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

Huang Yihong[1,†], Xu Zhaolong[1,†], Xu Ling[1], Wei Peipei[2], Zhang Dayong[1,*], He Xiaolan[1], Guo Shiwei[1], Peng Chen[1] and Ma Hongxiang[1]

[1]Jiangsu Key Laboratory for Bioresources of Saline Soils，Provincial Key Laboratory of Agrobiology, Institute of Biotechnology，Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China; [2]College of life science, Nanjing Agricultural University, Nanjing, 210095, China.
[*]Corresponding author's e-mail: cotton.z@126.com, zhan1658@purdue.edu
[†]Huang Yihong and Xu Zhaolong contributed equally.

MYB transcription factors in plant play a critical role in regulating response to abiotic stress or adverse environments. In our previous study, DGEP 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 *GmMYBj6* 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, *GmMYBJ6* of soybean is induced by ABA, GA_3 and NAA, and the *GmMYBJ7* is induced by ABA and NAA (Du *et al*., 2008). Therefore, plant anti-adversity capability can likely be modulated by controlling the expression of MYB transcription factors (Liao *et al*., 2008; Miyake *et al*., 2003; Yang *et al*., 2009).

GmMYB92, one of the 156 MYB transcription factors genes was identified by Liao (Liao *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 GmMYB92 can form homodimers or heterodimer with GmMYB76 to produce trans activity. Previously, our lab found that there is the differential expression of *GmMYB92* gene between wild and cultivated soybean under salt stress and cloned this gene. After 200mM NaCl treatment for two days, *GmMYB92* over -expressed composite transgenic soybean plants remained normal while the control seedlings was clearly wilt, indicating *GmMYB92* could improve soybean salt-resistance capability (Ali *et al*., 2012). Moreover, researches about GmMYB92 binding to the promoters of target genes have been reported, but the expression mechanism of *GmMYB92* and structure analysis of its promoter have not been reported so far.

In this study, *GmMYB92* expression pattern under normal and challenged circumstance were analyzed using semi-quantitative RT-PCR. *GmMYB92* promoter region with 1,951bp in length was cloned and used to drive GUS heterologous 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 tresses by fine regulation of *GmMYB92* via plant exogenous hormone, light and temperature in the future.

MATERIALS AND METHODS

***Plant material, bacteria and reagents*:** Soybean variety Willimas82, *Arabidopsis thaliana* (Col-0), strains of Escherichia coli *DH5α*, *Agrobacterium tumefaciens* 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 from Promega, the biological reagent was from Shanghai Hao Jia Technology Development Co., ltd respectively.

***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^{-1} NaCl, 20% PEG6000, 100 μmol L^{-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 manufacturer's instructions. Single-stranded cDNA was synthesized using 1μg of total RNA and Oligd (T) 18 primer with the Takara RT-PCR system in a total volume of 20μl according to the instructions.

***Gene expression analysis*:** *GmMYB92* Gene expression analysis in different tissues was performed by semi quantitative RT-PCR method using Soybean housekeeping gene *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^{-1} dNTPs 0.5 μL, 25mmol L^{-1} $MgCl_2$ 1.5 μl, each primer 1 μl, 5 U mL^{-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 *GmActin* is 5'-AACAGATGGTCCCTCAT AG-3', and the reverse primer is 5'-TAAATACATTGCCTTCACTC-3'. The primers of *GmMYB92* gene are 5'-AACAGATGGTCCCTCATAG-3'and 5'-TAAATACATTGCCTTCACTC-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 *GmMYBs* were carried out for detecting further Genome-wide analysis of the MYB transcription factor family in *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).

***GmMYB92 promoter cloning and analysis on cis-acting elements*:** To clone *GmMYB92* promoter region, a pair of primers was designed based on the 5' flanking sequence of *GmMYB92* from soybean genome online database http://www.phytozome.net/cgi-bin/gbrowse/soybean. The forward primer sequence is 5'-AGATTTCAGATTCAATTTCA-3' and the reverse primer sequence is 5'-TTTTTGGCACTCTCACTCAC-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^{-1} dNTPs 1 μl, 25 mmol L^{-1} $MgCl_2$ 3 μl, 10 mmol L^{-1} primers 3 μl, 5 U μL^{-1} high fidelity Taq DNA polymerase PRIME STAR 0.5 μl, ddH_2O 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-bro-mo-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).

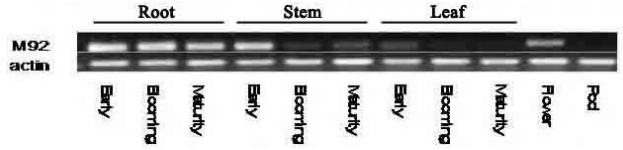


Figure 1. Semi-quantitative RT-PCR analysis of *GmMYB92* expression in different organs and different development stages.

***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 6h, 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.

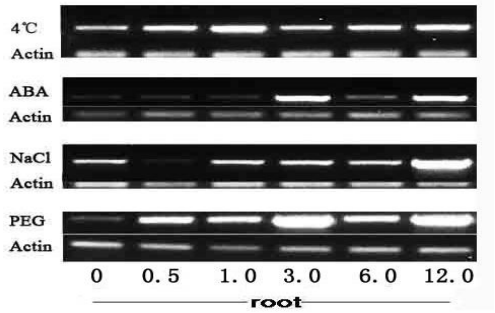
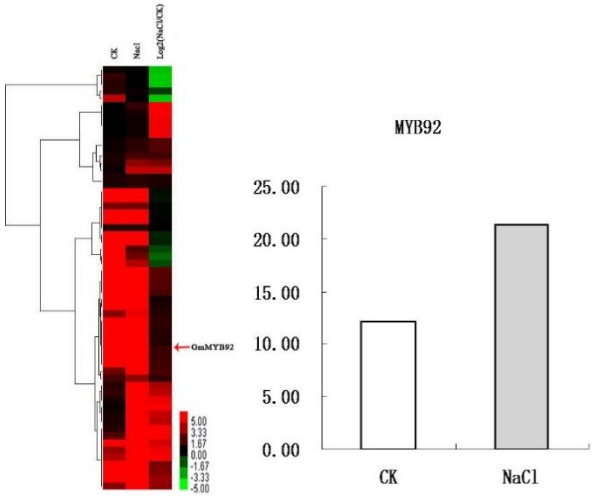


Figure 2. Semi- quantitative RT-PCR analysis of *GmMYB92* in response to ABA treatment and low temperature, high salt and drought stress.

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*.



(A) (B)

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

Table1. The cis-acting elements in *GmMYB92* promoter.

Cis-elements	Number	Position	Function
3-AF3 binding site	1	+	part of a conserved DNA module array (CMA3)
5UTR Py-rich stretch	1	-	cis-acting element conferring high transcription levels
AAGAA-motif	1	-	-
ARE	1	+	cis-acting regulatory element essential for the anaerobic induction
AT-rich element	1	+	binding site of AT-rich DNA binding protein (ATBP-1)
AT1-motif	1	-	part of a light responsive module
ATGCAAAT motif	1	-	cis-acting regulatory element associated to the TGAGTCA motif
Box 4	1	+	part of a conserved DNA module involved in light responsiveness
Box I	2	+	light responsive element
CAAT-box	32	+/-	common cis-acting element in promoter and enhancer regions
CAT-box	1	+	cis-acting regulatory element related to meristem expression
F-box	1	-	-
GA-motif	1	+	part of a light responsive element
GAG-motif	1	-	part of a light responsive element
GARE-motif	1	+	gibberellin-responsive element
GATA-motif	1	+	part of a light responsive element
GT1-motif	3	-	light responsive element
HSE	2	+/-	cis-acting element involved in heat stress responsiveness
Skn-1_motif	2	+	cis-acting regulatory element required for endosperm expression
TATA-box	132	+/-	core promoter element around -30 of transcription start
TC-rich repeats	2	-	cis-acting element involved in defense and stress responsiveness
TCT-motif	2	+	part of a light responsive element
Unnamed__4	2	+/-	-
as-2-box	1	-	involved in shoot-specific expression and light responsiveness
chs-CMA1a	1	-	part of a light responsive element
chs-CMA2a	1	-	part of a light responsive element
circadian	2	-	cis-acting regulatory element involved in circadian control

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.

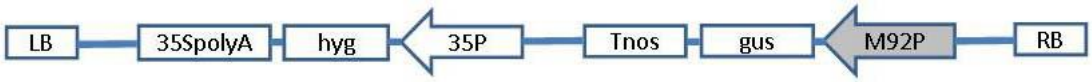


Figure 5. Schematic structure of the plant expression vector pCXGUS-M92P generated by insertion of *GmMYB92* promoter fragment (M92P).

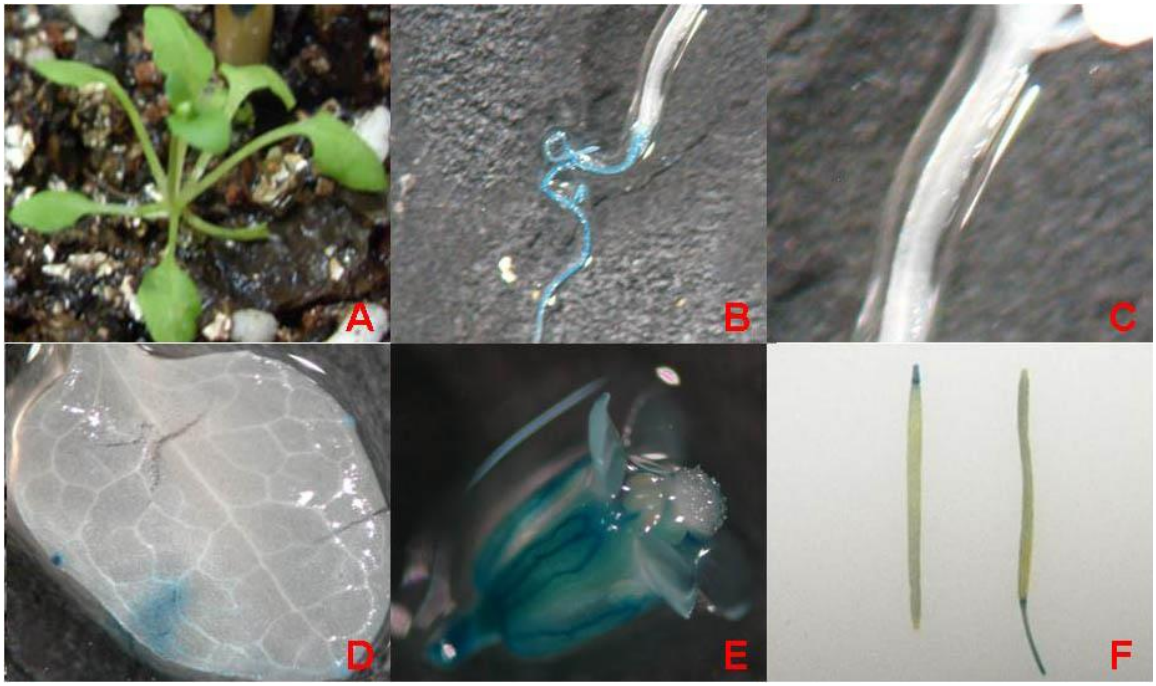


Figure 6. GUS staining in various tissues from transgenic *Arabidopsis thaliana* harboring *GUS* gene driven by *GmMYB92* promoter. A: 3-week-old transgenic seedling; B: root; C: stem; D: leaf of 3-week-old seedling; E: flower at blooming stage; F: pods of transgenic *Arabidopsis thaliana.*

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, TAACTG, the core sequence for MYB binding was identified (Urao *et al*., 1993). MYB transcription factor can also bind with MYBS I (conservative sequence T/CAACG/TGA/C/TA/C/T) , MYBS II , （conservative sequence TAACTAAC), CNGTTR and GKTWGTTRGKTWGGTR (N： A, G, C or

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 (TCTCACCTACC) and Mmre1 (CCGGAAAAAAGGAT). 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/rd29A* 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 Pl in corn has a role in the regulation of anthocyanin synthesis, but C1 is only expressed in the aleurone layer and flower organs, while Pl 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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