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Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) Function as Transcriptional Activators in Abscisic Acid Signaling

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In Arabidopsis, the induction of a dehydration-responsive gene, *rd22*, is mediated by abscisic acid (ABA). We reported previously that MYC and MYB recognition sites in the *rd22* promoter region function as *cis*-acting elements in the drought- and ABA-induced gene expression of *rd22*. bHLH- and MYB-related transcription factors, rd22BP1 (renamed AtMYC2) and AtMYB2, interact specifically with the MYC and MYB recognition sites, respectively, in vitro and activate the transcription of the *β-glucuronidase* reporter gene driven by the MYC and MYB recognition sites in Arabidopsis leaf protoplasts. Here, we show that transgenic plants overexpressing AtMYC2 and/or AtMYB2 cDNAs have higher sensitivity to ABA. The ABA-induced gene expression of *rd22* and *AtADH1* was enhanced in these transgenic plants. Microarray analysis of the transgenic plants overexpressing both AtMYC2 and AtMYB2 cDNAs revealed that several ABA-inducible genes also are upregulated in the transgenic plants. By contrast, a Ds insertion mutant of the *AtMYC2* gene was less sensitive to ABA and showed significantly decreased ABA-induced gene expression of *rd22* and *AtADH1*. These results indicate that both AtMYC2 and AtMYB2 proteins function as transcriptional activators in ABA-inducible gene expression under drought stress in plants.

INTRODUCTION

Drought and salt stress conditions retard plant growth and decrease crop productivity. Plant responses to these stresses have been analyzed at the molecular and cellular levels as well as at the physiological level. Various genes are induced by these stresses (reviewed by Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Hasegawa et al., 2000). Their gene products are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction of the response (reviewed by Bray, 1997; Hasegawa et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2000). The plant hormone abscisic acid (ABA) mediates a variety of physiological processes, including the response to drought and salt stress. ABA is produced under water deficit conditions, which causes stomata closure and tolerance to drought and salt stress (reviewed by Bray, 1997; Busk and Pages, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000).

Most of the drought-inducible genes studied to date also are induced by ABA. It appears that drought stress triggers the production of ABA, which, in turn, induces various genes. *Cis*- and *trans*-acting factors involved in ABA-induced gene expression have been analyzed (reviewed by Bray, 1997; Busk and Pages, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). Many ABA-inducible genes contain a conserved, ABA-responsive, *cis*-acting element named ABRE (ABA-responsive element; PyACGTGGC) in their promoter regions (Guiltinan et al., 1990; Mundy et al., 1990; Yamaguchi-Shinozaki et al., 1990). Recently, several groups isolated genes for the ABRE binding proteins that interact with ABRE and regulate gene expression (Hobo et al., 1999; Choi et al., 2000; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Uno et al., 2000). These ABRE binding proteins contain a similar DNA binding motif of basic domain/Leu zipper (bZIP) structure and three conserved regions in their N termini. These include rice TRAB and Arabidopsis AREB/ABF and ABI5 proteins. Phosphorylation of the proteins is required for their activation (Uno et al., 2000; Lopez-Molina et al., 2001).

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The *rd22* gene is a dehydration-responsive gene induced by the application of exogenous ABA to Arabidopsis plants (Yamaguchi-Shinozaki and Shinozaki, 1993). Because the induction of the *rd22* gene by ABA is inhibited by the addition of cycloheximide, an inhibitor of protein biosynthesis, the induction of this gene apparently requires de novo protein biosynthesis for its expression under drought stress (Yamaguchi-Shinozaki and Shinozaki, 1993). Although regulation of the expression of many ABA-inducible genes has been postulated to involve the ABRE sequence in their promoter regions, *rd22* does not contain any typical ABRE consensus sequence in its promoter. These results suggest the existence of a novel regulatory system for gene expression in response to ABA other than the ABRE-bZIP regulatory system in vegetative tissues under drought stress.

We have shown that a 67-bp promoter region of *rd22* can regulate drought-inducible gene expression (Iwasaki et al., 1995). There is a MYC and a MYB recognition site within this 67-bp region. We have reported that the MYC and MYB recognition sites function as *cis*-acting elements in the drought-induced expression of the *rd22* gene (Abe et al., 1997). We isolated cDNAs for a basic helix-loop-helix (bHLH)–related protein, rd22BP1 (renamed AtMYC2), and a MYB-related protein, AtMYB2 (Urao et al., 1993; Abe et al., 1997). The AtMYC2 protein and the AtMYB2 protein bound specifically to the MYC recognition site and the MYB recognition site, respectively, in the 67-bp region. Both *AtMYC2* and *AtMYB2* genes are induced by drought and by ABA treatment. A transient transactivation experiment using Arabidopsis leaf protoplasts demonstrated that both AtMYC2 and AtMYB2 activated the transcription of the *β-glucuronidase* reporter gene fused to the 67-bp region of the *rd22* promoter (Abe et al., 1997). Moreover, coexpression of both AtMYC2 and AtMYB2 further transactivated the *β-glucuronidase* fusion gene (Abe et al., 1997). Regulation of gene expression by the cooperation of bHLH and MYB proteins may be another regulatory system in the ABA signaling pathway under drought and salt stress.

In the present study, we analyzed transgenic plants overexpressing AtMYC2 and/or AtMYB2 cDNA. All of these transgenic plants showed some ABA hypersensitivity. The plants overexpressing both AtMYC2 and AtMYB2 cDNAs showed stronger ABA hypersensitivity than those overexpressing either AtMYC2 or AtMYB2 cDNA alone. In these transgenic plants, the ABA-inducible gene expression of *rd22* and *AtADH1* (*alcohol dehydrogenase1*) was increased markedly. In addition, microarray analysis of the transgenic plants overexpressing both AtMYC2 and AtMYB2 cDNAs revealed that several ABA-inducible genes also are upregulated in the transgenic plants. A knockout mutant of *AtMYC2* by Ds transposon was less sensitive to ABA. In this mutant, ABA-inducible expression of *rd22* and *AtADH1* was decreased significantly. These results indicate that both AtMYC2 and AtMYB2 proteins play important roles as transcription factors in ABA-regulated gene expression under drought and salt stress.

RESULTS

Creation of Transgenic Plants Overexpressing the rd22BP1/AtMYC2 and/or AtMYB2 cDNAs

We generated transgenic plants in which the rd22BP1/AtMYC2 or AtMYB2 cDNA was overexpressed (35S:AtMYC2 and 35S:AtMYB2). In each case, we used kanamycin as a selection marker (Figure 1A). The AtMYC2 and AtMYB2 cDNAs were overexpressed under the control of the 35S promoter of *Cauliflower mosaic virus*. The Ω sequence of *Tobacco mosaic virus* was inserted upstream of these cDNAs to increase their translation level. Twenty-four and six transgenic Arabidopsis plants for AtMYB2 and AtMYC2, respectively, were generated using a vacuum infiltration method (Bechtold et al., 1993). For each line, 70 to 80 of the T2 seeds were plated on germination medium (GM) kanamycin agar plates, and the lines in which all of the plated seeds showed kanamycin resistance were selected. The T3 progeny were used for further analyses. Expression levels of 3 and 11 lines of the 35S:AtMYC2 and 35S:AtMYB2 plants, respectively, were examined by RNA gel blot analysis using AtMYC2 and AtMYB2 cDNAs as probes. Most of the lines showed overexpression of the transgenes (data not shown). We selected two transgenic lines for each construct, which showed a higher level of overexpression of each transgene, and used them for further analyses. The transgenic plants with the kanamycin resistance gene were used as control wild-type plants (WT [Km]).

To create transgenic plants that overexpress both AtMYC2 and AtMYB2 cDNAs (35S:AtMYC2/AtMYB2), 35S:AtMYB2 plants were retransformed with the AtMYC2/35SΩHyg construct (Figure 1A). The 35S:AtMYC2/AtMYB2 transgenic plants were screened for kanamycin and hygromycin resistance. We plated 70 to 80 T2 seeds of each line on GM hygromycin/kanamycin agar plates and selected the lines whose plated seeds showed kanamycin/hygromycin resistance. We performed RNA gel blot analysis to determine the expression of the transgenes and selected two lines showing higher levels of overexpression of each transgene from the T3 progeny. These lines were used for further analyses. The transgenic plants with the kanamycin and hygromycin resistance genes were used as control wild-type plants (WT [Km/Hyg]).

The growth of the transgenic plants was compared with that of the wild-type plants at 2 weeks after sowing. The 35S:AtMYB2 plants growing on GM agar plates showed a phenotype similar to that of the wild-type plants (data not shown). By contrast, 35S:AtMYB2 plants growing on soil exhibited severe growth inhibition (Figure 1B). We observed more severe growth retardation in 35S:AtMYC2/AtMYB2 plants growing on soil. However, no morphological changes were observed in 35S:AtMYC2 plants grown either on GM agar or on soil. We compared the epidermal cells and palisade parenchyma of these transgenic plants with those of

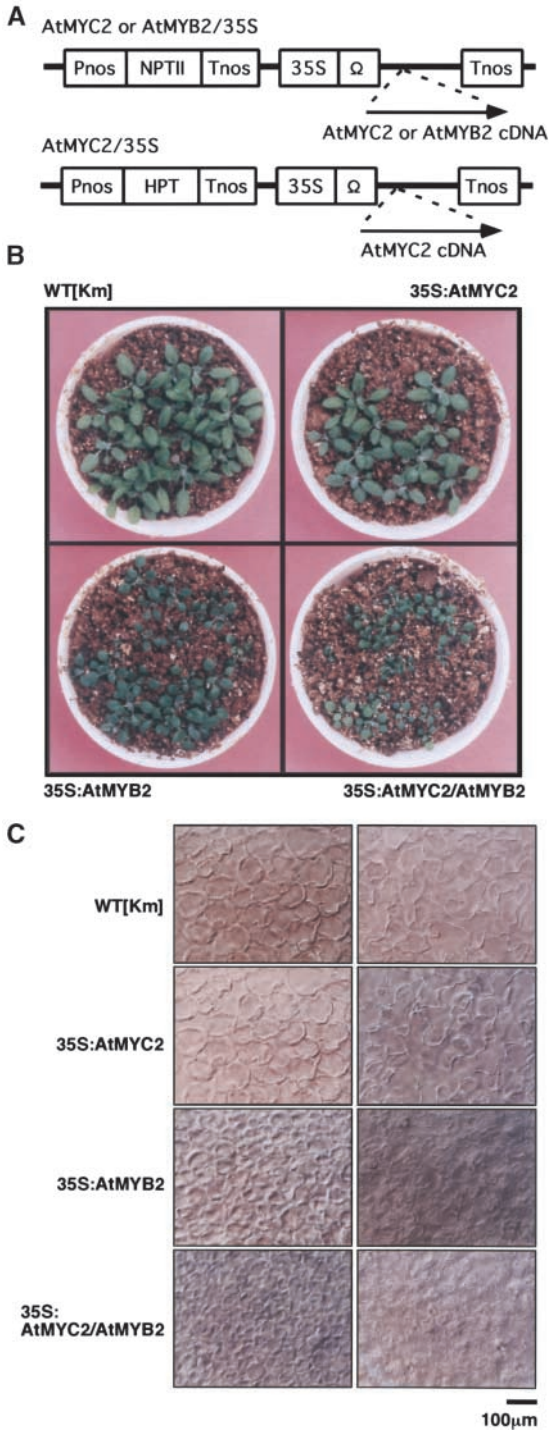

Figure 1. Establishment of the 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2 Plants.

(A) Scheme of the plant expression vectors. The constructs contain the 35S promoter of *Cauliflower mosaic virus* and *Tobacco mosaic virus* Ω sequence (Gallie et al., 1987) fused to AtMYC2 or AtMYB2 cDNAs. The AtMYC2 cDNA was cloned into the NotI site of the vector pBI121ΩKm or pBI121ΩHyg (AtMYC2/pBI121ΩKm or AtMYC2/pBI121ΩHyg), and AtMYB2 cDNA was cloned into the NotI site of the vector pBI121ΩKm (AtMYB2/pBI121ΩKm). Next, plasmids containing the AtMYC2 or AtMYB2 cDNA in the sense direction were identified by sequence analysis. AtMYC2/pBI121ΩKm and AtMYB2/pBI121ΩKm were used for the transformation of Arabidopsis. To make 35S:AtMYC2/AtMYB2 plants, 35S:AtMYB2 plants were retransformed by AtMYC2/pBI121ΩHyg. HPT, *hygromycin phosphotransferase*; NPTII, *neomycin phosphotransferase II*; Pnos and Tnos, *nopaline synthase* promoter and terminator, respectively.

(B) Morphology of 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2. Two-week-old seedlings of 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2 grown axenically in biopots are shown. WT [Km] indicates the control plants transformed with the *NPTII* gene.

(C) Paradermal micrographs of 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2. Micrographs of 2-week-old plants were taken using Nomarski optics. All micrographs were taken at the same scale. Left panels represent epidermal cells, and right panels represent the palisade parenchyma. Bar = 100 μm.

the wild-type plants using a microscope. We used leaves from the rosettes of transgenic plants for the microscopic analyses. The cells in the leaf palisade parenchyma of the wild-type and 35S:AtMYC2 plants had a bulbous shape, as shown in Figure 1C. The cells in the palisade parenchyma of 35S:AtMYB2 and 35S:AtMYC2/AtMYB2 transgenic leaves had a similar bulbous shape but were smaller. By contrast, the leaf epidermis of the wild-type and 35S:AtMYC2 plants contained cells with characteristically irregular shapes. The cells in the leaf epidermis of 35S:AtMYB2 and 35S:AtMYC2/AtMYB2 transgenic plants were similar to those of the wild type, but they were smaller than wild-type cells (Figure 1C). These results indicate that the dwarfed phenotype of 35S:AtMYB2 and 35S:AtMYC2/AtMYB2 transgenic plants probably is the result of a smaller cell size rather than a decreased cell number. Cell expansion probably is inhibited in these transgenic plants, but their cell division may not be.

35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 Transgenic Plants Show Hypersensitivity to ABA

The AtMYB2 and AtMYC2 proteins have been shown to be transcription factors that bind to the MYB and MYC recognition sites, respectively, leading to the ABA-inducible expression of *rd22*. Both *AtMYB2* and *AtMYC2* genes are induced by ABA. Therefore, we analyzed the sensitivity of 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 transgenic plants to ABA. First, we analyzed the effect of ABA on the growth of these transgenic plants compared with that of the wild-type plants. Transgenic plants with each construct were grown on GM agar plates containing 0, 0.3, 0.5, 0.7, 1, or 2 μM ABA without selection marker. Figure 2A shows the transgenic plants growing on GM plates containing 0.7 μM

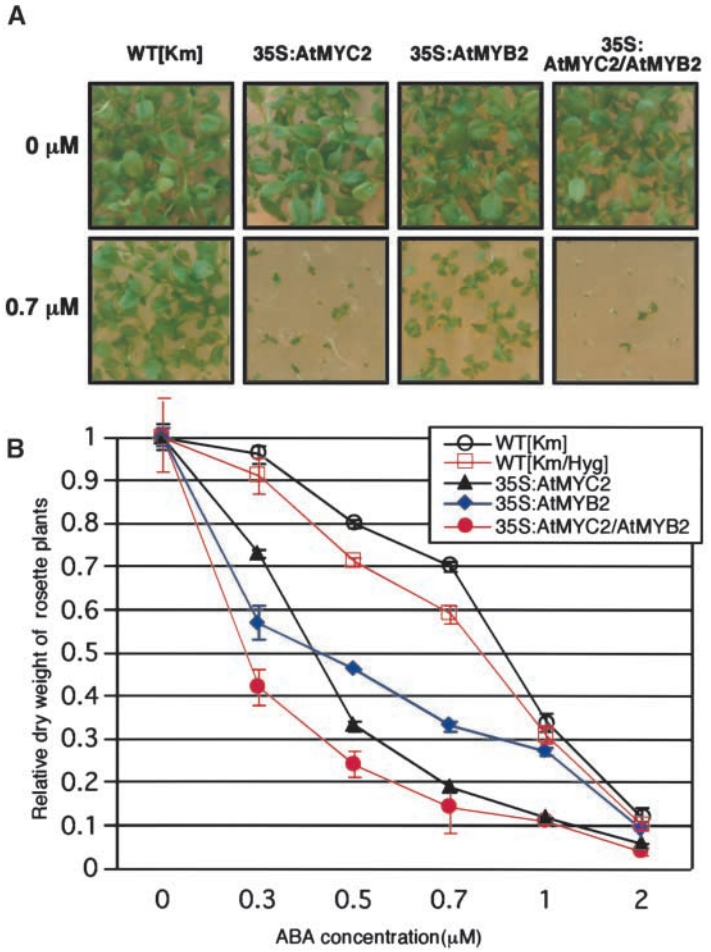

Figure 2. Comparison of Growth Inhibition by ABA in 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2 Plants.

(A) Three-week-old seedlings of 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2 grown on GM agar plates with or without 0.7 μM ABA. Transgenic plants with the *NPTII* gene (WT [Km]) were used as control wild-type plants.

(B) Dry weight of 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2 seedlings grown on GM agar plates containing ABA at 0, 300, 500, 700, or 1000 nM. The dry weight of each transgenic plant was measured at 30 days after the seeds were sown. Each value represents the average of >80 transformants with the standard error of at least two replicates. Transgenic plants with the *NPTII* gene (WT [Km]) and transgenic plants with the *NPTII* and *HPT* genes (WT [Km/Hyg]) were used as control wild-type plants.

ABA. The 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 plants showed significant hypersensitivity to ABA on GM agar plates. Figure 2B shows the relative dry weights of each transgenic plant after drying in an oven as described in Methods. Increasing concentrations of ABA resulted in greater growth retardation of the wild-type plants. The dry weights of 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 were lower than that of the wild-type plants. WT [Km/Hyg] also showed lower dry weight than WT [Km]. However, the loss of dry weight in 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 was more dramatic than that of WT [Km/Hyg]. Treatment with 300 nM ABA markedly decreased the dry weight of 35S:AtMYC2/AtMYB2 plants compared with that of 35S:AtMYC2 or 35S:AtMYB2 plants. These results were obtained with two independent lines of each transgenic plant (data not shown).

Next, we analyzed the germination rates (see Methods) of 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 plants on GM agar plates containing 0, 0.5, 1, 2, 3, or 5 μM ABA. ABA inhibited germination in all of the 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 plants at each concentration more severely than in the wild-type plants (Figure 3). In addition, ABA inhibition of germination in 35S:AtMYC2/AtMYB2 plants was more severe than that in

35S:AtMYB2 and 35S:AtMYC2 plants. The germination rates of 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 plants on the plates without ABA were almost the same as those of the wild-type plants. These results indicate that the reduced germination rates observed in 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 plants are dependent on ABA sensitivity.

RNA Gel Blot Analyses of Target Genes of the AtMYB2 and AtMYC2 Proteins

Previously, we reported that AtMYB2 and AtMYC2 function as transcriptional activators in the expression of *rd22* in a transient assay using Arabidopsis protoplasts. We analyzed the effects of the overexpression of AtMYB2 and AtMYC2

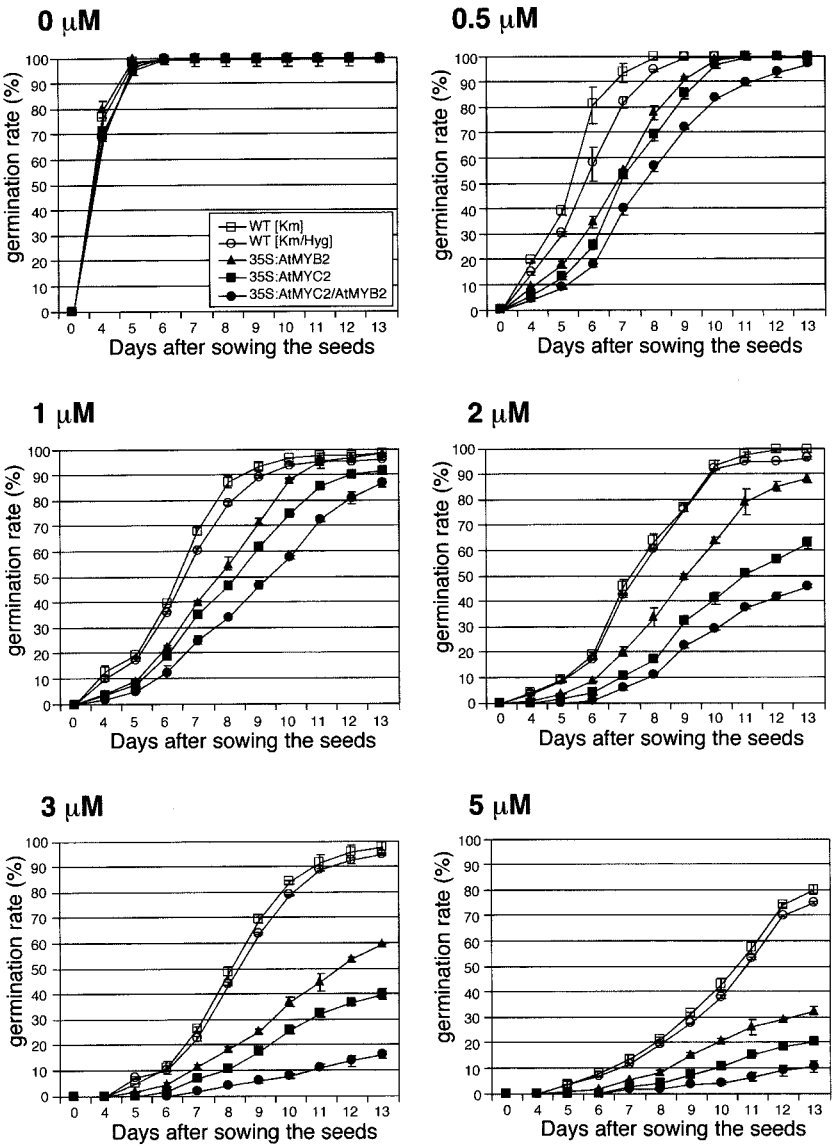

Figure 3. Effect of the ABA Sensitivity of 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2 Plants on Seed Germination Rate.

The seed germination rates of 35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2 were measured on GM agar plates containing 0, 0.5, 1, 2, 3, or 5 μM ABA at 4 to 13 days after sowing. Each value is the average of >80 transformants with the standard error of at least two replicates. Transgenic plants with the *NPTII* gene (WT [Km]) and transgenic plants with the *NPTII* and *HPT* genes (WT [Km/Hyg]) were used as control wild-type plants. Percentages of germinated seeds were obtained and scored as germination rates.

on the expression of *rd22* by RNA gel blotting (Figure 4). The 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 plants were treated with 0, 0.5, 0.7, 1, 5, or 10 μM ABA as described in Methods. The AtMYC2 cDNA was significantly overexpressed in 35S:AtMYC2 and 35S:AtMYC2/AtMYB2 plants, and AtMYB2 cDNA was similarly overexpressed in 35S:AtMYB2 and 35S:AtMYC2/AtMYB2 plants (Figures 4C and 4D). In 35S:AtMYC2 and 35S:AtMYC2/AtMYB2 plants, the expression of *rd22* apparently was increased by treatment with ABA at 500 and 700 nM compared with that in the wild-type plants (Figure 4A). However, no significant difference in the expression of *rd22* was detected in 35S:AtMYB2 plants.

Hoeren et al. (1998) reported that AtMYB2 functions as a transcription factor for the low-oxygen-inducible gene expression of *AtADH1* in Arabidopsis in a transient assay using particle bombardment. The *AtADH1* gene is well known as an ABA-inducible gene (Dolferus et al., 1994; de Bruxelles et al., 1996). Therefore, we analyzed the expression of *AtADH1* using transgenic plants. In 35S:AtMYB2 and 35S:AtMYC2, the expression of *AtADH1* was increased, and the expression level in 35S:AtMYC2 plants was higher than that in 35S:AtMYB2 plants (Figure 4B). In 35S:AtMYC2/AtMYB2 plants, the expression of *AtADH1* was increased dramatically compared with that in 35S:AtMYB2 and 35S:AtMYC2 plants. Interestingly, a high expression level of *AtADH1* was detected in 35S:AtMYC2/AtMYB2 plants without ABA treatment. The maximum induction of *AtADH1* was obtained in plants treated with 500 nM ABA, and the expression level was decreased gradually at higher ABA concentrations. These results demonstrate that AtMYC2 and AtMYB2 regulate the expression of the ABA-inducible target genes such as *rd22* and *AtADH1* in plants.

Identification of Target Genes of AtMYB2 and AtMYC2 Using a Full-Length cDNA Microarray

To identify novel genes upregulated in 35S:AtMYC2/AtMYB2 plants, we used a full-length cDNA microarray containing ~7000 Arabidopsis full-length cDNAs (Seki et al., 2002). mRNAs prepared from 35S:AtMYC2/AtMYB2 and wild-type plants were used for the generation of Cy3-labeled and Cy5-labeled cDNA probes, respectively. These cDNA probes were mixed and hybridized with the cDNA microarray. T4 progeny of 35S:AtMYC2/AtMYB2 plants treated with 500 nM ABA or water were used for mRNA preparation, and differences in the expression of ~7000 selected genes were analyzed. In the microarray analysis, AtMYB2 cDNA was overexpressed by approximately twofold in 35S:AtMYC2/AtMYB2 plants (control ratio, 2.03; ABA-treated ratio, 2.11). The AtMYC2 cDNA was highly overexpressed in 35S:AtMYC2/AtMYB2 plants (control ratio, 7.54; ABA-treated ratio, 11.77). The highly upregulated genes in 35S:AtMYC2/AtMYB2 plants are summarized in Table1. Among them, seven genes were found to be upregulated in both control and ABA-treated plants (Table 1, asterisks). The *AtADH1* gene was upregulated in both control plants (ratio, 4.00) and ABA-treated plants (ratio, 4.00). On the other hand, *rd22* was upregulated by ABA treatment (ratio, 2.25). These expression profile data obtained by microarray analysis corresponded with those obtained by RNA gel blot analysis in the *AtADH1* and *rd22* genes (Figures 4A and 4B). We searched the Arabidopsis genome database to obtain the promoter regions of 32 genes listed in Table 1. We searched for the MYC recognition sequence (CANNTG) and the MYB recognition sequences (A/TAACCA and C/TAACG/TG) located within the 10- to 600-bp upstream region

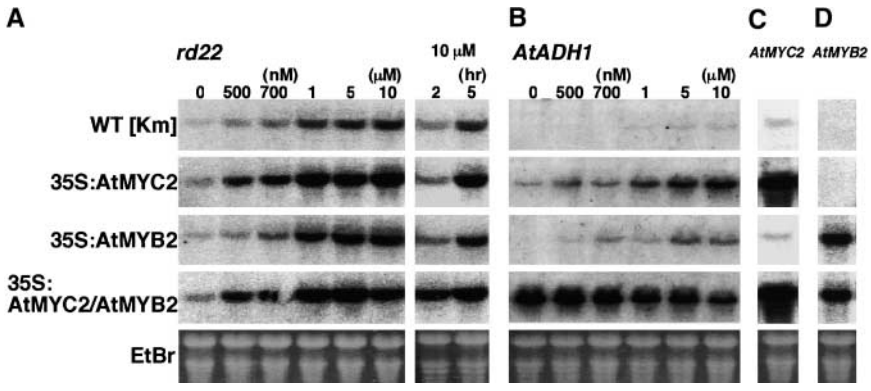

Figure 4. RNA Gel Blot Analysis of the Expression of the *rd22* and *AtADH1* Genes.

(A) and **(B)** To analyze the expression of the *rd22* **(A)** and *AtADH1* **(B)** genes, each lane was loaded with 30 μg of total RNA prepared from Arabidopsis plants (35S:AtMYC2, 35S:AtMYB2, 35S:AtMYC2/AtMYB2, and WT [Km]) that had been treated with ABA for 10 h at the indicated concentrations. The plants were transferred to water for 20 h and then treated with ABA. DNA fragments of the full-length rd22 and AtADH1 cDNAs were used as probes. EtBr, ethidium bromide.

(C) and **(D)** To analyze the expression of *AtMYC2* **(C)** and *AtMYB2* **(D)**, each lane was loaded with 30 μg of total RNAs prepared from untreated transgenic Arabidopsis plants. DNA fragments of the full-length AtMYC2 and AtMYB2 cDNAs were used as probes.

Table 1. Significantly Upregulated Transcripts in 35S:AtMYC2/AtMYB2

Gene Name[a]	Gene ID[b]	Ratio[c]	MYC Recognition Sequence[d]	MYB Recognition Sequence[d]	Annotation
Control					
RAFL06-82-G15	At5g44420	10.19	−527 (caattg), −341 (cacttg), −233 (catgtg), −140 (catgtg)	−272 (taacca), −116 (aaacca)	Antifungal protein-like protein
RAFL06-77-K06	At1g75830	5.07	−315 (tggttt), −202 (taacca), −185 (tagtta)	−354 (tggttt), −257 (taacca), −237 (tagtta)	Putative antifungal protein
*RAFL06-16-D08	At2g25510	4.93	−577 (cagatg), −485 (catctg), −224 (catatg)	−515 (tggttt)	Unknown protein
*RAFL07-10-G08	At3g03270	4.33	−446 (caagtg)	−342 (aaacca), −48 (aaacca)	Unknown protein
**ADH1*	At1g77120	4.00	−218 (cacgtg), −194 (caagtg), −218 (cagatg), −41 (cagctg)	−364 (aaacca), −162 (aaacca)	Alcohol dehydrogenase 1
*RAFL09-17-A07	At5g25980	3.52	−525 (caattg), −236 (cagttg)	−432 (taacca), −303 (caacgg), −236 (cagttg)	Myrosinase TGG2
*RAFL09-06-D04	At3g14210	3.50	−475 (catgtg), −443 (caattg), −257 (caattg), −137 (catatg)	−398 (taacca)	Myrosinase-associated protein
RAFL11-09-A12	At5g19550	2.95	−450 (caggtg), −720 (cagatg)	−529 (tggttt), −523 (tggtta)	Aspartate aminotransferase Asp2
RAFL07-10-G07	At1g52400	2.92	−205 (cacttg), −144 (cacgtg)	−294 (aaacca), −126 (taactg)	β-glucosidase homolog BG1
RAFL07-07-M09	At1g16410	2.88	−53 (cacatg)	Not found	P450-like protein
RAFL11-13-B13	At4g21830	2.80	−449 (caggtg), −255 (caaatg)	−528 (tggttt), −522 (tggtta)	Putative protein
*RAFL05-18-H22	At4g08870	2.75	−107 (caagtg)	−551 (taacca), −530 (taacca), −92 (taactg)	Putative arginase
RAFL09-06-K23	At1g07920	2.75	Not found	−599 (ccgtta), −563 (taacca), −553 (aaacca), −232 (taacca)	Elongation factor 1-α
kin2	At5g15970	2.72	−163 (cacgtg), −90 (cacgtg), −73 (cacgtg), −49 (catttg)	−764 (aaacca), −86 (tggttt)	Cold-regulated protein COR6.6
*RAFL06-08-C03	At1g31580	2.59	−486 (cacttg)	−368 (ccgtta), −291 (aaacca)	Pathogen-inducible protein CXc750
RAFL06-07-L14	At2g28000	2.58	Not found	−615 (taactg), −518 (tggttt), −100 (caacgg)	Putative Rubisco[e] subunit binding protein α-subunit
*RAFL04-13-P21	At5g42530	2.57	−266 (cacttg)	−254 (aaacca), −249 (aaacca)	Unknown protein
RAFL06-11-F24	At5g24770	2.54	−145 (catttg), −94 (catctg)	−454 (tggttt)	Vegetative storage protein VSP2
RAFL05-16-I09	At5g20830	2.54	−447 (cacttg), −235 (catgtg)	−356 (taacca), −244 (tggttt)	Suc synthase 1
RAFL05-09-P04	At2g19590	2.53	−607 (catttg), −262 (cacatg)	−302 (aaacca)	1-Aminocyclopropane-1-carboxylate oxidase
ABA treated					
*RAFL06-16-D08	At2g25510	6.13	−577 (cagatg), −485 (catctg), −224 (catatg)	−515 (tggttt)	Unknown protein
*RAFL05-18-H22	At4g08870	5.33	−107 (caagtg)	−551 (taacca), −530 (taacca), −92 (taactg)	Putative arginase
*RAFL09-17-A07	At5g25980	4.88	−525 (caattg), −236 (cagttg)	−432 (taacca), −303 (caacgg), −236 (cagttg)	Myrosinase TGG2
*RAFL09-06-D04	At3g14210	4.81	−475 (catgtg), −443 (caattg), −257 (caattg), −137 (catatg)	−398 (taacca)	Myrosinase-associated protein
*RAFL06-08-C03	At1g31580	4.59	−486 (cacttg)	−368 (ccgtta), −291 (aaacca)	Pathogen-inducible protein CXc750
**ADH1*	At1g77120	4.32	−218 (cacgtg), −194 (caagtg), −218 (cagatg), −41 (cagctg)	−364 (aaacca), −162 (aaacca)	Alcohol dehydrogenase 1
RAFL05-01-D08	At2g03760	4.09	Not found	−599 (aaacca)	Putative steroid sulfotransferase
RAFL11-02-P06	AAK43841	3.98	−614 (cagatg)	−451 (taacca), −417 (taacgg), −115 (aaacca)	Unknown protein
RAFL04-19-O21	At3g57050	3.92	−632 (catctg), −48 (cacgtg)	−571 (aaacca), −479 (ccgtta), −39 (taacca)	Cystathionine β-lyase
RAFL08-09-P03	At1g29930	3.73	−535 (caggtg), −171 (caattg)	−292 (tggttt), −142 (taacca)	Chlorophyll *a/b* binding protein
RAFL05-11-L07	At3g27690	3.53	−218 (cacttg), −132 (cacgtg)	−264 (aaacca), −158 (taacca), −105 (aaacca)	Putative chlorophyll *a/b* binding protein
RAFL06-07-C24	At3g21720	3.45	−589 (caaatg), −515 (caattg), −259 (caaatg), −234 (caaatg)	Not found	Putative isocitrate lyase
RAFL06-09-H06	At1g29920	3.39	−552 (cagatg), −522 (caattg), −439 (caactg), −201 (catgtg)	−574 (aaacca), −439 (caactg), −111 (aaacca)	Chlorophyll *a/b* binding protein
*RAFL07-10-G08	At3g03270	3.26	−446 (caagtg)	−342 (aaacca), −48 (aaacca)	Unknown protein
RAFL08-16-M12	At2g33380	3.25	−482 (caattg), −264 (catatg), −151 (cacgtg), −69 (cacgtg)	−216 (tggttt), −155 (taacca)	Calcium binding protein RD20
RAFL07-16-M19	At2g42220	3.25	−535 (caggtg), −171 (caattg)	−292 (tggttt), −142 (taacca)	Hypothetical protein
*RAFL04-13-P21	At5g42530	3.21	−266 (cacttg)	−254 (aaacca), −249 (aaacca)	Unknown protein
RAFL05-11-H09	At1g05680	3.21	−589 (cacgtg), −243 (cacatg), −229 (cacatg)	Not found	Putative indole-3-acetate β-glucosyltransferase
RAFL09-11-O13	At3g46780	3.14	−584 (catctg), −389 (cagttg), −363 (caattg)	−574 (tggtta), −521 (aaacca), −499 (taactg), −389 (cagttg)	Putative protein
RAFL07-12-M09	At2g21330	3.10	−151 (catatg), −101 (cacgtg), −41 (cacttg)	−538 (ccgtta), −387 (ccgttg), −184 (tggttt), −147 (tggttt)	Putative fructose bisphosphate aldolase

mRNAs from 35S:AtMYC2/AtMYB2 and WT [Km] plants with or without ABA treatment were used for the preparation of Cy3-labeled and Cy5-labeled cDNA probes. These cDNA probes were mixed and hybridized with the cDNA microarray. In this study, we used the λ-DNA as an internal control because its fluoresence level is almost the same in the two conditions. The first 20 genes with higher expression levels in 35S:AtMYC2/AtMYB2 than in WT [Km] plants are shown.

[a] Gene names are for full-length cDNA clones (Seki et al., 2001). Upregulated clones in 35S:AtMYC2/AtMYB2 in both the control and ABA-treated plants are indicated by asterisks.

[b] MIPS entry codes and GenBank identifiers for cDNA used in this study.

[c] $\frac{\text{Fluorescence intensity of each cDNA of 35S:AtMYC2/AtMYB2}}{\text{Fluorescence intensity of each cDNA of WT [Km]}} \div \frac{\text{Fluorescence intensity of λ-DNA of 35S:AtMYC2/AtMYB2}}{\text{Fluorescence intensity of λ-DNA of WT [Km]}}$

Ratios are average values from three independent experiments.

[d] Numbers indicate the nucleotide beginning at the 5′ terminus of the longest cDNA clone isolated. Minus signs indicate that the nucleotide exists upstream of the 5′ terminus of the putative transcription start site. MYC and MYB recognition sequences located 10 to 600 bp upstream from each TATA box are indicated.

[e] Ribulose-1,5-bisphosphate carboxylase/oxygenase.

Myrosinaseof each putative TATA box. We found that 29 genes had the MYC recognition sequence, 29 genes had the MYB recognition sequence, and 26 genes had both MYC and MYB recognition sequences in their promoter regions.

Improved Stress Tolerance in 35S:AtMYC2/AtMYB2 Transgenic Plants

Microarray analyses indicated that several ABA-inducible genes are upregulated in 35S:AtMYC2/AtMYB2 transgenic plants. To evaluate the effect of AtMYC2 and AtMYB2 overexpression on osmotic stress tolerance, the electrolyte leakage test was conducted using 35S:AtMYC2/AtMYB2 and wild-type plants under osmotic stress conditions treated with mannitol solution (Figure 5). Increasing concentrations of mannitol resulted in more electrolyte leakage in the wild-type control plants. Electrolyte leakage in 35S:AtMYC2/AtMYB2 plants was reduced significantly compared with that in the wild-type plants (0.4 M [0.54-fold lower], 0.5 M [0.41-fold lower], and 0.6 M [0.72-fold lower]). These results demonstrated that overexpression of AtMYC2 and AtMYB2 cDNAs resulted in improved stress tolerance.

Loss-of-Function Mutant of the *AtMYC2* Gene

Arabidopsis lines containing transposed Ds elements were generated using an Ac/Ds tagging system (Smith et al., 1996; Ito et al., 1999, 2002). Ds-transposed lines were generated from a Ds donor line (392-13). Genomic DNA was extracted from leaves of individual transposed lines, and Ds-flanking genomic DNA was obtained from 1000 lines using thermal asymmetric interlaced PCR. The partial sequences of the PCR products then were determined. Among these 1000 lines, we identified a Ds insertion mutant of *AtMYC2*. By sequence analysis, we determined the Ds insertion site in *AtMYC2*. The Ds element was inserted in the open reading frame of *AtMYC2*, between amino acids 107 and 108 (Figure 6A).

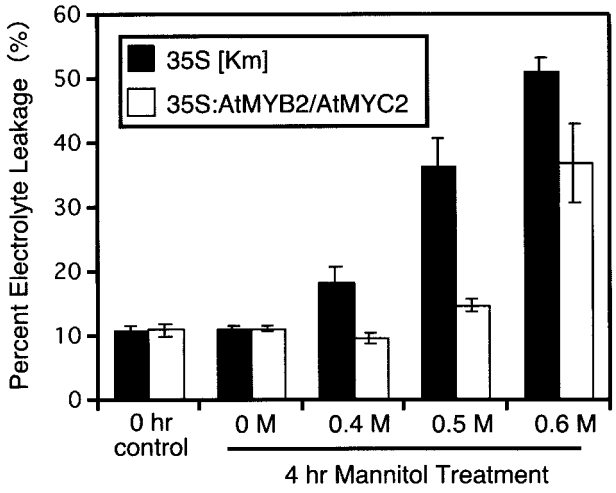

Figure 5. Comparison of the Stress Tolerance of 35S:AtMYC2/AtMYB2 and Wild-Type Plants Using Electrolyte Leakage.

Three-week-old Arabidopsis rosette-stage plants were used to quantify electrolyte leakage. The plants were removed from the agar plates and grown hydroponically in water for 20 h (0 h control) and subsequently grown in mannitol solution at various concentrations (0, 400, 500, and 600 mM) for 4 h.

Because AtMYC2 has been shown to function as a transcriptional activator in ABA signaling, we analyzed the sensitivity of the *atmyc2* mutant to ABA. We observed the effect of ABA on the growth of the *atmyc2* mutant. Seeds of the homozygous *atmyc2* mutant were plated onto GM agar plates containing ABA at 0, 1, 2, or 3 μM. Figure 6B shows these plants grown on a plate containing 2 μM ABA. Growth retardation by ABA was suppressed significantly in the *atmyc2* mutant compared with Nossen wild-type plants. The *atmyc2* mutant showed normal growth on an agar plate without ABA (Figure 6B). Figure 6C shows the relative dry weights of the wild-type plants and the *atmyc2* mutant. In both plants, the level of growth retardation correlated with the concentration of ABA. However, the dry weight of the *atmyc2* mutant was significantly higher than that of the wild-type plants (1 μM [1.9-fold higher], 2 μM [3.9-fold higher], and 3 μM [3.2-fold higher]). These results indicate clearly that AtMYC2 acts as a positive factor in ABA signaling.

Next, we compared the germination rates of the *atmyc2* mutant and the wild-type plants on GM agar plates containing 0, 1, 2, or 3 μM ABA (Figure 7). ABA inhibition of germination in the *atmyc2* mutant was less than that in the wild-type plants at each ABA concentration. The germination rates of these *atmyc2* and wild-type plants were almost the same on the GM agar plates without ABA. These results indicate that the increased germination rates observed in the *atmyc2* mutant are dependent on the existence of ABA.

We performed RNA gel blot analysis to determine the expression of *AtMYC2* in the *atmyc2* mutant. In wild-type plants, *AtMYC2* was expressed weakly under control conditions, and its expression apparently was induced by ABA. By contrast, we could not detect the expression of *AtMYC2* in the mutant at all (Figure 8A, *AtMYC2*). We analyzed the *atmyc2* mutant for the expression of *rd22* and *AtADH1*. The plants were treated with ABA at 0, 0.5, 0.7, 1, 5, 10, or 50 μM as described in Methods. In wild-type plants, the expression of both *rd22* and *AtADH1* was induced gradually by ABA treatment, and maximal induction was obtained at 50 μM ABA. The induction of these genes by ABA apparently decreased in the *atmyc2* mutant compared with that in the wild-type plants. These results indicate that AtMYC2 acts as a transcription factor in the ABA-induced expression of the *rd22* and *AtADH1* genes and that some AtMYC2 homologs may partially complement AtMYC2 function in ABA signaling.

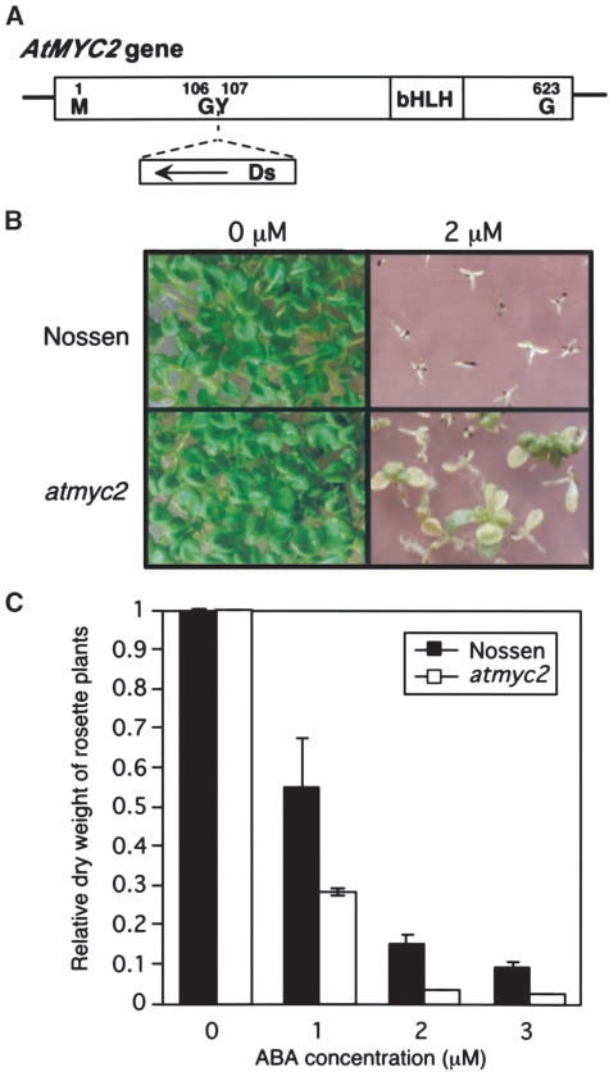

Figure 6. Disruption of the *AtMYC2* Gene by Transposon-Mediated Mutagenesis Causes Insensitivity to ABA.

(A) Scheme of the *AtMYC2* gene showing the Ds insertion site between amino acids 107 and 108. The arrow indicates the direction of the Ds insertion.

(B) Four-week-old *atmyc2* mutant and Nossen wild-type Arabidopsis seedlings grown on GM agar plates with or without 2 μM ABA.

(C) Dry weight of the *atmyc2* mutant and wild-type Arabidopsis seedlings grown on GM agar plates containing 0, 1, 2, or 3 μM ABA. The dry weight of each transgenic plant was measured at 30 days after sowing. Each value represents the average of >80 transformants.

Analysis of Novel AtMYC2-Related Genes in the Arabidopsis Genome

We searched the Arabidopsis genome database for amino acid sequences of the bHLH DNA binding domains of several bHLH-related proteins, including AtMYC2, and found >100 proteins with the bHLH DNA binding domain in Arabidopsis. We selected 57 genes whose ESTs are available and 16 genes obtained from the genome sequence that may belong to a specific subfamily in the bHLH gene family based on phylogenetic analysis. Next, we selected 43 distinct genes for the bHLH-related proteins from these 73 genes to simplify the phylogenetic tree. On the basis of similarities in the amino acid sequences of the bHLH DNA binding domain among 43 genes, we found seven proteins that may belong to the AtMYC2 subfamily (Figure 9A). In the seven AtMYC2-related proteins, we found two proteins showing high homology with AtMYC2 in the N-terminal conserved regions (62.7 and 59.9% identity) and the bHLH DNA binding domain (94.4 and 96.1% identity) (Figure 7B). These two AtMYC2-related proteins have high homology not only in these two conserved regions but also in the whole region (Figure 9B). We performed RNA gel blot analysis to determine the expression of these two genes. The *At4g17880* (*AtMYC4*) gene showed constitutive expression, whereas the expression of the *At5g46760* (*AtMYC3*) gene was induced slightly by ABA treatment and high-salt stress (Figure 9C). The AtMYC3 protein might have a function as a transcription factor, like the AtMYC2 protein, in ABA-inducible expression during osmotic stress.

DISCUSSION

We have shown that the rd22BP1/AtMYC2 and AtMYB2 proteins function as transcriptional activators in the ABA-responsive gene expression of the *rd22* gene (Abe et al., 1997). In the present study, we analyzed the biological functions of AtMYC2 and AtMYB2 not only in ABA-responsive gene expression but also in ABA signal transduction using transgenic Arabidopsis plants and Ds transposon insertion mutants. We analyzed the ABA-inducible expression of the *rd22* and *AtADH1* genes in 35S:AtMYB2, 35S:AtMYC2, and 35S:AtMYC2/AtMYB2 plants. Overexpression of AtMYC2 cDNA increased the ABA-responsive expression of *rd22*. In 35S:AtMYC2/AtMYB2 plants, the expression of *rd22* was induced by ABA significantly earlier than in 35S:AtMYB2 or 35S:AtMYC2 plants (Figure 4A). Although the effect on the expression of *rd22* was not as strong, AtMYB2 seems to function cooperatively with AtMYC2 in the ABA-responsive expression of *rd22*. On the other hand, in plants overexpressing both AtMYC2 and AtMYB2 cDNAs, the *AtADH1* transcript accumulated to a high level even under control conditions, and the level of ABA-responsive expression of *AtADH1* in these plants was higher than that in plants overexpressing either AtMYC2 or AtMYB2 cDNA alone. These results indicate that both AtMYC2 and AtMYB2 proteins act as transcription factors in the ABA-responsive expression of *AtADH1*.

There are MYC and MYB recognition sites in the promoter region of *AtADH1* as well as the *rd22* promoter. Promoter

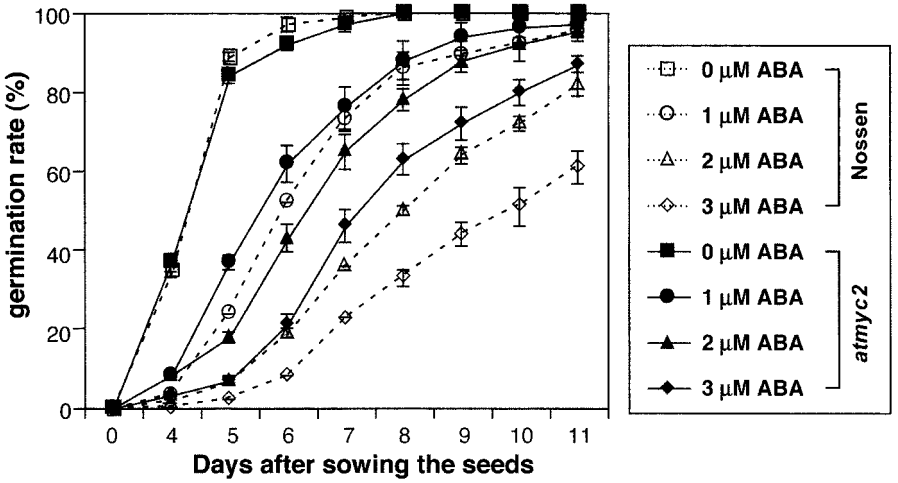

Figure 7. Effect of the ABA Sensitivity of the *atmyc2* Mutant and Wild-Type Plants on Seed Germination Rate.

The seed germination rates of *atmyc2* and wild-type plants were measured on GM agar plates containing 0, 1, 2, or 3 μM ABA at 4 to 11 days after sowing. Each value represents the average of >80 transformants with the standard error of at least two replicates. Percentages of germinated seeds were obtained and scored as germination rates.

analysis of *AtADH1* revealed that the G-box (CACGTG, a MYC recognition site) is necessary for the induction of this gene by ABA (de Bruxelles et al., 1996). Hoeren et al. (1998) reported that AtMYB2 binds to the MYB recognition site called MBS-1 in the *AtADH1* promoter and transactivates gene expression via MBS-1 in transient expression analysis. We have shown that AtMYC2 binds specifically to the CACATG sequence that acts as a dehydration-responsive *cis* element using the *rd22* promoter (Abe et al., 1997). de Pater et al. (1997) isolated a cDNA encoding RAP-1 that binds to G-box sequences, and this gene was identical to *AtMYC2*. Therefore, the AtMYC2/RAP-1 protein is likely to bind to the G-box sequence in the *AtADH1* promoter and also to function with AtMYB2 as a transcription factor that controls the ABA-responsive expression of *AtADH1*. Recently, Kang et al. (2002) reported that overexpression of the Arabidopsis bZIP factors ABF3 and ABF4 resulted in the overexpression of *AtADH1* under high-salt conditions. Stress-responsive expression of *AtADH1* might be regulated by both bHLH/MYB and bZIP transcription factors. Transactivation experiments using Arabidopsis protoplasts would help to elucidate the mechanism of *AtADH1* expression.

The *AtMYC2* and *AtMYB2* genes are expressed in seeds as well as in vegetative tissue (Abe et al., 1997), and overexpression of AtMYB2 and/or AtMYC2 proteins in transgenic plants showed significant hypersensitivity to ABA in both vegetative tissue and seeds (Figure 2). This finding suggests that AtMYC2 and AtMYB2 function as transcriptional activators in ABA signal transduction in seeds as well as in vegetative tissue. In Arabidopsis, the seed-specific ABA-insensitive mutants *abi3*, *abi4*, and *abi5* have been isolated by germination on medium containing ABA at concentrations that normally inhibit germination (Koornneef et al., 1984; Finkelstein, 1994). These *ABI3*, *ABI4*, and *ABI5* genes encode three different classes of transcription factors that may function in ABA-responsive gene expression in seeds (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000).

Recently, it was shown that ABI3 and ABI5 interact with each other in the yeast two-hybrid protein assay (Nakamura et al., 2001). Furthermore, ectopic expression of the *ABI3* or *ABI4* gene increases the accumulation of *ABI5* mRNA, and these genes could act cooperatively in vivo (Soderman et al., 2000). The ABI5 protein contains a bZIP domain and three conserved regions in its N termini such as rice TRAB and Arabidopsis AREB/ABF proteins. These bZIP proteins interact with ABRE in the promoter region of ABA-inducible genes and induce their expression. ABRE resembles the G-box, and AtMYC2 also can interact with the ABRE/G-box. However, a single copy of ABRE is not sufficient for ABA-responsive transcription, and at least one coupling element is required to specify the function of ABRE (Hobo et al., 1999). Although both bZIP and AtMYC2 proteins interact with ABRE, there may be coupling elements in the promoter regions that share their target ABA-inducible genes. Therefore, we suggest that AtMYC2 and AtMYB2 function as transcriptional activators for gene expression in response to ABA different from the ABRE-bZIP regulatory system in vegetative tissue and seeds.

Microarray analysis using 35S:AtMYC2/AtMYB2 transgenic plants showed that most of the upregulated genes have the MYC and MYB recognition sequences in their promoter regions. In addition to the *rd22* and *AtADH1* genes, the *Cor6.6*

and *rd20* genes were upregulated in 35S:AtMYC2/AtMYB2. These genes have been described previously as ABA-inducible genes (Wang et al., 1995; Takahashi et al., 2000). These results supported the hypothesis that AtMYC2 and AtMYB2 function in ABA signaling. Moreover, osmotic stress–inducible genes encoding Suc synthase 1 (SUS1), vegetative storage protein 2 (VSP2), β-glucosidase homolog (BG1), and myrosinase (TGG2) also were upregulated in 35S:AtMYC2/AtMYB2 (Dejardin et al., 1999; Gong et al., 2001) (Table 1). Upregulation of these osmotic stress–inducible genes tempted us to evaluate the stress tolerance of 35S:AtMYC2/AtMYB2. Electrolyte leakage analyses indicated that ionic leakage in 35S:AtMYC2/AtMYB2 was significantly less than that in the wild-type plants. These data suggested improvement of stress tolerance in 35S:AtMYC2/AtMYB2 plants.

The transgenic plants overexpressing AtMYB2 or both AtMYB2 and AtMYC2 showed a dwarf phenotype (Figure 1). This finding may be attributable to the overexpression of stress-inducible genes that are controlled by AtMYB2 under unstressed conditions. Interestingly, the transgenic plants grown on GM agar plates did not show a phenotype different from that of the wild-type plants (data not shown). On the other hand, the transgenic plants grown on soil showed a dwarf phenotype. ABA contents in rosette leaves of plants grown on soil were significantly higher than in rosette leaves of plants grown on GM agar plates (our unpublished results).

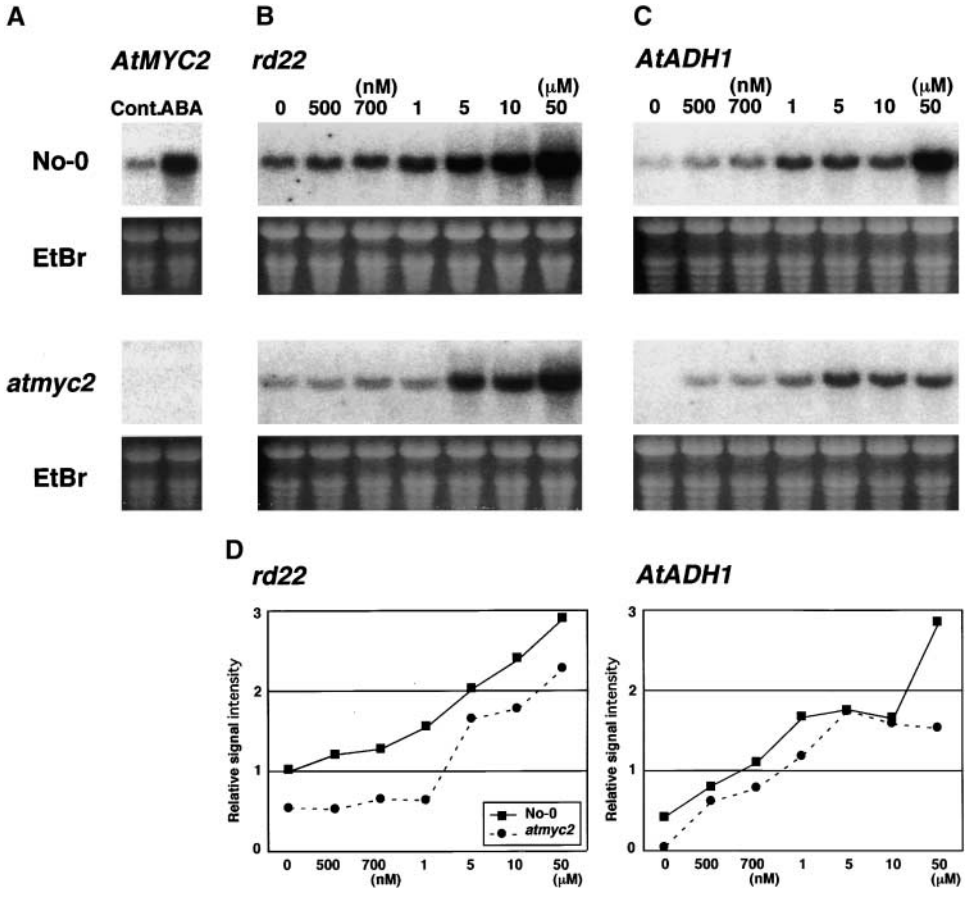

Figure 8. RNA Gel Blot Analysis of the Expression of the *rd22*, *AtMYC2*, and *AtADH1* Genes in *atmyc2* Mutant and Wild-Type Plants.

(A) To analyze the expression of *AtMYC2*, each lane was loaded with 30 μg of total RNA prepared from *atmyc2* mutant and Nossen wild-type plants (No-0) that had been treated for 5 h with 100 μM ABA. Cont., control treatment with water; EtBr, ethidium bromide.
(B) and **(C)** To analyze the expression of the *rd22* and *AtADH1* genes, each lane was loaded with 30 μg of total RNAs prepared from *atmyc2* mutant and Nossen plants that had been treated with ABA for 5 h at the designated concentrations. The plants were transferred to water for 20 h and then treated with ABA. DNA fragments of the full-length AtMYC2, rd22, and AtADH1 cDNAs were used as probes.
(D) To quantify the expression of *rd22* and *AtADH1* genes, the intensity of each band was quantified by densitometry.

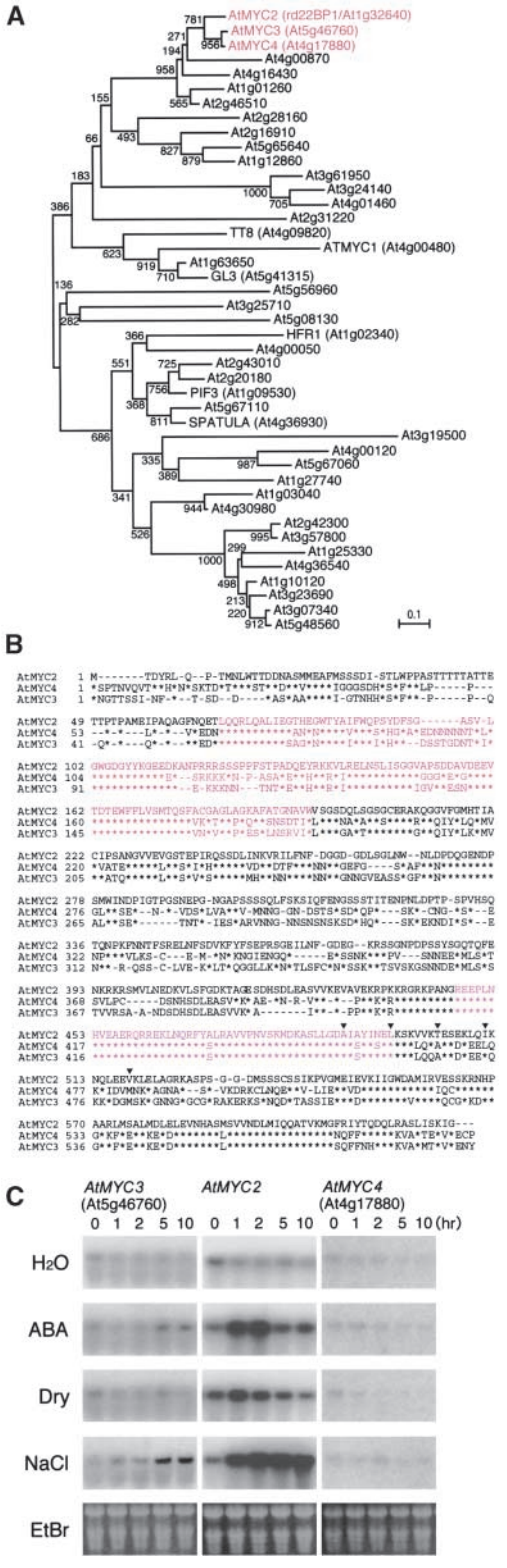

Figure 9. Phylogenic Tree of bHLH-Related Proteins and Comparison of Amino Acid Sequences of AtMYC2 and Its Homologs, AtMYC3 and AtMYC4.

(A) The bootstrapped tree file was produced by CLUSTAL W from sequences of bHLH-related proteins. We searched the Arabidopsis genome database for amino acid sequences of the bHLH DNA binding domains of several bHLH-related proteins including AtMYC2 as a query. MIPS identifiers are shown by their protein names. The AtMYC2, AtMYC3, and AtMYC4 proteins are indicated in red. We basically selected the genes whose ESTs were obtained to construct the phylogenic tree except for At1g25330, At5g56960, At1g12540, At4g00120, At5g67060, and At1g27740.

(B) The deduced amino acid sequence of AtMYC2 is compared with the sequences of AtMYC3 and AtMYC4. Asterisks represent identical amino acid residues, and dashes indicate gaps introduced to maximize alignment. The N-terminal unique domains of the bHLH family are highlighted in green, and N-terminal conserved regions and bHLH DNA binding domains are shown in red and purple. Triangles show repeated hydrophobic residues, which extend from helix II into the putative Leu zipper.

(C) To analyze the expression of *AtMYC2*, *AtMYC3*, and *AtMYC4*, each lane was loaded with 30 μg of total RNA prepared from unbolted wild-type (Columbia) plants that had been dehydrated (Dry) and transferred from agar plates to hydroponic growth conditions in 100 μM ABA, 250 mM NaCl, or water. The plants were transferred to water for 20 h and then given various treatments. The DNA fragments of the full-length AtMYC2 and partial AtMYC3 and AtMYC4 cDNAs were used as probes. Numbers above each lane indicate the time in hours after the initiation of treatment.

The dwarf phenotype of the transgenic plants may be related to the ABA contents of the Arabidopsis cells.

We isolated a Ds transposon–inserted Arabidopsis mutant of *AtMYC2* (Figure 6). In this mutant, expression of *AtMYC2* was not detected at all. The homozygote of the Ds insertion mutant was less sensitive to ABA in terms of growth. The results of the mutant analysis as well as the overexpression of AtMYC2 support the idea that AtMYC2 functions as a transcriptional activator in ABA signaling. Moreover, ABA-induced expression of the *rd22* and *AtADH1* genes was decreased in the mutant. Thus, less ABA sensitivity of the *AtMYC2* mutant was correlated with the expression of the *rd22* and *AtADH1* genes. Although the level of expression of the *rd22* and *AtADH1* genes was decreased significantly, these genes were induced by ABA in the mutant. Previously, we showed that a 67-bp DNA fragment between positions −207 and −141 of the *rd22* promoter is sufficient for drought- and ABA-induced expression, and base substitution in the MYC recognition site in the fragment resulted in apparently decreased induction by drought stress. However, some significant induction by drought stress remained. The mutant fragment with base substitutions in both MYC and MYB sites did not function at all in drought-induced expression (Abe et al., 1997). We are now screening Ds transposon or T-DNA insertion mutants by

PCR to isolate *atmyb2* mutant lines. A knockout mutant of the *AtMYB2* gene and a double knockout mutant of the *AtMYC2* and *AtMYB2* genes would help us elucidate the mechanism of the regulation of *rd22* gene expression in more detail.

Another possible explanation for the induction of the target genes by ABA in the *atmyc2* mutant is the existence of functional redundancy of other bHLH proteins. We searched the Arabidopsis genome database for amino acid sequences of the bHLH proteins and found two distinct genes encoding the bHLH proteins (AtMYC3/At5g46760 and AtMYC4/At4g17880) that showed high homology with AtMYC2 not only in the DNA binding domain but also in the N-terminal unique domain (Figures 9A and 9B). The bean PG1 protein has the same N-terminal domain sequence (Kawagoe and Murai, 1996), but we found no other bHLH proteins except AtMYC3 and AtMYC4 with this N-terminal domain in the Arabidopsis genome. Remarkably, the bHLH domains of both AtMYC3 and AtMYC4 proteins were nearly identical to the bHLH domain of AtMYC2 (94.1 and 96.1% identity, respectively). Recently, some bHLH-type transcription factors (SPATULA, TT8, and GL3) were reported in Arabidopsis (Nesi et al., 2000; Payne et al., 2000; Heisler et al., 2001). SPATULA is the regulator of carpel margin tissue development, whereas TT8 is a regulator of late flavonoid metabolism. GL3 functions as a regulator of trichome development. The bHLH domains of these three proteins have only 62.8, 37.3, and 37.9% identity, respectively, to that of AtMYC2 (Nesi et al., 2000; Payne et al., 2000; Heisler et al., 2001). The AtMYC3 and AtMYC4 proteins may have a function similar to that of AtMYC2 in Arabidopsis. Functional analysis of AtMYC3 and AtMYC4 may indicate whether these proteins act as transcriptional regulators redundantly to AtMYC2 in ABA signaling.

In conclusion, the transgenic plants overexpressing AtMYC2 or AtMYB2 cDNAs exhibited ABA hypersensitivity. ABA-induced gene expression of *rd22* and *AtADH1* was enhanced in these transgenic plants. Transgenic plants overexpressing both AtMYC2 and AtMYB2 cDNAs showed higher levels of ABA hypersensitivity and expression of many stress-inducible genes, including *rd22* and *AtADH1*. By contrast, the *atmyc2* mutant was insensitive to ABA and showed significantly decreased ABA-induced gene expression of *rd22* and *AtADH1*. These results indicate that both AtMYC2 and AtMYB2 function as transcriptional activators in the ABA signal transduction pathway under drought stress conditions in plants.

METHODS

Plant Materials

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates containing 1% Suc as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). In the case of plants growing in pots, seeds were sown on vermiculite:perlite (1:1) moistened with Hyponex diluted 1:1000 (Hyponex Japan, Osaka, Japan) and grown at 23°C as described previously (Nanjo et al., 1999).

Transgenic Plants Overexpressing AtMYC2 and/or AtMYB2 cDNAs

Plasmids used in the transformation of Arabidopsis were constructed with AtMYC2 or AtMYB2 full-length cDNA cloned into a polylinker site of binary vector pBI35SΩKm or pBI35SΩHyg, which was derived from pBI121 (Clontech, Palo Alto, CA), or pBIG-Hyg (Becker, 1990). For the construction of the pBI35SΩKm vector, pBI121 was digested with SmaI and SacI to delete the *β-glucuronidase* coding region and ligated with SmaI-NotI-SacI polylinker. Subsequently, the Ω sequence of *Tobacco mosaic virus* (Gallie et al., 1987), which was provided by H. Shinshi (National Institute of Advanced Industrial Science Technology, Tsukuba, Japan), was ligated with the BamHI site located downstream of a 35S promoter of *Cauliflower mosaic virus*. For the construction of the pBI35SΩHyg vector, pBIG-Hyg was digested with HindIII and EcoRI to delete the *β-glucuronidase* coding region and ligated with a HindIII and EcoRI fragment containing the 35S promoter, Ω sequence, and nopaline synthase terminator of pBE2113 (Kasuga et al., 1999). To construct AtMYC2/35SΩKm, AtMYB2/35SΩKm, and AtMYC2/35SΩHyg, we cloned a NotI fragment of AtMYC2 or AtMYB2 cDNA into the NotI site of the pBI35SΩKm or pBI35SΩHyg vector. The constructs were introduced into *Agrobacterium tumefaciens* C58 as described previously. Plants were transformed using the vacuum infiltration method described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

Microscopic Analysis

Rosette leaves used for microscopic analyses were obtained from 3-week-old plants grown axenically in the pots. Fully expanded fifth leaves were fixed, washed, and cleared as described by Tsuge et al. (1996) and then observed with a DCLIPSE E800 microscope (Nikon, Tokyo, Japan) equipped with Nomarski differential interference contrast optics.

Analyses of Sensitivity to Abscisic Acid

Germination Experiments

Arabidopsis seeds used in the germination assays were allowed to imbibe on germination medium plates at 4°C for 4 days to encourage synchronous germination and grown at 22°C. Seeds were regarded to have germinated when the radicle tip had fully expanded the seed coat. The percentage of germinated seeds was scored as the germination rate. The experiments were repeated more than twice.

Growth Inhibition Experiments

Arabidopsis plants were grown as described above. The dry weight of each transgenic plant was measured at 30 days after sowing the

seeds by baking >80 rosette-stage plants in one box at 160°C for 2 h and normalizing the dry weight for each plant. The experiments were repeated more than twice.

RNA Gel Blot Analysis

For abscisic acid (ABA) treatments, Arabidopsis plants were removed from the agar plates and grown hydroponically in water for 20 h to decrease the background expression of the *rd22* gene and then grown subsequently in ABA solution at various concentrations for 5 h (Nossen and *atmyc2* mutant) or for 2, 5, and 10 h (35S:AtMYC2, 35S:AtMYB2, and 35S:AtMYC2/AtMYB2). The plants subjected to the ABA treatments were frozen in liquid nitrogen for further analyses.

Isolation of total RNA and RNA gel blot hybridization were performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). The DNA fragments of the full-length rd22, AtMYC2, AtMYB2, and AtADH1 cDNAs were used as probes. Probes for *AtADH1* were obtained by PCR using cDNAs prepared from ABA-treated Arabidopsis plants as a template.

Arabidopsis Full-Length cDNA Microarray Analysis

Total RNA was isolated using Trizol regent (Gibco BRL). mRNA was prepared using MACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Glabach, Germany). Preparation of fluorescent probes, microarray hybridization, and scanning were as described previously (Seki et al., 2001). Data analysis also has been described previously (Seki et al., 2001). Lambda control template DNA fragment (Takara, Kyoto, Japan) was used as an external control to equalize hybridization signals generated from different samples. To assess the reproducibility of microarray analysis, each experiment was repeated three times.

Electrolyte Leakage Assay

Three-week-old Arabidopsis plants (35S:AtMYC2/AtMYB2 and wild type) were removed from the agar plates and grown hydroponically in water for 20 h and then grown subsequently in mannitol solution at various concentrations for 4 h. Electrolyte leakage assay was performed as described by Weigel et al. (2001).

Ds Transposon Tagging Mutant of the *AtMYC2* Gene

The Ds-transposed line (background ecotype Nossen) and thermal asymmetric interlaced PCR analyses were as described previously (Ito et al., 2000).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The GenBank accession numbers for the genes mentioned in this article are as follows: *AtMYC2* (AB000875), *AtMYB2* (D14712), *rd22* (D10703), *AtADH1* (D63461), *AtMYC3* (AF251690), and *AtMYC4* (T05074).

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