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Two Genes Encoding GmPM1, A Soybean Seed Maturation Protein

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Late embryogenesis abundant (LEA) proteins are synthesized during the late stages of seed development and have been widely reported in monocot and dicot plants. We have selected several cDNA clones of seed maturation proteins from a soybean pod-dried seed cDNA library by differential screening and these clones are designated GmPM clones, which stands for Glycine max physiological mature. In the present study, we study the characteristics and developmental regulation of a soybean gene family encoding GmPM1 and GmPM9, two typical type IV LEA proteins. Sequence alignment of the GmPM1/9 proteins with other similar proteins found in the database indicates extensive similarity at the N-terminus. The β-glucuronidase (GUS) reporter gene driven by the GmPM9 promoter was induced at significant levels in late developing seeds of the transgenic tobacco plants. Histochemical analysis of GUS activity revealed that the GmPM9 promoter functions in the mature seeds and pollen. To analyze the mechanism of GmPM9 regulation, the truncated promoter was introduced by bombardment into embryonic axis excised from maturing soybean seeds. With only 114 bp of promoter sequence, the GUS expression was stimulated by seed maturity.

Key words: Glycine max, late embryogenesis abundant (LEA), seed maturation, Western analysis, intron, exon

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Abbreviations: DAF, days after flowering; GmPM, *Glycine max* physiological mature; LEA, late embryogenesis abundant.

The nucleotide sequence data reported will appear in the EMBL, GenGank and DDBJ Nucleotide Sequence Databases under the accession numbers M80666, X63565, and M97285.

Introduction

There are dynamic changes in the synthesis of proteins during late maturation of seeds. Proteins synthesized during this late stage, which are correlated with desiccation tolerance [1], ABA content [2], or transition to seedling growth [3], are termed maturation proteins [3] or late embryogenesis abundant (LEA) proteins [4]. However, maturation proteins are slightly different from LEA proteins in that the level of maturation protein mRNAs is not necessarily very high at late embryogenesis.

Based on their common amino acid sequence domains, LEA proteins are divided into four groups [5, 6]. The group I LEA proteins, also known as D-19 family or Em proteins, contain a very high proportion of charged amino acids. The group II LEA proteins, also known as D-11 family or dehydrin, poss a tract of 6 to 9 contiguous Ser residues and a high Lys region of 14 residues. The group III LEA proteins, also known as D-7 family, contain many tandemly-repeated 11-mers. The last group (IV) of LEA proteins are D-113 family which contain a large number of Ala residues and the random-coil promoting residues Gly and Thr. Almost all of the LEA proteins studied as far are highly hydrophilic, contain no Cys or Trp residues, and are boiling soluble [e.g. 5, 6]. In addition to being expressed in the embryo, some LEA genes can also be induced in vegetative tissues by osmotic stress, including salt treatment and desiccation, and exogenous abscisic acid [7, 8]. LEA proteins are believed to play a role in cellular protection because they share the extremely hydrophilic property as well as the timing for accumulation. For example, using transgenic rice plants as the system, Wu and his colleagues demonstrated that expression of HVA1, a group 3 LEA protein from barley, conferred tolerance to water deficit or salt stress in transgenic rice [9].

For understanding the function of LEA proteins, it is also important to investigate the molecular mechanisms that regulate LEA genes. A number of LEA genes are thought to respond to ABA, and their promoters contain conserved motifs, also known as ABREs (ABA-responsive elements), would interact with nuclear protein factors. For instances, Guiltinan *et al.* [10] reported that EmBP-1, a leucine-zipper DNA binding protein, could bind to the ABRE sequence (CACGTGGC) of the wheat *Em* gene. In order to analyze the regulation mechanism of LEA genes, several promoters from different plants were fused to β-glucuronidase (GUS) reporter gene (*uirA*) [e.g. 11, 12, 13, 14, 15]. These studies indicated that the promoters of different LEA genes would respond to one or more stimuli, including ABA, cold, salt, or desiccation. These promoters could direct GUS expression to mature seeds, pollens, roots, stems or trichomes, with most of these LEA genes expressing in seeds. Promoter deletion analysis revealed a variety of cis-acting sequences within these promoters that conferred differential responsiveness to environmental stimuli [12, 16].

We have selected several cDNA clones of soybean seed maturation protein from a pod-dried seed cDNA library by differential screening [17, 18]. These are designated as pGmPM clones, denoting for *Glycine max* physiological mature. In this paper, we report the characterization and gene regulation of the two genes encoding a type IV LEA soybean seed maturation protein. These two genes, GmPM1 and GmPM9, belong to the same gene family. GmPM1 protein [19] had an apparent molecular mass of 22 kDa on SDS-PAGE. GmPM9 [20], another protein with apparent molecular mass of 20 kDa, shared high degree of sequence homology with GmPM1, but with a 23-amino acid deletion [20]. Hybrid select translation indicated that there were two protein bands corresponding to the pGmPM1 cDNA, with the apparent molecular weight of 20 kDa and 22 kDa, respectively [18]. The

promoter driven analysis of transgenic tobacco plant assay and transit expression analysis are presented in the current report while similar analysis using callus system [21] or transgenic *Arabidopsis* [22] were described in previous papers.

Materials and Methods

Plant materials

Soybean (*Glycine max* cv. Shi-shi) seeds were kindly provided by the Kaoshiung Agricultural Experimental Station, Pintong, Taiwan. These plants were grown to maturity in the Experimental Farm, Institute of Botany, Academia Sinica, Taiwan. *Nicotiana tabacum* cv. W38 was used in gene transformation experiments. After harvesting, plant samples were immediately frozen in liquid N_2 and stored at -70°C prior to extraction.

Isolation of cDNA and genomic clones, DNA sequencing and molecular analysis

A soybean dried-seed cDNA library was constructed and a series of GmPM cDNA clones selected by differential screening as previously described (Hsing and Wu, 1992). The sequences were determined with the dideoxy chain termination method [23]. Sequence data were analyzed using the Genetics Computer Group Sequences Analysis software package Version 9.0 [24]. The accession numbers for GmPM1 and GmPM9 cDNA are M80666 and X63565, respectively. The accession number for GmPM9 genomic DNA is M97285.

Construction of promoter/GUS fusion

Plasmids containing the GUS gene were used in this study. Figure 2 illustrates all constructs used in the transgenic plant assay or transient assay. The 1057bp XbaI-PvuII fragment (-966 bp to +91 bp)–containing the 966 bp upstream region from the initiation site of transcription, 48 bp of the untranslated sequence and 43 bp of the coding region of genomic clone gGmPM9–was ligated into the XbaI-SmaI site of pBluescript SK-. This clone was digested with PstI followed with klenow fragment then cut by XbaI. The purified fragment was ligated into XbaI-SmaI digested binary vector pBI101 (Clontech) and designated pZP966 or construct 1. The Sau3AI fragment (-572 bp to +41 bp) from gGmPM9 was ligated into the BamHI site of pBluescript SK-. The new clone was cut with XbaI and SmaI and ligated into the XbaI-SmaI site of pBI101, designated pZP572 or construct 2. The EcoRV-Sau3AI fragment (-328 bp to +41 bp) was cloned similarly and designated pZ328 or construct 5. The pZP510 (-510 bp to +41 bp) and ZP114 (-114 bp to +91 bp) were generated from exonuclease III deletion of pZP966 and pZP572, respectively and designated construct 3 and 6. The two PmlI sites in the promoter region, i.e. -179 and -69 bp, was excised and self-ligated from pZP572 and designated construct 4.

DNA delivery

For DNA delivery, batches of 15 (5 X 3 rows) embryonic axis from soybean 45 DAF seeds were incubated at 27°C on petri dishes containing half strength of Murashige and Skoog (MS) medium supplemented with 10 μg ABA and 0.8% agar. The DNA bombardment was carried out as described by Barcelo and Lazzeri [25]. The axis was bombarded with submicron gold particles (1.0 μm gold particle, BioRad), coated with precipitated plasmid DNA. Particle bombardments were carried out using a PDS-1000/He gun (BioRad) with a target distance of 9 cm from the stopping plate at helium pressure of 450 psi for two shots. Following bombardment, the embryos were incubated at 27°C under darkness for a further 48 for on the same medium before the GUS assay.

Plant transformation

The vectors containing the GmPM9/GUS gene fusion, including construct 1 and 4, were transferred from *E. coli* DH5α into *Agrobacterium tumefaciens strain* LBA4404 (Clontech). The chimaeric gene constructs were transferred to tobacco plants via leaf disc transformation as described by Holsters *et al.* [26]. Development of small plantlets was achieved by transferring knamycin-resistant shoots onto a semisolid MS medium with 1% sucrose. Plantlets were maintained in a medium containing 100 (μg/ml kanamycin throughout this period. Plants were grown in culture for 1 month before being transferred to soil. Plants were then transferred to greenhouse conditions where they matured, flowered and set seeds which were harvested for later analysis.

Fluorometric GUS assay

Fluorometric analysis of GUS activity was carried out as described by Jefferson *et al.* [27]. Tissues were ground in extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 10 mM

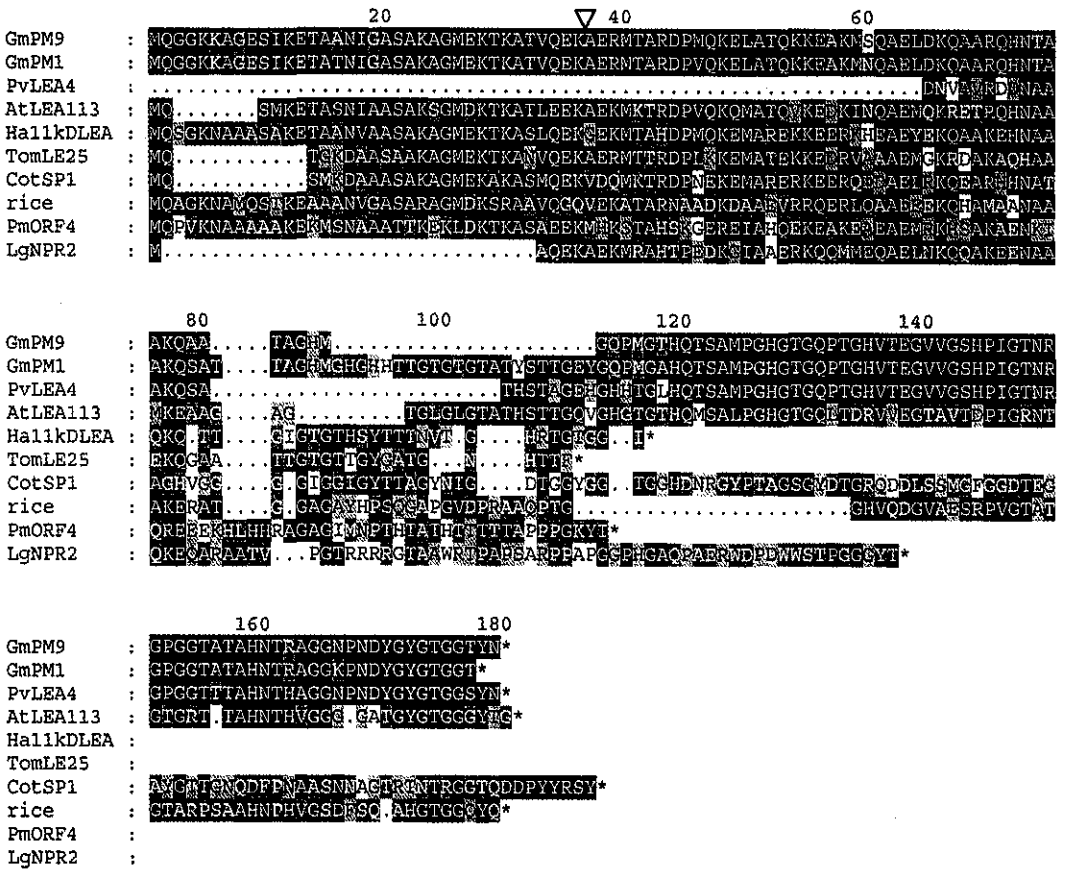

Fig. 1 Comparison of GmPM1 with homologs in database. Dashes were introduced to maximize sequence alignment. Stars indicate the stop codons. Arrow indicates the position of the only intron from the available genomic sequences. Sequences are: GmPM1 (M80666, soybean), GmPM9 (X63565, soybean), PvLEA4 (U72767, common bean), AtLEA113 (X91915, *Arabidopsis*), Hal1kDLEA (X59700, sunflower), TomLE25 (M76552, tomato), CotSP1 (M19406, cotton), rice (AP003867, HTGs data), PmORF4 (Z49711, Douglas fir), LgNPR2 (X64145, duckweed).

β-mercaptoethanol, 0.1% Triton X-100 and 0.1% sarkosyl. The extracts were centrifuged at 10,000 rpm in eppendorfs for 10 min, and the supernatants were used for fluorometric assay. Reactions were started by addition of 100 μl 1mM 4-MUG (BRL) to the supernatants and incubated at 37°C; one reaction was terminated at the time zero and the other was quenched at 60 min by adding 0.2 M Na_2CO_3 into each reaction (final volume 2 ml). Fluorometric quantitation of 4-methylumbelliferone (4-MU) was measured in a calibrated Hoeffer TKO-100 fluorometer. Protein concentration was determined with a protein assay kit from BioRad. GUS activity was expressed as pmol 4-MU.min^{-1}.mg^{-1} protein.

Histochemical localization of GUS reporter gene expression

Histochemical staining of GUS activity with 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) (BRL) was performed using a modified procedure described by Jefferson *et al.* [27]. The bombarded soybean embryonic axis or tissues of transgenic tobacco plants were treated directly or were cut by hand with a razor blade before being immersed in 1 mM X-gluc, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 20% methanol. Penetration of the X-gluc solution into the samples was facilitated by centrifugation for 10 min. The histochemical reaction was carried out overnight in the dark, at 37°C. Then the samples were observed and photographed under a dissecting microscope.

Results and Discussion

Comparison of GmPM1/9-like genes

The amino acid sequences encoded by GmPM1 or GmPM9 genes were compared with similar proteins in the database (Fig. 1). They are PvLEA4 from common bean [28], AtLEA113 from *Arabidopsis* [29], Ha11kDLEA from sunflower [30], TomL225 from tomato [31], CotSP1 from cotton [32], PmORF4 from Douglas fir [33], LgNPR2 from duckweed [34] and a rice protein predicted from a chromosome 8 HTGs genome sequencing data (AP003867, Sasaki et al., 2002). Most of these proteins are of the similar length and give homology at the N-terminal part where many charged amino acid residues are located. The genomic sequences are available for several of these proteins, i.e. GmPM1, GmPM9, AtLEA113, TomLe25, CotSP1, NPR2, and rice. There was only one intron in the genes from dicot species while no intron in the genes from monocot species, i.e. rice and duckweed. The position of this single intron is reserved as indicated at Fig. 1. When GmPM1 or GmPM9 protein were compared with all the available sequences, the percentage identity of corresponding regions ranged from 36% to 84%, and the similarity between GmPM1 and the other genes is in good agreement with the phylogenetic position of the various species. Our results also indicate that the intron 1 appeared after the separation of monocot and dicots.

The amino acid composition of four of these proteins was analyzed (Table 1). There were no Cys or Trp residues in all proteins, i.e. GmPM1, GMPM9, AtLEA113 and rice, as most of the other LEA proteins. Ala and Gly residues are most abundant ones in all four of them. For the charged residues, there are more Lys and Arg compared with Asp and Glu, which account for the high pI values of these proteins. Ser and Thr are usually abundant in LEA proteins, however, there are very few Ser in these four proteins. There are many Thr residues in GmPM1, GmPM9 and AtLEA113, but not in the rice protein. As to the aromatic amino acids, there are very few, if any, Phe and few Tyr residues in these proteins.

Analysis of GmPM9 promoter in transgenic tobacco plants

Table 1. Amino acid compositions of four GmPM1/9 type proteins.

Residue	GmPM1	GmPM9	AtLEA113	Rice
A	23	23	18	36
C	0	0	0	0
D	3	3	5	6
E	10	9	9	9
F	0	0	0	1
G	30	24	27	18
H	10	7	7	6
I	3	3	3	0
K	14	13	12	7
L	2	2	5	1
M	7	8	8	4
N	6	7	5	5
P	7	7	3	7
Q	11	11	10	13
R	5	5	6	10
S	6	5	6	8
T	27	18	25	8
V	5	4	7	9
W	0	0	0	0
Y	4	3	2	2

Sequence analysis of the 966 bases of the region upstream of the GmPM9 transcription initiation sites indicated several regulation motifs, in addition to the TATA and CAAT boxes. Six chimeric constructs were produced with varying lengths of the promoter, with increasing numbers of these motifs, and fused to the GUS gene to investigate their contribution to transcriptional regulation. Various length of the promoter was fused to the GUS reporter gene, as indicated in Fig. 2. The resulting clones - construct 1 (-966 bp to +91 bp), construct 2 (-572 bp to +41 bp), construct 3 (-510 bp to +41 bp), construct 4 (construct 2 with the deletion of a PmlI fragment from -179 bp to -69 bp), construct 5 (-328 bp to +41 bp) and construct 6 (-114 bp to +41 bp) - were assayed by transient gene expression or transgenic plant assays of GUS reporter gene, as indicated in Materials and Methods. The constructs 1 and 4 were transferred to tobacco using *Agrobacterium tumenfaciens*-mediated transformation and between 10 to 20 plants were regenerated per construct. Selected transgenic plants were analyzed by southern blots to assure that they contained the complete insert. GUS activity was measured in various tissues of the primary transformants and in offspring generations. The results are summarized in Fig. 3. Expression of the GUS gene driven by construct 1 was detected in seeds while not in other tissues tested. On the contrary, the expression was quite high in roots and was detectable in stem, leaf and seeds for the plants driven by 35S promoter (Panel A). In whole seeds, GUS activity was also determined throughout embryogenesis. For construct 1, it was not detected in the early stage of seed development, up to 15 DAF. A dramatic increase in GUS activity was observed in seeds at 20 DAF, which was maximum at the mature, or 30 DAF, seeds (Panel B). No GUS activity was observed in plants with construct 4, where a PmlI fragment was excised from construct 2. The PmlI restriction site is CACGTG and is exactly the ABRE motifs at position -179 bp and -69 bp. This fragment was eliminated in

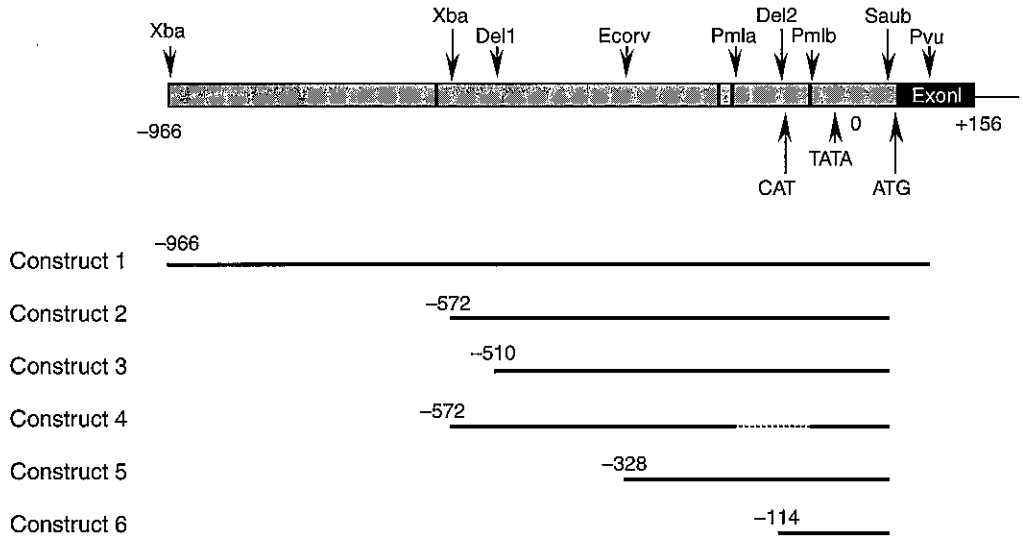

Fig. 2 Schematic illustration of the different GmPM9 gene fusions analyzed. Top, The GmPM9 gene. Black box indicates first exon, gray box indicate the promoter region, and the line indicates the intron. Top. The arrows on the top indicate the restriction enzyme sites or Exo nuclease digested sites, the arrows on the bottom indicate CAT box, TATA box and translation starting sites. The numbers on the bottom indicate the nucleotide position, with transcription starting point as 0. The four bars in the gray box indicate the ABRE in the promoter region. Bottom. Six constructs used for promoter analysis.

order to receive a construct with one less ABRE, however, the CATT box was also included in this fragment and thus was deleted also. The deletion of the CATT box should be the reason for the abolishment of GUS activity driven by the construct 4.

GmPM9 promoter activity was analyzed in transgenic *Arabidopsis* system previously [22]. Construct 1, 2, 3 and 6 were used in that study. GUS enzyme activities were detected in mature seeds and cotyledons and hypocotyls of seedlings in transgenic plants containing any of these constructs; they were not detected in other tissues at different developmental stages. Thus, both tobacco system and *Arabidopsis* system gave similar results.

The spatial distribution of the GmPM9 promoter-driven gene expression was analyzed during seed and another development in tobacco plants transformed with construct 1. Fig. 4 shows the histochemical analysis, and it indicates that GUS activity are detected in both mature seeds (Panel A) and mature pollens (Panel B). In the mature seeds, GUS activity is confined to the embryonic tissue while not present in the surrounding endosperm. The segregation of 3:1 ratio for blue/white staining of these pollens also indicates the isolation of the GUS gene in the offspring.

Transient gene expression analysis of GmPM9 promoter

For transient gene expression, construct DNA was delivered using Biolistic particle bombardment into the embryonic axis tissue dissected from 45 DAF soybean seeds. These axis were incubated with the presence of 10 μM ABA during the bombardment and incubation period to reduce the growth of the intact embryos. The full-length promoter, or contruct 1, which contained all four ABRE motifs gave relatively high GUS activity in the excised embryos (Fig. 5). Constructs 2, 3 or 5 did not give the same level of GUS

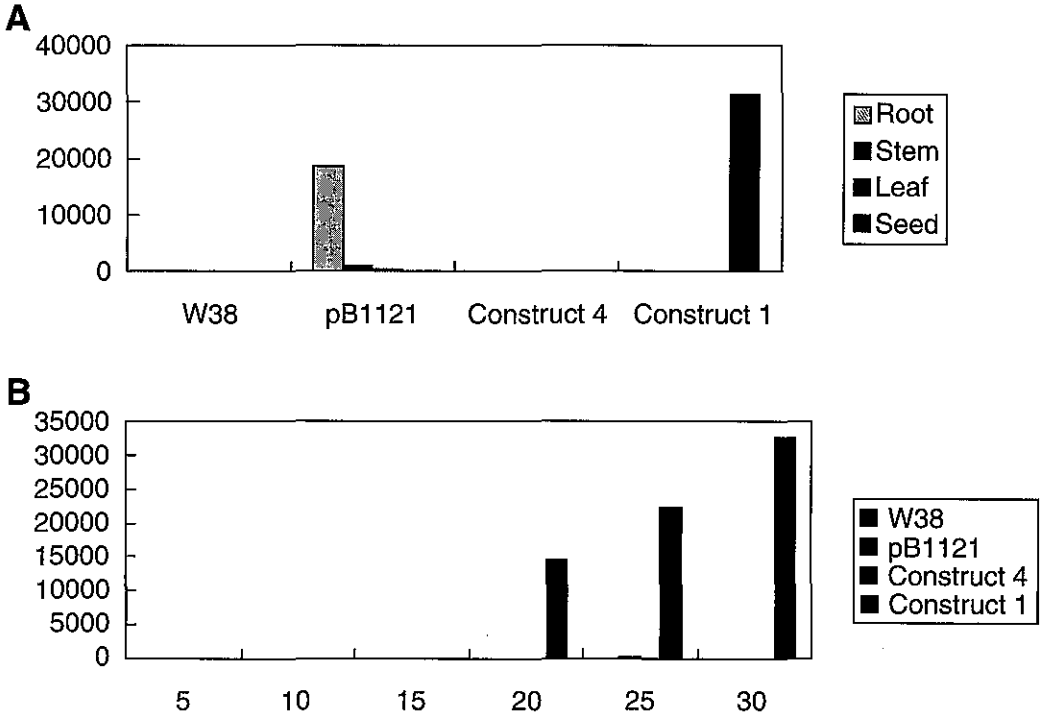

Fig. 3 The GUS activity in transgenic tobacco plants. Construct 1 or construct 4 indicated in Fig. 2 was fused to the coding region of the GUS reporter gene, pBI101. Top. Average GUS activities in transgenic tobacco root, stem, leaf or seed. Bottom. Average GUS activities in the developing seeds of transgenic tobacco. Numbers on the abscissa indicate the days after flowering (DAF) of the seeds. The untransformed (W38) plants and 35S cauliflower promoter (pBI121) were used as the control. GUS activity was expressed as pmols 4 MU.min^{-1}.mg^{-1} protein, and measured in five independently obtained transgenic plants.

Table 2. Digital Northern analysis of GmPM1 occurrence in soybean EST libraries.

Libray ID	Description	Total reads	Similar to GmPM1
gm-c1036	Somatic embryoids D20	3207	1
gm-c1075	Embryoids MAC	1621	1
gm-c1029	Young coty	1540	0
gm-c1040	Seedlings	3251	0
gmaxsc	Seed coat	2076	1
gm-c1051	Floral meristem	6591	0
gm-c1055	Mature pod	3565	0
gm-c1063	Seedlings	1738	0
gm-c1007	Mature coty	2396	0
gm-c1008	Young pod	2005	0
gm-c1016	Immature flower	9146	1
gm-c1019	Seed coat	5460	0
gm-c1015	Mature flower	5827	0

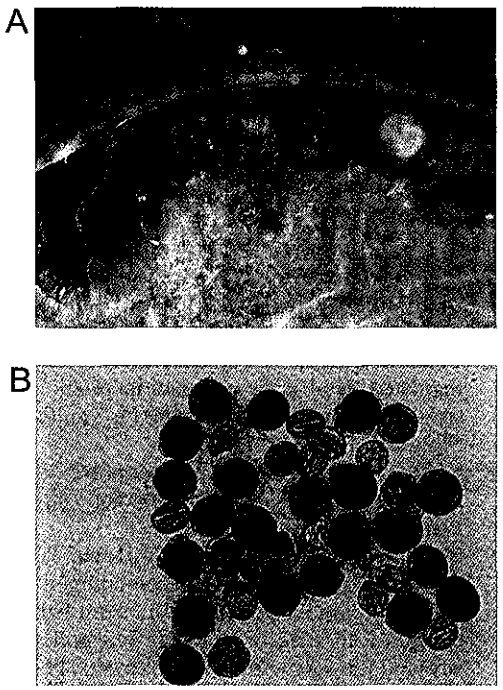

Fig. 4 Histochemical analysis of GUS activity in transgenic tobacco plants. Transgenic tobacco plants containing construct 1/GUS fusion construct were grown to maturity in the greenhouse. GUS activity is shown in mature seeds (A) and pollens (B).

activity although they all contained 3 ABRE motifs. The construct 6, or -114 truncated one, which containing only one ABRE motifs gave the most dramatic GUS activity, highlighting the potential importance of enhancer elements in a region of the promoter distal from the start of transcription. Similar phenomenon had been reported in *blt 4.9*, a barley low-temperature-responsive gene [35]. Fig 5 also demonstrates the presence of negative regulatory element between construct 2 and 3 or –572 to –510 bp. These results suggest that promoter region responsible for maintaining high levels of GUS activity in the seeds include positively and negatively regulated motifs and that –114 bp fragment is sufficient for the activity.

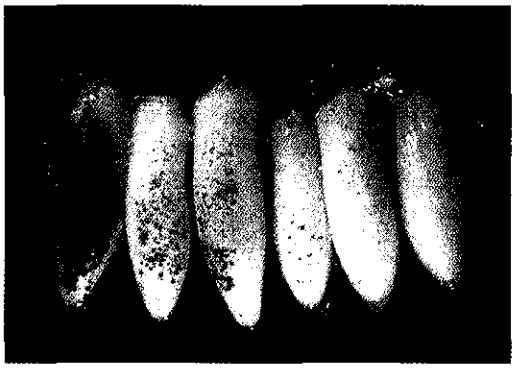

Fig. 5 Transient reporter gene expression analysis of GmPM9 promoter using bombardment treatments. From left to right: construct 6, construct 1, construct 2, construct 3, construct 5, and the control (no plasmid DNA). The length and feature of each construct are indicated in Fig. 2.

Constructs 1, 2, 3, and 6 were also used to drive the GUS activity in transgenic *Arabidopsis* system in previous study [22]. In that system, dry seeds with the longest, Construct 1 expressed the highest GUS activity. The activity promoted by Construct 2 and 3 was only 23% and 25% of that promoted by the longest construct, respectively. The lowest GUS activity was observed in transgenic seeds containing Construct 6 although this –114 bp fragment was sufficient to drive the GUS expression in mature seeds. The results between these two systems, i.e. transient expression analysis using excised embryonic axis and stable transgenic plant assay using *Arabidopsis*, gave different results for the Construct 6.

Digital Northern analysis of GmPM1/9 gene expression

Public databases now include vast amounts of recently acquired DNA sequences, for instances, there are about eleven millions EST (expressed sequence tags) entries in the NCBI database. In this huge database, soybean contains 245,767 entries and is the biggest one among all the plant species [39]. The Digital Northern

analysis for gene expression profile has been used recently in both plants [e.g. 36] and animals [e.g. 37]. In this kind of study, the EST database was used and the abundance of the query sequence in each set of EST data was counted.

We pulled out 13 soybean EST libraries, including seed, flower, pod, somatic embryoids and so on, and performed the digital Northern analysis of GmPM1 occurrence (Table 2). The read counts ranged from 1540 to 9146 in these libraries. GmPM1 was present with only one counts in gm-c1036, gm-c1075, gm-c1015 and gmaxsc which represent somatic embryoids, immature flower and seed coat, respectively. However, there was no similar sequence in all the other libraries tested. This means that these GmPM1 messages were present at very low level in these tissues and thus digital Northern analysis would not give reasonable results.

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