels of GUS activity were observed in leaf epidermal guard cells (Fig. 2B). This concurs with the results of microarray analyses, which showed that *AtMYB44* was induced by ABA preferentially in guard cells compared to mesophyll cells (Leonhardt et al., 2004). Guard cells respond to various environmental conditions, such as humidity, temperature, light, CO_2, and ABA exposure, resulting in the opening or closing of the stomata (Roelfsema and Hedrich, 2005). Drought causes stomata to close, thereby limiting water loss through transpiration. The rate of water loss from *35S:AtMYB44* plants was lower than that from wild-type plants (Fig. 6A).

The stomata of *35S:AtMYB44* plants had smaller guard cells and apertures that were approximately 80% of the size of those in wild-type plants (Fig. 5B). By contrast, overexpression of genes that encode vacuolar Ca^{2+}-activated channel TPC1, which is involved in stomatal movement (Peiter et al., 2005), and TMAC2, which is a negative regulator of ABA and salinity responses (Huang and Wu, 2007), did not affect the size of the stomatal apertures. As demonstrated in all of these cases, the pBI121 vector (CLONTECH), which was used to carry the genes, including *AtMYB44*, into transgenic Arabidopsis did not affect the size of the stomatal apertures.

Reduced stomatal size has been observed in many transgenic or mutant Arabidopsis plants in which the genes that modulate the stomatal aperture have been manipulated. For instance, overexpression of *AtMYB61* (Liang et al., 2005), which controls dark-induced stomatal closure, resulted in smaller stomatal apertures in transgenic Arabidopsis. Mutations on *AtMYB60*, which controls stomatal opening (Cominelli et al., 2005), *OST1*, which encodes a protein kinase involved in ABA-mediated stomatal closure (Xie et al., 2006), and *HT1*, which encodes a kinase involved in stomatal movements in response to CO_2 (Hashimoto et al., 2006), also resulted in smaller stomatal apertures, respectively. Therefore, overexpression or mutation of the genes involved directly or indirectly in structural movements of the stomata might affect morphology of the guard cells in transgenic plants.

Similar to transgenic Arabidopsis overexpressing the genes that modulate the stomatal aperture, stomatal closure was increased in *35S:AtMYB44* plants in response to ABA compared to wild-type plants (Fig. 5B). Furthermore, transgenic plants showed enhanced dehydration and salinity resistance compared to wild-type plants (Fig. 6). Therefore, AtMYB44 functions as a positive regulator of ABA-mediated stomatal closure.

Huang et al. (2007) used *AtMYB44*-overexpressing plants and a knockout mutant to show that AtMYB44 functions as a negative regulator of (+)-ABA signal transduction. These results contradict ours, which were obtained from experiments in which a mixture of the plus (+)- and minus (−)-ABA enantiomers were used. In their experiment, overexpression resulted in seeds that were insensitive to 3.3 μM natural (+)-ABA and had increased germination relative to the wild type. The *atmyb44* knockout mutant had reduced germination compared to wild-type plants under the same conditions. In our experiments, by contrast, *AtMYB44*-overexpressing plants were hypersensitive to ABA treatment, whereas ABA sensitivity of the

knockout mutant was comparable to that of wild-type plants (Fig. 5A). In general, (−)-ABA has been found to be as effective as (+)-ABA. Experiments with the aquatic fern *Marsilea quadrifolia* suggest that (−)-ABA is either intrinsically active or its activity is caused by the stimulation of (+)-ABA biosynthesis (Lin et al., 2005). Huang et al. (2007) reported that the expression of *AtMYB44* was not induced by (+)-ABA, but we observed that it was rapidly induced by (±)-ABA.

On salt treatment, compared to wild-type plants, *35S*:*AtMYB44* plants exhibited significantly altered gene expression patterns (Supplemental Table S3). This could be primary or secondary effects of AtMYB44 overproduction and could explain the cause and consequence of the enhanced salt stress tolerance of *35S*:*AtMYB44* plants. Genes showing much higher increased expression levels in the *35S*:*AtMYB44* plants on salt treatment included those involved in water transport, auxin response, cell wall biosynthesis or modification, chlorophyll biosynthesis, transcriptional regulation, and protein phosphorylation.

Aquaporins are water-channel proteins of intracellular (tonoplast) and plasma membranes and play a crucial role in plant-water relationships triggered by various abiotic stresses, such as drought, high salinity, and cold (Daniels et al., 1996; Jang et al., 2004). Auxin-induced genes encoding IAAs and SAURs were also up-regulated in *35S*:*AtMYB44* plants, suggesting a salt stress response and auxin-signaling cross-talk at the level of transcriptional regulation. Several AGPs were up-regulated in salt-treated *35S*:*AtMY44* plants, supporting observations that salt stress severely affects the maintenance of cell wall structure in seedling roots and ABA-induced seed dormancy (Van Hengel and Roberts, 2003; Lamport et al., 2006). Altered expression of several types of genes encoding chlorophyll biosynthetic enzymes, chlorophyll-binding proteins, thylakoid proteins, and other chloroplast-related proteins might be correlated with salt-induced chlorophyll disorganization and degradation (chlorosis; Hernandez et al., 1999). Enhanced expression of genes encoding subunits of magnesium-protoporphyrin-IX chelatase (Mg-chelatase), including CHLH, was also notable. CHLH specifically binds ABA and thereby mediates plastid-to-nucleus signaling as a positive regulator in seed germination, postgermination growth, and stomatal movement (Nott et al., 2006; Shen et al., 2006).

Microarray analysis (Table I) and northern blots (Fig. 7) revealed that expression of major abiotic stress-responsive genes, including *RD29A*, *RD22*, and *RAB18*, was not reinforced in *35S*:*AtMYB44* plants under salt stress. This suggests that drought/salt stress tolerance exhibited by *AtMYB44* transgenic plants was not conferred by the proteins that are encoded by these genes. Instead, expression of genes that encode a group of Ser/Thr PP2Cs, such as ABI1, ABI2, AtPP2CA, HAB1, and HAB2, was suppressed in *35S*:*AtMYB44* plants and enhanced in *atmyb44* knockout plants. These proteins belong to group A PP2Cs (Schweighofer et al., 2004) and have been described as negative regulators of the ABA signal transduction cascade (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001; Sáez et al., 2004; Kuhn et al., 2006; Yoshida et al., 2006). The *abi1* and *abi2* mutations lead to phenotypic alterations in ABA-resistant seed germination and seedling growth, reduced seed dormancy, abnormal stomatal regulation, and defects in various responses to drought stress (Leung et al., 1997; Merlot et al., 2001). Overexpression of *HAB1* impaired stomatal closure (Sáez et al., 2004, 2006). In addition, a T-DNA disruption mutation in PP2C AtP2C-HA (HAB1) confers ABA hypersensitivity in the regulation of stomatal closure and seed germination (Leonhardt et al., 2004).

Enhanced salt stress tolerance of *35S*:*AtMYB44* plants appears, at least in part, to be conferred by reduced ABI2 activity triggering a negative feedback loop of the SOS2-mediated stress tolerance response. ABI2 interacts with SOS2 (Ohta et al., 2003), which is a Ser/Thr protein kinase required for salt tolerance (Liu et al., 2000). The *abi2* mutation disrupts the protein kinase-phosphatase interaction, causing increased tolerance to salt shock and ABA insensitivity (Ohta et al., 2003). Upon salt stress, SOS2 is activated by interacting with SOS3, a calcium-binding protein (Halfter et al., 2000), and the SOS2-SOS3 kinase complex is required for activation of SOS1, a plasma membrane Na^+/H^+ antiporter (Shi et al., 2000; Qui et al., 2002; Quintero et al., 2002). Interestingly, in the Arabidopsis *sos2* mutant, expression of *AtMYB44* was significantly up-regulated under salt stress, whereas the transcript levels of *RD29A*, *COR47*, *COR15A*, *KIN1*, and *RD22* were similar to those in wild-type plants (Kamei et al., 2005). One-half of the approximately 60 genes that showed increased salt-induced expression in the *sos2* mutant also showed enhanced transcription in salt-treated *35S*:*AtMYB44* plants.

As described, expression of *AtMYB44* was suppressed in the *abi3* mutant (Kirik et al., 1998). Upon overexpression of the maize (*Zea mays*) transcription factor gene *VIVIPAROUS1* (*VP1*), the ortholog of Arabidopsis ABI3, ABA-induced activation of *ABI1* and *ABI2* was strongly inhibited (Suzuki et al., 2003). Therefore, the proposed roles of AtMYB44 could be expanded to the feed-forward regulation of the ABI3-mediated ABA-signaling pathway through repression of the group A PP2C genes.

The *atmyb44* knockout line showed somewhat reduced drought/salt stress tolerance (Fig. 6) and enhanced salt-induced expression of PP2C-encoding genes compared to wild-type plants (Fig. 7). However, the overall phenotype of the mutant was not obviously different from that of wild-type plants. This is presumably because of the functional redundancy of transcription factors. In particular, other R2R3 MYB subgroup 22 genes respond to environmental stresses very similarly, as indicated by significant up-regulation in the *sos2* mutant (Kamei et al., 2005) and by cold (Fowler and Thomashow, 2002). In many studies, double-knockout mutants of MYB genes resulted in more severe defects than the parental single mutants,

as observed in anther and stomatal development (Lai et al., 2005; Mandaokar et al., 2006). Some pairs of similar MYB genes, such as GL1-WEREWOLF and FLP-MYB88, are capable of reciprocally complementing loss-of-function mutations in each locus (Lee and Schiefelbein, 2001; Kirik et al., 2005; Lai et al., 2005). Therefore, a future study should be performed using double or multiple mutants of R2R3 MYB subgroup 22 genes. Without salt treatment, *35S:AtMYB44* plants did not show significant alteration in the overall expression patterns (Supplemental Table S1). Therefore, overproduction of AtMYB44 does not appear to be sufficient to induce gene activation. Rather, the transcription factor may induce the expression of a group of specific target genes, either through salt-induced structural modification or by working cooperatively with other salt-activated transcription factors. In many cases, MYB transcription factors interact with basic helix-loop-helix (bHLH) transcription factors to exert their specific roles (Grotewold et al., 2000; Zimmermann et al., 2004; Quattrocchio et al., 2006). Further studies to identify the target genes, binding sites on promoters, and interacting proteins would clearly define the biological roles of the AtMYB44 transcription factor.

MATERIALS AND METHODS

Plant Materials and Treatments

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) was used throughout this study. Seeds of the *atmyb44* T-DNA insertion line (SALK_039074) were obtained from The Arabidopsis Information Resource (TAIR). A homozygous *atmyb44* knockout line was isolated from TAIR seeds. Plants were grown on soil or one-half-strength Murashige and Skoog agar medium (Duchefa) in a growth chamber maintained at 22°C to 24°C and 60% relative humidity under long-day conditions (16-h-light/8-h-dark cycle).

For chemical treatment, a solution of 100 μM (±)-ABA (Sigma product no. A-1049) was applied to the surface of solid Murashige and Skoog agar medium in which 2-week-old seedlings were growing. Petri dishes were then sealed with parafilm. Abiotic stresses were applied to 2-week-old seedlings either by drying on Whatman 3MM paper (dehydration treatment), treating with 250 mM NaCl (salt treatment), or incubating at 4°C under continuous light (cold treatment). After each treatment, sample seedlings or leaves were harvested and frozen immediately in liquid nitrogen until use in northern blotting.

Seed Germination Test

For the germination assays, approximately 50 seeds were placed on one-half-strength Murashige and Skoog agar medium containing 1% Suc and different concentrations of ABA. To break dormancy, seeds were incubated at 4°C for 4 d in the dark before germination and were subsequently grown in a growth chamber as described above. Seed germination was followed for 7 d. Seeds were counted as germinated when the radicles had emerged by 1 mm. The germination rate was calculated as a percentage of the total number of seeds plated.

Manipulation of *AtMYB44* Transcription

A full-length *AtMYB44* cDNA (EST 119B8) was obtained from TAIR. For the transformation, a DNA fragment containing the entire coding region plus the 3′-untranslated region was amplified from the EST clone by PCR. The cDNA fragment was inserted into the pBI121 vector (CLONTECH) from which the GUS gene had been removed at the *Xba*I and *Bam*HI sites, fusing the fragment downstream from the CaMV 35S promoter.

For gene transformation, a DNA construct was transformed into 5-week-old Arabidopsis using *Agrobacterium tumefaciens* strain C58C1 and the floral-dip method (Clough and Bent, 1998). Transformed seeds were selected on Murashige and Skoog agar medium containing the appropriate antibiotics: 40 μg/mL kanamycin (Sigma), 30 μg/mL hygromycin (Duchefa), and/or 100 μg/mL cefotaxime (Duchefa).

The *atmyb44* T-DNA insertion line (SALK_039074) was obtained from the SALK collection. Plant lines homozygous for the T-DNA insertion were selected by PCR following a standard procedure (Alonso et al., 2003). The position of the T-DNA insert was confirmed by nucleotide sequencing. The absence of *AtMYB44* expression in the homozygous plants was confirmed by northern blotting.

Histochemical GUS Assay

To investigate *AtMYB44* gene expression, approximately 3.0 kb of the promoter (−2,976 to −1 from the translation initiation codon) was amplified by PCR from genomic DNA. The PCR product was inserted into the pCAMBIA 1391Z vector at the *Pst*I and *Bam*HI sites upstream from the GUS gene. Twenty-five hygromycin-resistant transgenic (T_1) plants were obtained. Four single-copy insertion lines were identified by Southern blotting (data not shown). Histochemical assays for GUS activity in transgenic plants were performed as described by Jefferson et al. (1987). Tissues were visualized using an Axiophot microscope (Carl Zeiss) coupled to a CCD camera.

Subcellular Localization

For subcellular localization, the cDNA fragment containing the *AtMYB44* coding region without stop codon was amplified from the TAIR EST 119B8 clone by PCR. The PCR product was then inserted downstream from the CaMV 35S promoter and in frame with the 5′ terminus of the GFP gene in the pGWB5 vector (obtained from Dr. Tsuyoshi Nakagawa) using the Gateway (Invitrogen) system, according to the manufacturer's instructions. Thirty-two and 12 kanamycin- and hygromycin-resistant transgenic (T_1) plants integrating *35S:AtMYB44-GFP* and *35S:GFP*, respectively, were obtained. At least five individual T_3 transgenic lines were used for the subcellular localization experiment. Young roots of 2-week-old transgenic plants were examined for GFP fluorescence under a confocal laser-scanning microscope LSM510 (Carl Zeiss).

Stress Tolerance Tests

For the drought tolerance test, plants were initially grown on soil under a normal watering regime for 4 weeks. Watering was then halted and observations were taken after a further 12 d without water. When wild-type plants exhibited lethal effects of dehydration, watering was resumed and the plants were allowed to grow for a subsequent 3 d. For the salt tolerance test, 4-week-old plants were watered for 12 d at 4-d intervals with increasing concentrations of NaCl: 100, 200, and 300 mM.

To measure stomatal closure, stomata were fully opened prior to ABA treatment. Rosette leaves of 5-week-old plants were detached and floated (abaxial side down) on opening solution containing 10 mM MES-KOH (pH 6.15), 30 mM KCl, and 1 mM $CaCl_2$, and incubated under lights for 2 h. Leaves were then treated with ABA for 2 h by adding it to the solution to the required concentration. Stomatal apertures in epidermal peels were observed under an Axiophot (Carl Zeiss) microscope coupled to a CCD camera. The size of the stomatal apertures was measured using a digital ruler. The sizes of at least 50 stomatal apertures were measured for each treatment.

For transpiration (water loss) measurements, aerial parts of the plants were separated from the roots, placed on weighing dishes, and allowed to dry slowly on the laboratory bench (25°C, 60% relative humidity). Weights of the samples were recorded at regular intervals.

Blot Analyses

For genomic Southern blots, 5 μg of genomic DNA were digested with restriction enzymes, separated on 0.8% agarose gels, and transferred to nylon membranes. Northern-blot analysis was performed with total RNA extracted from frozen, ground samples using the phenol-SDS-LiCl method (Carpenter and Simon, 1998). Total RNA (5 μg) was separated on 1.3% agarose formaldehyde gels and transferred to GeneScreen Plus hybridization transfer mem-

branes (Perkin-Elmer). cDNA probes used in Southern and northern blotting were EST clones obtained from TAIR.

For antibody production, an *AtMYB44* cDNA fragment encoding a carboxy-terminal region of the protein (AtMYB44ΔN) was amplified by PCR using the primers CGGATCCTACGACCATCGGGGTTAC (for the N-terminal side of the protein) and GGAATTCCTACTCGATTCTCCCAAC (for the C-terminal side). The PCR product was inserted into the pRSET A expression vector (Invitrogen) at the *Bam*HI and *Eco*RI sites (restriction sites are underlined in the primer sequences) and transformed into *Escherichia coli* strain BL21 (DE3) pLysS. Protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 3 h. His-tagged AtMYB44ΔN protein was purified using nickel nitrilotriacetic acid agarose resin (Invitrogen) according to the manufacturer's instructions. Polyclonal antibodies were raised against the purified His-AtMYB44ΔN protein in rabbits (LabFrontier) and used in western-blot analysis.

Microarray Analysis

Two independent biological replicates of microarray experiments were performed using 5-week-old wild-type, *35S:AtMYB44*, and *atmyb44* knockout plants treated with or without 250 mM NaCl. Twenty-four hours after treatment, total RNA was isolated from the rosette leaves using Concert plant RNA purification reagent (Invitrogen) and purified using the RNeasy mini kit (Qiagen). Using 10 μg of total RNA, double-stranded cDNA was synthesized with a T7 promoter-containing oligo(dT) primer using a GeneChip one-cycle cDNA synthesis kit (Affymetrix), followed by in vitro transcription using a GeneChip IVT labeling kit (Affymetrix). Resulting cRNA was fragmented for hybridization to Affymetrix ATH1 genome arrays using an Affymetrix Fluidics Station 450 according to the manufacturer's instructions (GeneChip Expression Analysis Technical Manual). The microarray was scanned using an Agilent GeneArray Scanner (Affymetrix). Scanned images were processed and analyzed using Microarray Suite 5.0 software (Affymetrix), as described previously (Leonhardt et al., 2004). Genes exhibiting more than 2-fold enhanced or reduced transcription level in both experiments were considered to show significant alterations in expression.

Supplemental Data

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

Supplemental Table S1. Genes showing more than 2-fold enhanced or reduced transcript level in *35S:AtMYB44* Arabidopsis.

Supplemental Table S2. Genes showing more than 2-fold enhanced or reduced transcript level in *atmyb44* knockout Arabidopsis.

Supplemental Table S3. Genes showing more than 2-fold enhanced or reduced transcript level in *35S:AtMYB44* Arabidopsis treated with 250 mM NaCl.

Supplemental Table S4. Genes showing more than 2-fold enhanced or reduced transcript level in *atmyb44* knockout Arabidopsis treated with 250 mM NaCl.

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