

Stress Tolerance and Glucose Insensitive Phenotypes in Arabidopsis Overexpressing the *CpMYB10* Transcription Factor Gene¹

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The resurrection plant *Craterostigma plantagineum* has the ability to survive complete dehydration. In an attempt to further understand desiccation tolerance in this plant, the *CpMYB10* transcription factor gene was functionally characterized. *CpMYB10* is rapidly induced by dehydration and abscisic acid (ABA) treatments in leaves and roots, but no expression was detected in fully hydrated tissues. Electrophoretic mobility shift assay experiments showed binding of rCpMYB10 to specific *mybRE* elements within the LEA *Cp11-24* and *CpMYB10* promoters. Localization of *CpMYB10* transcript by in situ reverse transcription-PCR reactions showed expression in vascular tissues, parenchyma, and epidermis both in leaves and roots in response to ABA. Transgenic Arabidopsis plants transformed with *CpMYB10* promoter fused to *GUS* gene showed reporter expression under ABA and stress conditions in several organs. Overexpression of *CpMYB10* cDNA in Arabidopsis led to desiccation and salt tolerance of transgenics lines. Interestingly, it was found that plants overexpressing *CpMYB10* exhibited Glc-insensitive and ABA hypersensitive phenotypes. Therefore, our results indicate that *CpMYB10* in Arabidopsis is mediating stress tolerance and altering ABA and Glc signaling responses.

Diurnal and seasonal environmental fluctuations as well as extreme conditions have been a major selective pressure for plant evolution. Plants are sessile organisms that cannot move to escape from adverse environmental cues, thus complex metabolic and anatomical adaptations have been developed to cope with abiotic stresses. Availability of water is probably the most limiting factor for crop productivity and yield, compromising economical output and human food supply. Therefore, there is a strong need to understand plant adaptation mechanisms against adverse environmental conditions to improve stress tolerance.

Plant stress responses involve the expression of a plethora of genes with an adaptive role. Among the products of these genes are enzymes catalyzing the synthesis of osmoprotectants or antioxidants, late-embryogenesis abundant (LEA) proteins, chaperones and heat shock proteins, lipid desaturases, water channels, and ion transporters, representing some of the best characterized examples (Ingram and Bartels, 1996). Abscisic acid (ABA) plays a major role in transducing stress responses (Knight and Knight, 2001).

Rapid stress responses are in most cases ABA-independent, and there is growing evidence that ABA-dependent and independent pathways cross-talk (Shinozaki and Yamaguchi-Shinozaki, 2000). Signal transduction components include protein kinases such as calcium-dependent protein kinases (CDPK) and mitogen-activated protein (MAP) kinases, G-proteins, phosphatase 2C, and second messengers such as Ca²⁺ and phosphoinositides. A phospholipase D raises its activity minutes after dehydration (Frank et al., 2000). Also, within 1 min after osmotic shock, inositol 1,4,5-P₃, a breakdown product of phospholipase C, dramatically increases its concentration, and rapid changes in cytosolic free Ca²⁺ concentrations are triggered during this process (DeWald et al., 2001). It has been claimed that nitric oxide is involved in ABA-induced response to stomatal closure and requires cGMP and cADPR (Neill et al., 2002). An Arabidopsis transmembrane His kinase functions as an osmosensor in a yeast mutant, suggesting a similar role in plants (Urao et al., 1999).

Several plant model systems have been used to study responses to water deficit, according to the severity of the stress. Upon a mild water deficit, plants reduce water loss by closing stomata, retain water by osmotic adjustment, and increase water uptake. These responses have been thoroughly studied in Arabidopsis and other mesophytes (Tabaeizadeh, 1998). A different situation occurs in the so-called resurrection plants that exhibit protoplasmic desiccation tolerance. These organisms withstand long periods with air of 0% (v/v) relative humidity, reviving a few hours after

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exposure to water. The best characterized example is *Craterostigma plantagineum*, a South African plant living on rocks in shallow soil (Gaff, 1971). An important question is whether the biochemical and molecular mechanisms to cope with dehydration stress that are present in *Arabidopsis*, crops, or seeds are also in *Craterostigma*. Although some common molecular components have been found in all these plants, in mature seeds or *Craterostigma* some differences with other systems have been uncovered, such as the presence of large concentrations of sugars (Bartels and Salamini, 2001). The C8 sugar octulose is very abundant in fully hydrated leaves of *Craterostigma*, but as soon as desiccation proceeds its concentration drastically drops down and concomitantly Suc reaches high levels. Disaccharides such as Suc or trehalose have been shown to protect enzymes and membrane structures under the dehydrated state and are abundant in anhydrobiotic organisms (Crowe et al., 1998; Hoekstra et al., 2001).

Another potential difference between *Craterostigma* and *Arabidopsis* might be the pattern of gene regulation coordinated by transcriptional activators and their tissue-specificity. Genetic and molecular approaches have identified transcription factors that modulate gene expression in response to abiotic stress and ABA. The transcription activators DREB1A, DREB2A, and CBF1, involved in ABA-independent stress response, bind to the consensus dehydration-responsive element (DRE) TACCGACAT, which is present in promoter regions of genes induced by osmotic, saline, and cold stresses (Stockinger et al., 1997; Liu et al., 1998). Genes responsive to ABA usually contain ABA-responsive elements consisting of the (C/T)ACGTGGC consensus sequence and are transactivated by bZIP transcription factors (Choi et al., 2000). Three classes of transcription factors have been characterized in *Craterostigma*: a heat shock transcription factor (Bockel et al., 1998), two members of the homeodomain Leu zipper family (Frank et al., 1998), and three MYB genes (Iturriaga et al., 1996). Here we show that *Arabidopsis* transgenic

lines overexpressing the heterologous MYB transcription factor gene *CpMYB10* are stress tolerant, Glc-insensitive, and ABA hypersensitive. The expression pattern of *CpMYB10* in *Craterostigma* suggests a key role in desiccation tolerance.

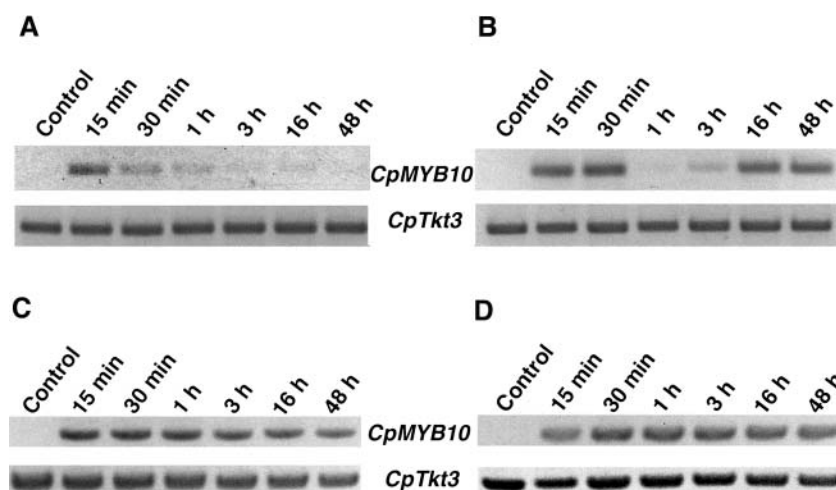
RESULTS

CpMYB10 Gene Is Induced by Desiccation and ABA and Is Under Repression Control in Unstressed Conditions

We have previously reported the cloning of a cDNA and two genomic MYB genes from *Craterostigma* (Iturriaga et al., 1996). The *CpMYB7* cDNA and *CpMYB5* and *CpMYB10* genomic clones share between them 96% to 98% identity and have the canonical MYB DNA-binding domain with the R2R3-type structure that is predominant in plants (Stracke et al., 2001). In *Arabidopsis*, the closest homolog to these MYB genes from *Craterostigma* is *AtMYB2*, which is induced by ABA, dehydration, and salt stresses (Urao et al., 1993). Although the DNA-binding domain of *CpMYB10* and *AtMYB2* shares 92% similarity, the 230-amino acid C terminus of *CpMYB10* is different from the corresponding *AtMYB2* region, except for a stretch of 31 amino acids that shows 87% similarity (Iturriaga et al., 1996).

To test whether *CpMYB10* is expressed in leaves and roots, a reverse transcription coupled to PCR (RT-PCR) analysis was used. Specific oligonucleotides for the 5' and 3' untranslated region of *CpMYB10* gene were used in this expression analysis. To confirm specific amplification of *CpMYB10* cDNA, the amplification product was cloned and sequenced. As shown in Figure 1, A and B, *CpMYB10* is induced upon desiccation in leaves and roots, respectively. No expression could be detected in unstressed organs. Since detached leaves were used for these experiments, the absence of expression also suggests that *CpMYB10* is not induced by wounding. Both in leaves and roots the *CpMYB10* transcript is detected at significant levels 15

Figure 1. Expression of *CpMYB10* is regulated by drought and ABA. A, Total RNA was isolated from dehydrated *Craterostigma* leaves at the indicated times and the expression of *CpMYB10* was determined by coupled RT and PCR. The PCR product of *Cptkt3* gene was included as a cDNA loading control. B, RNA from dehydrated roots. C, RNA from leaves treated with 100 μ M ABA. D, RNA from roots treated with 100 μ M ABA. Specific primers were used to amplify *CpMYB10* and *Cptkt3* gene transcripts. The lengths of the PCR products are 1,117 and 733 bp, respectively. The linear phase of the exponential PCR reaction was corroborated for each gene (data not shown). A representative experiment from three biologically independent experiments is shown.



min after dehydration suggesting that its expression began earlier. In leaves, *CpMYB10* reaches its maximum level at about 15 min, sharply declining thereafter and is absent 48 h after stress treatment began (Fig. 1A). The *CpTkt3* gene that encodes a transketolase (Bernacchia et al., 1995) was used as a control for constitutive expression.

In desiccated roots, *CpMYB10* has a biphasic expression pattern where a first transcript peak is observed from 15 to 30 min after stress was initiated and drastically declining after 1 h (Fig. 1B). A second burst of *CpMYB10* expression was observed 16 h after desiccation treatment at similarly high levels as the first peak, and it is maintained during the 48 h of the experimental time course. To determine if *CpMYB10* expression is induced by ABA, fully hydrated plants were treated with 100 μ M ABA at same time points as above (Fig. 1, C and D). In contrast to desiccation treatment, ABA switches on *CpMYB10* at constant levels both in leaves and roots after 15 min of treatment and continues up through 48 h. These two sets of experiments show that *CpMYB10* is induced by desiccation and ABA, although its differential expression pattern upon ABA treatment suggests that the endogenous and exogenous ABA signals are sensed differently during the desiccation treatment.

To test whether *CpMYB10* expression depends on de novo protein synthesis, fully hydrated *Craterostigma* leaves were incubated in the presence of the protein synthesis inhibitor cycloheximide (CHX) and/or ABA at different time points. The LEA *Cp11-24* gene was used as a control of ABA treatment (Velasco et al., 1998). As shown in Figure 2A, CHX treatment blocked completely *Cp11-24* gene expression, suggesting its dependence on de novo protein synthesis. Surprisingly, *CpMYB10* expression was not inhibited but rather induced with CHX treatment (Fig. 2A) at relatively higher levels after comparison to its induction by ABA (Fig. 2B). Control experiments showed that ABA induced expression of *CpMYB10* was present 15 min after treatment, whereas *Cp11-24* was not detected until 1 h after ABA exposure (Fig. 2B). This delay in *Cp11-24* expression may be due to the fact that it requires de novo protein synthesis, as suggested by Figure 2A. Incubation of *Craterostigma* leaves in the presence of both CHX and ABA resulted in higher levels of *CpMYB10* expression than in ABA alone, whereas LEA *Cp11-24* transcription was restored although only after 16 h of ABA treatment (Fig. 2C). These results showed that *CpMYB10* does not require prior protein synthesis for its expression, instead inhibition of translation triggers *CpMYB10* transcription, suggesting that somehow it is repressed under unstressed conditions.

***CpMYB10* Protein Binds to *Cp11-24* and *CpMYB10* Promoters**

To study the DNA-binding properties of *CpMYB10* protein, its cDNA was expressed in *Escherichia coli* as

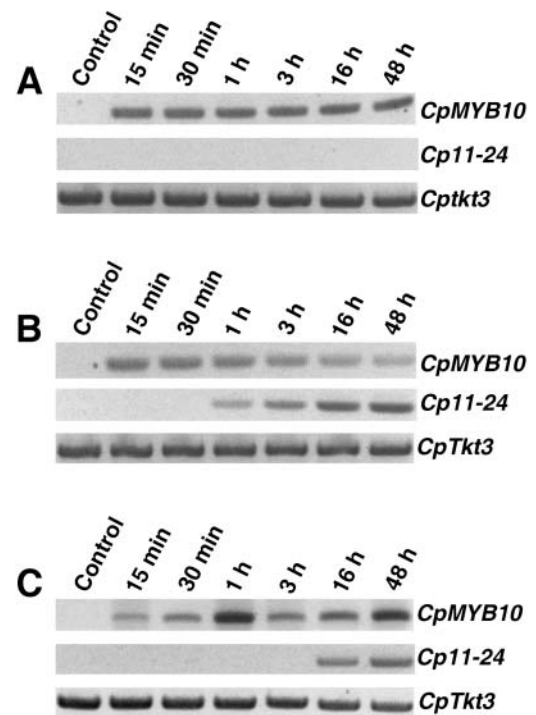


Figure 2. Expression of *CpMYB10* in the presence of cycloheximide. A, Total RNA was isolated from *Craterostigma* leaves treated with 10 μ M cycloheximide at the indicated times and the expression of *CpMYB10* was determined by coupled RT and PCR. The *Craterostigma* LEA gene *Cp11-24* was included in the experiment as a control of ABA treatment, whereas *CpTkt3* was used as a cDNA loading control. B, *Craterostigma* leaves were treated with 100 μ M ABA. C, *Craterostigma* leaves were treated with 100 μ M ABA and 10 μ M cycloheximide. Specific primers were used to amplify *CpMYB10*, *Cp11-24*, and *CpTkt3* gene transcripts. The lengths of the PCR products are 1,117, 629, and 733 bp, respectively. The linear phase of the exponential PCR reaction was corroborated for each gene (data not shown). A representative experiment from three biologically independent experiments is shown.

a fusion protein to a hexapeptide of His residues. The purified recombinant protein was used to perform gel mobility shift assays with double-strand oligonucleotide probes 32 P labeled. These probes contained the MYB binding sequence found in the LEA *Cp11-24* (Velasco et al., 1998) and *CpMYB10* (Iturriaga et al., 1996) gene promoters of *Craterostigma*. The consensus DNA-binding recognition sequence of several plant MYB genes including *AtMYB2* has been defined as T/GAAGT/GA (Urao et al., 1993; Higo and Ugawa, 1999). Three TAACTG elements and a GAAGTA sequence were found in *CpMYB10* and *Cp11-24* promoter regions, respectively. Each DNA motif was included in either MYB-P10 or MYB-P11-24 oligonucleotide probes. To analyze the binding affinity of recombinant MYB protein (rCpMYB10) to MYB-P10 and MYB-P11-24 probes, the optimal concentration of required protein was determined (data not shown). Figure 3A shows binding of rMYB10 protein to MYB-P10 probe. To analyze the specificity of the DNA binding activity, a mutated form of MYB-P10 was used

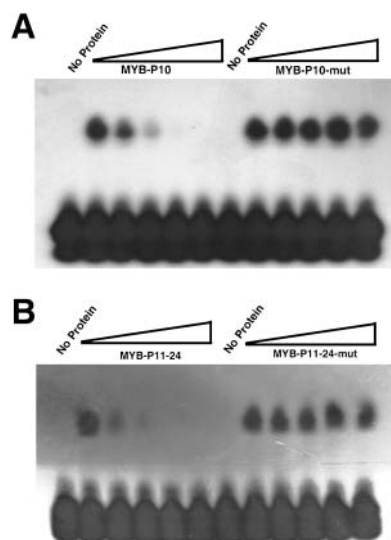


Figure 3. DNA-binding assays of rCpMYB10 protein. A, Purified recombinant rCpMYB10 protein expressed in *E. coli* was used in EMSA assays. Complementary 20-nucleotide length primers corresponding to the MYB-P10 DNA-binding region were used as 32 P-labeled probe. Unlabeled mutant (MYB-P10-mut) and unmodified versions of primers were used to compete radioactive probe. The concentrations used in the competition represented by the triangles were 0, 5, 25, 125, and 625 ng. B, EMSA assays using MYB-P11-24 DNA-binding region as a 32 P-labeled probe. Unlabeled mutant (MYB-P11-24-mut) probe was used for competition.

(MYB-P10-mut) as well as MYB-P10 as probes (Fig. 3A). The MYB-P10-mut oligonucleotide contained two point mutations in the MYB binding site (TCCCTG instead of TAACTG). In the electrophoretic mobility shift assay (EMSA), the DNA binding activity rMYB10 protein was reduced by the addition of excess unlabeled MYB-P10 but not by that of unlabeled MYB-P10-mut. Thus, rMYB10 protein bound to MYB-P10 but not to MYB-P10-mut. These results indicate that rMYB10 binds sequence specifically to the MYB-P10 oligonucleotide probe and suggests that CpMYB10 protein binds to its own promoter.

To further characterize the DNA binding properties of rCpMYB10 protein, it was incubated with MYB-P11-24 probe and used to perform an EMSA, as shown in Figure 3B. Unlabeled competitor MYB-P11-24 decreased rCpMYB10 protein binding activity, whereas a mutated form of MYB-P11-24, MYB-P11-24-mut (GCCCTA instead of GAACTA), was unable to compete for protein binding. Therefore, rCpMYB10 protein also binds specifically to MYB-P11-24 probe, suggesting that the LEA 11-24 might be a possible target gene.

Expression of *CpMYB10* Is Localized in Discrete Tissues in *Craterostigma*

The expression pattern of *CpMYB10* was further investigated by in situ RT-PCR analysis in *Craterostigma*. Leaf and root tissue sections obtained from

control or 100 μ M ABA pretreated plants were processed for in situ RT-PCR reactions, which yields high-resolution specific mRNA amplification signals in plant tissues (Xoconostle-Cázares et al., 1999). Red chlorophyll autofluorescence was superimposed with the green signal produced by the in situ RT-PCR reaction, leading to a yellow fluorescence (Fig. 4). In untreated leaves, *CpMYB10* amplification products were visible at low levels in epidermis, in some parenchyma cells, and in the vascular bundle central region (Fig. 4A). In contrast, PCR products in ABA treated leaves were detected at higher levels in epidermis, palisade, and spongy parenchyma, and vascular bundle and undetectable in trichoma (Fig. 4B). ABA treated roots showed a more discrete pattern of *CpMYB10* expression, limited to vascular cylinder and some isolated cortex cells (Fig. 4D). No fluorescent PCR products were detected in untreated roots (Fig. 4C). It was not possible to perform in situ RT-PCR assays from dehydrated plants since tissue sections were difficult to handle.

CpMYB10 Promoter Is Regulated in Transgenic Arabidopsis

More detailed expression patterns of *CpMYB10* under stress and ABA treatments were determined by histochemical β -glucuronidase (GUS) staining of Arabidopsis transgenic plants that harbored a *CpMYB10* promoter-GUS fusion construct. Three independent T3 homozygous (9.2, 11.1, and 13.5) lines with similar performance in response to ABA and dehydration were analyzed. In unstressed transgenic plants, GUS activity was detected in apical shoot meristem at low levels (Fig. 5A) and in pollen grains (Fig. 5B). After subjecting transgenic plants to 100 μ M ABA treatment, a strong staining was observed in all vegetative tissues (Fig. 5C), although staining was stronger in the subapical region of roots and no staining was visible in root tip (Fig. 5D). Dehydration treatment led to a more localized staining, mainly in leaf vascular tissues and conspicuously strong in apical shoot meristem, emerging leaves, and roots (Fig. 5E). In contrast to ABA treatment, dehydration led to a stronger GUS activity in the root tip (Fig. 5F). These results strongly suggest that in transgenic Arabidopsis the *CpMYB10* promoter is regulated by ABA and dehydration as in *Craterostigma*.

To have a quantitative analysis of *CpMYB10* promoter strength in Arabidopsis, GUS activity was determined by fluorimetric assays. Transgenic plants 3, 6, 15, and 21 d after germination were dehydrated, treated with 100 μ M ABA, or unstressed to measure GUS activity. No significant GUS activities were detected in untransformed Arabidopsis plants (dehydrated, ABA treated, or unstressed) at the developmental stages analyzed. GUS activity in unstressed transgenic plants exhibited similarly low levels at different ages, basal expression was around 10% to 30% of that observed for ABA or dehydrated plants of

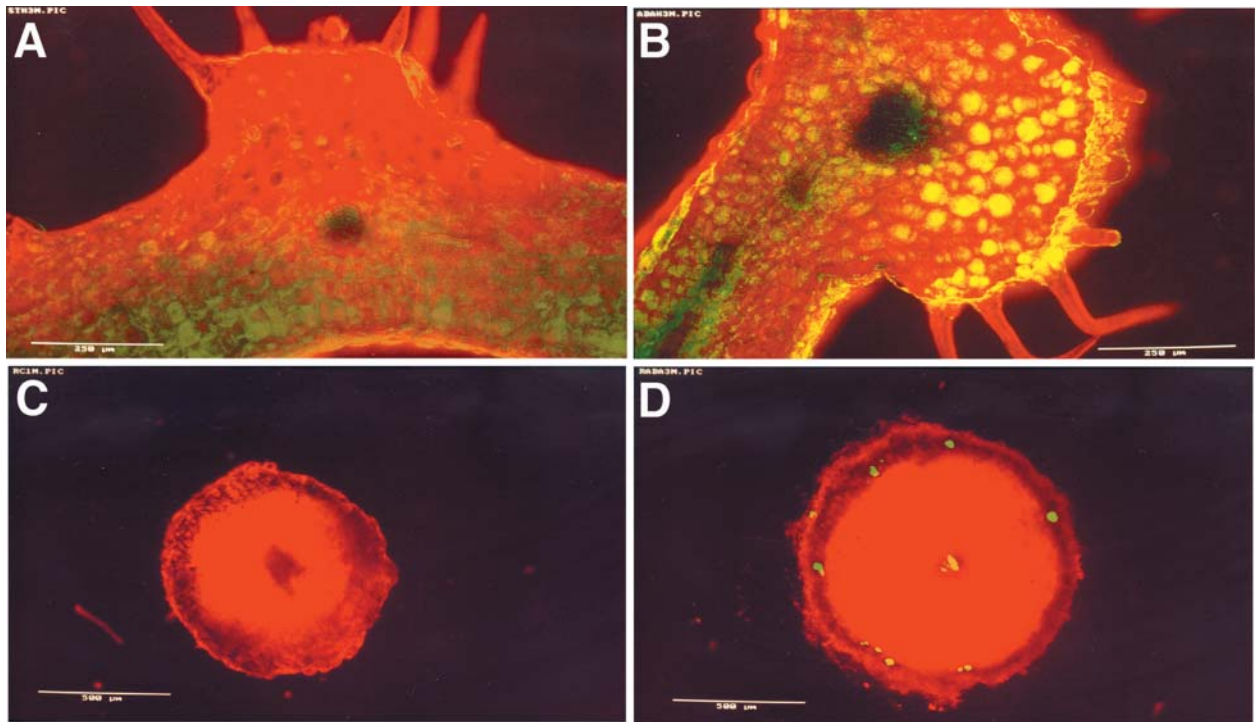


Figure 4. Tissue localization of *CpMYB10* expression in *Craterostigma*. A, Confocal laser scanning microscope images of transverse sections from fully hydrated *Craterostigma* leaves or B, treated with 100 μM ABA. C, Tissue sections of fully hydrated *Craterostigma* roots or D, treated with 100 μM ABA. The sections were processed for in situ coupled RT and PCR using specific *CpMYB10* primers. Overlaps of green and red fluorescence from the same field of view are presented. Bars = 500 μm . A representative experiment from at least three biologically independent experiments is shown.

3 to 21 d old. This basal β -glucuronidase activity is consistent with the histochemical GUS staining described above (Fig. 5A). As shown in Figure 5G, ABA treatment induced the highest levels of GUS activity, reaching a peak in 6-d-old plants. In 15-d-old plants, ABA induced GUS activity was 25% greater than that observed in dehydration stress. The maximum level of GUS activity induced by dehydration was seen in the leaves of 21-d-old plants. Only in roots were levels caused by dehydration greater than those of ABA treatment (Fig. 5G).

Ectopic Expression of *CpMYB10* Gene Confers Stress Tolerance in Arabidopsis

To investigate the in vivo function of *CpMYB10*, its cDNA was overexpressed in Arabidopsis using the 35S promoter. Thirty independent T2 lines, named 35S-*CpMYB10*, were recovered and checked by RT-PCR for *CpMYB10* expression and 10 T3 homozygous lines were corroborated by RNA gel blot (Fig. 6). Eight T3 homozygous lines showing transgene expression were used for stress tolerance tests. Representative lines 11.5, 17.1, 9.3, 22.5, and 7.6 with decreasing levels of *CpMYB10* transcript were further analyzed. Comparison of 35S-*CpMYB10* lines with wild-type plants showed no morphological alterations or growth re-

tardation except for a bulky root system in transgenic lines that was clearly visible in 3-week-old plants (data not shown). The germination rate of wild-type and 35S-*CpMYB10* lines was assayed in Murashige and Skoog (MS) (1962) media containing different concentrations of osmoticum compounds. Germination was defined as radicle emergence. The sharpest differences were observed in media containing 200 mM NaCl or 400 mM sorbitol (Fig. 7). After 2 d in 200 mM NaCl, 60% to 70% of 5 selected 35S-*CpMYB10* lines had already germinated compared to 30% of wild-type plants (Fig. 7A). In media containing 400 mM sorbitol, 90% of 35S-*CpMYB10* lines germinated in contrast to only 40% of wild-type seedlings (Fig. 7B). After 5 d, close to 100% and around 90% of transgenic and wild-type seedlings, respectively, germinated in both treatments. All lines germinated at the same rate in the absence of osmoticum compounds. These results showed a faster rate of germination of Arabidopsis lines overexpressing *CpMYB10* gene in osmotic stress conditions. In a further experiment, transgenic seeds were germinated media and grown for 4 weeks in high osmoticum media. As shown in Figure 8A, plants from the representative 11.5 line grew normally in 250 mM NaCl, whereas wild-type plants became chlorotic and died. In 500 mM sorbitol, transgenic line 11.5 grew poorly but better than wild-type plants (Fig. 8A).

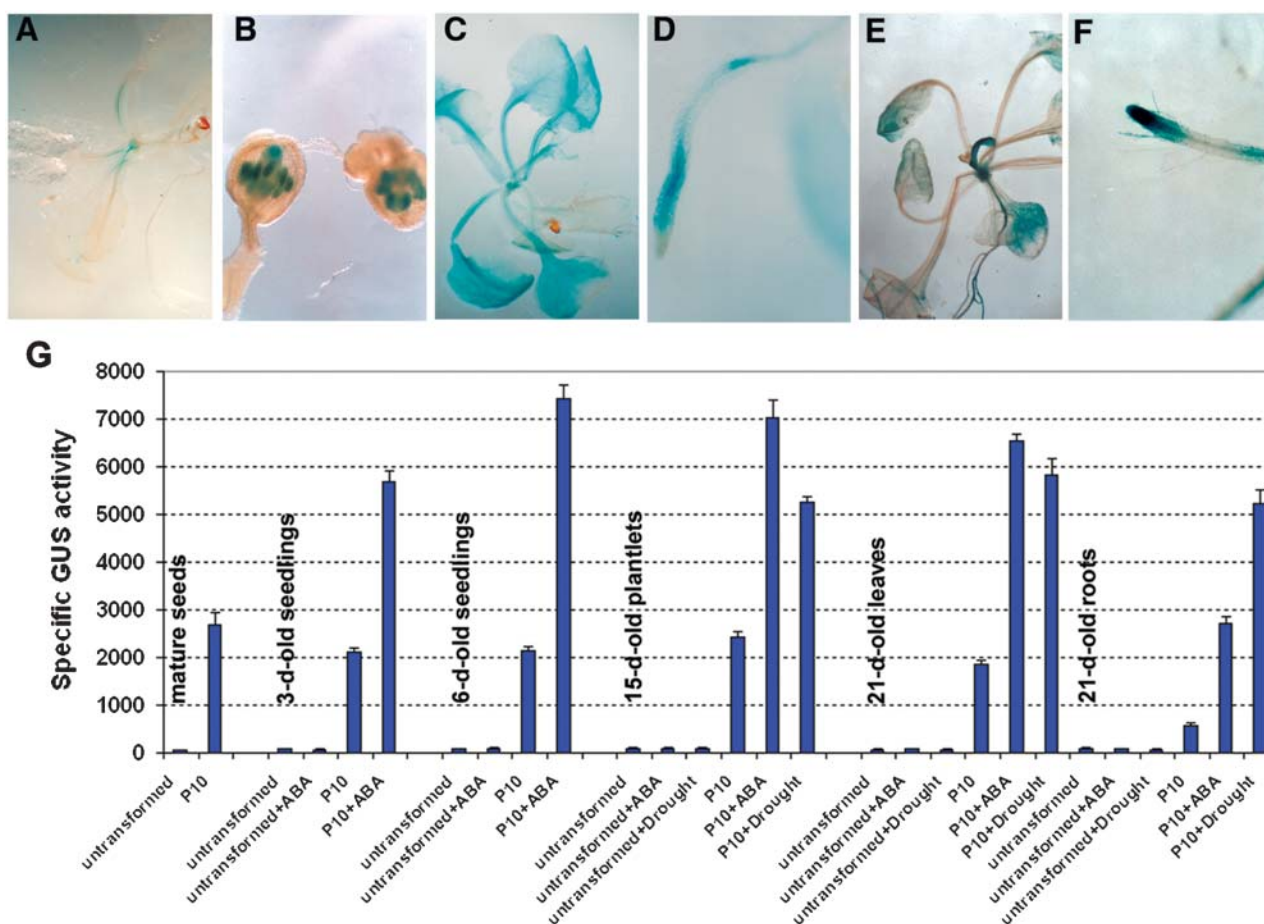


Figure 5. Expression pattern of *CpMYB10* in transgenic Arabidopsis. Three-week-old plants of a representative transgenic Arabidopsis line (9.2) carrying a 1.5-kb *CpMYB10* promoter-GUS construct were processed for histochemical GUS staining (A–F). β -Glucuronidase activity is visualized by the blue color. A, Unstressed plants. B, Anthers from unstressed plants. C, Plants treated with 100 μ M ABA. D, Roots from plants treated with 100 μ M ABA. E, Dehydrated plants. F, Roots from dehydrated plants. G, Quantification of β -Glucuronidase activity in mature seeds, and different developmental stages of untreated or 100- μ M ABA-treated seedlings for 16 h or seedlings subjected to dehydration. Treated and untreated untransformed plants were included as negative controls. The results are means of GUS activities from three independent experiments with a repetition in each experiment. Specific GUS activities are expressed as pmol of 4-methylumbelliferone per mg of total protein per min.

To assess if the overexpression of the *CpMYB10* gene conferred stress tolerance, drought and salt tolerance tests in adult plants grown in soil were performed. Plants from five selected 35S lines were grown for 4 weeks under fully watered conditions followed by 2 weeks of water deprivation. As shown in Figure 8B, most 35S-*CpMYB10* lines recovered water deprivation after rewatering, whereas wild-type plants did not survive this treatment. To test for salt-stress tolerance, transgenic plants overexpressing *CpMYB10* were grown with increasing concentrations of salt up to 250 mM NaCl. Figure 8C shows that transgenic plants grew well, whereas wild-type plants are wilted and chlorotic. Plants from transgenic lines under both treatments continued normal growth and set viable seeds. These results suggest that the overexpression of *CpMYB10* gene in Arabidopsis could be up-regulating genes involved in stress tolerance.

Altered Response to Glc and ABA in 35S-*CpMYB10* Plants

Several reports have documented that mutations in ABA-insensitive-4 (*ABI4*) transcription factor gene led to a Glc-insensitive phenotype (Arenas-Huertero et al., 2000) and displayed salt and osmotic stress tolerance during germination (Quesada et al., 2000), thus suggesting a cross-talk between signaling pathways for stress tolerance, ABA, and Glc. In an effort to further characterize our transgenic plants overexpressing *CpMYB10* and test their germination in response to Glc, seeds from 7.6, 9.3, 11.5, 17.1 and 22.5 *CpMYB10* transgenic lines were germinated in MS media supplemented with various concentrations of Glc and allowed to grow for 10 d. In Figure 9A it can be observed that MS medium supplemented with 6.5% or 7.5% Glc, or 2% Glc without MS salts, have a

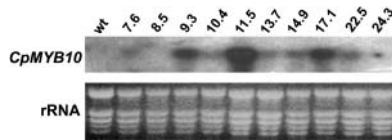


Figure 6. Analysis of *CpMYB10* expression in transgenic Arabidopsis. Northern blot from Col wild-type and several transgenic lines (line number on top of the figure) grown for 15 d on MS medium. Each lane contains 10 μ g of total RNA. The complete 1.1-kb *CpMYB10* cDNA was used as a probe. On bottom part of the figure rRNAs are shown as a loading control.

detrimental effect in development of wild-type seedlings, whereas no effect could be detected in the representative 11.5 transgenic line that showed cotyledon expansion and greening. A low concentration of Glc without MS salts was tested to discard a possible effect of nitrate on germination since it is known to antagonize Glc responses (Moore et al., 2003). Thus, germination of *CpMYB10* transgenic lines on 2% Glc without MS supports the observation that it is caused by Glc and it is not an osmotic effect. This Glc-insensitive phenomenon was further characterized by comparing the germination rate on different Glc concentrations, of wild-type and five selected 35S-*CpMYB10* transgenic lines, as shown in Figure 9B. The largest differences in germination rate were observed after 2 d on media containing 6.5% Glc, where more than 50% of transgenic lines initiated root emergence and elongation, whereas less than 5% of wild-type seedlings had germinated on the same conditions. On 7.5% Glc, germination rate of transgenic lines after 2 d was roughly 30%, whereas the germination rate in wild-type seedlings was near 5%. All these results indicated that overexpression of *CpMYB10* gene confers Glc insensitivity in Arabidopsis.

Glc analogs have been used to discriminate the sugar signaling pathway involved in specific responses such as germination, cotyledon expansion, and greening as well as gene expression. 2-deoxy-Glc (2-DG) has been shown to trigger a potent sugar response even at very low concentrations compared to Glc. For example, 2-DG strongly represses photosynthetic gene expression as well as photosynthetic efficiency (Jang and Sheen, 1994; Van Oosten et al., 1997) in an HXK mediated manner. To get insight into the sugar signaling pathway altered in *CpMYB10* overexpressing lines, we examined greening and expansion of cotyledons, known to be inhibited by a low 2-DG concentration (0.8 mM; Jang et al., 1997). As in the Glc assay, the 35S-*CpMYB10* lines were 2-DG hyposensitive and appeared green and had more expanded cotyledons compared to wild-type plants (Fig. 9A). The rest of the transgenic lines expressing *CpMYB10* showed similar phenotypes, although at different degrees, most likely due to variations in transgene expression. Additionally, the *ABI4* gene, involved in sugar and ABA signaling (Arenas-Huertero et al., 2000), has been shown to participate in the

HXK-dependent sugar signaling (Pego et al., 1999; Van Oosten et al., 1997); thus we included the *abi4* mutant in our sugar bioassays. Interestingly, this mutant also showed 2-DG hyposensitivity in this assay, confirming its participation in this sugar signaling pathway. Together, these data suggest that the *CpMYB10* overexpressing lines are affected at least in part in the hexose phosphorylation-dependent sugar signaling, and also indicate that Glc resistance of these lines can be separated from their osmotic resistance.

As mentioned above, there is strong evidence for a cross-talk of Glc and ABA signaling pathways (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). Therefore, we decided to investigate the ABA sensitivity of *CpMYB10* transgenic lines. As shown in Figure 10A, an ABA dose response curve in germination was conducted, which showed that 3 μ M ABA was sufficient to inhibit 35S-*CpMYB10* lines germination efficiency to 20% or less, whereas wild-type plants retained 50% germination under the same

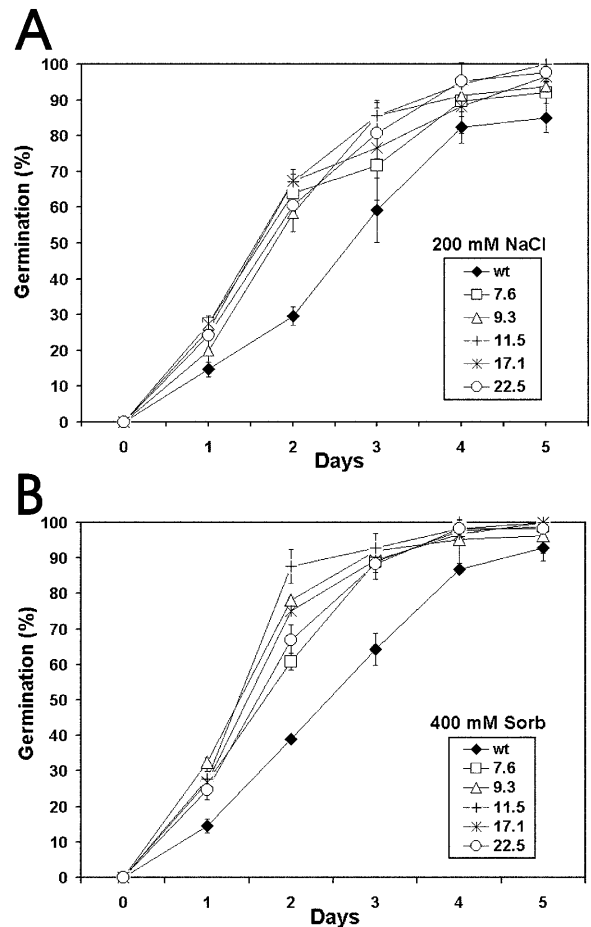


Figure 7. Kinetics of germination of 35S-*CpMYB10* lines. A, Growth of transgenic plants on MS media added with 200 mM NaCl. B, Growth of transgenic plants on MS media added with 400 mM sorbitol. The figure represents the average of 3 replicates of 100 seeds from wild-type and 5 independent transgenic (T3 generation) homozygous lines. Error bar represents sds. Germination was defined as complete protrusion of the radicle.

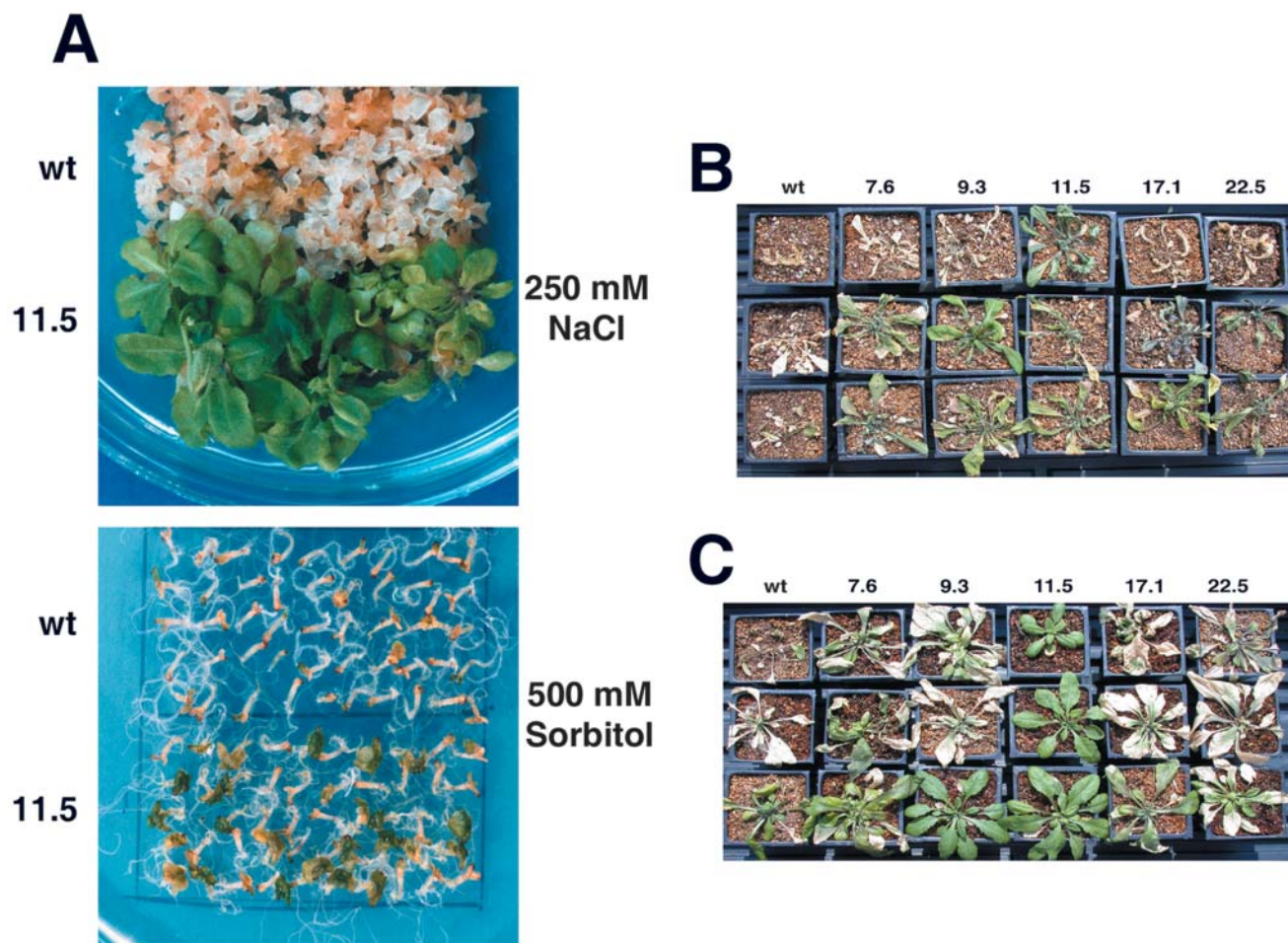


Figure 8. Salt and drought tolerance of *35S-CpMYB10* plants. A, Wild-type and 11.5 transgenic seeds were germinated on MS media containing 250 mM NaCl or 500 mM sorbitol during 4 weeks. B, Wild-type and five independent (T3 generation) homozygous lines expressing the *35S CpMYB10* transcript were withheld from water for 14 d and then rewatered before being photographed. C, Wild-type and five independent *35S-CpMYB10* lines were stressed with increasing concentrations of NaCl (50, 100, 150, 200, and 250 mM). Three plants per line are shown. See “Materials and Methods” section for details.

conditions. The line 11.5 showed 10% of germination rate at $3 \mu\text{M}$ ABA. Additional evidence was gathered by a germination kinetics experiment using $3 \mu\text{M}$ ABA, where selected lines of *CpMYB10* seeds had only about 10% germination after 1 d. In contrast, 50% germination was observed in wild-type seeds, as shown in Figure 10B. These results indicated that overexpression of *CpMYB10* gene in *Arabidopsis* conferred ABA hypersensitivity.

CpMYB10 Regulates Stress Related Genes in *Arabidopsis*

So far, we have shown that overexpression of *CpMYB10* confers osmotic stress tolerance, Glc insensitivity, and ABA hypersensitive phenotypes in *Arabidopsis*. Since *CpMYB10* is a transcription factor, it might be modulating the expression of genes involved in these processes. To test this hypothesis, gene expression analysis of possible regulated genes in 11.5 and 22.5 transgenic lines was carried out by RNA

gel blot (Fig. 11). We selected the following genes for expression analysis: *AtEM6* (Vicent et al., 2000) and *ERD10* (Kiyosue et al., 1994), which correspond to LEA group I and group II genes, respectively; dehydration- and ABA-responsive genes *RD29A*, *RD29B*, and *RD22* (Yamaguchi-Shinozaki and Shinozaki, 1993, 1994); cold-responsive gene *COR15a* (Baker et al., 1994), Δ^1 -pyrroline-5-carboxylate synthetase gene *P5CS1* (Yoshida et al., 1999), alcohol dehydrogenase gene *ADH1* (de Bruxelles et al., 1996), and transcription factor genes *AtMYB2* (Urao et al., 1993), *AtMYC2* (Abe et al., 1997), *DREB1A*, and *DREB2A* (Liu et al., 1998) as they are involved in stress response; hexokinase gene *HXK2* (Jang et al., 1997) and Suc synthase gene *SUS1* (Martin et al., 1993) for their role in sugar sensing and metabolism, respectively; and ABA-insensitive transcription factor genes *ABI4* (Finkelstein et al., 1998) and *ABI5* (Finkelstein and Lynch, 2000) and zeaxanthine epoxidase gene *ZEP1* (*Arabidopsis* Genome Initiative, 2000) involved in ABA signaling and

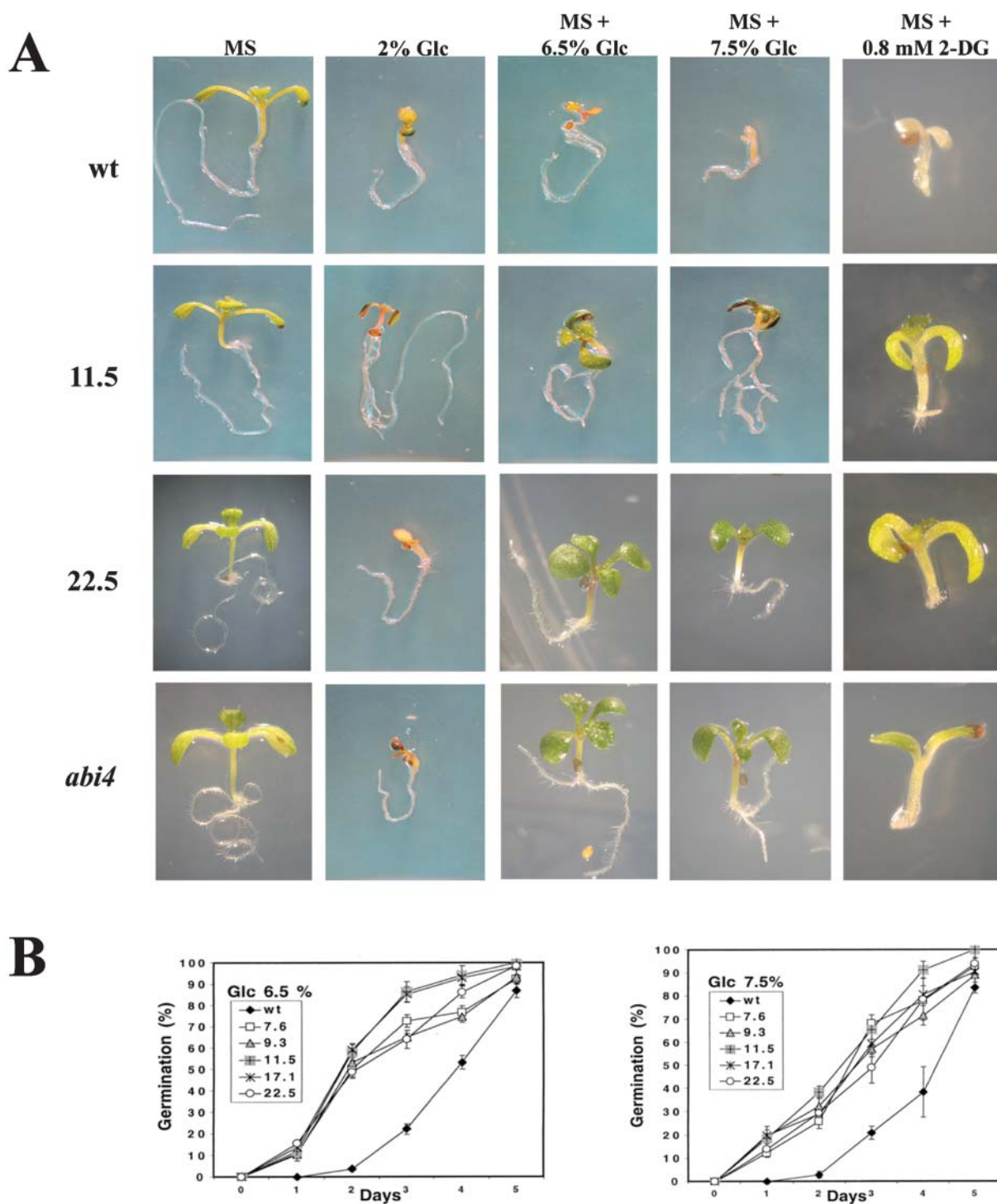


Figure 9. Glc sensitivity of 35S-CpMYB10 plants. A, Transgenic lines overexpressing CpMYB10 gene were germinated on MS salts added with 6.5 or 7.5% Glc, or 2% Glc without MS, or 0.8 mM 2-DG, or MS medium as a control, and growth for 10 d. Representative lines 11.5 and 22.5 are shown in comparison to wild type and *abi4* mutant. B, Germination of 35S-CpMYB10 transgenic lines in MS with 6.5% or 7.5% Glc. Error bar represents sds. The figure represents the average of 3 or more replicates of 100 seeds. Germination was defined as complete protrusion of the radicle.

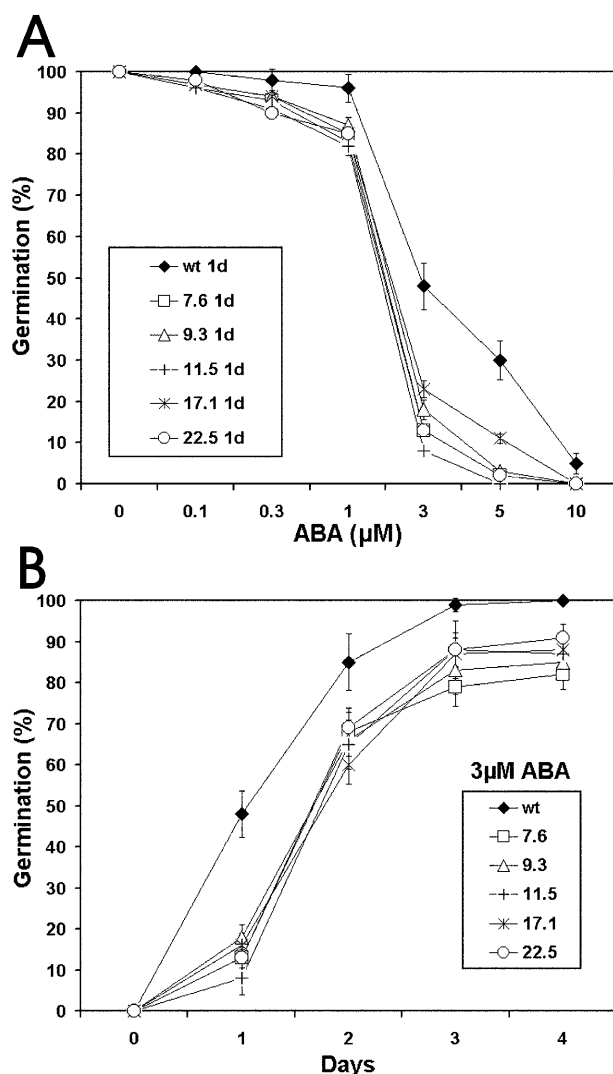


Figure 10. ABA sensitivity of *35S-CpMYB10* plants. A, ABA dose response in germination on MS media supplemented with 0.1, 0.3, 1, 3, 5, or 10 μM ABA. B, Kinetic of germination of the *CpMYB10* transgenic lines on MS containing 3 μM ABA. The data correspond to the average of 3 different experiments each containing 50 seeds per data point. Five independent transgenic (T3 generation) homozygous lines were used in the analysis. Germination was defined as complete protrusion of the radicle.

biosynthesis. Two-week-old wild-type, 11.5, and 22.5 transgenic lines were treated for 16 h with 7.5% Glc, 100 μM ABA, or untreated before RNA-gel blot analysis. It can be observed in Figure 11 that *RD29A*, *COR15a*, and *ADH1* genes are overexpressed in transgenic lines upon ABA treatment, whereas without treatment *RD22*, *COR15a*, and *P5CS1* are repressed in both 11.5 and 22.5 transgenics after comparison to wild-type plants. Under Glc treatment, *RD29A*, *RD22*, *P5CS1*, and *AtMYC2* transcripts were up-regulated only in 11.5 transgenic line. In the case of *ABI4* no differences in gene expression could be observed after comparison with wild-type plants except for a lower

level of induction upon Glc treatment in 11.5 line. Also, the *AtMYB2* gene was partially repressed upon ABA treatment in 11.5 transgenic plants. None of the other analyzed genes (*AtEM6*, *ERD10*, *RD29B*, *SUS1*, *H XK2*, *ABI5*, *ZEP1*, *DREB1A*, and *DREB2A*) were up- or down-regulated under the described conditions in both 11.5 and 22.5 transgenic lines (data not shown).

DISCUSSION

In this work, we analyzed the function of a MYB transcription factor gene, *CpMYB10*, from the resurrection plant *Craterostigma* using different techniques including overexpression in a heterologous back-

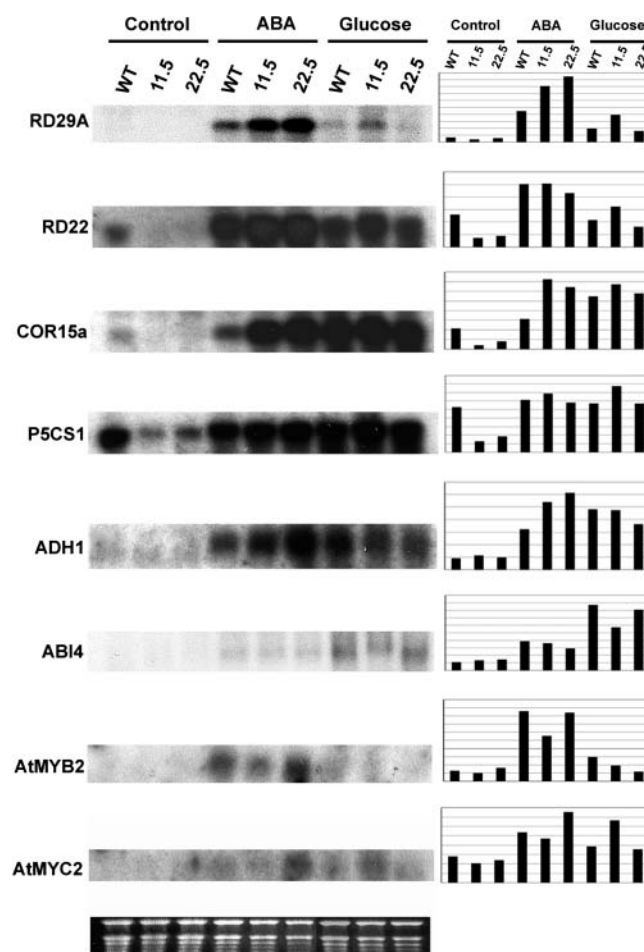


Figure 11. Expression of *CpMYB10* target gene mRNAs in *35S-CpMYB10* plants. RNA gel blotting was conducted to measure the amount of *RD29A*, *RD22*, *COR15a*, *P5CS1*, *ADH1*, *ABI4*, *AtMYB2*, and *AtMYC2* mRNA in transgenic Arabidopsis plants. Each lane was loaded with 10 μg of total RNA from 2-week-old plants grown in normal MS medium and transferred to MS plates containing the indicated compound 16 h before collecting tissue. Graphics show the densitometric quantification of shown bands after normalization against loaded RNA according to Image 1.61 software from NIH. Ethidium bromide-stained rRNAs were used as a loading control (bottom section).

ground. First of all, the expression pattern of the *CpMYB10* gene in *Craterostigma* was addressed by coupled RT and PCR since RNA blot experiments did not detect gene expression, suggesting that *CpMYB10* could be a low-abundance transcript. Here we showed that *CpMYB10* gene is induced by desiccation and ABA treatments in leaves and roots a few minutes after treatment began. In leaves, maximum *CpMYB10* expression was observed around 15 min after desiccation, and thereafter sharply declined. This early induction and rapid shut off suggests a key role of *CpMYB10* in gene activation for stress tolerance. In roots, a biphasic pattern of *CpMYB10* expression was observed, raising the question of whether the same or a different set of genes are transactivated at early (30 min) and late (16 h and thereafter) desiccation stages, which physiologically represent quite different water status levels. ABA turns on *CpMYB10* in leaves and roots also around 15 min after dehydration, maintaining the gene expression for as long as 48 h, the duration of the experiment. Upon dehydration, within the first 30 min, *Craterostigma* leaves have a relative water content of 90% and several genes are already being expressed (Bartels et al., 1990). In contrast, 16 h after dehydration the relative water content drops to only 20% and it goes down to 2.5% in plants desiccated for 48 h (Bartels et al., 1990). After 2 d, *Craterostigma* leaves have lost their chlorophyll, and ultrastructural changes are evident such as convoluted cell walls, shrunk organelles freely suspended in the cytosol, and loss of chloroplast grana (Schneider et al., 1993). Therefore, it is likely that two set of genes for early and late desiccation stages are transactivated by *CpMYB10* in *Craterostigma*.

The expression pattern of *CpMYB10* gene was consistent with its role in stress tolerance. Using *in situ* coupled RT and PCR analysis in *Craterostigma* tissue sections, *CpMYB10* was found in epidermis, palisade, and spongy parenchyma, and vascular bundle of ABA treated leaves, whereas in ABA treated roots *CpMYB10* expression was limited to the vascular cylinder and some isolated cortex cells. In untreated leaves, *CpMYB10* was faintly expressed in epidermis and in some parenchyma and vascular bundle cells and totally undetectable in roots. Therefore, *CpMYB10* is expressed at basal levels in unstressed leaves and shortly after stress its transcript increases dramatically in a translation-independent manner as mentioned above, suggesting a rapid posttranslational activation mechanism.

The promoter analysis of *CpMYB10* gene in *Arabidopsis* further supported its role in stress tolerance. These experiments showed *GUS* gene induction after dehydration in leaf vascular tissues and conspicuously strong expression in root tip, apical shoot meristem, and emerging leaves. Roots and root cap sense water deficit triggering ABA translocation from roots to shoots (Zhang et al., 1987), thus *CpMYB10* gene might play a major role on this process. Treatment with ABA led to a strong *GUS* staining in all vegetative tissues,

although no staining was visible in root tip. Quantification of *GUS* activity showed that although ABA treatment induced the highest levels of *GUS* at the whole plant level, in roots it was induced 2-fold higher upon dehydration. Also, *CpMYB10* promoter induction by ABA reached its maximum in 6-d-old plants, whereas upon dehydration *GUS* activity peaked in 21-d-old plants. On the other hand, in unstressed plants *GUS* activity was detected at low levels in apical shoot meristem and in pollen grains. Expression of *CpMYB10* promoter in mature pollen grains probably reflects that this tissue is developmentally programmed for desiccation tolerance and hence prone to regulate genes responsive to stress. In addition, since 5' untranslated region leader sequence was present in the construct, posttranscriptional regulatory events cannot be excluded. Other regions, such as intergenic or 3' untranslated region, could be also required for regulation.

The use of protein synthesis inhibitors in transcription analysis in animal and plant cells allowed characterization of genes whose induction is stimulated in the absence of protein synthesis as primary response genes and many of them correspond to transcription factor genes (Herschman, 1991; Fujimoto et al., 2000). Those genes whose induction requires protein synthesis are termed secondary response genes. When unstressed tissues are pretreated with cycloheximide (CHX), *CpMYB10* was highly expressed but not the *LEA* encoding-gene *Cp11-24*, suggesting that the former gene is independent on prior protein synthesis for its expression. The induction of *CpMYB10* expression in the presence of CHX suggests that translation of a putative repressor protein that could act on its promoter is inhibited, thus releasing *CpMYB10* transcription. Alternatively, it could indicate protection of *CpMYB10* mRNA from degradation by inhibition of labile mRNases. Similar experimental criteria using CHX has been widely used to characterize several plant genes as primary responsive to stimuli (Koshiba et al., 1995; Fujimoto et al., 2000; Bouquin et al., 2001; Laskowski et al., 2002).

A role of *CpMYB10* as a transcription factor was elucidated after analyzing the DNA binding properties of a recombinant *CpMYB10* protein by using EMSA assays. Our results showed that rCpMYB10 protein has DNA binding recognition for two specific MYB motifs, namely TAACTG and GAACTA sequences present in *CpMYB10* and *Cp11-24* promoter regions, respectively. The TAACTG sequence is also the recognition site of AtMYB2 protein (Urao et al., 1993). Binding of rCpMYB10 protein to *Cp11-24* promoter element suggests that a possible target gene of *CpMYB10* transcription factor is the *Cp11-24* gene which has been detected 30 min after dehydration of *Craterostigma* leaves (Bartels et al., 1990). The *Cp11-24* gene encodes a LEA (late-embryogenesis abundant) protein which is among other proteins involved in stress tolerance (Ingram and Bartels, 1996). rCpMYB10 protein also binds to a MYB responsive element

present in its own promoter. The binding of rCpMYB10 to its own promoter could be indicating a possible autoregulation of *CpMYB10* gene. This mechanism has been reported in other plant genes (Schwarz-Sommer et al., 1992; Finkelstein and Lynch, 2000), although it cannot be ruled out that another MYB homolog might regulate *CpMYB10* promoter. Although *CpMYB10* encoded sequence has three potential phosphorylation sites (Iturriaga et al., 1996), apparently no such protein modification is required for DNA binding in vitro.

A major finding of the present study was that overexpression of *CpMYB10* in Arabidopsis using the 35S promoter confers both osmotic stress tolerance of transgenic seedlings germinated in tissue culture and desiccation and salt tolerance in adult plants grown in soil. Other reports have shown that overexpression of transcription factor genes improves stress tolerance. Freezing tolerance was shown in Arabidopsis overexpressing *CBF1* (Jaglo-Ottosen et al., 1998). Plants overexpressing *DREB1A* gene improved their freezing, drought, and salt stress tolerance, although constitutive expression of this gene led to growth retardation, whereas minimal effects were observed when a stress-inducible promoter was used (Kasuga et al., 1999). On the other hand, except for a bulky root system, no other morphological or growth alterations in all transgenic lines overexpressing *CpMYB10* compared to wild-type plants were observed, suggesting that *CpMYB10* may also play an important role in promoting root growth and differentiation. In contrast, overexpression of *AtMYB2* in Arabidopsis showed a dwarf phenotype and no stress tolerance was reported, only coexpression of both *AtMYB2* and *AtMYC2* conferred moderate stress tolerance (measured in an electrolyte leakage test in the presence of mannitol) and plants exhibited severe growth retardation as well (Abe et al., 2003). In contrast, in this study it was found that overexpression of the *CpMYB10* gene alone led to an improved stress tolerance to severe drought and salt conditions in planta.

An important question was to determine which genes are responsible for the stress tolerance phenotype in Arabidopsis overexpressing the *CpMYB10* gene. In an effort to find downstream regulated genes by *CpMYB10* in Arabidopsis, we analyzed the expression pattern of 17 genes involved in either stress tolerance, sugar sensing and metabolism, or ABA signaling and biosynthesis by RNA gel blot. Only 8 of these genes showed altered gene expression in plants overexpressing *CpMYB10*. Two genes encoding hydrophilic proteins, *RD29A* and *Cor15A*, and the alcohol dehydrogenase *ADH1* gene were up-regulated in 35S-*CpMYB10* plants upon ABA treatment. Overexpression of *DREB1A* or *CBF1* transcription factor genes in Arabidopsis led to up-regulation of several hydrophilic protein genes including *RD29A* and *COR15a* (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). In contrast, the overexpression of *AtMYB2* in Arabidopsis only activates *ADH1*, although coexpression of

both *AtMYB2* and *AtMYC2* up-regulates several other genes, including *RD22*, *COR6.6*, and *SUS1* (Abe et al., 2003). We also found that *RD22*, *Cor15a*, and *P5CS1* are down-regulated in 35S-*CpMYB10* plants without stress but not under ABA or Glc treatments, suggesting that *CpMYB10* might be acting on these genes as a repressor and activator. A transcriptomics profile would reveal other target genes of *CpMYB10* in Arabidopsis. Noteworthy is the presence of multiple plant *myb* binding sites in the 1-kb upstream sequence of the putative promoter region of all the putative target genes analyzed in this study, as revealed by PLACE computer analysis program (Higo and Ugawa, 1999), supporting a possible transactivation by *CpMYB10* upon them.

Taking together these results with the EMSA analysis, it is tempting to speculate whether this dual role is also present in *Craterostigma*, *CpMYB10* acting as a repressor in unstressed tissues and as an activator upon dehydration. For instance, it could be involved in maintaining *CpMYB10* gene shut off in unstressed conditions, and could also activate *Cp11-24* and other target genes upon dehydration. This dual mechanism could allow a fast down-regulation of target genes responsive to abiotic stress. This shift in activity could well be modulated by a kinase or phosphatase in order to rapidly activate gene response for adapting cells to abiotic stress.

Besides having an essential function in plant metabolism, sugars play a role in regulating developmental and physiological processes such as photosynthesis, photomorphogenesis, flowering, and germination (Smeekens, 2000). The isolation of Arabidopsis mutants exhibiting normal development and greening when germinated on high Glc concentrations has been reported. The molecular identity of some mutants with a similar phenotype corresponded to the *ABI4* and *ABI5* transcription factors required for ABA signaling, suggesting a link between sugar- and ABA-signaling pathways (Arenas-Huertero et al., 2000; Finkelstein and Lynch, 2000). The molecular mechanisms by which *abi4* and *abi5* mutants affect sugar and stress responses are currently unknown. In this work it was found that overexpression of *CpMYB10* gene in Arabidopsis conferred insensitivity to high Glc concentrations and also ABA hypersensitive response. To correlate these phenotypes with a possible altered expression pattern of well-characterized genes involved in Glc and ABA responses, the transcription of *ABI4*, *ABI5*, and *HXX2* genes was analyzed. *ABI5* and *HXX2* transcription pattern was not modified; only *ABI4* was partially repressed in 11.5 line but not in 22.5 under Glc treatment. Overexpression of *AtMYB2* also conferred ABA hypersensitive response but no Glc response was reported (Abe et al., 2003).

The use of 2-DG analog allows us to suggest that these transgenic lines are affected at least in part in the hexose phosphorylation-dependent sugar signaling, commonly interpreted as the HXK-dependent pathway. These data correlate with the *abi4* phenotype

observed in presence of this Glc analog, which has been demonstrated to participate in the HXK-dependent sugar signaling (Pego et al., 1999). In fact, *sun6* mutant, an *abi4* allele, was shown to be less sensitive to photosynthesis inhibition by 2-DG than wild-type plants in adult stages (Van Oosten et al., 1997), further supporting our observations at earlier developmental stages. However, interpretations based on the use of Glc analogs must be taken carefully as no specific studies about their metabolism and effects on different tissues and plant species have been performed (Gibson, 2000). A direct link between the sugar response and stress resistance of the transgenic lines expressing *CpMYB10* is still difficult to establish. Data about the molecular bases for both phenotypes in these transgenics remains to be determined in the future. However, osmotolerant phenotypes on sugar response mutants have been reported (Gibson, 2000), which correlates with our results.

In summary, in this study it is shown that a *MYB* homolog (*CpMYB10*) from the resurrection plant *Craterostigma plantagineum* is induced by dehydration and ABA treatments in leaves and roots and in transgenic Arabidopsis as well. The expression of *CpMYB10* is induced early and could be activated by CHX treatment alone, suggesting its regulation by unknown and short-lived repressor or RNase. *CpMYB10* might regulate its own promoter and transactivate the LEA *Cp11-24* gene. We propose that this *MYB* gene might account in part for the differences between *Craterostigma* and Arabidopsis regarding drought tolerance. This is substantiated by the ectopic expression of *CpMYB10* in Arabidopsis that confers salt and drought tolerance and led to an altered expression of several stress-responsive genes. Additionally, overexpression of *CpMYB10* in Arabidopsis led to ABA hypersensitive and Glc insensitive phenotypes by an unknown mechanism. This is the first report of a transgenic plant with improved stress tolerance using a gene from a resurrection plant. Additionally, heterologous expression of *CpMYB10* transcription factor gene represents a potential approach to improve stress tolerance in crops avoiding endogenous mechanisms that often cosuppress the transgene of interest.

MATERIALS AND METHODS

Plant Growth Conditions and Stress Treatments

Craterostigma plantagineum Hochst. and Arabidopsis ecotype Columbia (wild type or transgenic) were grown at 24°C/20°C with 16 h light/8 h dark cycle on sterile MS medium supplemented with 2.0% and 1.0% Suc, respectively, and solidified with 0.8% phytoagar. The carbon source was as mentioned, unless another is indicated. To break dormancy, seeds were incubated at 4°C for 4 d before germination. Fully grown *Craterostigma* plants (before flowering) were used to perform dehydration and ABA treatments. For *Craterostigma* dehydration experiments the plants were placed on filter paper in a growth chamber under described conditions. For ABA treatment, *Craterostigma* plants were submerged in a solution containing 100 μ M ABA (Sigma-Aldrich, St. Louis). For dehydration treatment of Arabidopsis, 4-week-old plants grown on a 1:1:1 mixture of vermiculite, perlite, and peat moss were used. At week 5, watering was stopped for 2 weeks and then rewatered, and

allowed to grow one more week before being photographed. For salt stress treatment in pots, 4-week-old Arabidopsis plants were watered every 4 d with increasing concentrations of NaCl, starting from 50 mM, 100 mM, 150 mM, and 200 mM, and twice with 250 mM. For salt, sugar, ABA, and osmotic stress treatments performed in plates, the wild-type and transgenic Arabidopsis seeds were germinated in MS media containing the indicated salt, Glc, 2-DG analog, or osmotic agent concentration. For treatments in GUS experiments, 3-week-old plants were transferred from standard MS medium to plates containing the indicated compounds.

RT-PCR Expression Analysis

RT-PCR experiments were performed using 5 μ g of total RNA extracted from *Craterostigma* by standard procedures and used for first strand cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen, Gaithersburg, MD) and oligo(dT). PCR program consisted of 25 to 40 cycles of amplification (1 min, 95°C; 30 s, 52°C; and 2 min, 72°C) using Taq polymerase (Roche Diagnostics, Indianapolis) and sequence-specific primers for each gene. The specific oligonucleotides designed to amplify the cDNA corresponding to *CpMYB10* have the following sequence: primer *CpMYB10*-sense 5'-AGGCAT-CAGCTTTTCTT-3', and *CpMYB10*-antisense 5'-ATGGTACGTCCTTGATT-3'. The expected 1.1-kb PCR product was cloned and sequenced, and corresponded to the *CpMYB10* cDNA after comparison with the corresponding genomic clone (Iturriaga et al., 1996). Specific oligonucleotides to amplify *Cp11-24* (forward 5'-GAAGTTTCGATGCTAACGA-3', reverse 5'-TGCTCATC-CGCAGCAGCAGC-3') and *Cptk3* (forward 5'-TGGATGGGAAAAAGCTC-3', reverse 5'-AAACAACCCTCACTCCC-3') gene transcripts of *Craterostigma* yielded 629- and 733-bp fragments, respectively. Each RT-PCR result was confirmed in three biologically independent experiments. Aliquots at different cycles of PCR reactions were analyzed to select for the linearity phase of the exponential PCR reaction. RT-PCR products were resolved in 1 $\times$ Tris-acetate EDTA, 1% agarose gels stained with ethidium bromide. The figures present the negatives of the fluorescent images.

Purification of Recombinant *CpMYB10* Protein

To express a hexahistidine-*CpMYB10* fusion protein in bacteria, the *CpMYB10* cDNA was amplified by PCR using *Bgl*II-CPM10.5 (5'-GAAGATC-TATGAACCAACAGCAGGTGA-3') and CPM10.3-*Kpn*I (5'-GGGGTACCTT-CGTATATCTAAAAGCAGC-3') primers and cloned in the pQE30 vector (Quiagen, Valencia, CA) on *Bam*HI and *Kpn*I restriction sites. DNA fusion was sequenced to confirm the in-frame cloning and used to transform BL21 *Escherichia coli* strain. Protein expression was achieved by adding 1 mM isopropylthio- β -galactoside to bacterial cultures and let grown for 2 h at 37°C. The soluble protein was purified according to manufacturer's instructions by affinity chromatography in Ni-NTA resin (Quiagen) and concentrated by Centricon-10 centrifugal concentrators (Amicon, Billerica, MA), before diluting in 3 mL of binding buffer (15 mM HEPES, 8 mM Tris, 120 mM KCl, 0.14 mM EDTA, 7 mM β -ME, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol). The protein concentration was determined with Bradford reagent (Bio-Rad Laboratories, Hercules, CA) and checked by SDS-PAGE.

EMSA

The EMSA protocol was essentially as previously described by Armstrong et al. (1992). The double-strand complementary oligonucleotides used as probes were radioactive labeled using α -³²P dATP and α -³²P dCTP and Klenow enzyme (Roche Diagnostics), and diluted to 1 ng/ μ L containing at least 50,000 cpm. For each reaction, around 100 ng of pure recombinant protein was used, 1.0 μ g of salmon sperm DNA, and 1 μ L milk powder suspension (20 mg/mL low fat milk powder). The reaction mixture was loaded on a native polyacrylamide gel (4% acrylamide, 0.1% N,N'-methylenebisacrylamide, 0.2 $\times$ Tris-acetate EDTA, 7% glycerol, 0.06% ammonium persulfate, and 0.06% N,N,N',N'-tetramethylethylenediamine). The gel was prerun at 100 V for 1 h at room temperature and run at 150 V for 3 to 4 h at 4°C. After electrophoresis, the gel was dried and exposed to x-ray film. The primer sequences used were: MYB-P10 forward 5'-GTGTATATAGTTAACTG-AAACTGC-3', MYB-P10 reverse 5'-GTGTGCAGTTTCAGTTAACTATAT-3', MYB-P10-mut forward 5'-GTGTATATAGTTCCCTGAACTGC-3', MYB-P10-mut reverse 5'-GTGTGCAGTTTCAGGGAATATAT-3', MYB11-24 forward 5'-GTGTACAGAGGTGAACCTACCGAATC-3', MYB11-24 reverse 5'-GTGT-GATTCCGGTAGTTACCTCTGT-3', MYB11-24-mut forward 5'-GTGTACA-

GAGGTGCCCTACGAATC-3', MYB11-24-mut reverse 5'-GTGTGATTCGG-TAGGGCACCTCTGT-3'.

RT-PCR in Situ

Transversal leaf or root hand-cut sections (100–200 μm) of *Craterostigma* plants pretreated with 100 μM ABA for 1 h were tested for RT-PCR in situ technique (Xoconostle-Cázares et al., 1999) using Oregon Green 488-5-dUTP as fluorescent label (Molecular Probes, Eugene, OR) and GeneAmp EZ rTth RNA PCR kit (Perkin Elmer, San Jose, CA). The primers used in the amplification were the same as described before for *CpMYB10* cDNA in the expression analysis section. The fluorescent signal was visualized in a confocal laser scanning microscope.

Plant Transformation

Two binary vector constructs were used to transform *Arabidopsis*. For the overexpression of *CpMYB10*, its 1.1-kb cDNA was amplified by RT-PCR with primers *CpMYB10*-sense and *CpMYB10*-antisense, cloned in pBluescript SK[−] (Stratagene, La Jolla, CA) and the DNA sequence was determined before subcloning in pBin19 vector (Bevan, 1984) containing the 0.8-kb 35S-promoter and 0.3-kb NOS polyadenylation site. This construct was introduced by electroporation in *Agrobacterium tumefaciens* C58C1 strain containing the pGV2260 plasmid. The second construct consisted of the 1.5 kb *CpMYB10* promoter region (P10) fused to the *uidA* (*GUS*) gene (Jefferson, 1987). The promoter fragment was amplified by PCR using Expand High Fidelity PCR System (Roche) using as a template the genomic CPM10 clone (Iturriaga et al., 1996), and primers MVP.5 (5'-GGGGTACCTATATGGCTAGGATAGGAT-AC-3') and MVP.3 (5'-CGAATTCATGTCTGTATTTTCTTCTTCC-3'), cloned in pBluescript SK⁺ and sequenced. A translational fusion between the first putative ATG of *CpMYB10* and the first codon of *GUS* gene was created before subcloning in pBin19 vector. This construct was introduced by electroporation in *A. tumefaciens* C58C1 strain. The resulting bacteria were used to transform wild-type *Arabidopsis* (Col) by in planta vacuum infiltration (Bechtold et al., 1993). Seedlings were grown on MS medium supplemented with 1% Suc and for selection of transgenic plants 50 $\mu\text{g mL}^{-1}$ kanamycin (Sigma-Aldrich) was added to the medium. One-week-old plantlets were transferred to pots under described conditions until plants formed seeds. To select homozygous lines, T2 generation seeds were analyzed for germination on kanamycin. Only T3 plants with a 3:1 segregation ratio were used. Ten independent homozygous lines for 35S-*CpMYB10* and three for P10-*GUS* construct were isolated.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

GUS Histochemical and Fluorimetric Assays

The histochemical staining of GUS activity in transgenic plants was performed as described by Jefferson et al. (1987). Seedlings were fixed in a 0.3 (w/v) formaldehyde solution containing 10 mM MES, pH 7.5, and 0.3 M mannitol for 30 min on ice prior to staining. Stained samples were rinsed extensively in 70% ethanol to remove chlorophyll. The stained plants were analyzed using a bright-field microscopy (Type 104, Nikon, Tokyo). Crude plant extracts for fluorimetric assay of GUS enzyme activity were prepared with an extraction buffer consisting of 50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% (w/v) Triton X-100, 0.1% (w/v) sodium lauryl sarcosine, and 10 mM β -mercaptoethanol. GUS activity was assayed as described by Jefferson et al. (1987) with 4-methylumbelliferyl- β -D-glucuronide (Sigma-Aldrich) as a substrate.

Gel Blot Analysis

Total RNA was isolated (Ausubel et al., 1989) from seedlings grown as indicated using standard protocols. For northern blots, total RNA was fractionated by electrophoresis in 1.2% (w/v) agarose gels and transferred onto Hybond N+ nylon membrane (Amersham, Buckinghamshire, UK).

Hybridizations and washes were performed at high stringency conditions according to standard procedures, using ³²P-radiolabeled probes. Probes for all the used genes were obtained by PCR using specific primers and cDNAs prepared from ABA-treated *Arabidopsis* plants. The graphic representation of densitometric quantification of the northern experiments were done using the public domain NIH Image program (developed at the United States National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image>) and normalized in each case by the amount of rRNA in the gel.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AF510112 for the *CpMYB10* cDNA.

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