PROMOTER CLONING AND EXPRESSION ANALYSIS OF TRANSCRIPTION FACTOR GENE GMMYB92 IN SOYBEAN (Glycine max. L)

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MYB transcription factors in plant play a critical role in regulating response to abiotic stress or adverse environments. In our previous study, DGE data revealed GmMYB92 is involved in soybean tolerance to abiotic stress. Here, we analyzed the spatial and temporal expression of GmMYB92 and found GmMYB92 mainly expressed in roots, young stem and flower tissues during the whole growth stages, and significantly induced by ABA, salt, low temperature (4°C) and PEG6000 (drought) treatments, and the time points for induction expression varied among different treatments. Bioinformatic analysis of GmMYB92 promoter region contained many stress-related cis-elements, such as, HSE for heat stress, ARE for anaerobic induction, TC-rich repeat element for defense and stress responsiveness and a CAT-box for specific-expression element in meristem expression. Hereafter, the GmMYB92 promoter region with a 1,951bp DNA fragment including start codon was cloned to drive GUS gene expression to form a chimeric expression cassette used for Arabidopsis Thaliana transformation by means of floral dip. GUS histochemical staining was conducted to characterize the GmMYB92 promoter activity in different tissues including root, stem, leaf, flower and pod from transgenic Arabidopsis Thaliana. The results showed that the gus gene under the control of GmMYB92 promoter mainly expressed in root, young stem and flower, which was consistent with the expression pattern in soybean plant. Our study supply a basis support to further insight into MYB transcription factor’s role in the complicated regulation network related to different genes and the mechanism in stress-resistance in plants.

Keywords: Glycine max, GmMYB92, promoter analysis, expression, GUS staining.

INTRODUCTION

Abiotic stress factors, such as salt, drought, high/low temperature cause the world’s major crop yield loss of about 50% annually (http://www.isaaa.org/), especially, the salinization is one of the most important factors causing the global food production reduction (Tuteja et al., 2007; Vij et al., 2007). In plant anti-adversity study, manipulation of single function gene rarely produces reliable stress tolerance, however, modulation or improvement of the activity of a key transcription factor is a more efficient way. Therefore, application of the transcription factor to improve the stress resistance of plants has become one of the research hotspots in recent years (Sun et al., 2009; Liu et al., 2008).

The MYB transcription factor family is one of the most numerous and functionally diverse family in plants (Riechmann et al., 2000; Lipsick et al., 1996). In soybean, 252 MYB genes have been identified so far, accounting for about 4% of all transcription factors. Among of them, 244 are R2R3-MYB proteins, which can be further grouped into 48 sub-families. Members in each sub-families are functionally conservative. Chromosome distribution model analysis indicates that MYB family are fragmented or tandem-repeated in all 20 chromosomes (Du et al., 2012).

Many studies indicate that MYB transcription factors play important roles in plant secondary metabolism (Uimari and Strommer, 1997; Du et al., 2008), cell differentiation and cycles (Payne et al., 2000; Suo et al., 2003), leaf morphogenesis (Lee and Schiefelbein, 2002; Legay et al., 2007; Yang et al., 2007) as well as the color formation in organ such as pericarp, fruit flesh, leaves and flower (Azuma et al., 2008; Ban et al., 2007; Espley et al., 2007; Takos et al., 2006). MYB transcription factors are also critical for plant hormone response and regulation to environmental stresses (Vannini et al., 2004; Liu et al., 2008; Chen et al., 2003; Hoeren et al., 1998; Lea et al., 2007; Gubler et al., 1997).

Increasing evidence suggest the function of MYB genes in response to abiotic stresses. Yang et al. (2009) treated soybean cultivar Zhong-dou27 with UV-B, drought and salt, and found the GmMYB6 expression is increased; Gm02g01300 and Gm03g38040 are strongly induced by drought, Gm03g38040 is also significantly enhanced in low temperature and high salt. Gm10g01340 and Gm19g40650 are also associated with the plant resistance to high salt,
drought, and low temperature. In hormone response research, 
\textit{GmMYB}96 of soybean is induced by ABA, GA3 and NAA, 
and the \textit{GmMYB}7 is induced by ABA and NAA (Du \textit{et al.}, 2008).
Therefore, plant anti-adversity capability can likely be 
modulated by controlling the expression of MYB 
transcription factors (Liao \textit{et al.}, 2008; Miyake \textit{et al.}, 2003; 
Yang \textit{et al.}, 2009).

\textit{GmMYB}92, one of the 156 MYB transcription factors genes 
was identified by Liao (Liao \textit{et al.}, 2008) in soybean in 2008. 
The gene locates on chromosome 16 and contains 1138bp in 
full length, with a 256bp intron, and encodes a 293 amino acid peptide. 
Their research showed that the \textit{GmMYB}92 can form 
homodimers or heterodimer with \textit{GmMYB}76 to produce trans 
activity. Previously, our lab found that there is the differential 
expression of \textit{GmMYB}92 gene between wild and cultivated 
soybean under salt stress and cloned this gene. After 200mM 
NaCl treatment for two days, \textit{GmMYB}92 over-expressed 
composite transgenic soybean plants remained normal while 
the control seedlings were clearly wilt, indicating 
\textit{GmMYB}92 could improve soybean salt-resistance capability (Ali \textit{et al.}, 2012). Moreover, researches about \textit{GmMYB}92 binding to the 
promoters of target genes have been reported, but the 
expression mechanism of \textit{GmMYB}92 and structure analysis of 
its promoter have not been reported so far.

In this study, \textit{GmMYB}92 expression pattern under normal and 
challenged circumstance were analyzed using semi- 
quantiative RT-PCR. \textit{GmMYB}92 promoter region with 1,951bp in length was cloned and used to drive GUS 
expression in Arabidopsis thaliana. Multiple 
elements were identified in the promoter region. The present 
study provides molecular cues for how to enhance plant 
tolerance to abiotic stresses by fine regulation of \textit{GmMYB}92 
via plant exogenous hormone, light and temperature in the 
future.

\textbf{MATERIALS AND METHODS}

\textbf{Plant material, bacteria and reagents:} Soybean variety 
Williams82, \textit{Arabidopsis thaliana} (Col-0), strains of 
Escherichia coli \textit{DH}5\textit{a}, \textit{Agrobacterium tumefaciens} \textit{EHA105} 
are preserved in our laboratory. pGEM-T Easy Vector T 
cloning kit, TaqDNA polymerase PRIME STAR, DNA 
marker, Trizol reagent, T4 DNA ligase were purchased from 
Fermentas; reverse transcription kit was from Shanghai 
Generay Bioengineering Co. Ltd; DNA purification kit was 
furnished by Shanghai Hao 
Jia Technology Development Co., Ltd respectively.

\textbf{Total RNA extraction and cDNA synthesis:} The samples of 
roots, stems, leaves from the Soybean Seeding of three-leaf 
stage, blossom and fruiting period, flowers, pod and the root of 
seedling of three-leaves stage were treated with 200 mmol 
L\textsuperscript{-1} NaCl, 20% PEG6000, 100 μmol L\textsuperscript{-1} ABA solution and 
4°C for 0 (control), 0.5, 3, 6 and 12 h were collected, grinded 
in liquid nitrogen, and then total RNA was extracted using a 
Plant RNA Kit (Promega, Beijing, China) according to the 
maker’s instructions. Single-stranded cDNA was 
synthesized using 1μg of total RNA and Oligod (T) 18 primer 
with the Takara RT-PCR system in a total volume of 20μl 
according to the instructions.

\textbf{Gene expression analysis:} \textit{GmMYB}92 Gene expression 
analysis in different tissues was performed by semi 
quantiative RT-PCR method using Soybean housekeeping 
gene \textit{GmActin} (Glycine max (actin-1-like), Genebank 
Accession No: XM_003552652) as a control, with 1 μl of 
cDNAs extracted from young root, stems, leaves, flowers and 
pods respectively in 25 μl of PCR mix containing 10×PCR 
buffer 2.5 μl, 10 mmol L\textsuperscript{-1} dNTPs 0.5 μl, 25mmol L\textsuperscript{-1} MgCl\textsubscript{2} 
1.5 μl, each primer 1 μl, 5 U mL\textsuperscript{-1} TaqDNA polymerase 0.2 
μl. For gene expression analysis in response to different 
stress, the cDNAs samples from the roots treated with NaCl, 
PEG6000, low temperature and ABA describe above were 
used for template. The forward primer sequence of \textit{GmActin} 
is 5'-AACAGATGTCCTCATAG-3', and the reverse 
primer is 5'-TAAATACATTGCTTACTC-3'. The primers of 
\textit{GmMYB}92 gene are 5'-AACAGATGTCCTCATAG- 
3' and 5'-TAAATACATTGCTTACTC-3'. PCR reaction 
was initial at 94°C for 3 min, followed by 26–29 cycles of 45 
seconds denaturation at 94°C, 45 seconds primer annealing at 
55 °C and 30s extension at 72 °C, with a final extension at 
72°C for 10 min, repeated three times. In silico transcript 
profiling and identification of salt responsive \textit{GmMYBs} 
were carried out for detecting further Genome-wide analysis of 
the MYB transcription factor family in \textit{Glycine max}. The DGEP 
data were obtained by developing a rigorous algorithm to 
detect differentially expressed genes (DEGs) among the NaCl 
treated and control samples (Audic and Claverie, 1997).

\textit{GmMYB}92 promoter cloning and analysis on cis-acting 
elements: To clone \textit{GmMYB}92 promoter region, a pair of 
primers was designed based on the 5' flanking sequence of 
\textit{GmMYB}92 from soybean genome online database 
http://www.phytozome.net/cgi-bin/gbrowse/soybean. The forward 
primer sequence is 5'-AGATTTCAATTCTAATTCTCA-3' 
and the reverse primer sequence is 5'-TTTTTGCACCTCTCAC-3'. Using 
soybean genome DNA as template, the PCR reaction was 
carried out in 50μl of system with 10×PCR buffer 5 μl, 10 
mmol L\textsuperscript{-1} dNTPs 1 μl, 25 mmol L\textsuperscript{-1} MgCl\textsubscript{2} 3 μl, 10 mmol L\textsuperscript{-1} 
primers 3 μl, 5 U mL\textsuperscript{-1} high fidelity Taq DNA polymerase 
PRIME STAR 0.5 μl, ddH\textsubscript{2}O up to 50 μl. PCR program was 
as follows: 98 °C pre-degeneration for 3 min; followed by 39 
cycles of denaturation at 98°C for 10 s, primer annealing at 
55°C for 20 s and primer extension at 72°C for 2 min, with a 
final extension at 72°C for 10 min. The PCR product was 
cloned into pGEM-T Easy Vector for sequencing. The 
promoter elements analysis was performed using online tool 
PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/ 
plantcare/html/).
Plasmid construct and plant transformation: To characterize activation of GmMYB92 promoter in various organs, the 1,951bp promoter fragments from GmMYB92 was cloned into the upstream of the β-glucuronidase (GUS) reporter gene in the vector pCXGUS-P (Chen et al., 2009) to form a new construct pCXGUS-M92P, which was transformed into Agrobacterium tumefaciens strain EHA105 and further transformed into Arabidopsis thaliana plant by flower dip as described by Clough and Bent (1998).

GUS histochemical staining: Transgenic seeds of Arabidopsis thaliana plants were selected on media containing 50 mg/L of kanamycin. For β-glucuronidase (GUS) staining, various tissues from positive lines including root, stem, leaves, flower and pod were incubated in 50 mM sodium phosphate, at a pH of 7.2, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 2 mM 5-bromo-4-chloro-3-indolyl-bD-glucuronic acid at 37°C for 4–6 h (Jefferson., 1987). Samples were stored in 70% ethanol before microscopic examination then observed under a microscope (Zeiss Stemi 2000-C, Germany).

RESULTS

GmMYB92 are mainly expressed in roots, young stems and flowers: To detect the spatial and temporal expression pattern of GmMYB92, semi quantitative RT-PCR method was carried out using different organs in different growth and developmental stages. The results showed that GmMYB92 was highly expressed in root, and the expression levels were maintained at a high level during the whole growing period. High expression levels were also detected in flowers. In the early stem development, GmMYB92 expression was higher in young stem, but very low at flowering and maturity stages, and no expression in leaves and pods (Fig.1).

Figure 1. Semi-quantitative RT-PCR analysis of GmMYB92 expression in different organs and different development stages.

GmMYB92 expression can be induced by NaCl, PEG and ABA treatments: GmMYB92 gene expression in the root tissue was also examined with ABA treatment and other treatments, including low temperature, high salt and drought simulation (20% PEG6000). Compared to the control (no treatment), the expression level of GmMYB92 gene gradually increased and reached the maximum value from 0-1 h under low temperature treatment (4°C), but it was not significantly different compared to NaCl, PEG6000 and ABA treatments. The GmMYB92 expression level increased sharply at 3 h, then the expression level decreased slightly at 6 h, but upregulated again at 12 h after treatment with ABA and PEG6000. The expression of GmMYB92 gene was significantly increased with 200 mM NaCl treatment for 12 hours and remained high thereafter. Therefore, the expression profiles in response to ABA, low temperature, high salt and drought indicate that GmMYB92 gene is involved in regulation under stresses.

Figure 2. Semi-quantitative RT-PCR analysis of GmMYB92 in response to ABA treatment and low temperature, high salt and drought stress.

Difference of MYB genes expression in Glycine max samples treated with 0 or 200 mM NaCl were analyzed using soybean Tag sequencing data generated via DGEP. The result showed that a total of 59 GmMYBs with higher expression level were found in Glycine max and 44 MYBs were up-regulated. The expression of GmMYB92 was 1.77 fold higher than of the control in up-regulated MYBs, which indicated GmMYB92 is response to NaCl stress and may be related to enhance salt tolerance of Glycine max.

Figure 3. Expression analysis of GmMYBs at 12h of 200 mM NaCl stress in root of trifoliate stage soybean seedlings. (A) Expression profile of 59 GmMYBs based on DGEP data. (B) GmMYB92 expression pattern in high salt stress, the ordinate based on the normalized expression data from DGEP.
The isolation of GmMYB92 promoter and cis-acting elements analysis:

GmMYB92 promoter region with 1,951 bp in length was cloned by PCR method. The cis acting elements in this promoter were analyzed using an online promoter analysis tool PLANTCARE (Table 1 and Fig. 4). As shown in the

<table>
<thead>
<tr>
<th>Cis-elements</th>
<th>Number</th>
<th>Position</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-AF3 binding site</td>
<td>1</td>
<td>+</td>
<td>part of a conserved DNA module array (CMA3)</td>
</tr>
<tr>
<td>5UTR Py-rich stretch</td>
<td>1</td>
<td>-</td>
<td>cis-acting element conferring high transcription levels</td>
</tr>
<tr>
<td>AAGAA-motif</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ARE</td>
<td>1</td>
<td>+</td>
<td>cis-acting regulatory element essential for the anaerobic induction</td>
</tr>
<tr>
<td>AT-rich element</td>
<td>1</td>
<td>+</td>
<td>binding site of AT-rich DNA binding protein (ATBP-1)</td>
</tr>
<tr>
<td>AT1-motif</td>
<td>1</td>
<td>-</td>
<td>part of a light responsive module</td>
</tr>
<tr>
<td>ATGCAAAT motif</td>
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<td>-</td>
<td>cis-acting regulatory element associated to the TGAGTCA motif</td>
</tr>
<tr>
<td>Box 4</td>
<td>1</td>
<td>+</td>
<td>part of a conserved DNA module involved in light responsiveness</td>
</tr>
<tr>
<td>Box 1</td>
<td>2</td>
<td>+</td>
<td>light responsive element</td>
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<tr>
<td>CAAT-box</td>
<td>32</td>
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<td>common cis-acting element in promoter and enhancer regions</td>
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<td>1</td>
<td>+</td>
<td>cis-acting regulatory element related to meristem expression</td>
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<tr>
<td>F-box</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>GA-motif</td>
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<td>+</td>
<td>part of a light responsive element</td>
</tr>
<tr>
<td>GAG-motif</td>
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</tr>
<tr>
<td>GARE-motif</td>
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<td>+</td>
<td>gibberellin-responsive element</td>
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<tr>
<td>GATA-motif</td>
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<td>part of a light responsive element</td>
</tr>
<tr>
<td>GT1-motif</td>
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<td>-</td>
<td>light responsive element</td>
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<tr>
<td>HSE</td>
<td>2</td>
<td>+/-</td>
<td>cis-acting element involved in heat stress responsiveness</td>
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<tr>
<td>Skn-1_motif</td>
<td>2</td>
<td>+</td>
<td>cis-acting regulatory element required for endosperm expression</td>
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<tr>
<td>TATA-box</td>
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<td>+/-</td>
<td>core promoter element around -30 of transcription start</td>
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<tr>
<td>TC-rich repeats</td>
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<td>+/-</td>
<td>-</td>
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<tr>
<td>as-2-box</td>
<td>1</td>
<td>-</td>
<td>involved in shoot-specific expression and light responsiveness</td>
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<td>chs-CMA2a</td>
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<td>part of a light responsive element</td>
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<tr>
<td>circadian</td>
<td>2</td>
<td>-</td>
<td>cis-acting regulatory element involved in circadian control</td>
</tr>
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Figure 4. Complete nucleotide sequence of the GmMYB92 promoter region. Nucleotide numbers are indicated to the both sides of each lane. The putative transcriptional start site (asterisk) is indicated as well as the start codon ATG (dark-colored box) of GmMYB92 gene. The TATA box (light-colored box), CAAT box (light-colored box) and other main cis-elements (underlined and bold) are also showed.
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Table, it contains five types of cis acting elements including transcription-related, light reaction, tissue specific expression, abiotic stress response and hormone response elements respectively. Transcription-related components often contain, in addition to normal promoter, TATA frame, CAAT frame, 5UTR Py-rich stretch elements. Total 15 Light response elements are exist in the area, belonging to 11 groups. There are three tissue specific expression elements, CAT box for meristem tissue, Skn-1 for endosperm and as-2-box for bud, respectively. Stress response elements with heat shock element HSE, hypoxia inducible element ARE and the stress response and defense element TC-rich repeat are contained. GARE, the gibberellic acid response element, is also. These elements are distributed in both sense and antisense strands. Among them, the CAAT box, TATA box and light responsive elements have multiple copies, while others only have one or two copies.

GmMYB92 promoter drove GUS expression analysis in transgenic Arabidopsis thaliana: Reporter expression cassette was constructed by fusing the GmMYB92 promoter and GUS gene (Fig.5), and transformed into Arabidopsis thaliana using the flower dip method. GUS histochemical staining were carried out in transgenic seedlings of T1 generation. The results showed that high GUS expression was found in the root and flower. No GUS expression was found in stems and pod (Fig.6), which was consistent with the organ expression pattern in soybean seedling.

DISCUSSION

The promoters of different stress-related gene often contain the same cis acting elements, being regulated in a similar way or through similar molecular mechanism. In other words, the transcriptional regulatory gene can regulate the expression level of a series of stress related genes, thereby enhance the resistance capability to the stress (Liu et al., 2008). Through the analysis of the Arabidopsis thaliana drought, high salt and ABA inducible promoter region of genes, TAACGTG, the core sequence for MYB binding was identified (Urano et al., 1993). MYB transcription factor can also bind with MYBS I (conservative sequence T/CAACG/TGA/C/TAC/T), MYBS II, (conservative sequence TAACCTAAC), CNGTTR and GKTWTGTRGKTWGGTR (N: A, G, C or

Figure 5. Schematic structure of the plant expression vector pCXGUS-M92P generated by insertion of GmMYB92 promoter fragment (M92P).

Figure 6. GUS staining in various tissues from transgenic Arabidopsis thaliana harboring GUS gene driven by GmMYB92 promoter. A: 3-week-old transgenic seedling; B: root; C: stem; D: leaf of 3-week-old seedling; E: flower at blooming stage; F: pods of transgenic Arabidopsis thaliana.
T; K: G or T; R: A or G; W: A or T), (Romero et al., 1998). Liao et al. (2008) showed that GmMYB92 can recognize and bind MBSI recognition sequence (TATAACGGTTTTTT), MRE4 (TCTCACCCTAC) and Mmre1 (CCGAAAAAAGGAT). Plant stress tolerance related genes such as DREB2A, RD17, RD29A, RD29B, P5CS, COR66 and COR78 are downstream genes of MYB transcription factor. In GmMYB92 transgenic Arabidopsis, DREB2A, RD17 and P5CS are increased while COR66 and COR78 expression level remain low, suggesting that GmMYB92 regulation on the downstream target genes is rather complicated. It may, alone or synergistically with other transcription factors, up-regulate or down-regulate target gene transcription level, eventually improves the stress tolerance of plants.

The interaction among genes forms a complex gene regulatory network in cell. While MYB transcription factor regulate target gene expression, its own activity is regulated by other proteins at all levels, in another word, it is the intermediate target of other regulatory factors (Gonzalez et al., 2008). RNA and protein are two kinds of molecules regulate the expression of transcription factor MYB gene. MYB gene is a common target of small RNAs (miRNAs) and trans silencing of RNAs (ta-siRNAs). For instance, miR159 acts on AtMYB33, AtMYB35, in AtMYB65 and AtMYB101, and thus regulates anther and pollen development (Gonzalez et al., 2008). In the MADS family, embryo development related AGL15 protein can bind 29 different MYB genes (Zheng et al., 2009). In addition to proteins, these genes directly regulate MYB transcription factor. For example, AtMYB123, AtMYB2, AtMYB66, AtMYB0 and AtMYB33 are direct regulatory sites for up to 552 genes. The regulation process also involves bHLH chaperone proteins (Koshino-Kimura et al., 2005). In general, MYB transcription factors can be divided into two categories. One is the ABA related transcription factors, promoter of many ABA induced genes contain ABRE consensus sequence. The other is ABA independent MYB transcription factors. Their promoter region contains CRT/DRE or other elements, acting as MYB protein recognition sites, and thus regulated by the corresponding factors (Liu et al., 2008). GmMYB92 promoter contain neither ABRE, nor CRT/DRE cis acting element, but in state of gibberellic acid response element GARE, suggesting that it belongs to ABA independent MYB transcription factors, and is related to gibberellic acid signaling pathway. Sequence analysis shows GmMYB92 promoter region contains the thermal stress components HSE, anaerobic stress inducible elements ARE, and indicating that the gene is involved in plant stress responses. In addition, the promoter region also contains multiple light response elements, some other elements that are rarely found in other MYB promoters, including 3’-AF3, AT rich element binding site, AT motif, F-box and chs-CMA2a etc. The roles of these elements in the regulation of GmMYB92 transcription are not clear so far. Further experimental studies are required to understand their functions.

MYB gene family has the differential temporal and spatial expression patterns. For example, AtMYB33 and AtMYB65 genes in Arabidopsis thaliana express in a variety of organs and tissues (Gocal et al., 2001). C1 and PI in corn has a role in the regulation of anthocyanin synthesis, but C1 is only expressed in the aleurone layer and flower organs, while PI is restricted in vegetative tissues (Piaaza et al., 2002). Northern blot results show that, GmMYBJ6 expression is only detected in the leaves, suggesting tissue specific expression of GmMYBJ6 (Yang et al., 2009). In this study, the GmMYB92 gene expression and its promoter driving GUS expression in Arabidopsis show that the expression in roots and young stems, leaves and flowers is high, but is low in fully developed response tissues and organs. We suspected that the stable, high level expression is conducive to its ability to regulate the stress responsive genes in order to improve the resistance ability of plants. Expression in young tissues may be related to existence of Meristem specific expression element CAT box, suggesting GmMYB92 plays a role in root, stem growth and flower development and tolerance to stresses.

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