ORIGINAL ARTICLE

Cell Research (2008) 18:1047-1060.
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Soybean *GmMYB76*, *GmMYB92*, and *GmMYB177* genes confer stress tolerance in transgenic *Arabidopsis* plants

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MYB-type transcription factors contain the conserved MYB DNA-binding domain of approximately 50 amino acids and are involved in the regulation of many aspects of plant growth, development, metabolism and stress responses. From soybean plants, we identified 156 *GmMYB* genes using our previously obtained 206 *MYB* unigenes, and 48 were found to have full-length open-reading frames. Expressions of all these identified genes were examined, and we found that expressions of 43 genes were changed upon treatment with ABA, salt, drought and/or cold stress. Three *GmMYB* genes, *GmMYB76*, *GmMYB92* and *GmMYB177*, were chosen for further analysis. Using the yeast assay system, GmMYB76 and GmMYB92 were found to have transactivation activity and can form homodimers. GmMYB177 did not appear to have transactivation activity but can form heterodimers with GmMYB76. Yeast one-hybrid assay revealed that all the three GmMYBs could bind to *cis*-elements TAT AAC GGT TTT TT and CCG GAA AAA AGG AT, but with different affinity, and GmMYB92 could also bind to TCT CAC CTA CC. The transgenic Arabidopsis plants overexpressing *GmMYB76* or *GmMYB177* showed better performance than the *GmMYB92*-transgenic plants in salt and freezing tolerance. However, these transgenic plants exhibited reduced sensitivity to ABA treatment at germination stage in comparison with the wild-type plants. The three *GmMYB* genes differentially affected a subset of stress-responsive genes in addition to their regulation of a common subset of stress-responsive genes. These results indicate that the three *GmMYB* genes may play differential roles in stress tolerance, possibly through regulation of stress-responsive genes.

Keywords: soybean, MYB transcription factor, abiotic stress tolerance

Cell Research (2008) **18**:1047-1060. doi: 10.1038/cr.2008.280; published online 26 August 2008

Introduction

Since the first *MYB* gene, the oncogene v-*myb*, was identified from the avian myeloblastosis virus [1], many *MYB* genes have been found in nearly all eukaryotes. MYB proteins can be classified into three subfamilies depending on the numbers (one, two or three) of adjacent repeats in the MYB domain [2-4], and *MYB* genes encoding two repeats (i.e. *R2R3-MYB*) constitute the largest *MYB* subfamily in plants [5, 6]. Members of the MYB family have been found to be involved in diverse processes [7], including developmental control and determination of cell fate and identity [8, 9], plant responses to environmental factors and hormones [3, 10-12], signal transduction in plant growth processes [13, 14], pathogen defense [15], and xylogenesis and lignin biosynthesis [16-21].

When plants are exposed to extreme conditions such as high salt, cold, and drought, plant growth and development are delayed. Analysis of gene expressions in *Arabidopsis* has revealed that a number of genes including effector genes and regulatory genes are involved in responses to various stresses. These genes belong to several independent signaling pathways [22, 23]. Because multiple stress responses are necessary for plants to endure severe stress conditions, the engineering of a single enzyme may not be sufficient [24, 25]. However, it is possible for a single transcription factor to control the expression of multiple target genes through specific binding

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Received 27 November 2007; revised 12 March 2008; accepted 20 March 2008; published online 26 August 2008

of the transcription factor to a *cis*-acting element in the promoters of their respective target genes [26]. Promoter analyses of these stress-inducible genes have identified at least four independent regulatory systems for gene expression [27-29]. There are two major *cis*-acting elements: ABRE and DRE/CRT. ABRE is involved in ABA-dependent gene expression in osmotic and cold stress responses, and DRE/CRT is involved in ABA-independent gene expression in osmotic and cold stress responses [30]. However, these two regulatory systems of gene expression are interdependent in the stress-responsive expression of the *rd29A* gene [31].

Soybean (*Glycine max*) is the major source of edible vegetable oil, and the dominant source of high-quality protein for livestock and humans. Its growth and yield were affected by various abiotic stresses. Several abiotic stress-inducible genes have been identified from soybean plants and their physiological functions have been characterized [32-38]. However, few soybean MYB transcription factor genes related to abiotic stresses were reported. In the present study, the expressions of soybean MYB transcription factor genes were analyzed in response to drought, salt, and cold treatments. Three stress-inducible genes, *GmMYB76*, *GmMYB92*, and *GmMYB177*, were further characterized for their roles in stress tolerance. We find that the transgenic *Arabidopsis* plants overexpressing *GmMYB76*, *GmMYB92*, or *GmMYB177* show reduced sensitivity to ABA and exhibit better tolerance to salt and freezing treatments when compared with the wild-type (WT) plants.

Results

Identification and phylogenetic analysis of MYB genes from soybean

Our previous study has identified 206 *MYB* genes from 56 147 soybean unigenes [39]. After further analysis by blasting these sequences against those from Genbank and through comparison of the amino acid sequences, 156 *MYB* genes were confirmed in the present study. These genes were named as *GmMYB17*-*GmMYB187* because the *GmMYB1*-*GmMYB16* have been used by others for *MYB*-related expressed sequence tags (ESTs) (Supplementary information, Table S1). In our nomenclature, some genes are not named in sequential numbers due to the removal of sequences of low quality. Twelve additional *MYB* genes from soybean plants, including eight genes with full-length open reading frames, have been given other names (Supplementary information, Table S1). In our analysis, 48 genes with full-length open reading frames were identified. Totally 184 *MYB* genes from soybean are listed in Supplementary information, Table S1.

The evolutionary relationships among 36 MYB proteins from *Arabidopsis* and 55 full-length MYB proteins available from soybean were analyzed using their full-length amino acid sequences (Figure 1). The 36 MYB proteins from *Arabidopsis* are selected from different subgroups described previously [4, 40]. Figure 1 shows that most of the GmMYB proteins clustered with the *Arabidopsis* members, which belong to different categories. However, a few GmMYB members cannot be classified into any categories due to absence of conserved motifs.

Expression of GmMYB genes under various stresses

Expression of the *GmMYB* genes in response to abiotic stresses was investigated using RT-PCR. Among 156 *GmMYBs*, the expression of 43 members responded to at least one of the four treatments including salt, cold, drought, and ABA (Supplementary information, Table S1). Expression of six genes was altered upon all four treatments. Ten genes were responsive to all the three abiotic stresses (Figure 2A). The overall expression of 26.3% of the *GmMYB* genes was changed upon treatment with at least one of the three abiotic stresses.

Three *GmMYB* genes *GmMyb76*, *GmMYB92*, and *GmMYB177*, which were responsive to abiotic stresses, were cloned and further analyzed. GmMYB76 and GmMYB92 belong to group 20 and 7, respectively, whereas the GmMYB177 belongs to one MYB domain CCA1-like type (Figure 1). The expressions of the three *GmMYBs* were monitored in cotyledons, roots, stems, leaves, and flowers of soybean plants. As shown in Figure 2B, the expression of *GmMYB76* was higher in cotyledons, roots, stems, and flowers than in leaves. The expression of *GmMYB92* was high in cotyledons and roots but very low in stems, flowers, or leaves. The expression of *GmMYB177* was higher in cotyledons and flowers than in stems, roots, or leaves.

The expressions of the three *GmMYB* genes under various treatments were also investigated. Figure 2C showed that *GmMYB76* was induced by NaCl treatment but not by other treatments. *GmMYB92* was induced by both cold and salt treatments, whereas *GmMYB177* was induced by drought and NaCl treatments. It should be noted that none of the three genes were affected by ABA treatment.

Analysis of transactivation and dimerization abilities of the three GmMYBs

The transactivation abilities of GmMYB76, GmMYB92, and GmMYB177 were analyzed using a yeast assay system. GAL4 DNA binding domain (BD)-GmMYBs fusion plasmids were transformed into yeast cells and assayed for their ability to activate transcription of

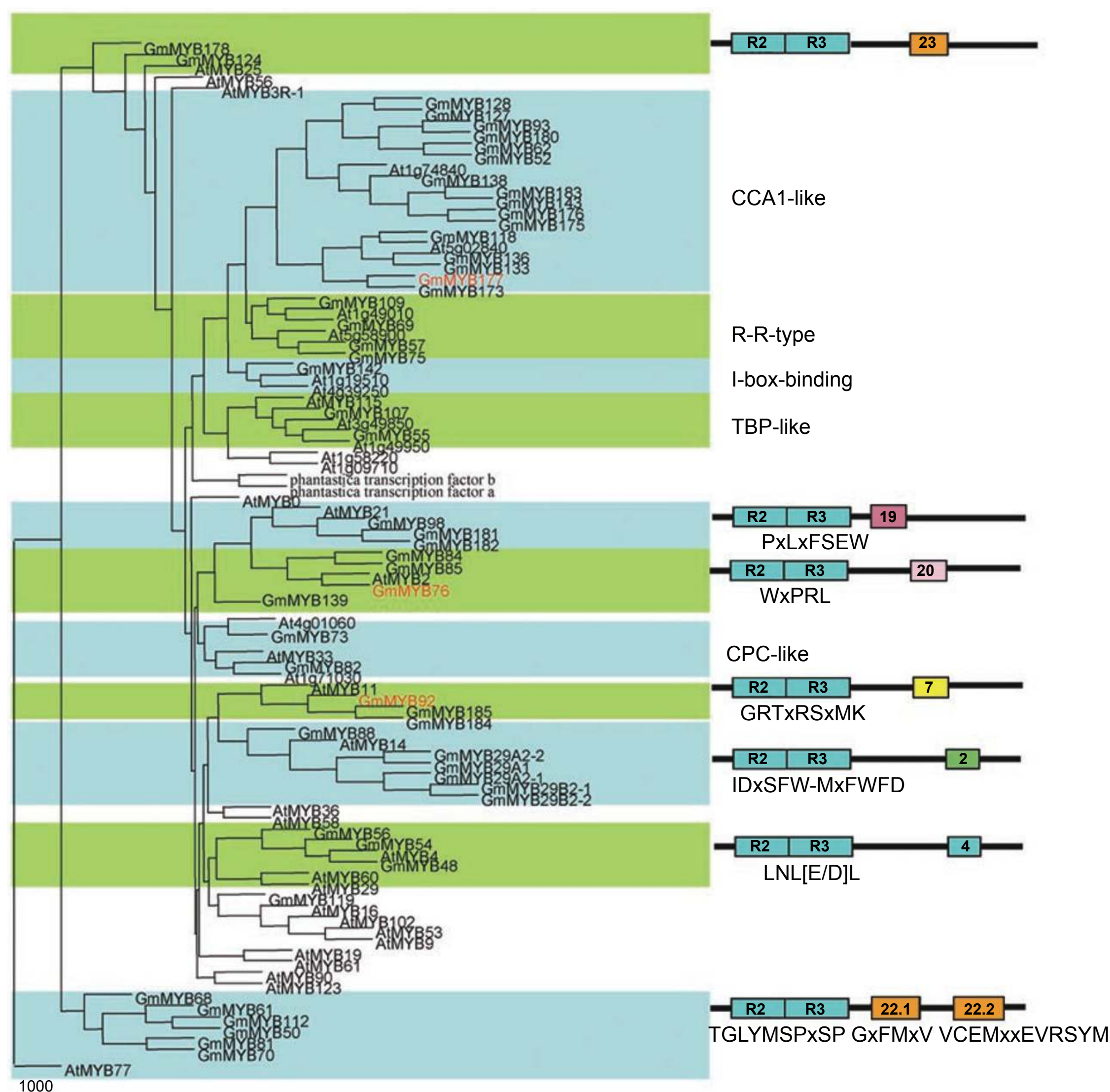

Figure 1 Cluster analysis of 55 soybean full-length GmMYB proteins with 36 *Arabidopsis* MYB proteins. The 36 MYB proteins from *Arabidopsis* are selected from different subgroups described previously, and the consensus sequences of each group were also shown based on the analysis of *Arabidopsis* MYB proteins [4, 40].

the marker *HIS3* gene controlled by the GAL4 upstream activation sequence and their ability to promote yeast growth in the absence of histidine. β-Galactosidase activity was also examined for the marker *LacZ* gene expression. Figure 3A showed that full-length GmMYB76 and GmMYB92 had transactivation capacity whereas GmMYB177 did not.

MYB proteins can form homo- and/or heterodimers to exert their physical and biological functions, as in the case of RTBP1 (rice telomere-binding protein1), which can form homodimers to bind telomeric DNA [41]. In order to analyze the dimerization abilities between these three GmMYB proteins, the yeast two-hybrid system was employed using two different reporters, *lacZ* and *HIS3*, in YRG-2 yeast cells. The full-length GmMYB proteins were expressed as fusions to the yeast GAL4 activation domain (AD) and GAL4 DNA-BD. The yeast strain was co-transformed with the pAD-GmMYB and the pBD-GmMYB. Then the transformants and controls were selected on SD/–Trp–Leu–His+10 mM 3-AT and examined for β-galactosidase activity. Figure 3B showed that GmMYB76 can form a homodimer whereas GmMYB76 and GmMYB177 can form a heterodimer. Because cells harboring pBD-GmMYB92 plus pAD vector can grow on SD/–Trp–Leu–His+10 mM 3-AT (data not shown) and this will generate false positive response, we removed the

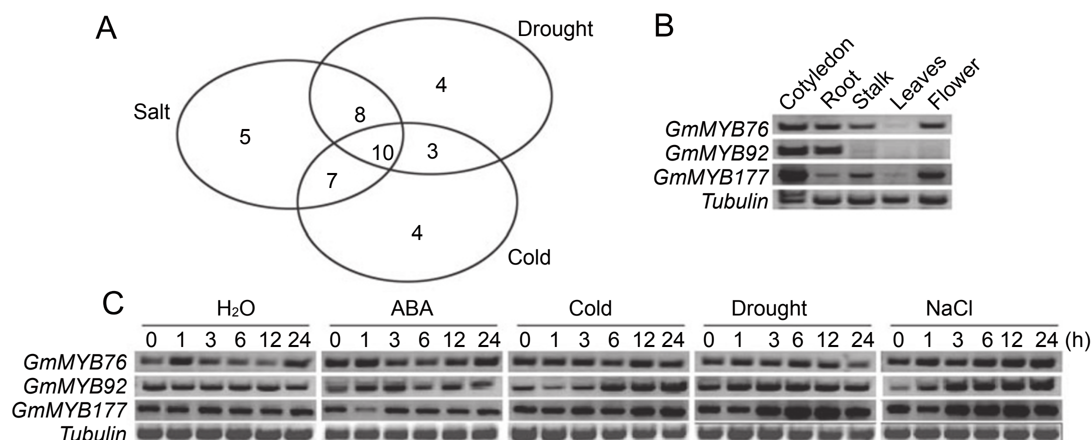

Figure 2 Expressions of *GmMYB76*, *GmMYB92*, and *GmMYB177* in soybean plants. **(A)** Comparison of numbers of soybean *GmMYB* genes that are responsive to salt, drought, and cold treatments. **(B)** Expression of *GmMYB76*, *GmMYB92*, and *GmMYB177* in different soybean organs. **(C)** Expression of *GmMYB76*, *GmMYB92*, and *GmMYB177* in response to ABA, cold, drought, and salt treatments. Water treatment was performed as a control treatment. *Tubulin* gene was amplified as a control.

C-terminal portion of the GmMYB92 to make a pBD-GmMYB92-N. The cells harboring pBD-GmMYB92-N or pBD-GmMYB92-N plus pAD vector could not grow on SD(–His) or SD/–Trp–Leu–His+10 mM 3-AT (Figure 3A; data not shown), and thus pBD-GmMYB92-N can be used for examination of dimerization with other proteins. Figure 3B showed that cells harboring pBD-GmMYB92-N plus the pAD-GmMYB92 can grow on selection medium, suggesting that GmMYB92 can form a homodimer and the N-terminal portion is responsible for the dimerization. GmMYB92-N cannot form dimers with other proteins (data not shown).

MYB proteins can interact with other transcriptional regulators [42-44]. We analyzed the interaction between these GmMYBs and five GmbZIP proteins, whose genes were identified from soybean and were responsive to abiotic stresses (Liao Y, Zhang JS and Chen SY, unpublished results). We observed an interaction between GmMYB76 and GmbZIP46 (Figure 3B). There were no interactions between GmMYBs and other GmbZIPs (data not shown).

DNA-binding ability of the three GmMYBs

The *cis*-DNA elements for several MYB proteins have been identified [45]. We investigated if GmMYB76, GmMYB92, and GmMYB177 can bind to these elements in yeast one-hybrid assay system. Four *cis*-DNA elements MRE1 (CCG GCA GTT AGG AT), MBSI (TAT AAC GGT TTT TTA), MRE3 (TCT AAC CTA CCA) and MRE4 (TCT CAC CTA CC) and their corresponding mutant sequences mMRE1(CCG GAA AAA AGG AT), mMBSI (TAT AAA AAT TTT TTT A), mMRE3 (TCT AAA AAA CCA), and mMRE4 (TCT CGA TCA CC) were synthesized and inserted into the reporter plasmid pHIS2 containing the reporter gene *HIS3* [46, 47]. A minimal promoter was present downstream of the cis-elements but upstream of the HIS3 gene. Each of the reporter plasmid harboring different elements plus the reporter gene *HIS3*, together with the effector plasmid pAD-GmMYB76, 92 and 177, was transformed into yeast (Y187) cells. The transformants and controls were selected on SD/–Trp–Leu–His plus 3-AT, and growth of the transformants indicates binding of the protein to the corresponding *cis*-DNA elements. Figure 3C showed that all the three proteins GmMYB76, GmMYB92, and GmMYB177 could bind to the sequence MBSI (TAT AACGGT TTT TT). GmMYB92 could also bind to the MRE4 sequence (TCT CACCTA CC) and mMRE1 (CCG GAAAAAAGG AT). GmMYB76 and GmMYB177 bound to the element mMRE1 with a weak affinity. None of the proteins could bind to MRE1, mMBSI, mMRE3, MRE3, or mMRE4. These results indicate that the three proteins can differentially bind to the *cis*-DNA elements examined.

ABA sensitivity of the transgenic Arabidopsis plants overexpressing the three GmMYBs

To investigate the *in vivo* function in plants, the *GmMYB76*, *GmMYB92*, and *GmMYB177* genes were inserted in sense orientation into a binary vector pBIN438 in which the *GmMYBs* were under the control of the 2× CaMV 35S promoter. The genes were transformed into *Arabidopsis* by using the vacuum infiltration method. The homozygous T3 lines, with relatively higher expres-

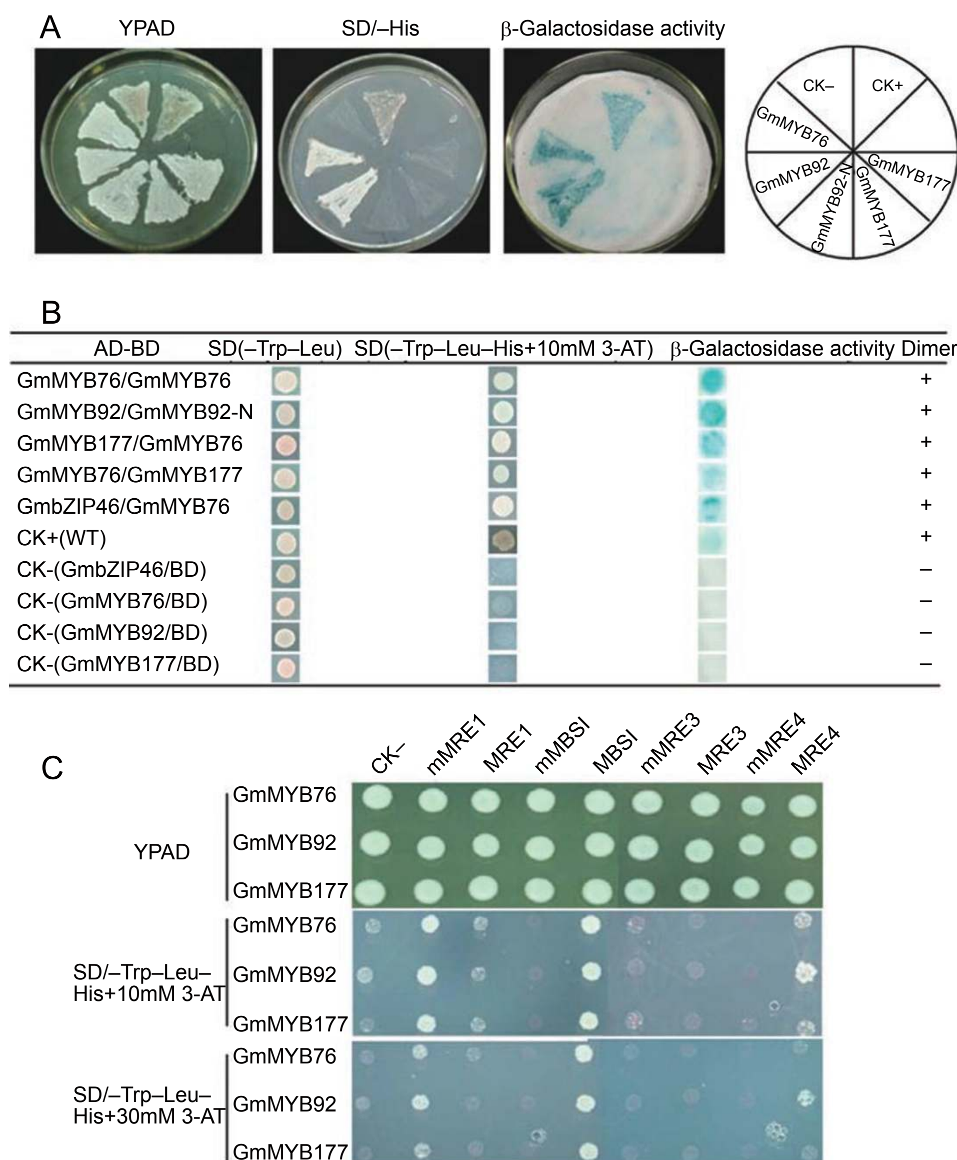

Figure 3 Transcriptional activation, dimerization, and DNA-binding analysis of GmMYB76, GmMYB92, and GmMYB177. **(A)** Transactivation ability of GmMYB76, GmMYB92, and GmMYB177. Growth of the transformants on SD/–His and blue color in β-gal assay indicate that the corresponding gene has transactivation activity. **(B)** Dimerization analysis among the three GmMYBs or between the three GmMYBs and GmbZIP46. Only the positive interactions were shown. The CK+(WT) containing pAD-WT/pBD-WT pair indicates positive interaction control and the pAD-GmMYBs/pBD or pAD-GmbZIP46/pBD pairs indicates negative controls. Growth of the transformants on SD(–Trp–Leu–His plus 3-AT) and the blue color in β-gal assay indicate that the corresponding protein pairs have the ability to form dimers. **(C)** DNA-binding ability of the GmMYB76, GmMYB92, and GmMYB177 by using yeast one-hybrid system. CK– indicates growth of cells harboring the effector plasmids pAD-GmMYBs and the reporter plasmid pHIS2 (without *cis*-DNA elements). Growth of the transformants on SD(–Trp–Leu–His plus 3-AT) indicates that the GmMYB proteins can bind to the corresponding *cis*-DNA elements. MRE1 (CCG GCA GTT AGG AT), mMRE1 (CCG GAA AAA AGG AT); MBSI (TAT AAC GGT TTT TTA), mMBSI (TAT AAA AAT TTT TTT A); MRE3 (TCT AAC CTA CCA), mMRE3 (TCT AAA AAA CCA); MRE4 (TCT CAC CTA CC), mMRE4 (TCT CGA TCA CC).

sion of *GmMYB76*, *GmMYB92*, or *GmMYB177*, were used for further analysis (Figure 4A). No obvious differences were observed in the phenotypes between the transgenic plans harboring *GmMYB76*, *GmMYB92*, or *GmMYB177* and the WT plants grown on MS agar plates and in the soil (Supplementary information, Figure S1; data not shown).

To investigate whether overexpression of *GmMYB76*,

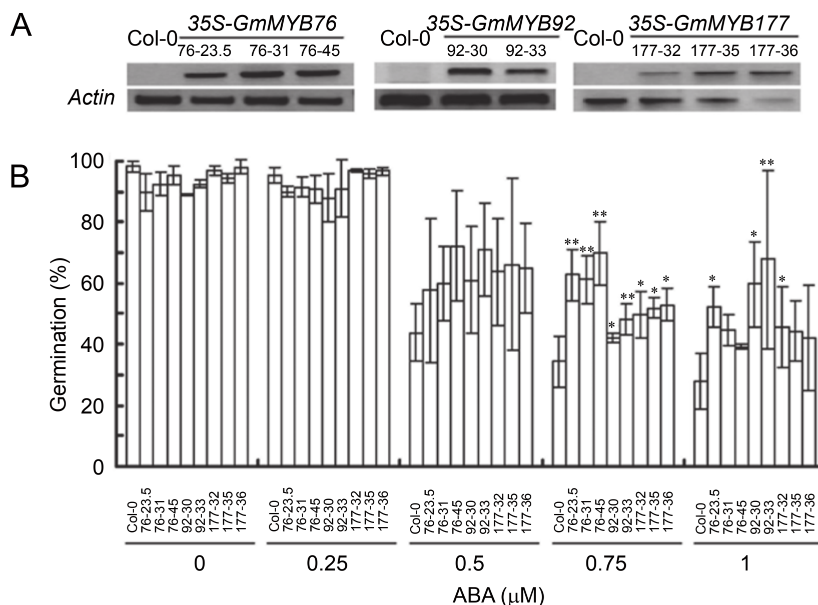

Figure 4 ABA sensitivity of the transgenic plants overexpressing the *GmMYB76*, *GmMYB92*, and *GmMYB177*. **(A)** Expression of *GmMYB76*, *GmMYB92*, and *GmMYB177* in various transgenic lines as revealed by RT-PCR. *Actin* gene was amplified as a control. **(B)** Germination of the *GmMYB*-transgenic seeds under various ABA concentrations. Each data point represents the mean of triplicates and each measurement has more than 100 seeds. The bars indicate SD. * indicates that the differences between the transgenic line and the Col-0 are significant ($P<0.05$). ** indicate that the differences between the transgenic line and the Col are highly significant ($P<0.01$).

GmMYB92, and *GmMYB177* affects ABA sensitivity of the transgenic plants, the seeds from the transgenic plants and WT plants were germinated in the medium containing ABA and the germination rates were compared. Figure 4B showed that, at 0.75 μM or higher concentrations of ABA, the germination rates of all the transgenic lines were substantially higher than those of the WT plants. At lower ABA concentrations, the germination rates were not significantly affected in transgenic seeds. These results indicate that the transgenic seeds were less sensitive to ABA treatment.

Performance of the GmMYB-transgenic plants under salt stress

Because the expression of *GmMYB76*, *GmMYB92*, and *GmMYB177* was induced under salt stress, these genes may participate in salt tolerance in plants. The *GmMYB*-transgenic seeds were germinated on NaCl medium and their germination rates were compared. Figure 5A showed that, at 100 mM NaCl, the germination rates of the *GmMYB177*-transgenic plants were higher than those of WT plants and other transgenic plants. At 150 mM NaCl no WT seeds could germinate, whereas around 10%, 5%, and 15% of the *GmMYB76*-, *GmMYB92*-, and *GmMYB177*-transgenic seeds, respectively, could germinate. The plate-grown seedlings were also transferred to soil and treated with stepwise increasing concentrations of NaCl. Figure 5B and 5C showed that, with this treatment, the *GmMYB76*- and *GmMYB177*-trangenic plants grew better and higher than WT plants. However, the growth of *GmMYB92*-transgenic plants showed no significant difference in comparison with WT plants under the stress. In the absence of stress, all the transgenic plants showed no significant difference compared to WT plants (Supplementary information, Figure S1). The plate-grown seedlings were also transferred to soil and further treated with high salt (600 mM NaCl) for 12 days. The plant survival was scored and compared. Figure 5D and 5E showed that the survival rates of the *GmMYB76*-, *GmMYB92*-, and *GmMYB177*-transgenic plants were significantly higher than those of WT plants. All these results indicate that the transgenic plants show better tolerance to salt stress than WT plants. However, each of the three *GmMYB* genes may confer differential tolerance to salt stress at different developmental stages and with different treatments.

Performance of the GmMYB-transgenic plants under freezing stress

Because the expression of *GmMYB92* was induced by cold treatment in soybean plants, we examined the effect of freezing on the performance of the *GmMYB*-

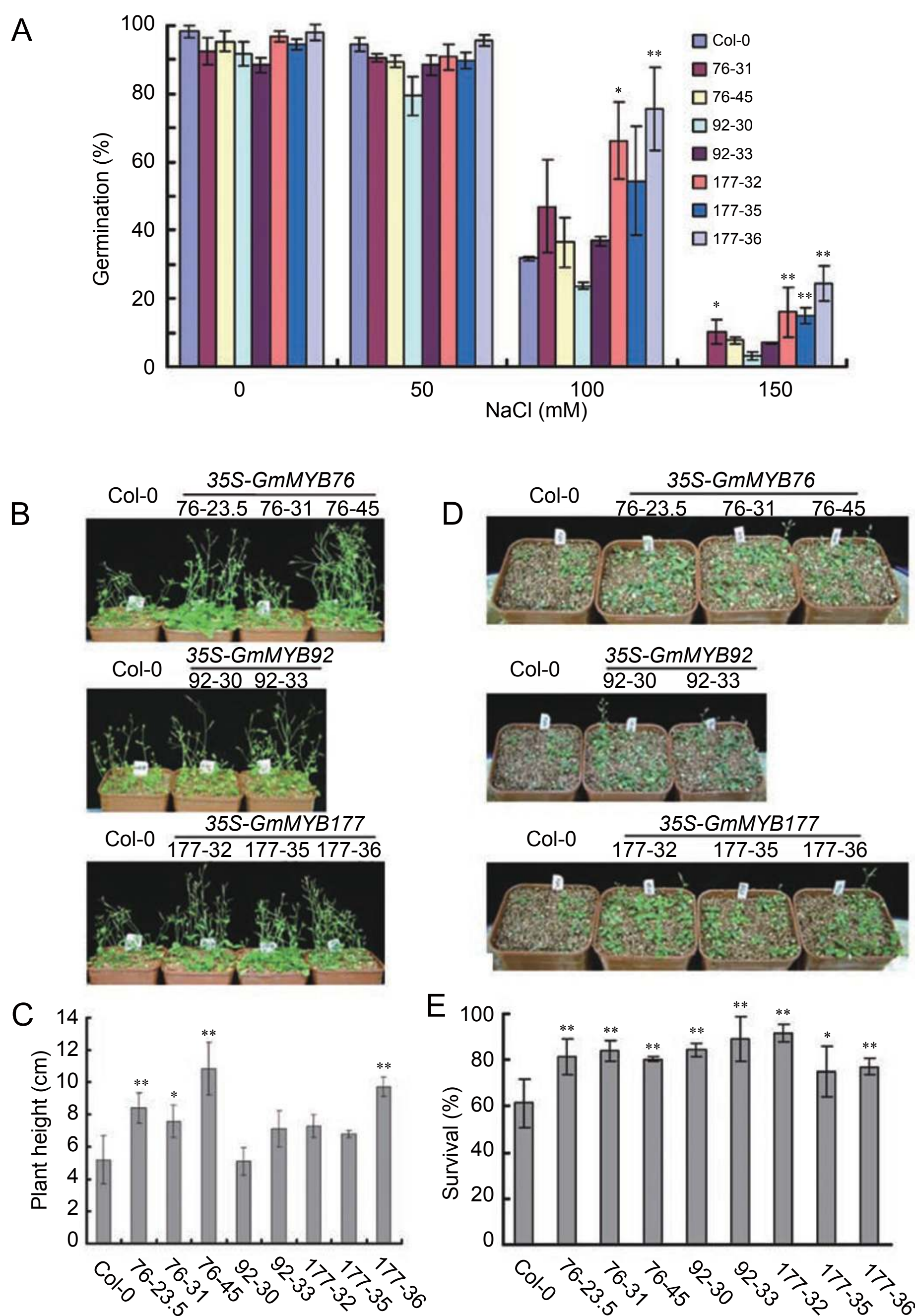

Figure 5 Effects of salt stress on the transgenic plants overexpressing the *GmMYB76*, *Gm92*, and *GmMYB177*. Transgenic lines 76-23.5, 76-31, or 76-45 for *GmMYB76*, 92-30 and 92-33 for *GmMYB92*, and 177-32, 177-35, 177-36 for *GmMYB177* were used. **(A)** Comparison of the germination rates of the transgenic seeds under salt stress. Experiments were performed in triplicate and bars indicate SD. **(B)** Phenotypic comparison of the plant growth at bolting stage under salt stress. Two-week-old seedlings were transferred to soil for one week, and then treated with increasing concentrations of NaCl. Photographs were taken after 14 days treatment. The plant height was measured after treatment for 20 days. **(C)** Effect of salt stress on the inflorescence height of the plant grown in soil. Plant height in (B) was measured. Each data point represents the mean of triplicates and each measurement has 11 plants. Bars indicate SD. **(D)** Survival of the transgenic plants under high salt (600 mM NaCl) treatment for 12 days. **(E)** Comparison of the survival rate of the transgenic plants in (D). Each data point represents the mean of triplicate tests and each test has 18 plants. Bars indicate SD. For **(C** and **E)**, * indicates that the differences between the transgenic line and the Col-0 are significant (P<0.05). ** indicate that the differences between the transgenic line and the Col-0 are highly significant (P<0.01).

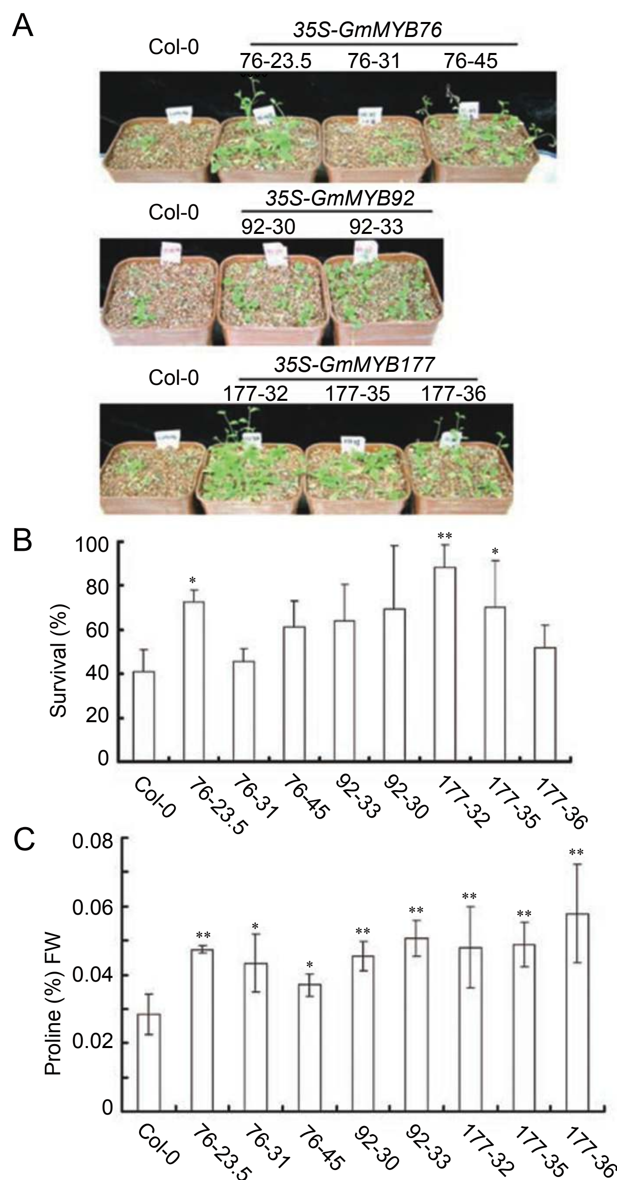

Figure 6 Performance of the *GmMYB*-transgenic plants upon freezing and comparison of proline contents. Transgenic lines 76-23.5, 76-31, and 76-45 for *GmMYB76*, 92-30 and 92-33 for *GmMYB92*, and 177-32, 177-35, and 177-36 for *GmMYB177* were used for the analysis. **(A)** Phenotype of the *GmMYB*-transgenic plants after freezing treatment. Two-week-old seedlings were transferred to soil for one week, and treated at –6 ºC for 2 days. The photograph was taken after recovery for 7 days, and survival rate was also calculated. **(B)** Comparison of the survival of the transgenic plants in (A). Each data point represents the mean of triplicate tests and each test has 18 plants. Bars indicate SD. **(C)** Proline content in the *GmMYB*-transgenic lines. Each data point represents the mean of three replicates and bars indicate SD. For (**B** and **C**), * indicates that the differences between the transgenic line and the Col are significant (P<0.05). ** indicate that the differences between the transgenic line and the Col are highly significant (P<0.01).

transgenic plants. Soil-grown seedlings were subjected to treatment at –6 ºC for 2 d, and the survival was evaluated after recovery for 3 days under normal conditions. The line 76-23.5 for the *GmMYB76*, and the lines 177-32 and 177-35 for the *GmMYB177* had a survival percentage significantly higher than that of WT plants (Figure 6A and 6B). Other lines showed no significant difference or only slightly higher survival percentage compared to WT. These results imply that the *GmMYB177* gene may confer freezing tolerance in the transgenic *Arabidopsis* plants whereas *GmMYB76* may play partial roles in freezing tolerance.

Proline is one of the osmolytes that copes with cellular dehydration and, in several plant species, the increase in proline content during cold correlates with freezing tolerance [48, 49]. The proline levels were measured in 16-day-old WT plants and the transgenic plants grown under normal condition. Figure 6C showed that the level of free proline was significantly higher in all the *GmMYB*-transgenic plants than in the WT plants. The higher proline levels may contribute to the freezing tolerance of the *GmMYB*-transgenic plants.

Altered gene expression in the GmMYB-transgenic plants

Because overexpression of *GmMYB76*, *92*, and *177* led to stress tolerance and changed ABA response in transgenic *Arabidopsis* plants, we examined if abiotic stress- and ABA-responsive genes were altered in these

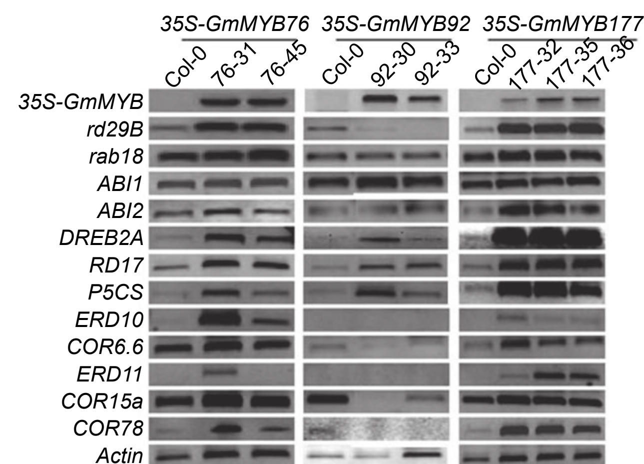

Figure 7 Expression of ABA- and abiotic stress-responsive genes in *GmMYB*-transgenic plants. Total RNAs were isolated from two-week-old plants grown on MS plates and then subjected to RT-PCR analysis. Transgenic lines 76-31 and 76-45 for *GmMYB76*, 92-30 and 92-33 for *GmMYB92*, and 177-32, 177-35, and 177-36 for *GmMYB177* were used for the examination.

plants. As shown in Figure 7, the transcript levels of a number of ABA- or abiotic stress-responsive genes were changed between the transgenic plants and WT plants. The expression levels of *rd29B* [50], *DREB2A* [51, 52], *P5CS* [53, 54], *RD17* [55], *ERD10* [55, 56], and *COR78/rd29A* [57, 58] were enhanced in the *GmMYB76*-transgenic plants. The expressions of *DREB2A*, *RD17*, and *P5CS* were higher in *GmMYB92*-transgenic plants than in WT plants. However, the expressions of *rd29B*, *COR6.6*, *COR15a* [59], and *COR78/rd29A* were lower in these *GmMYB92*-transgenic plants. In the *GmMYB177*-transgenic plants, genes including *rd29B*, *ABI2* [60], *DREB2A*, *RD17*, *P5CS*, *ERD10*, *COR6.6* [55, 61], *ERD11* [62], and *COR78* were upregulated. The expressions of *rab18* [63] and *ABI1*, which were induced by ABA, were not significantly affected in the three *GmMYB*-transgenic plants when compared with the WT plants. These results suggest that each of the three *GmMYB* genes may differentially regulate the expressions of the downstream genes, which are related to abiotic stresses and/or ABA responses.

Discussion

The MYB family comprises a large group of transcription factors involved in plant development and tissue differentiation, and are found in nearly all eukaryotes. They contain a DNA-binding MYB domain in the N-terminal part of the protein and bind DNA in a sequence-specific manner. MYB proteins are classified into three subfamilies, namely MYB1R, MYBR2R3, and MYB3R, and most plant MYB proteins are the R2R3 type [4]. The R2R3-type MYB factors encoded by the *AtMYB* genes from *Arabidopsis* have been categorized into 22 subgroups on the basis of conserved amino-acid sequence motifs present carboxyterminal to the MYB domain [40, 4]. There are 203 MYB members in *Arabidopsis* and 206 MYB members in soybean [39]. Further analysis confirmed 156 MYB members of soybean, and 55 full-length MYB proteins of soybean clustered, with some but not all the subgroups of MYB proteins from *Arabidopsis*. Among the 156 *GmMYBs*, 27% were regulated by at least one of the four treatments including ABA, salt, drought, and low temperature. Three genes, *GmMYB76*, *GmMYB92*, and *GmMYB177*, which were induced by abiotic stresses, were chosen for further investigation.

Most MYB proteins are presumed to be transcriptional activators with ADs in the C-terminal region to the DNA-BD since c-MYB has an acidic AD C-terminal to its DNA-BD [64]. However, the sequences in the C-terminal regions of MYB proteins are not strongly conserved, presumably because the structural determinants for ADs are fairly flexible. Not all MYB-related proteins need to be transcriptional activators. A survey of the predicted C-terminal sequences of the *Arabidopsis* R2R3 MYB family revealed 22 different subgroups, and these subgroups showed limited sequence conservation within their C-terminal regions [40]. The moderately conserved motifs in each subgroup might represent ADs, an idea supported by the fact that some are relatively acidic, and others are rich in amino acids (glutamine and proline) frequently associated with ADs. Alternatively, these regions with sequence conservation may represent repression domains or domains for interaction with other transcription factors [42]. We show that GmMYB76 and GmMYB92 have transactivation activities in yeast assay system, whereas GmMYB177 has no transactivation ability. These observations need to be examined further in plant system. Both GmMYB76 and GmMYB92 can form homodimers, and GmMYB76 can form heterodimers with GmMYB177 and GmbZIP46, respectively. However, the biological function of these interactions needs to be further investigated.

Sequence-specific DNA-binding has been demonstrated for several MYB proteins, in agreement with their regulation in transcription, and the proteins can bind to one or more *cis*-DNA elements. Different target recognition sites for different groups of MYB proteins have been identified. Mammalian repeat MYBs such as c-MYB, A-MYB, and B-MYB and closely related proteins from invertebrates and cellular slime moulds all bind to the cognate site T/CAACG/TGA/C/TA/C/T (MBSI). Some of the two repeat R2R3-type proteins in plants can recognize this binding site while others cannot. Several plant MYB proteins that bind to MBSI will also bind to a second site, TAACTAAC (MBSII), which is a *cis*-DNA element recognized by the majority of plant R2R3 MYB proteins [5]. GmMYB76, GmMYB92, and GmMYB177 all could bind to the sequence MBSI (TAT AACGGT TTT TT), which is the recognition site of AtMYB2 [42, 10]. GmMYB92 can also bind to the *cis*-DNA sequences MRE4 (TCT CACCTA CC) [47] and mMREI (CCG GAAAAAAGG AT), while GmMYB76 and GmMYB177 can only interact with mMREI weakly. The mMREI (CCG GAAAAAAGG AT) is similar to aaaAaaCG/CGTTA, which is bound by MmMYB, PhMYB3, and AtMYB77 [5]. The differential binding ability suggests that the three GmMYBs may regulate different sets of downstream genes in addition to the common sets of genes. This speculation appears to be consistent with the analysis of altered gene expressions in the *GmMYB*-transgenic plants (Figure 7). Whereas all the three *GmMYB* genes promoted the expression of *DREB2A*, *RD17*, and *P5CS* genes, the regulation of

rd29B, *ERD10*, and *COR78* seemed different in the *GmMYB*-transgenic plants. GmMYB76 and GmMYB177 can enhance the expression of *rd29B*, *ERD10*, and *COR78* genes. On the contrary, GmMYB92 inhibited *rd29B* or *COR78* but did not affect the *ERD10* gene.

Functional analyses of plant MYBs indicate that they regulate numerous processes including responses to environmental stress [40, 65, 66]. MYBs have been found to be involved in ABA-dependent or ABA-independent signaling pathways. The genes *AtMYB2* [10], *CpMYB10* [67], and *HOS10* [68] are induced by ABA, and also induced by drought, salt, chilling, or heat treatments. However, the genes *HPPBF-1* [69], *Osmyb4* [49], and *AtMYB61* [70] are not induced by ABA, but can enhance drought, salt, or freezing tolerances. *AtMYB60* is repressed by ABA and desiccation, and a null mutation in *AtMYB60* leads to reduction in stomatal opening and water stress tolerance [71]. In the present study, the three MYB transcription factor genes, *GmMYB76*, *GmMYB92*, and *GmMYB177*, from the soybean can be induced by various abiotic stresses but are not significantly affected by ABA treatment. However, the transgenic *Arabidopsis* plants overexpressing these three genes exhibited reduced ABA sensitivity, suggesting that the three *GmMYB* genes could function as negative regulators of ABA signaling.

Studies have shown that overexpression of MYB transcription factor genes improves stress tolerance. Overexpression of *CpMYB10* in *Arabidopsis* can enhance osmotic stress tolerance of transgenic seedlings germinated in plates, and improve desiccation and salt tolerance in adult plants grown in soil [67]. Overexpression of *Osmyb4* in *Arabidopsis* increases plant tolerance to chilling and freezing [49]. Several MYB proteins have relatively high similarity (45-84%) with GmMYB76, GmMYB92, and GmMYB177. AtMYB2, AtMYB112, and a homeodomain-related protein from *Medicago truncatula* (ABE78836) share similarity with GmMYB76. AtMYB12, AtMYB3/4/5, and MYB56 from Vitis Vinifera (AAX51291) show similarity with GmMYB92. A homeodomain-related protein from *Medicago truncatula* (ABE83274) is homologous to GmMYB177. Among these homologous genes, only *AtMYB2* is induced by dehydration, high salt stress, and exogenous ABA. However, no stress tolerance was reported in transgenic plants overexpressing this gene [10]. Coexpression of both *AtMYB2* and *AtMYC2* conferred moderate stress tolerance [72]. Overexpression of *GmMYB76*, a close homologue of *AtMYB2*, in *Arabidopsis* plants improved salt tolerance and partial freezing tolerance in the transgenic plants, implying that *GmMYB76* may have a stronger role than the *AtMYB2* in regulation of stress tolerance. *GmMYB92* and *GmMYB177* also showed dominant roles in conferring plant salt and freezing tolerance. These analyses support that multiple MYB proteins are functional in plant responses to various stresses.

The *GmMYB76*, *GmMYB92*, and *GmMYB177* genes may confer stress tolerance through upregulation of downstream genes *DREB2A*, *RD17*, and *P5CS*. *DREB2A,* an AP2 domain transcription factor, is also induced by dehydration and salt stress. Overexpression of *DREB2A* induced weak expression of the target genes under unstressed conditions [51]. However, its constitutive active form caused significance stress tolerance in transgenic plants [73]. *RD17* contains the DRE or related motif in its promoter region and is induced by dehydration, salt, and cold. Its overexpression increased the freezing tolerance of transgenic plants [74]. DREB2A can promote the expression of *RD17* [73]. *P5CS* encoding delta-pyrroline-5-C synthase was upregulated in *35S-GmMYBs* transgenic plants, leading to proline accumulation (Figure 6C). Free proline was increased in plants in response to many stresses [75], and its accumulation could contribute to the increase of freezing tolerance [76] and salt tolerance [77]. Therefore, *GmMYB76*, *GmMYB92*, and *GmMYB177* may contribute to the basal salt tolerance at least via activation of the above three genes. However, it should be noted that the levels of *DREB2A*, *RD17*, and *P5CS* transcripts are much higher in the *GmMYB177*-transgenic plants than those in the *GmMYB76*- and *GmMYB92*-transgenic plants, consistent with the good performance of the *GmMYB177*-transgenic plants in germination test and freezing treatment (Figures 5A, 6A and 6B). In addition to the common set of genes, *GmMYB76* and *GmMYB177* also promoted expressions of a subset of genes including *rd29B*, *ERD10*, and *COR78*, which are involved in stress tolerance. The enhanced expression of the three genes was not found in the *GmMYB92*-transgenic plants. These genes may potentially contribute to the better performance of the *GmMYB76*- and *GmMYB177*-transgenic plants during germination test (Figure 5A), stepwise salt treatment (Figure 5B and 5C), and freezing treatment (Figure 6A and 6B). The differential expression of the downstream genes may depend on the differential DNA-binding ability of the three GmMYBs (Figure 3C), which is determined by the MYB domain structures. It should be noted that, although GmMYB177 and GmMYB76 regulated similar genes, their structures are different, with GmMYB177 being the 1R-type MYB protein and GmMYB76 being the R2R3-type MYB protein. It is possible that the MYB repeat in GmMYB177 is functionally equivalent to the MYB repeat in GmMYB76 in regulation of gene expressions. The relationship between structure and function remains to be further

investigated.

Overall, we identified three stress-responsive *GmMYB* genes: *GmMYB76*, *GmMYB92*, and *GmMYB177*. Overexpression of these genes in transgenic *Arabidopsis* plants conferred basal salt tolerance through differential regulation of a series of downstream genes. *GmMYB177* and *GmMYB76* may play larger roles than *GmMYB92* in salt and freezing tolerance. However, the three genes appear to be negative regulators of ABA signaling. Further analysis should be performed to study the mechanisms by which the three *GmMYB* genes regulate plant tolerance response to various abiotic stresses.

Materials and Methods

Phylogenetic analysis of soybean MYB proteins

Previously we have identified different ESTs encoding soybean MYB transcription factors using BLASTX Network Service [39]. These ESTs were used as queries to search against the data in GenBank for further confirmation. Alignments, tree construction of predicted amino acid sequences by the neighbor-joining method, and its bootstrapping (1 000 samples) were performed with CLUSTAL X (1.8). In parsimony method, the PHYLIP 3.65 package was used. Multiple most parsimonious trees were found and the consensus tree was built with the CONSENSUS program of PHYLIP.

Growth of soybean seedlings and stress treatments

Soybean (*Glycine max* L. Merr.) plants were grown on vermiculite under 16/8 h light/dark at 25 ºC for two weeks and used for stress treatments. Soybean seedlings were carefully pulled out from vermiculite, rinsed in water, and then dehydrated on Whatman No. 3 MM paper at 25 ºC with 70% humidity for various times. For ABA and salt stress treatments, two-week-old seedlings were placed hydroponically in 100 μM ABA or 150 mM NaCl for various times. Cold treatment was performed by exposing plants at 4 ºC. After these treatments, the leaves of plants were collected, frozen in liquid nitrogen, and stored at –80 ºC for RNA analysis.

RNA analysis, reverse transcription, and PCR (RT-PCR)

RNA isolation from soybean leaves and homozygous *Arabidopsis* transgenic seedlings, and RNA gel blot analysis were performed as described [78]. RT-PCR was performed in 25 μl reaction volume containing 1-3 μl cDNA, 2.5 μl 10× PCR reaction buffer, 0.5 μl dNTP (10 mM/each), 0.25 μl Primer R (25 μM), and 0.25 μl Primer L (25 μM), 1 U Taq, with 30 cycles of denaturation at 94 ºC for 30 s, annealing at 54-58 ºC for 60 s, and extension at 72 ºC for 1 min. *GmMYB* genes, their accession numbers, and primers used for RT-PCR analysis are listed in Supplementary information, Tables S1 and S2.

Transactivation and dimerization analysis in yeast

GmMYB76, *GmMYB92*, and *GmMYB177* coding sequences were cloned into the DNA-BD vector pBD-GAL4 (HybriZAP-2.1 XR library construction kit and hybriZAP-2.1 XR cDNA synthesis kit; Stratagene, La Jolla, CA) to construct GAL4 DNA-BD-GmMYB fusion plasmids pBD-GmMYBs. These plasmids were transformed into the yeast strain YRG-2 harboring the *LacZ* and *HIS3* reporter genes. The transformed yeast culture was dropped onto SD plates with or without histidine. The plates were incubated for 3 days and applied to a β-gal assay for examination of the transactivation ability.

GmMYB coding sequence was also cloned into the AD vector pAD-GAL4-2.1 to generate a GAL4-activation-GmMYB fusion plasmid pAD-GmMYB. pAD-GmMYB and pBD-GmMYB were co-transformed into the yeast strain YRG-2. The transformed yeast culture was dropped onto SD/–Trp–Leu or SD/–Trp–Leu–His plus 3-AT. The plates were incubated for 3 days and also applied to β-gal assay for examination of dimerization ability. For pBD vector construction, 5′-CCG gaa ttc ATG GAG ACC ACG AAT GTT CA-3′ and 5′-TCC ccc ggg GTG TAA ATT GCA AAA CTG GA-3′ were used for GmMYB76; 5′-ACG Cgt cga cAA ATG GGA AGG GCT CCT TGT-3′ plus 5′-AAA ctg cag TCT TTG TTG ATA TTC TTT TA-3′ or plus 5′-AAA ctg cag ATA CAT TGC CTT CAC TCT AA-3′ were used for GmMYB92 or GmMYB-N, respectively. For pAD vector construction, 5′-CGC gga tcc ATG GAG ACC ACG AAT GTT CA-3′ and 5′-CCG gaa ttc GTG TAA ATT GCA AAA CTG GA-3′ were used for GmMYB76; 5′-CGC gga tcc ATG GGA AGG GCT CCT TGT-3′ and 5′-AAA ctg cag TCT TTG TTG ATA TTC TTT TA-3′ were used for GmMYB92. For pAD and pBD vector construction, 5′-CCG gaa ttc ATG GCG ATA CAA GGC CAA AA-3′ and 5′-AAA ctg cag GAA AAG CCA ATA AGA GGG TA-3′ were used for GmMYB177.

DNA-binding ability of GmMYBs in yeast one-hybrid assay system

Three copies of each of the *cis*-acting DNA elements MRE1 (CCG GCA GTT AGG AT), MBSI (TAT AAC GGT TTT TTA), MRE3 (TCT AAC CTA CCA), MRE4 (TCT CAC CTA CC), and their corresponding mutant sequences mMRE1 (CCG GAA AAA AGG AT), mMBSI (TAT AAA AAT TTT TTT A), mMRE3 (TCT AAA AAA CCA), and mMRE4 (TCT CGA TCA CC) were synthesized (Sengon of Shanghai, China), annealed, and cloned into the *Sac*I and *Mlu*I sites of reporter plasmid pHIS2, a reporter vector that contains the nutritional reporter gene *HIS3*. The sequence of the inserts was confirmed by sequencing. pAD-GmMYB76, pAD-GmMYB92, and pAD-GmMYB177, together with the reporter plasmid pHIS2 containing different *cis*-DNA elements, were co-transformed into yeast cells (Y187) and selected on selection medium. For negative Control, pAD-GmMYBs construct and pHIS2 were co-transformed into yeast cells (Y187) and growth of the transformants was examined on selection medium.

Generation of the GmMYB-transgenic Arabidopsis plants and stress treatments

The coding region of *GmMYBs* was cloned into the *BamH*I and *Kpn*I sites of the plant expression vector pBin438 and confirmed by sequencing. The transgene was driven by the 35S CaMV promoter and an Ω translation enhancer. Transformation of *Arabidopsis* was performed by the vacuum infiltration method [79], using *Agrobacterium tumefaciens* strain GV3101. For *GmMYB76*, primers 5′-CGC GGA TCC GGG CAA ATT AGT ATA ATA TG-3′ and 5′-CGG GGT ACC TGC AAA GCC TAG TGT AAA TT-3′ were used. For *GmMYB92*, 5′-CGG GGA TCC TCT AGA GAT TAA ATG GGA AG-3′ and 5′-TAT GGT ACC GAC GAT TGC AAT GTG ATC TTC A-3′ were used. For *GmMYB177*, 5′-CGC GGA

TCC GCC AAC CAC AGA GGA GAA GA-3′ and 5′-CGG GGT ACC CAA GGT CAT CCA GCA ACA GA-3′ were used.

For NaCl or ABA treatments, seeds (>100) of the transgenic *Arabidopsis* plants were planted in triplicate on filter papers saturated with different concentrations of ABA or NaCl, and incubated at 4 ºC for 4 days before being placed at 22 ºC for germination under a photoperiod of 16 h/8 h (light/dark). Germination (emergence of radicals) was scored after 3 days. The experiment was repeated twice and the results were consistent. Results from one set of experiments are shown.

For NaCl treatment in soil, 14-day-old seedlings of WT and transgenic seedlings from MS agar media were grown in potted soil for 7 days. Then the plants were watered with 100, 150, and 200 mM NaCl, and each concentration lasted for 4 days. The inflorescence height was measured after the treatment. The 14-day-old seedlings were also grown in pots for 7 days and then treated with high salt by placing the pots in a tray containing 600 mM NaCl. After 12 days, the survival of the plants was examined. The experiments were repeated independently three times and the results were consistent. Results from one set of experiments are shown.

For freezing tolerance assay, 14-day-old WT and transgenic seedlings from MS agar media were grown in potted soil for 7 days, and the plants were subjected to –6 ºC for 2 days. After that, the plants were placed under normal condition. The survival of the seedlings was scored after 7 days and photograph was taken. The experiments were repeated independently for three times and the results were consistent. Results from one set of experiments are shown.

Determination of proline

Free proline content in plants was estimated following the methods of [80]. Plant samples (100 mg) were homogenized in 1 ml of sulfosalicylic acid (3%) using a mortar, and the mixture was mixed and centrifuged at 13 000 rpm for 15 min at 4 ºC. The supernatant (200 μl) was transferred to a new tube, and 200 μl of acid ninhydrin and 200 μl of acetic acid were added. The reaction mixture was boiled in a water bath at 100 ºC for 1 h, and then stored at 4 ºC for 30 min. After 800 μl toluene was added to extracts, the mixture was vortexed for 15 s and then 700 μl of the toluene phase was removed for measurement of the absorbance at 520 nm on a spectrophotometer. The results from three samples are averaged. Vertical bars represent standard deviation for n=3 ($^{*}P$<0.05 and $^{**}P$<0.01).

Acknowledgments

This work was supported by the National Natural Science Foundation of China (30490254, 30671316), the National Basic Research Program of China (2006CB100102), and the Hi-Tech Research and Development Program of China (2006AA10Z113, 2006AA10A111).

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