Running head: Arabidopsis AtMYB44 Transcription Factor

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Overexpression of *AtMYB44* enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis

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AtMYB44 belongs to the R2R3 MYB subgroup 22 transcription factor family in 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. The 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 the salt-induced activation of the genes that encode a group of Ser/Thr protein phosphatase 2Cs (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, the enhanced abiotic stress tolerance of transgenic Arabidopsis overexpressing AtMYB44 was conferred by the 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 1600 transcription factors, representing approximately 6% of the total number of genes, have been identified in the Arabidopsis genome (The 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 50–53-amino acid imperfect repeats that form the helix–turn–helix motifs R1, R2, and R3 (Rosinsky et al., 1998). MYB proteins in animals generally contain the 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; Stracke et al., 2001) 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).

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 ABA-inducible expression of RD22 (responsive to dehydration 22; Urao et al., 1993; Abe et al., 2003). AtMYB102 is a regulatory component that integrates the dehydration, osmotic, or salinity stress, ABA application, and wound signaling pathways (Denekamp and
Smeekens, 2003). In addition, the Arabidopsis mutant *hos10-1* (confering high expression of osmotically responsive genes) exhibits altered expression of ABA-responsive genes, showing a 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 the 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). The 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 sensitive 2*) mutants (Kamei et al., 2005). In addition, *AtMYB44* and *AtMYB77* expression is reduced in *fus3*, *lec1*, and *abi3* 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 base pairs, encoding a putative 305-amino acid polypeptide with a predicted molecular weight of 33.3 kDa. 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 abscisic acid (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 abscisic acid (ABA), 100 µM methyl jasmonate (MeJA), or 50 µM ethylene to Arabidopsis rosette leaves (Fig. 1A). AtMYB44 transcript levels also increased when Arabidopsis were exposed to dehydration, high salt levels, or cold (Fig. 1B). The increase in AtMYB44 transcript 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 β-glucuronidase (GUS) reporter gene driven by the AtMYB44 promoter (~3.0 kb), GUS activity was observed in all tissues examined in the 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 MS 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 green fluorescent protein (GFP) marker gene and expressed in transgenic Arabidopsis under the control of the CaMV35S 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 T3 or T4 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 (~33 kDa) accumulation in the transgenic plants and the absence of the protein in the atmyb44 knockout plants (SALK_039074) (Fig. 3C).

The 35S:AtMYB44 plants germinated uniformly, as measured 1 week after growing.
on MS medium (Fig. 4A). In early stage of vegetative growth, however, the rosette leaves of 35S:AtMYB44 plants were smaller, but became longer and wider than those of wild-type plants after flowering (Fig. 4B). The 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 days after sowing (DAS), whereas the AtMYB44-transgenic plants took 36 to 37 DAS to reach the same stage (Fig. 4D). At this time point, all of the 35S:AtMYB44 plants had 16 to 18 leaves, whereas the wild-type and atmyb44 knockout plants had 13 leaves on average per rosette. Thus, the delay of flowering was not merely caused by slower overall growth rate, but reflected developmental retardation in the flowering process.

When flowering, i.e., 6 weeks after sowing, the heights of transgenic plants were comparable to that of wild-type plants. The adult 35:AtMYB44 plants had much shorter petioles and smaller seeds than wild-type plants. The 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 the germination of 35S:AtMYB44 plants more severely than that of wild-type plants, indicating ABA-hypersensitivity of the 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 the wild-type plants in the ABA-germination experiment.

The 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 (numbers 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 non-treated plants. In 35S:AtMYB44 plants, the same treatment reduced stomatal apertures to 60–70% of those of non-treated plants. Therefore, transgenic plants overexpressing AtMYB44 exhibited more rapid ABA-induced stomatal closure than did wild-type plants. The stomatal apertures of atmyb44 knockout plants were slightly larger (~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 re-watering after 12 days 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, the 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 9 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.
Expression of salt-induced genes in transgenic plant

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 (Affymetrix, Santa Clara, CA). The microarray experiments using the synthetic oligonucleotide chip demonstrated a high degree of reproducibility between the two sets of independent experiments. The 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 the overall expression patterns (Supplemental Tables S1 and S2). Based on the twofold 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 twofold in 35S:AtMYB44 transgenic plants, while 496 genes (2.2% of the total) had transcription levels reduced by more than twofold (Supplemental Tables S3). In atmyb44 knockout plants, with the salt treatment, 102 genes (0.5% of the total) had transcription levels enhanced by more than twofold, while 38 genes (0.2% of the total) had twofold lower levels, compared to wild-type plants (Supplemental Tables S4).

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

The microarray analysis revealed that the 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/CFB and AREB, which bind to the dehydration-responsive element (DRE/CRT) and ABA-responsive element (ABRE), respectively, were not enriched or rather reduced in some cases.

Instead, the salt-induced activation of the genes encoding Ser/Thr protein phosphatase 2Cs (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 Tables 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 the expression of these genes is related to abiotic stresses (Narusaka et al., 2004).

The result from microarray experiments on the 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 abscisic acid (ABA), methyl jasmonate, or ethylene was applied to *Arabidopsis* rosette leaves (Fig. 1A). The 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 \textit{AtMYB44} transcripts 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, \textit{AtMYB44} was identified as a jasmonate-inducible gene (Jung et al., 2007). Signaling mechanism leading to the multi-hormonal activation of \textit{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 \textit{AtMYB44} promoter. The ABI3 and FUS3 transcription factors are associated with ABA action (Nambara et al., 2000; Gazzarrini et al., 2004). This explains the lower levels of \textit{AtMYB44} transcripts that were observed in the \textit{fus3} (\textit{fusca 3}), \textit{lec1} (\textit{leafy cotyledon 1}), and \textit{abi3} (\textit{ABA insensitive 3}) 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 \textit{AtMYB44} (35S:AtMYB44) was hypersensitive to ABA during seed germination, was dwarfed in the early stages of growth, and was delayed in flowering (Fig. 4). Similar phenotypes have been observed in Arabidopsis lines that overexpress well-known ABA-dependent, drought-response genes such as \textit{DREB1A/CBF3} (Kasuga et al. 1999; Gilmour et al. 2000), \textit{DREB2A} (Sakuma et al. 2006), \textit{ABF3} (Kang et al. 2002), and \textit{ABF4} (Kang et al. 2002). This suggests that \textit{AtMYB44} plays a role in ABA-mediated responses to abiotic stresses such as drought, high salinity, and low temperature.

In the \textit{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 \textit{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, the
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, Palo Alto, CA), which was used to carry the genes, including \textit{AtMYB44}, into the transgenic Arabidopsis, did not affect the size of the stomatal apertures.

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

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

Huang et al. (2007) used \textit{AtMYB44}-overexpressing plants and a knockout mutant to show that \textit{AtMYB44} functions as a negative regulator of (+)-ABA signal transduction. These results contradict ours that 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 \textit{atmyb44} knockout mutant had reduced germination compared to the wild-type plants under the same conditions. In our experiments, by contrast, \textit{AtMYB44}-overexpressing plants were hypersensitive to ABA treatment, whereas the ABA sensitivity of the knockout mutant was comparable to that
of the 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 Tables S3). This could be primary or secondary effects of AtMYB44 overproduction, and explain the cause and consequence of the enhanced salt stress tolerance of *35S:AtMYB44* plants. The genes showing much higher increased expression levels in the *35S:AtMYB44* plants on salt treatment included those involved in water transport, the 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 upregulated in *35S:AtMYB44* plants, suggesting a salt stress response and auxin signaling crosstalk at the level of transcriptional regulation. Several arabinogalactan proteins (AGPs) were upregulated in the salt-treated *35S:AtMY44* plants, supporting the 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). The altered expression of several types of gene encoding chlorophyll biosynthetic enzymes, chlorophyll binding proteins, thylakoid proteins, and other chloroplast-related proteins might be correlated with the salt-induced chlorophyll disorganization and degradation (chlorosis) (Hernandez et al., 1999). Enhanced expression of the 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, post-germination growth, and stomatal movement (Shen et al., 2006; Nott et al., 2006).
Microarray analysis (Table I) and Northern blots (Fig. 7) revealed that the expression of major abiotic stress-responsive genes, including \textit{RD29A}, \textit{RD22}, and \textit{RAB18} was not reinforced in \textit{35S:AtMYB44} plants under salt stress. This suggests that the drought/salt stress tolerance exhibited by \textit{AtMYB44} transgenic plants was not conferred by the proteins that are encoded by these genes. Instead, the expression of genes that encode a group of Ser/Thr protein phosphatase 2Cs (PP2Cs) such as ABI1, ABI2, AtPP2CA, HAB1, and HAB2 was suppressed in \textit{35S:AtMYB44} plants and enhanced in \textit{atmyb44} knockout plants. These proteins belong to the ‘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 \textit{abi1} and \textit{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). The overexpression of \textit{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).

The enhanced salt stress tolerance of \textit{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 (Salt Overly Sensitive 2; Ohta et al., 2003), which is a Ser/Thr protein kinase required for salt tolerance (Liu et al., 2000). The \textit{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 the 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 \textit{sos2} mutant, the expression of \textit{AtMYB44} was significantly up-regulated under salt stress, whereas the transcript levels of \textit{RD29A}, \textit{COR47}, \textit{COR15A}, \textit{KIN1}, and \textit{RD22} were similar to those in wild-type plants (Kamei et al., 2005). Half of the ~60 genes that showed increased salt-induced expression in the \textit{sos2} mutant also showed enhanced transcription in salt-
treated 35S:AtMYB44 plants.

As described, the expression of AtMYB44 was suppressed in the abi3 mutant (Kirik et al., 1998). Upon overexpression of maize 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 ABI3-mediated ABA signaling pathway through the 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 the 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, the 35S:AtMYB44 plants did not show significant alteration in the overall expression patterns (Supplemental Tables S1 and S2). Therefore, the 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 role(s) of the AtMYB44 transcription factor.
MATERIALS AND METHODS

Plant Materials and Treatments

The *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 the TAIR seeds. Plants were grown on soil or half-strength Murashige-Skoog (MS) agar medium (Duchefa, The Netherlands) in a growth chamber maintained at 22–24°C and 60% relative humidity under long-day conditions (16 h light/8 h dark cycle).

For the chemical treatment, solution of 100 µM (±)-ABA (Sigma product no. A-1049, St. Louis, MO) was applied to the surface of a solid MS agar medium in which 2-week-old seedlings were growing. The 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 half-strength MS agar medium containing 1% sucrose and the different concentrations of ABA. To break dormancy, seeds were incubated at 4°C for 4 days in the dark before germination and were subsequently grown in a growth chamber as described above. Seed germination was followed for 7 days. 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 polymerase chain reaction (PCR). The cDNA fragment was inserted into the pBI121 vector (Clontech, Palo Alto, CA) from which the GUS gene had been removed at the XbaI and BamHI sites, fusing the fragment downstream from the cauliflower mosaic virus 35S (CaMV35S) 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 MS 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 (–2976 to –1 from the translation initiation codon) was amplified by PCR from genomic DNA. The PCR product was inserted into the pCAMBIA 1391Z vector (Cambia, Australia) at the PstI and BamHI sites upstream from the β-glucuronidase (GUS) gene. Twenty-five hygromycin-resistant transgenic (T1) 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, Germany) 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 CaMV35S promoter and in frame with the 5' terminus of the green fluorescent protein (GFP) gene in the pGWB5 vector (obtained from Dr. Tsuyoshi Nakagawa, Shimane University, Japan) using the Gateway (Invitrogen) system, according to the manufacturer’s instructions. Thirty-two and twelve kanamycin- and hygromycin-resistant transgenic (T1) plants integrating 35S:AtMYB44-GFP and 35S:GFP, respectively, were obtained. At least five individual T3 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, Germany).

**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 days 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 days. For the salt tolerance test, 4-week-old plants were watered for 12 days at 4-day intervals with increasing concentrations of NaCl: 100 mM, 200 mM, 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 CaCl₂, and incubated under lights for 2 h. The 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, Germany) 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 the transpiration (water loss) measurements, aerial part of the plants were
Blot Analyses

For genomic Southern blots, 5 µg of genomic DNA was 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 membranes (PerkinElmer, Boston, MA). The 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, CGGATCCTACGACCATTGGTGTTAC (for the N-terminal side of the protein) and GGAATTCCATCGATTCTCCTCAAC (for the C-terminal side). The PCR product was inserted into the pRSET A expression vector (Invitrogen) at the BamHI and EcoRI sites (restriction sites are underlined in the primer sequences) and transformed into the 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. The His-tagged AtMYB44ΔN protein was purified using Ni²⁺-NTA resin (Invitrogen) according to the manufacturer’s instructions. Polyclonal antibodies were raised against the purified His-AtMYB44ΔN protein in rabbits (LabFrontier, Korea) 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, Carlsbad, CA, USA) and purified using the RNeasy mini kit (QIAGEN, Valencia, CA,
USA). Using 10 \mu g of total RNA, double-stranded cDNA was synthesized with a T7 promoter-containing oligo(dT) primer using GeneChip® One Cycle cDNA Synthesis Kit (Affymetrix), followed by in vitro transcription using GeneChip® IVT Labeling Kit (Affymetrix). Resulting cRNA was fragmented for hybridization to Affymetrix ATH1 genome arrays (Affymetrix, Santa Clara, CA) 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). The scanned images were processed and analyzed using Microarray Suite 5.0 (MAS) software (Affymetrix), as described previously (Leonhardt et al., 2004). Genes exhibiting a more than twofold 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 twofold enhanced or reduced transcript level in 35S:AtMYB44 Arabidopsis.

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

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

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

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LITERATURE CITED

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15: 63-78

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Kanes M, Mulholland C, Nubakuro R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeu E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653-657

Carpenter CD, Simon AE (1998) Preparation of RNA. Methods Mol Biol 82: 85-89

Cheong YH, Chang HS, Gupta R, Wang X, Zhu T, Luan S (2002) Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol 129: 661-677

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-743

Cominelli E, Galbiati M, Vavasseur A, Conti L, Sala T, Vuylsteke M, Leonhardt N, Dellaporta SL, Tonelli C (2005) A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. Curr Biol 15: 1196-1200

Daniels MJ, Chaumont F, Mirkov TE, Chrispeels MJ (1996) Characterization of a new vacuolar membrane aquaporin sensitive to mercury at a unique site. Plant Cell 8: 589-599

Denekamp M, Smeekens SC (2003) Integration of wounding and osmotic stress signals determines the expression of the AtMYB102 transcription factor gene. Plant Physiol 132: 1415-1423

Fowler S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14: 1675-1690
Gazzarrini S, Tsuchiya Y, Lumba S, Okamoto M, McCourt P (2004) The transcription factor FUSCA3 controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid. Dev Cell 7: 373-385

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol 124: 1854-1865

Gong W, Shen YP, Ma LG, Pan Y, Du YL, Wang DH, Yang JY, Hu LD, Liu XF, Dong CX, Ma L, Chen YH, Yang XY, Gao Y, Zhu D, Tan X, Mu JY, Zhang DB, Liu YL, Dinesh-Kumar SP, Li Y, Wang XP, Gu HY, Qu LJ, Bai SN, Lu YT, Li JY, Zhao JD, Zuo J, Huang H, Deng XW, Zhu YX (2004) Genome-wide ORFeome cloning and analysis of Arabidopsis transcription factor genes. Plant Physiol 135: 773-782

Gosti F, Beaudoin N, Serizet C, Webb AA, Vartanian N, Giraudat J (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid. Plant Cell 11: 1897-1910

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL (2000) Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. Proc Natl Acad Sci USA 97: 13579-13584

Halfter U, Ishitani M, Zhu J-K (2000) The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc Natl Acad Sci USA 97: 3735-3740

Hashimoto M, Negi J, Young J, Israelsson M, Schroeder JJ, Iba K (2006) Arabidopsis HT1 kinase controls stomatal movements in response to CO2. Nature Cell Biol 8: 391-397

Hernandez JA, Campillo A, Jimenez A, Alacon JJ, Sevilla F (1999). Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. New Phytol 141: 241–251

Hoth S, Morgante M, Sanchez JP, Hanafey MK, Tingey SV, Chua NH (2002) Genome-wide gene expression profiling in Arabidopsis thaliana reveals new targets of abscisic acid and largely impaired gene regulation in the abi1-1 mutant. J Cell Sci 115: 4891-4900

Huang D, Jaradat MR, Wu W, Ambrose SJ, Ross AR, Abrams SR, Cutler AJ
(2007) Structural analogs of ABA reveal novel features of ABA perception and signaling in Arabidopsis. Plant J 50: 414-428

Huang M-D, Wu W-L (2007) Overexpression of TMAC2, a novel negative regulator of abscisic acid and salinity responses, has pleiotropic effects in Arabidopsis thaliana. Plant Mol Biol 63: 557-569

Jang JY, Kim DG, Kim YO, Kim JS, Kang H (2004) An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic responses in Arabidopsis thaliana. Plant Mol Biol 54: 713-725

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907

Jin H, Martin C (1999) Multifunctionality and diversity within the plant MYB-gene family. Plant Mol Biol 41: 577-585

Jung C, Lyou SH, Yeu SY, Kim MA, Rhee S, Kim M, Lee JS, Choi YD, Cheong J-J (2007) Microarray-based screening of jasmonate-responsive genes in Arabidopsis thaliana. Plant Cell Rep 26: 1053-1063

Kamei A, Seki M, Umezawa T, Ishida J, Satou M, Akiyama K, Zhu J-K, Shinozaki K (2005) Analysis of gene expression profiles in Arabidopsis salt overly sensitive mutants sos2-1 and sos3-1. Plant Cell Environ 28: 1267-1275

Kang J-Y, Choi H-I, Im M-Y, Kim SY (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell 14: 343-357

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnol 17: 287-291

Kirik V, Kölle K, Miséra S, Bäumlein H (1998) Two novel MYB homologues with changed expression in late embryogenesis-defective Arabidopsis mutants. Plant Mol Biol 37: 819-827

Kirik V, Lee MM, Wester K, Herrmann U, Zheng Z, Oppenheimer D, Schiefelbein J, Hulskamp M (2005) Functional diversification of MYB23 and GL1 genes in trichome morphogenesis and initiation. Development 132: 1477-1485

Kranz HD, Denekamp M, Greco R, Jin H, Leyva A, Meissner RC, Petroni K, Urzainqui A, Bevan M, Martin C, Smeekens S, Tonelli C, Paz-Ares J, Weisshaar
B (1998) Towards functional characterization of the members of the R2R3-MYB gene family from Arabidopsis thaliana. Plant J 16: 263-276

Kuhn JM, Boisson-Dernier A, Dizon MB, Maktabi MH, Schroeder JI (2006) The protein phosphatase AtPP2CA negatively regulates abscisic acid signal transduction in Arabidopsis, and effects of abh1 on AtPP2CA mRNA. Plant Physiol 140: 127–139

Lai LB, Nadeau JA, Lucas J, Lee EK, Nakagawa T, Zhao L, Geisler M, Sack FD (2005) The Arabidopsis R2R3 MYB proteins FOUR LIPS and MYB88 restrict divisions late in the stomatal cell lineage. Plant Cell 17: 2754-2767

Lamport DTA, Kieliszewski MJ, Showalter AM (2006) Salt stress upregulates periplasmic arabinogalactan proteins: using salt stress to analyse AGP function. New Phytol 169: 479-492

Lee MM, Schiefelbein J (2001) Developmentally distinct MYB genes encode functionally equivalent proteins in Arabidopsis. Development 128: 1539-46

Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JI (2004) Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell 16: 596-615

Leung J, Merlot S, Giraudat J (1997) The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatase 2C involved in abscisic acid signal transduction. Plant Cell 9: 759-771

Liang Y-K, Dubos C, Dodd IC, Holroyd GH, Hetherington AM, Campbell MM (2005) AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in Arabidopsis thaliana. Curr Biol 15: 1201-1206

Lin B-L, Wang H-J, Wang J-S, Zaharia I, Abrams SR (2005) Abscisic acid regulation of heterophyllly in Marsilea quadrifolia L.: effects of R-(-) and S-(+) isomers. J Exp Bot 56: 2935-2948

Lipstick JS (1996) One billion years of Myb. Oncogene 13: 223-235

Liu J, Ishitani M, Halfter U, Kim C-S, Zhu J-K (2000) The Arabidopsis thaliana SO2 gene encodes a protein kinase that is required for salt tolerance. Proc Natl Acad Sci USA 97: 3730-3734

Ma L, Sun N, Liu X, Jiao Y, Zhao H, Deng XW (2005) Organ-specific expression of Arabidopsis genome during development. Plant Physiol 138: 80-91

Majewska-Sawka A, Nothnagel NA (2000) The multiple roles of arabinogalactan
proteins in plant development. Plant Physiol **122**: 3-10

**Mandaokar A, Thines B, Shin B, Markus Lange B, Choi G, Koo YJ, Yoo YJ, Choi YD, Choi G, Browse J** (2006) Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. Plant J **46**: 984-1008

**Martin C, Paz-Ares J** (1997) MYB transcription factors in plants. Trends Genet **13**: 67-73

**Meissner RC, Jin H, Cominelli E, Denekamp M, Fuertes A, Greco R, Kranz HD, Penfield S, Petroni K, Urzainqui A, Martin C, Paz-Ares J, Smeekens S, Tonelli C, Weisshaar B, Baumann E, Klimyuk V, Marillonnet S, Patel K, Speulman E, Tissier AF, Bouchez D, Jones JJD, Pereira A, Wisman E, Bevan M** (1999) Function serach in a large transcription factor gene family in Arabidopsis: Assessing the potential of reverse genetics to identify insertional mutations in R2R3 MYB genes. Plant Cell **11**: 1827-1840

**Merlot S, Gosti F, Guretto D, Vavasseur A, Giraudat J** (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid pathway. Plant J **25**: 295–303

**Mönke G, Altschmied L, Tewes A, Reidt W, Mock H-P, Bäumlein H, Conrad U** (2004) Seed-specific transcription factors ABI3 and FUS3: molecular interaction with DNA. Planta **219**: 158-166

**Nambara E, Hayama R, Tsuchiya Y, Nishimura M, Kawaide H, Kamiya Y, Naito S** (2000) The role of ABI3 and FUS3 loci in Arabidopsis thaliana on phase transition from late embryo development to germination. Dev Biol **220**: 412-423

**Narusaka Y, Narusaka M, Seki M, Umezawa T, Ishida J, Nakajima M, Enju A, Shinozaki K** (2004) Crosstalk in the responses to abiotic and biotic stresses in Arabidopsis: Analysis of gene expression in cytochrome P450 gene superfamily by cDNA microarray. Plant Mol Biol **55**: 327-342

**Nott A, Jung HS, Koussevitzky S, Chory J** (2006) Plastid-to-nucleus retrograde signaling. Annu Rev Plant Biol **57**: 730-759

**Ohta M, Guo Y, Halfter U, Zhu J-K** (2003) A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. Proc Natl Acad Sci USA **100**: 11771-11776

**Peiter E, Maathuis FJM, Mills LN, Knight H, Pelloux J, Hetherington AM,**
Sanders (2005) The vacuolar Ca\(^{2+}\)-activated channel TPC1 regulates germination and stomatal movement. Nature 434: 404-408

Quattrocchio F, Verweij W, Kroon A, Spelt C, Mol J, Koes R (2006) PH4 of petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. Plant Cell 18: 1274-1291

Qui Q-S, Guo Y, Dietrich MA, Schumaker KS, Zhu J-K (2002) Regulation of SOS1, a plasma membrane Na\(^{+}\)/H\(^{+}\) exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proc Natl Acad Sci USA 99: 8436-8441

Quintero FJ, Ohta M, Shi H, Zhu J-K, Pardo JM (2002) reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na\(^{+}\) homeostasis. Proc Natl Acad Sci USA 99: 9061-9066

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C-Z, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G-L (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science 290: 2105-2110

Roelfsema MRG, Hedrich R (2005) In the light of stomatal opening: new insights into 'the Watergate'. New Phytol 167: 665-691

Romero I, Fuertes A, Benito MJ, Malpica JM, Leyva A, Paz-Ares J (1998) More than 80 R2R3-MYB regulatory genes in the genome of Arabidopsis thaliana. Plant J 14: 273-284

Rosinsky JA, Atchley WR (1998) Molecular evolution of the Myb family of transcription factors: evidence for polyphyletic origin. J Mol Evol 46: 74-83

Sáez A, Apostolova N, González-Guzmán M, González-García MP, Nicolás C, Lorenzo O, Rodríguez PL (2004) Gain of function and loss of function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid. Plant J 37: 354–369

Saez A, Robert N, Maktabi MH, Schroeder JI, Serrano R, Rodriguez PL (2006) Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatase type 2C ABI1 and HAB1. Plant Physiol 141: 1389-1399

Sakuma K, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-
Shinozaki K (2006) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. Plant Cell 18: 1292-1309

Schwechheimer C, Zourelidou M, Bevan MW (1998) Plant transcription factor studies. Annu Rev Plant Physiol Plant Mol Biol 49: 127-150

Schweighofer A, Hirt H, Meskiene I (2004) Plant PP2C phosphatases: emerging functions in stress signaling. Trends in Plant Science 9: 236-243.

Shen YY, Wang XF, Wu FQ, Du SY, Cao Z, Shang Y, Wang XL, Peng CC, Yu XC, Zhu SY, Fan RC, Xu YH, Zhang DP (2006) The Mg-chelatase H subunit is an abscisic acid receptor. Nature 443: 823-826

Shi H, Ishitani M, Kim C, Zhu J-K (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na⁺/H⁺ antiporter. Proc Natl Acad Sci USA 97: 6896-6901

Singh K, Foley RC, Oñate-Sánchez L (2002) Transcription factors in plant defense and stress responses. Curr Opin Plant Biol 5: 430-436

Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol 4: 447-456

Suzuki M, Ketterling MG, Li Q-B, McCarty R (2003) *Viviparous1* alters global gene expression patterns through regulation of abscisic acid signaling. Plant Physiol 132: 1664-1677

Tahtiharju S, Palva T (2001) Antisense inhibition of protein phosphatase 2C accelerates cold acclimation in *Arabidopsis thaliana*. Plant J 26: 461–470

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature. 408: 796-815.

To A, Valon C, Savino G, Guilleminet J, Devic M, Giraudat J, Parcy F (2006) A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. Plant Cell 18: 1642-1651

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An *Arabidopsis myb* homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5: 1529–1539

Van Hengel AJ, Roberts K (2003) AtAGP30, an arabinogalactan-protein in the cell walls of the primary root, plays a role in root regeneration and seed germination. Plant J 36: 256-270
Xie X, Wang Y, Williamson L, Holroyd GH, Tagliavia C, Murchie E, Theobald J, Knight MR, Davies WJ, Leyser HMO, Hetherington AM (2006) The identification of genes involved in the stomatal response to reduced atmospheric relative humidity. Curr Biol 16: 882-887

Xiong L, Schumaker KS, Zhu J-K (2002) Cell signaling during cold, drought, and salt stress. Plant Cell Supplement: S165-S183

Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L, Zhaofeng G, Zhiqiang L, Yunfei Z, Xiaoxiao W, Xiaoming Q, Yunping S, Li Z, Xiaohui D, Jingchu L, Xing-Wang D, Zhangliang C, Hongya G, Li-Jia Q (2006) The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol 60: 107-124

Yoshida T, Nishimura N, Kitahata N, Kuromori T, Ito T, Asami T, Shinozaki K, Hirayama T (2006) ABA-Hypersensitive Germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs. Plant Physiol 140: 115-126

Zhu J, Verslues PE, Zheng X, Lee BH, Zhan X, Manabe Y, Sokolchik I, Zhu Y, Dong C-H, Zhu JK, Hasegawa PM, Bressan RA (2005) HOS10 encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants. Proc Natl Acad Sci USA 102: 9966-9971

Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF (2004) Comprehensive identification of Arabidopsis thaliana MYB transcription factors interacting with R/B-like BHLH proteins. Plant J 40: 22-34
| Probe ID | AGI No. | Description | No treatment | Salt treatment |
|---------|---------|-------------|--------------|---------------|
| 247025_at | At3g5030 | Zeaxanthin epoxidase (ABA1) | NC | NC |
| 259699_at | At1g52340 | Short chain alcohol dehydrogenase (ABA2) | NC | NC |
| 246325_at | At1g56540 | Aldehyde oxidase (ABA3) | NC | NC |
| 254098_at | At2g24650 | ABA insensitive protein 3 (AB13) | NC | NC |
| 263377_at | At2g0220 | AP2 domain transcription factor (AB14) | NC | NC |
| 263907_at | At2g2920 | ABA insensitive 5 (AB15) | NC | NC |
| 246814_at | At3g50850 | ABRE binding factor (ABRE3, DPBF3) | NC | NC |
| 253263_at | At4g4000 | ABRE binding factor 3 (ABF3) | NC | NC |
| 254026_at | At1g2920 | ABRE binding factor 4 (ABF3, AREB2) | NC | NC |
| 248487_at | At5g3107 | CIP protease ATP binding subunit (ERD1) | -1.7 | -1.7 |
| 258258_at | At3g2679 | B3 DNA binding factor (FUS3) | NC | NC |
| 260854_at | At1g2970 | CCAAT box binding factor HAP3 homolog (LEC1) | NC | NC |
| 248511_at | At3g5960 | Cold and ABA inducible protein (KIN1) | -1.3 | -1.3 |
| 263947_at | At2g2450 | Cold regulated protein (COR15a) | -1.7 | -1.7 |
| 264995_at | At2g4250 | Cold regulated protein (COR15b) | -4.0 | -4.0 |
| 245303_at | At1g7412 | Cysteine protease (RD2A1) | NC | NC |
| 246908_at | At5g5610 | Dehydration induced protein (RD22) | NC | NC |
| 259570_at | At2g0440 | Deyshlin (COR47) | NC | NC |
| 248352_at | At5g52300 | Low temperature induced protein 65 (RD29B) | NC | NC |
| 248357_at | At5g52310 | Low temperature induced protein 78 (RD29A) | NC | NC |
| 247095_at | At5g6400 | Dehydrin (RAB18) | -1.8 | -1.8 |
| 251775_at | At3g5610 | Delta-1-pyroline-5-carboxylate synthase (P5CS) | NC | NC |
| 254966_at | At2g5480 | DRE/CRT binding factor 1A (DREB1A, CBF3) | -1.7 | -1.7 |
| 254974_at | At2g5490 | DRE/CRT binding factor 1B (DREB1B, CBF1) | NC | NC |
| 254975_at | At2g4270 | DRE/CRT binding factor 1C (DREB1C, CBF2) | NC | NC |
| 248369_at | At5g5990 | DRE/CRT binding factor 1D (DREB1D, CBF4) | NC | NC |
| 250781_at | At5g0410 | DRE/CRT binding factor 2A (DREB2A) | -2.1 | -2.1 |
| 254630_at | At3g1020 | DRE/CRT binding factor 2B (DREB2B) | NC | NC |
| 259381_at | At1g69600 | Zinc finger homodomain (ZFP1) | NC | NC |
| 261713_at | At1g3260 | bHLH transcription factor (AMY2C, JIN1) | NC | NC |
| 258310_at | At2g6744 | bHLH transcription factor (ICE1) | NC | NC |
| 260971_at | At2g7910 | R2R3 MYB transcription factor (AMY1B2) | NC | NC |
| 264782_at | At1g8810 | R2R3 MYB transcription factor (MYB60) | NC | NC |
| 264556_at | At1g8540 | R2R3 MYB transcription factor (MYB61) | -1.6 | -1.6 |
| 261648_at | At1g7730 | Salt-tolerance zinc finger protein (STZ, ZAT10) | -2.8 | -2.8 |
| 245120_at | At2g3918 | E3 ubiquitin ligase (HOS1) | NC | NC |
| 247113_at | At5g3980 | 3(2),5-bisphosphate nucleotidase (HOS2, FRY1) | 1.1 | 1.1 |
| 249405_at | At5g4028 | Beta subunit of protein farnesyl transferase (ERFA1) | NC | NC |
| 262030_at | At3g2890 | NAM like protein (ANAC019) | -3.0 | -3.0 |
| 258995_at | At1g35500 | NAM like protein (ANAC055) | -2.1 | -2.1 |
| 253872_at | At2g7410 | NAM like protein (ANAC072) | -3.2 | -3.2 |
| 253405_at | At3g2200 | Potassium channel protein (AKT2/3) | NC | NC |
| 248088_at | At5g6240 | Potassium channel protein (KAT1) | NC | NC |
| 256710_at | At2g8650 | Protein transporter (APT) | NC | NC |
| 253264_at | At4g3590 | ABA activated protein kinase (OST1) | NC | NC |
| 256633_at | At1g2400 | Protein kinase (HII) | NC | NC |
| 265252_at | At2g3980 | NTHF antipporter (SOS1) | NC | NC |
| 248614_at | At5g4540 | Serine/threonine protein kinase (SOS2) | NC | NC |
| 249783_at | At2g2470 | Calcineurin B like protein 4 (CBIA, SOS3) | NC | NC |
| 255731_at | At1g2940 | Protein phosphatase 2A, subunit A (RCP1) | NC | NC |

Table I. Comparison of the transcription levels of selected genes in 35:AtMYB44 plants treated with 250 mM NaCl.
| Probe Set ID  | Gene Name                        | Description          | NC  | Fold Change 1 | P-value 1   | Fold Change 2 | P-value 2   | Fold Change 3 | P-value 3   | Fold Change 4 | P-value 4   |
|--------------|----------------------------------|----------------------|-----|--------------|-------------|--------------|-------------|--------------|-------------|--------------|-------------|
| 253994_at    | At4g26080 Protein phosphatase 2C (ABI1) | NC                   | 0.88199 | -2.0         | 0.99998     | -1.4         | 0.99931     | -3.2         | 0.99998     |              |             |
| 247957_at    | At5g57050 Protein phosphatase 2C (ABI2) | NC                   | 0.66941 | -1.6         | 0.99938     | -2.0         | 0.99981     | -6.9         | 0.99973     |              |             |
| 259231_at    | At3g14410 Protein phosphatase 2C (AtPP2CA) | -2.0                 | 0.99998 | -1.6         | 0.99998     | -1.2         | 0.99979     | -6.9         | 0.99998     |              |             |
| 259922_at    | At1g72770 Protein phosphatase 2C (HAB1) | NC                   | 0.96903 | -1.6         | 0.99997     | -2.2         | 0.99998     | -7.4         | 0.99998     |              |             |
| 260712_at    | At1g17550 Protein phosphatase 2C (HAB2) | NC                   | 0.58000 | -1.4         | 0.99593     | -1.5         | 0.99973     | -1.8         | 0.99797     |              |             |
| 247723_at    | At5g59220 Protein phosphatase 2C | -2.1                 | 0.99956 | -4.0         | 0.99998     | -3.7         | 0.99998     | -19.6        | 0.99998     |              |             |
| 261077_at    | At1g07430 Protein phosphatase 2C | -1.8                 | 0.99923 | -4.5         | 0.99998     | -3.0         | 0.99998     | -29.8        | 0.99998     |              |             |
| 266274_at    | At2g29380 Protein phosphatase 2C | NC                   | 0.42914 | NC           | 0.60259     | NC           | 0.50000     | NC           | 0.85432     |              |             |
| 248428_at    | At5g51760 Protein phosphatase 2C | NC                   | 0.35444 | NC           | 0.23255     | NC           | 0.99350     | NC           | 0.06422     |              |             |

**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** No change.
(Figure legends)

**Figure 1.** Northern blots of AtMYB44 expression. A, Induction of AtMYB44 by abscisic acid. Sterilized water (non-treatment; NT), 100 µM methyl jasmonate (MJ), 100 µM abscisic acid (ABA), or 50 µM ethephon (ET) was applied to the surface of a solid MS 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).

**Figure 2.** Localization of AtMYB44 expression. A, Histochemical β-glucuronidase (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 MS 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. The scale bar indicates 20 µm. C, Subcellular localization of AtMYB44 protein. The AtMYB44 cDNA was fused to the green fluorescent protein (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, DIC (Differential Interference Contrast; optical microscopic image); 3, merged image (GFP+DIC); 4, GFP from 35S:GFP control plant. The scale bars indicate 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
cauliflower mosaic virus 35S (CaMV35S) 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 the copy numbers of the inserted T-DNA. Genomic DNA was digested with XbaI (X) and EcoRI (E), and the blot was hybridized with a NPTII (neomycin phosphotransferase II) probe. B, Northern blot demonstrating the constitutive expression of AtMYB44 in the transgenic plants. C, Western blot showing the AtMYB44 protein levels in the transgenic plants. The asterisk indicates the AtMYB44 protein band (~33 kDa); arrowheads indicate bands of two unknown cross-reacted proteins (~40 and ~29 kDa, respectively).

Figure 4. Growth of transgenic Arabidopsis overexpressing AtMYB44. A, One-week-old seedlings grown on MS medium. The scale bar indicates 1 cm. B, The growth of rosette leaves after growing on soil. The scale bars indicate 1 cm for all the images. C, The appearance of transgenic plants 5 weeks after sowing. D, Flowering time of 35S:AtMYB44 plants. The time (days after sowing; DAS) at which the main inflorescence shoot had elongated to 1 was recorded. In addition, the number of rosette leaves when the plants were flowering was counted. In all cases, 20 plants were counted to calculate the average ± standard deviation.

Figure 5. Responses of 35S:AtMYB44 and atmyb44 knockout plants to ABA. A, Germination rate. Seeds were germinated and grown on MS agar plates with or without ABA for 7 days. 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. The 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.

Figure 6. Abiotic stress tolerance tests of 35S:AtMYB44 plants. A, Transpiration rates. For water-loss measurements, aerial part of 5-week-old plants were separated from the
roots, placed on weighing dishes, and allowed to dry slowly on the laboratory bench (25°C, 60% relative humidity). The weights of the samples were recorded at regular intervals. B, Drought tolerance test. Watering of 4-week-old plants was stopped for 12 days and then resumed for 3 days. C, Salt tolerance test. Four-week-old plants were watered for 12 days at 4-day 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.

**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. The cDNA probes used were EST clones obtained from TAIR.
Jung et al. Fig. 3
ABA Concentration (x10^-6 M)

Germination (%)

WT
T-17
T-21
atmyb44

ABA Concentration (x10^-6 M)

Stomatal Aperture (x10^-6 m)

WT
T-17
T-18
T-21
atmyb44

ABA Concentration (x10^-6 M)
A. Leaf weight (%) over time for WT, T-18, T-21, and atmyb44.

B. Phenotype of 35S:AtMYB44 plants. Numbers in parentheses indicate the number of plants with the specified phenotype out of the total number of plants.

C. Phenotype of WT, T-17, T-18, T-21, and atmyb44 plants. Numbers in parentheses indicate the number of plants with the specified phenotype out of the total number of plants.

Jung et al. Fig. 6
