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Regulation of the Soybean GmPM9 Promoter in Callus Tissue

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Late embryogenesis abundant (Lea) proteins, also known as seed maturation proteins, are synthesized during seed maturation stage, and widely exist in monocot and dicot plants. They are also induced at vegetative tissues under dehydration condition or exogenous abscisic acid (ABA) treatment. The soybean gGmPM9 clone corresponding to a gene encodes a 16 kDa group IV Lea protein. After analyzing the 5' sequence of GmPM9 gene, several predicted cis-elements, including four abscisic acid-responsive elements (ABREs), are found. The consensus sequence of ABRE is -C/TACGTG-. Previous reports indicated that ABREs, which were present in the promoter region of several genes responding to a variety of environmental and physiological cues, were necessary or sufficient to ABA responses. To understand the regulation mechanism of GmPM9 gene, we constructed a series fusion genes with various length of GmPM9 promoter and uidA gene, which encoded β-glucuronidase (GUS). For the transient assay, the soybean callus tissue were bombarded with five fusion genes, including pGUS966, pGUS573Δ, pGUS573, pGUS510, pGUS224 and pGUS75, and the GUS activity were then measured. Two constructs, pGUS573 and pGUS510, starting from the middle of the promoter region exhibited the highest GUS activity under ABA or salt treatment. Construct pGUS966 with full-length GmPM9 promoter showed the spatial silence. For the transgenic assay, the calli were induced from transgenic tobacco plants with full-length promoter fusion gene (pZP966), and the GUS activity was then measured by fluorometric assay and histochemical staining. The GUS activity was highly active by ABA or salt treatment, but dehydration and temperature stress would not give the same results. These findings indicated the promoter of gGmPM9 could be regulated by ABA and salt directly or indirectly in callus system.

Key words: late embryogenesis abundant, promoter, transient assay, bombardment, soybean callus, transgenic tobacco plants.

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Introduction

ABA affects a variety of plant physiology phenomenon in different development stage and environment stress. For instance, in seed germination stage, exogenous ABA appears to maintain seed dormancy and prevent germination process. Besides, environment stress such as salt, chilling or drought could increase endogenous ABA level in plant tissues (reviewed by [1-2]). In general, these physiology responses to ABA accompany with the expression of many newly syntheses genes [2,3]. Previous studies showed that some of these expressed genes belonged to Lea proteins, which accumulate in the late stage of seed development [4-5]. Lea proteins have been isolated from various monocot and dicot plants, and classified into at least six groups by their conserved sequence or the conformation of amino acid [6]. The function of Lea proteins still remained unclear. However, according to the proteins chemical characteristics and the timing for accumulation, Lea proteins were demonstrated to be acted as desiccation protectants [7] or to maintain the seed dormancy [8].

In addition to the studies on the function of Lea proteins, it is important to investigate the molecular mechanism of ABA or other environment factors. For these purpose, promoter analysis would be a critical strategy. The 5' upstream sequence of ABA-responsive genes, including *Lea* and some storage proteins genes, were compared. The conserved sequences (-C/TACGTG-) were identified to be the putative ABA-responsive elements, and designated ABRE [9]. ABRE

Table 1. The ABREs in various promoters of *lea* genes.

Species and genes	Sequence of ABREs			Reference
Soybean				
gGmPM9	-75 CTGAC	ACGT	GTAAGA	Lee *et al*, 1992
	-181 CAATT	ACGT	GTAAGA	
	-184 TTGCC	ACGT	CTCCCT	
	-681 ACACT	ACGT	GCGATG	
Cotton				
D113	-80 TGTAT	ACGT	GGCAGC	Baker *et al*, 1988
	-101 AGCAT	ACGT	GTCAGA	
	-130 CTGAA	ACGT	GTAAGC	
	-461 CCTAC	ACGT	ATTTTC	
Tomato				
Le25	-64 TATTT	ACGT	GGCATC	Cohen *et al*, 1991
	-85 CGCGT	ACGT	T T A T A	
	-132 ACAAA	ACGT	GTCATG	
	-1032 ACGCT	ACGT	GTTGGC	
Arabidopsis				
Pap260	-161 CACTG	ACGT	GTCGTC	Raynal, 1995
	-242 CCAAC	ACGT	G T A G G	

Consensus sequence: C/TACGTG

is one of the best-characterized ABA-responsive *cis*-elements, and was studied using many approaches. Transient expression studies using wheat *Em* (group I *Lea*) or rice *rab16A* (group II *Lea*) promoters with reporter genes suggested that ABREs were sufficient to confer ABA-dependent regulation pathway in rice protoplasts [10-11]. One the other hand, several *Lea* gene promoters from different plant species were fused to GUS reporter gene, and transformed to tobacco or Arabidopsis plants. The results with transgenic plants also indicated that ABREs could response to ABA or other environment stimuli [e.g. 9,12-14].

According to gel-retardation assay and DNaseI footprinting experiment, some DNA-binding proteins should interact with the ABREs. Guiltinan *et al.* [15] reported a basic leucine-zipper (bZIP) transcription factor, EmBP-1, which bond to the ABRE (CACGTGGC) in the –75 position of wheat *Em* gene. ABREs with ACGT-core sequence were very similar to the G-box that existed in the promoter region of a variety of genes responding to other environmental and physiological cures, such as light or auxin (reviewed by [16]). However, G-box binding factors (GBFs) had less affinity to ABREs [17]. Therefore, it was indicated that the EmBP-1, or other *trans*-acting factors responding to ABREs, contained the specificity to ABA/osmotic-responsive genes. According to the literatures, there were three possibilities causing the specificity, i.e. the franking sequence [18], dimerization status [17] and associated cis-elements [19]. For example, studies on the promoter of the barley ABA-responsive *HVA22* gene indicated an ABA-responsive complex (ABRC) consisting an ABRE and a novel coupling element (CE), which appeared to be necessary and sufficient for high-level ABA induction [20].

We have selected several cDNA clones of seed maturation proteins from a soybean pod-dried seed cDNA library by differential screening [21,22]. These are designated GmPM clones, which stands for *Glycine max* physiological mature. GmPM1 [23] and GmPM9 [24] belong to group IV, or so-called D-113, Lea protein, which was first identified in cotton [5]. Group IV Lea proteins usually contain a large number of Ala residues and the random-coil promoting residues Gly and Thr. The putative amino acid sequences of GmPM1 and GmPM9 had 95% homology except GmPM1 contained a sector of 23 amino acids that was deleted in GmPM9 proteins. The *gGmPM9* genomic clone for *GmPM9* contained a 1 kb 5' untranscripted sequence, a 0.3 kb 3' untranslated sequence, and a complete open reading frame that encode a 16 kDa Lea protein. Several group IV Lea proteins had been identified from various plant species. One of them, tomato *Le25* gene, was induced in roots and leaves tissue by ABA or drought [25]. Imai et al. used yeast as a model system, and found Le25 proteins might play the role for ion scavenger [26]. In our previous study, *GmPM1/9* mRNA was not accumulated in ABA- or drought-treated soybean seedlings, but also in ABA- or chilling-treated soybean callus [unpublished data]. Various lengths of *GmPM9* promoter region were fused to GUS reporter gene, and then transformed to Arabdiopsis. In these transgenic plants, the GUS activities were detected in the mature seeds and seedling treated with NaCl or PEG, but not with ABA or chilling [14]. In the present study, the relationship between *GmPM9* promoter and ABA or environment stimuli was investigated using the callus system. For transient assay, soybean callus was derived from hypocotyls, and bombarded with a series *GmPM9* fusion genes. Callus induced from the transgenic tobacco plants with full-length *GmPM9* promoter were used. GUS activities were measured in both transient and transgenic calli in order to reveal the *GmPM9* gene regulation mechanism in callus system.

Table 2.Comparison of SEF binding sites at the promoter region of several soybean seed proteins

Genes		Sequence of SEF binding site			Accession number
SEF1					
gGmPM9	-328	TTTAA	ATATTTAT	ACAAA	M97285
	-966	AAATA	ATATTTAT	ACAAA	
β-conglycinin	-719	TGACT	ATATTTAT	CTGCA	M13759
	-842	ATAAT	ATATTTAT	ATTTT	
Lectin	-674	CAGAT	ATATTTAT	TTGTG	K00821
SEF4					
gGmPM9	-524	TTCAT	GTTTTTA	TCTGC	
	-760	ATAAT	ATTTTTA	AAATA	
	-781	TTGCG	ATTTTTG	TACTG	
β-conglycinin	-175	ACCTC	ATTTTTG	TTTAT	
	-749	TTTGC	ATTTTTA	TCAAT	
	-774	AAAAT	ATTTTTA	TATCT	
Lectin	-195	GTTTA	GTTTTTA	AATTT	
	-591	TTTTT	ATTTTTA	AGTCA	
	-597	TTTTT	ATTTTTA	TTTTT	

Consensus sequence: SEF1 ATATTTAT, SEF4 A/GTTTTTA/G

Materials and Methods

Plant material

Soybean (*Glycine max* [L.] Merr. cv. Shi-shi) and tobacco (*Nicotiana tabacum*, L. cv. W38) plants were used in this research. Mature soybean seeds were surface sterilized in 70% ethanol for 1 min and 10% bleach for 15 min, followed by 3 to 4 times rinses with sterile water. Sterilized seeds were germinated under non-bacterial environment until hypocotyls expanding. Surface sterilized tobacco seeds were grown on half Murashige and Skoog (MS) medium [27] contained 1% (w/v) sucrose and 0.2% (w/v) Gellan Gum. After germination, the seedlings were transferred to greenhouse until flowering.

DNA construct preparation

A 1057bp *Xba*I-*Pvu*II fragment (-966bp to +91bp), containing the full-length promoter region and 43bp of the coding region of gGmPM9 genomic clone, was cloned into *Xba*I-*Pvu*II site of pBluescript SK-. This clone was digested with *Pst*I followed with klenow treatment then cut by *Xba*I. The purified fragment was ligated into *Xba*I-*Sma*I digested vector, pUC18-GUS and pBI101 (Clontech) respectively, and designated pUGS966 and pZP966. The 610bp *Sau3*A fragment (-573bp to +37bp) was ligated into the *Bam*HI site of pBluescript SK-. This clone was disgeted by *Xba*I and *Sma*I, and then cloned into *Xba*I-*Sma*I site of pUC18-GUS, designated pGUS573. The *Sau3*A fragment with the remove of an 111bp *Pml*I fragment (-178bp to -68bp) was ligated into pUC18-GUS vector and designated pUGS573Δ. Besides, *Sau3*A fragment was used to generate the exonuclease III deletion fragments. Three deletion fragments were cloned into pUC18-GUS vector, and designated pUGS510 (-510bp to +37bp), pUGS224 (-224bp to +37bp) and pGUS75 (-75bp to +37bp),

respectively.

Production of transgenic plants

Plasmid pZP966, and positive control (pBI121) (Clontech), were transferred from Escherichia coli strain XL-1Blue to *Agrobacterium tumefaciens* strain LBA4404 via the freeze-thaw method [28]. Tobacco transformation was carried out by leaf disc infection that described by Holsters et al. [28]. Regenerated seedling was screened on half MS medium contained 1% (w/v) sucrose, 0.2% (w/v) Gellan Gum, and 50 μg/ml kanamycin. Kanamycin-resistant seedling (T1) were then transferred to soil and grown in greenhouse under the same condition as described above. Self-pollinated seeds from T1 plants were collected, and screened by kanamycin to get the homozygous T2 seed for further experiments.

Production of soybean and transgenic tobacco callus

The expanded hypocotyls of soybean seedling were wounded and cultured on callus induction medium consisted of MS medium supplemented with 1 mg/L 2,4-D, 3% (w/v) sucrose and 0.2% (w/v) Gellan Gum at room temperature under darkness. Callus was subcultured every three weeks. The tobacco callus were generated from the pith or leaf tissue of T2 transgenic or un-transgenic tobacco plants on the medium consisted of MS medium supplemented with 1 mg/L NAA, 0.1 mg/L BA, 3% (w/v) sucrose and 0.2% (w/v) Gellan Gum at room temperature under darkness.

DNA bombardment

The DNA bombardment was carried out as described by Barcelo and Lazzeri [29]. The subcultured soybean calli were bombarded with submicron gold particles (1.0 μm gold particle) (Bio-Rad), coated with precipitated plasmid DNA. Particle bombardments were carried out using a PDS-1000/He gun (Bio-Rad) with a target distance of 9 cm from the stopping plate at helium pressures of 450 psi.

Treatment and GUS activity assay

After the bombardment, soybean calli were incubated with 10^{-5} M ABA or 1% NaCl under darkness for 48 hours. Besides, the transgenic and non-transgenic tobacco calli ware treated with 10^{-5} and 10^{-6} M ABA, 1% NaCl or 2% PEG under darkness for 24 hours; or incubated in 4°C or 42°C growth chamber for 6 hours.

GUS fluorometric assay was carried out as described by Jefferson [30]. Proteins extracts were prepared from callus tissue in the extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, and 0.1% sarkosyl). Protein concentration of the crude extracts was determined by DC-protein assay kit (Bio-Rad). The 4-methylumbelliferyl glucuronide (4-MUG) was used as the substrate for quantitative determination of GUS activity. Reaction samples that incubated at 37°C were taken at 0 and 60 min and the reaction was stopped by adding 0.2M Na_2CO_3. The fluorometric quantity of 4-methylumbelliferone (4-MU) was measured by F4010 Fluorometer (Hitachi). The GUS activity was expressed as pmol 4-MU per minute per mg protein. All the experiments were performed at least three duplications.

The procedure of histochemical staining of GUS activity with 5-bromo-4- chloro-3-indolyl glucuronide (X-Gluc) (BRL) was modified from Jefferson et al. [31]. Calli were immersed in GUS histochemical buffer (1 mM X-Gluc, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 20% methanol), and facilitated by vacuum for 10 min. The histochemical reaction was carried out overnight under darkness at 37°C before the stained sample was recorded by photograph.

Result

The effects of four ABREs in the GmPM9 promoter

A database search revealed that the *GmPM9* promoter contained several *cis*-elements, which were identified from other plant promoters. Besides the TATA- and CAAT-box, several ABREs, light-responsive elements (e.g. GATA-motif) [32], soybean embryo factor (SEF) motif [33], and cell-type specific element [e.g. (CA)n] [34] existed. The four ABREs were found, and three of them were located at proximal region of TATA box between –178bp to –68bp (Figure 1). To assess the spatial regulation mechanism of the *GmPM9* promoter, various length of the promoter were fused to the GUS reporter genes (Figure 1). The resulting constructs, including pUGS966 (-966bp to +91bp), pGUS573 (-573bp to +37bp), pUGS573Δ(-573bp to +37bp, with the deletion of an internal 111bp *Pm*/I fragment from -178bp to -68bp), pUGS510 (-510bp to +37bp), pUGS224 (-224bp to +37bp) and pGUS75 (-75bp to +37bp), were used for transient assay by particle gun bombardment.

Bombarded calli were treated with 10^{-5} M ABA or 1% NaCl, and the effects of each promoter constructs ABREs were quantified by GUS fluormetric assay (Figure 2). Clearly, the pGUS573 and pUGS510 were able to confer a higher level of ABA induction. The GUS activity of the pUGS966 gave a GUS activity with only 60% of that by pGUS573, and thus revealed the presence of silent

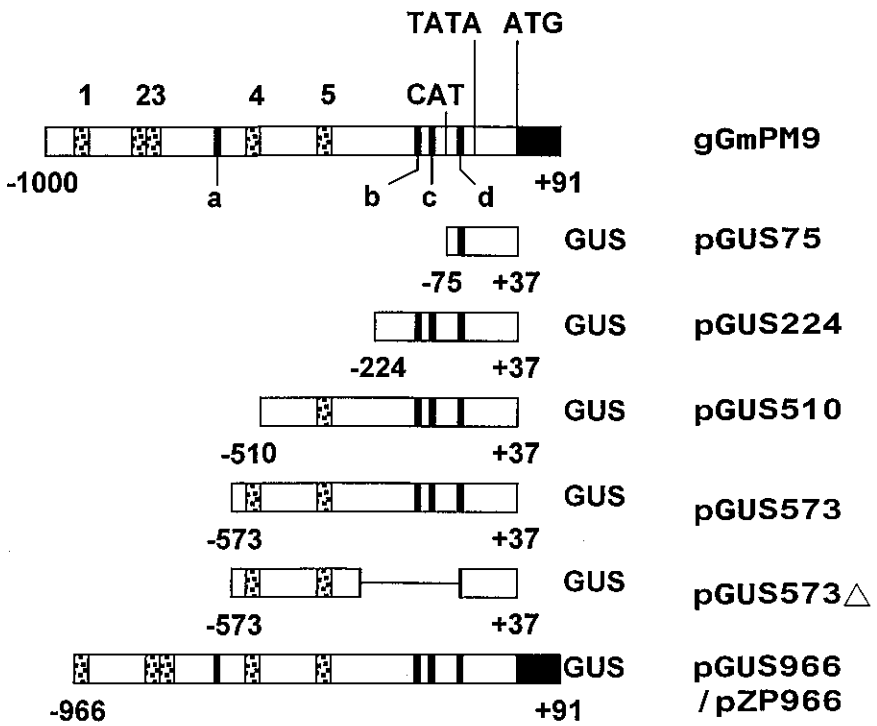

Fig. 1. Schematic representation of *GmPM9* promoter and chimeric *GmPM9/GUS* constructs. The overview of GmPM9 promoter's structure is illustrated in the top line. Translation start position (ATG), TATA box (TATA), CAT box (CAT), ABRE (a, b, c, d), and SEF binding site (1, 2, 3, 4, 5) are marked. The structure of chimeric constructs is also illustrated. The 5' ends of the GmPM9 promoter in these constructs were at -966, -573, -510, -224, and -75, respectively.

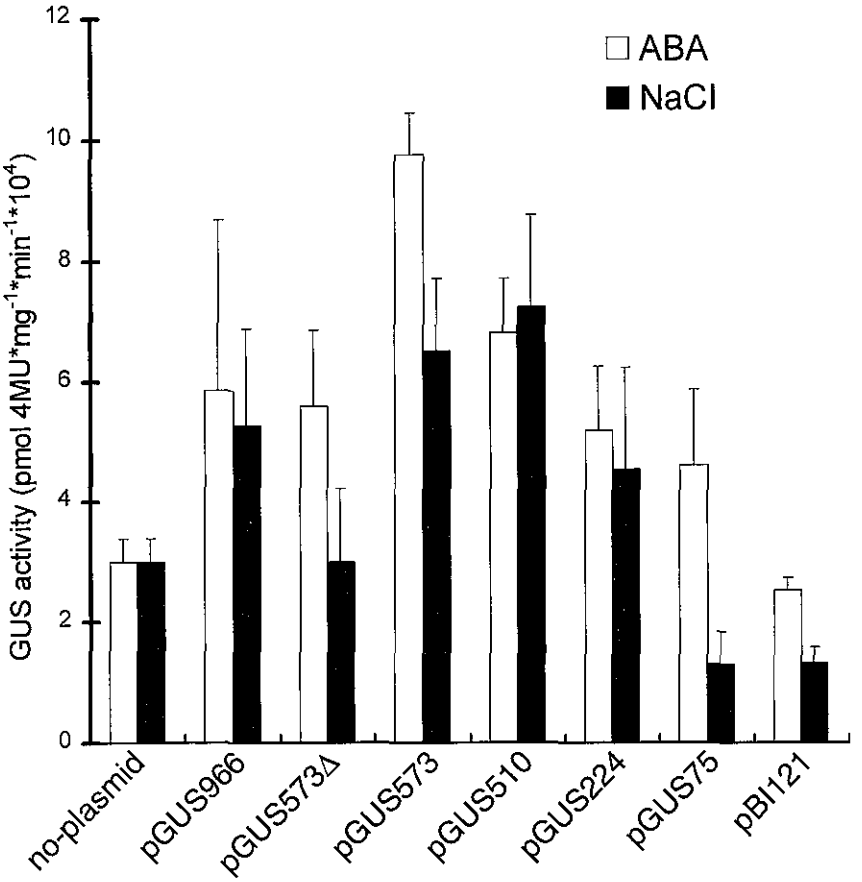

Fig. 2. Transient assay analysis for the five different chimeric *GmPM9/GUS* constructs in soybean callus. The GUS activities were quantified by fluorometric analysis. Values are the average GUS activity from three independent experiments.

sequences between –966bp and –573bp. The pUGS573Δ that containing one remaining ACGT-core had about the 60% GUS activity compared with that of pGUS573. There were similar GUS activities in pUGS224 and pGUS75. The GUS activity promoted by these constructs was about 50% of that by pGUS573, indicating one ABRE might be enough to drive the GUS activity in soybean callus system. In contrast, the pGUS573 and pUGS510 were also able to confer a higher level of salt induction. But the GUS activities promoted by the pUGS573Δ and pGUS75 was only 45% and 20% of that by pGUS573. This result indicated the three ABREs, which located at the proximal region of the TATA-box, are necessary for NaCl stimulus.

Analysis of the GmPM9 promoter in transgenic tobacco callus

The pZP966, which contained the full-length GmPM9 promoter and *uidA* reporter gene, was introduced into tobacco. The induced callus was then treated with osmotic- or temperature-stress, including ABA (10^{-5}, 10^{-6} M), NaCl (1%), PEG (2%), chilling (4°C), and heat shock (42°C). The GUS activities were measured after treatment (see Materials and Methods), and the fluormetric results are shown in Figure. 3. Similar to the transient assay results, ABA and salt significantly induced the expression

Table. 3 The search results from EST database using the GmPM9 protein sequence

EST Database	Accession number	Properties
Soybean	BF 325229	Leaf, drought stressed, 1 month old plants
	BE 660624	Seed coats
	BF 324643	Leaf and shoot tip, salt stressed, 2 week old seedling
	AW 568142	Immature cotyledons
	AW 756319	Somatic embryos (age ranging from 2 months to 9 months) cultured on MSD 20
	BF 425103	Leaf, drought stressed, 1 month old plants
	BF 325000	Leaf and shoot tip, salt stressed, 2 week old seedling
	BF 325489	Leaf, drought stressed, 1 month old plants
	AW 202085	Cotyledons of 3- and 7-day-old seedlings
	BF 324804	Leaf, drought stressed, 1 month old plants
	BG 551446	Differentiating somatic embryos cultured on MSM6AC
Barrel medic	BF 634767	Drought plantlets
	BG 454018	Developing leaf
	BG 454578	Developing leaf
	BF 633496	Drought plantlets
	BG 453141	Developing leaf
	BG 450987	Drought plantlets
	BF 633965	Drought plantlets
	BI 270486	Developing flower
	BG 452393	Developing leaf
	BG 454407	Developing leaf
	BG 453106	Developing leaf
Arabidopsis	BE 038529	Leaf after 20 h 200mM NaCl, 12 weeks old plants
	BE 521009	Developing seed after 5-13 days after flowering
	BE 522152	Developing seed after 5-13 days after flowering
Rice	AU 174479	Seed
Sorghum	AW 924296	Mix of 5-week old plants on days 7 & 8 after water was withheld

of GUS gene in transgenic tobacco callus. The GUS activities in ABA- or salt- treated calli were higher by 17 or 12 fold than non-transgenic tobacco callus. Besides, the GUS activity in the MS medium with ABA and salt treatment had strong additive effects (data not shown). These results indicated that ABA and salt could interact with the promoter of GmPM9. To test the dosage effect of ABA, callus was treated with 10^{-5} M or 10^{-6} M ABA. The result appeared that the GUS activity was enhanced about 3 times by high level of ABA, and indicated the significant dosage effect. On the other hand, desiccation and temperature stress just slightly induced (PEG- or chilling- treatment), or almost non-induced (heat shock-treatment) GUS activity. The relative GUS activity increased to only two fold. These results indicated the promoter of *GmPM9* might not response to desiccation or temperature stress in tobacco callus.

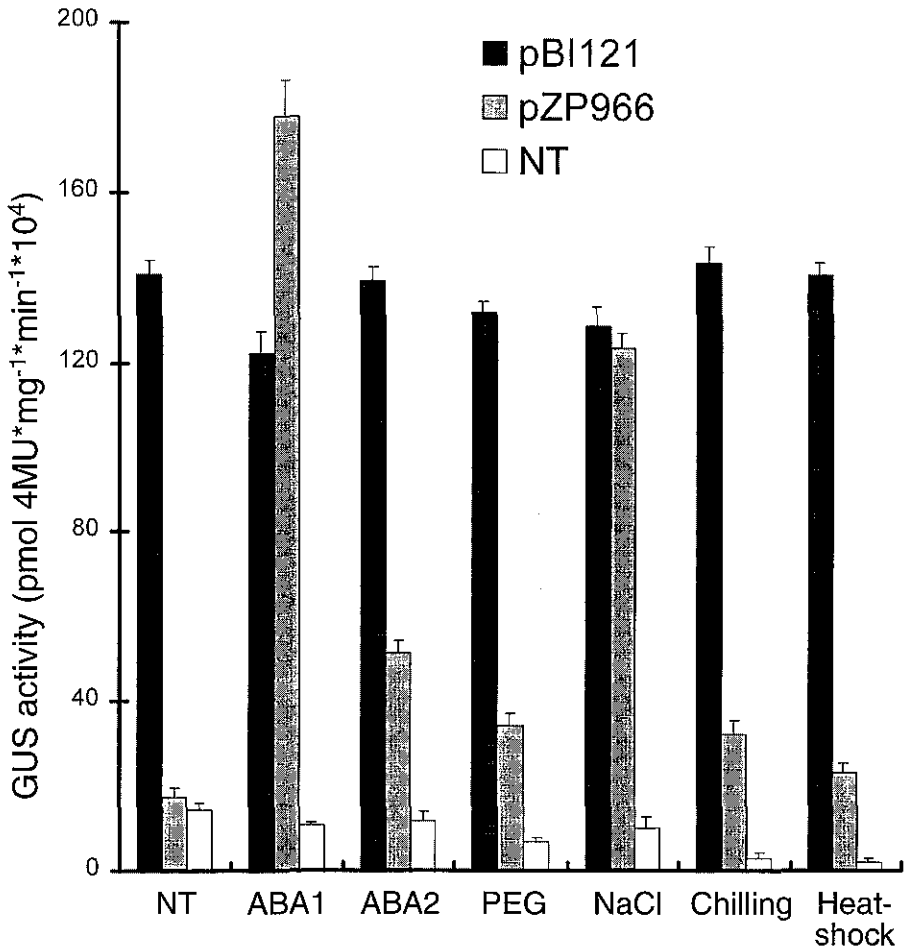

Fig. 3. GUS activity in transgenic tobacco callus. The activity was determined by fluorometric analysis. Calli were derived from transgenic tobacco with pBI121 (positive control), full-length *GmPM9* fusion gene, and non-transgenic tobacco plants. Each pieces of calli were treated with 10^{-5} M ABA (ABA1), 10^{-6} M ABA (ABA2), 2% PEG (PEG), 1% NaCl (NaCl), chilling (Chilling), and heat-shock (Heat-shock). Values are the average GUS activities from three independent duplicates.

Histochemical staining also showed similar results as fluormetric assay (Figure 4). No GUS activity was observed in non-transgenic callus under all treatment. Strong GUS activity was observed in the transgenic callus under ABA or salt treatment, and by both. Desiccation or temperature treatment just gave light GUS staining. Thus, it is suggested that *GmPM9* promoter would be respond to ABA and salt in the tobacco callus system.

Discussion

The regulation mechanism of ice *rab16A* gene and wheat *Em* gene were studied in very detail using transient assay [9,10]. The construct with full-length promoter was not as sufficient to up-regulate as that with shorter (about 500bp) promoter fragment. Thus, it was suggested that the ABREs located at proximal region of TATA-box were necessary to ABA-response, and the full-

length promoter might contain the silence sequence. In transgenic study, however, it gave different results [9, 12-14]. In these studies, the GUS fusion genes with full-length promoter from several Lea genes were introduced into tobacco or Arabidopsis plants. The full-length promoter, but not short or deleted promoter, would strongly activate the GUS activities under different stress conditions. These results suggested that another factors might involve in the ABRE mediated regulation pathway.

The sequences flanking to the ACGT-core were studied intensively by several group. These sequences seemed to be important for ABA-response since some DNA-binding proteins that interacted with ABRE/G-box-like element, such as wheat EmBP-1 or Arabidopsis GBF1-4, used the flanking sequence to recognize the ACGT-core [18]. It was indicated that the difference of flanking sequence might cause the specificity. For example, Arabidopsis light-responsive trans-acting factor, GBF1, would interact with the G-box that located at the promoter of rubisco small subunit (*rbcS*) gene, but not with ABREs [17,18]. The CE of barley *HVA22* genes showed another style for regulating the ABA-mediated responses. Several ABREs were present in the *HVA22* promoter, and the transient assay study indicated that ABREs seemed to be necessary while not sufficient for ABA response. However, the ABRC that consisting ABRE and CE was necessary and sufficient for high-level ABA induction [19,20].

In the present study, the callus system that derived from soybean or tobacco was used for the experiments. According to the previous work, the *GmPM1/9* mRNA were accumulated in soybean callus under ABA, salt or chilling treatment. Several putative *cis*-elements were identified in the *GmPM9* promoter. For the four ABREs in this region, one locatized at –618 and the other three localized at the 5' upstream proximal region

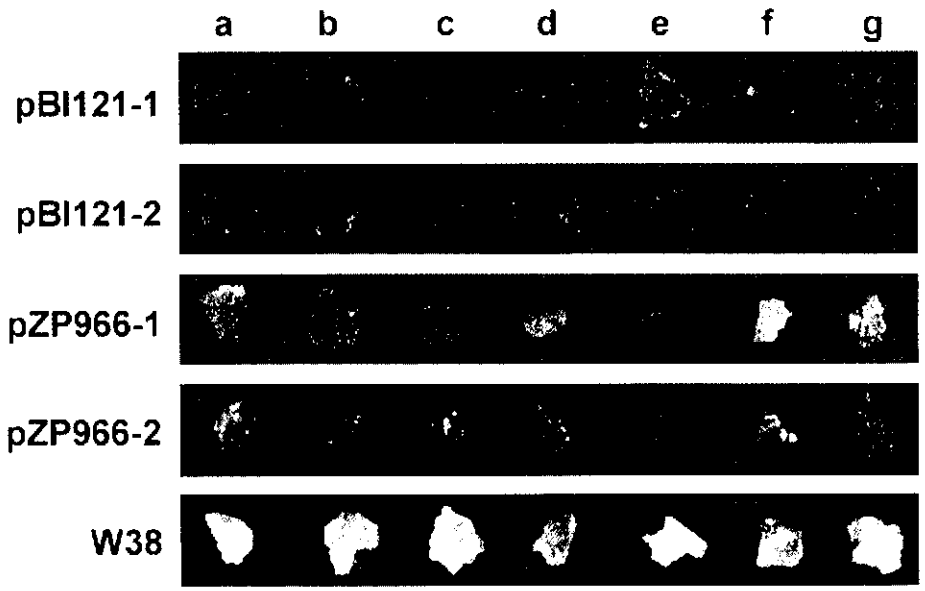

Fig. 4. Histochemical staining in transgenic tobacco callus. Lane a to g are non-treatment, 10^{-5} M ABA, 10^{-6} M ABA, 2% PEG, 1% NaCl, chilling, and heat-shock, respectively. These calli tissues were derived from four individual transgenic tobacco plants and one non-transgenic tobacco plant. Two of transgenic tobacco plants are transformed with pBI 121, the other two are transformed with pZP966 fusion gene.

of TATA-box between –200 and +1, as shown in Figure 1. Similar situations were also discovered in other plant species. Five AT-rice sequences (i.e. SEF motifs) were localized at the region between –966bp and –300bp. It contained the opposite distribution manner between ABRE and AT-rich element: four of five AT-rice elements were localized between –966bp and –573bp. It was reported that AT-rice element might involve in tissue specificity, or enhancing the gene expression and tissue specificity [33, 35]. The $(CA)_n$ element was found between –172bp and –114bp, and proposed to be important for seed-specific expression [34].

Various length of *GmPM9* promoter was constructed according to these elements. Transient assay showed that the pGUS966 would not give the highest GUS activities; thus, the most upstream AT-rich sequences might play a role as silent sequence through the spatial factors. This result was similar to the previous researches. However, the GUS activity derived from pGUS224 was still lower than that of pGUS573 and pGUS510, indicated that AT-rich element located at the proximal region played the role for enhancing the gene expression in callus system. The shortest construct, pGUS75, showed the medium GUS activity in ABA treatment, but the lowest GUS activity in salt treatment. The pUGS573Δ showed similar situation but had higher GUS activity in salt stress than construct pGUS75 had. These two constructs contained one ABRE, but pUGS573Δ also contained one AT-rice sequences and $(CA)_n$ element. It was indicated that a truncated *GmPM9* promoter with only one ABRE was still sufficient to activate the GUS activity in ABA treatment. On the other hand, the proximal ABREs and AT-rice sequences might be important in response to salt stimuli in callus system.

Lee *et al.* found that the GmPM9 promoter was induced by salt or drought treatment, but not by ABA or chilling treatment in transgenic Arabidopsis system [14]. Indeed, recent studies had suggested that several genes, such as 15a and 26g of pea [36], *AtDi19* and *AtDr4* of Arabidopsis [37], were induced by drought or osmotic stress, but not by exogenous ABA. Several ABA-independent gene regulation pathways had been identified. The dehydration response element (DRE) in Arabidopsis was interacted with desiccation, cold, or salt stimuli, but not mediated by ABA. From all of these statements, the *GmPM9* promoter might involve in the ABA-independent regulation pathway [38]. In tobacco callus system, however, it was revealed that the expression of *GmPM9* was induced by ABA or salt treatment; in contrast, drought and chilling just gave slight induction effects on *GmPM9* promoter. These results, especially in ABA-response, were similar the regulation studies of several other *Lea* or ABA-responsive genes. Bostock and Quantrano [39] had reported that the mRNA of wheat *Em* gene was accumulated in ABA and salt combined treatment, and indicated the ABA-dependent and ABA-independent regulation pathway might affect to the *Em* promoter at the same time. In our trangenic callus system, the *GmPM9* promoter would respond to ABA and salt stress, but not to desiccation and temperature stress. Comparing with the transgenic Arabidopsis system, it was proposed that *GmPM9* promoter might contain the spiral effects, and would be affected by ABA-dependent and ABA-independent regulation pathway.

The Expression Sequence Tag (EST) databases increase dramatically in the last several years, and provide an excellent tool for gene functional analysis. For example, the soybean EST database, the biggest plant EST database, contains over 170,000 entire at the end of July 2001. TBLASTX was used to search the current plant EST databases, using GmPM9 protein sequence as the query. Several EST sequences were found in soybean, Arabidopsis, rice, and barrel medic (*Medicago truncatula*) EST databases

(Table 3). In the case of soybean, the GmPM1/9 expressed in the drought stressed leaf, salt stressed leaf or root tip, somatic embryo, seed coat, immature cotyledons, and cotyledons of 3- and 7-day-old seedlings. In barrel medic, a new Leguminosae model system, *GmPM1/9* homolog were expressed in drought plantlets, developing leaf and developing flower. In Abrabidopsis, the transcripts of *GmPM1/9* homolog were found in salt stressed leaf, and developing seed. There were also GmPM1/9 homolog present in rice and sorghum, and they were expressed in seed or water stressed plant. These data demonstrated that *GmPM1/9* genes responded to environment stimuli. Further work is necessary to dissect the promoter region and to establish the relationship between the environmental signal and the *cis*-element.

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