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Promoter activity of a soybean gene encoding a seed maturation protein, GmPM9

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Abstract. Late embryogenesis abundant (LEA) proteins are synthesized during the late stages of seed development and have been widely reported in monocot and dicot plants. In order to understand the developmental regulation of the promoter of a soybean gene encoding LEA protein GmPM9, we fused a series of fragments of the promoter region to the β-glucuronidase (GUS) reporter gene (*uirA*) and transformed the resulting constructs (pZP966, pZP572, pZP510, pZP294 and pZP114) into *Arabidopsis thaliana*. GUS enzyme activities were detected in mature seeds and cotyledons and hypocotyls of seedlings in transgenic plants containing any one of the five constructs; they were not detected in other tissues at different developmental stages. During seed development, GUS began to appear at 10 days after flowering (DAF) and increased rapidly to a maximum in the mature seeds at 14 DAF. The longest promoter construct (pZP966) enabled the transgenic plants to exhibit the highest GUS activity, whereas the shortest construct (pZP114) was sufficient to direct the expression of the GUS gene at a detectable level. The above findings indicate that the promoter of gGmPM9 can be used to express desirable genes in seeds during late seed maturation. The expression of the GUS gene could be induced in the leaves of the transgenic plants containing pZP966 by salt and desiccation, but not by ABA treatment.

Keywords: β-glucuronidase; Late embryogenesis abundant; Promoter; Transgenic *Arabidopsis.*

Abbreviations: ABA, abscisic acid; **DAF,** days after flowering; **GUS,** β-glucuronidase; **LEA,** late embryogenesis abundant; **MU,** methylumbelliferone; **MUG,** methylumbelliferyl glucuronide; **PEG,** polyethylene glycol.

Introduction

Late embryogenesis abundant (*LEA*) genes are highly expressed at the late stage during seed development. They have been studied extensively and isolated from many plants, including monocots and dicots (Baker et al., 1988; Close et al., 1989; Gaubier et al., 1993; Hsing et al., 1995; Mundy and Chua, 1988; Pla et al., 1991). LEA proteins have been classified into at least six groups based on their conserved sequences (Dure, 1993). In addition to being expressed in the embryo, some *LEA* genes can be induced in vegetative tissues by osmotic stress, including salt treatment and desiccation, and exogenous abscisic acid (Chandler and Robertson, 1994; Skriver and Mundy, 1990). 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, expression of the group 3 LEA protein, HVA1, from barley, conferred a tolerance to water deficit and salt stress in transgenic rice (Xu et al., 1996).

In addition to determining the function of LEA proteins, it is 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 called ABREs (ABA-responsive elements), which interact with nuclear protein factors. Guiltinan et al. (1990) 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*) (for instance, Goupil et al., 1992; Hull et al., 1996; Michel et al., 1994; Rouse et al., 1996; Yamaguchi-Shinozaki and Shinozaki, 1993). 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 (Giraudat et al., 1994; Hull et al., 1996).

We isolated and characterized gGmPM9 (GmPM, *Glycine max* physiological mature) (Lee et al., 1992), a genomic sequence from soybean. This sequence contained: a 1 kb, 5' untranscripted sequence, a 0.3 kb, 3' untranslated sequence, and coded for the 16 kDa seed maturation protein, GmPM9, which was highly homologous to

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GmPM1. Both proteins belonged to the D113 group and were similar to LEA protein D113 in cotton (Baker et al., 1988) and LE 25 in tomato (Cohen and Bray, 1992). Here we describe the results of a functional analysis of the promoter region in gGmPM9 by transgenic *Arabidopsis* using the GUS reporter gene. A series of fragments containing different lengths of gGmPM9 promoter region fused to the GUS gene were used to investigate the spatial and temporal expression. The results indicated that this promoter directs the GUS expression in seeds and the enzyme activity accumulated at the late stage during seed development. It is also suggested the GmPM9 promoter contains cis-elements that confer the gene expression and respond to salt treatment as well as desiccation, but not to ABA or cold.

Materials and Methods

Plant Material

Arabidopsis thaliana ecotype columbia was used in all gene transformation experiments. Seeds were surface sterilized in 10% bleach for 15 min followed by 3 to 4 times rinses with sterile water. Sterilized seeds were grown on half Murashige and Skoog medium (BRL) in the presence of 1% sucrose and 0.8% agar. Plated seeds were incubated at 4°C for 48 h and then maintained under a 16-h light/8-h dark photoperiod at 24°C.

Construction of Promoter/GUS Fusion

A 1057bp *Xba*I-*Pvu*II 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 *Xba*I-*Sma*I site of pBluescript SK-. This clone was digested with *Pst*I followed with klenow fragment then cut by *Xba*I. The purified fragment was ligated into *Xba*I-*Sma*I digested binary vector pBI101 (Clontech) and designated pZP966. The *Sau*3AI fragment (-572 bp to +41 bp) from gGmPM9 was ligated into the *Bam*HI site of pBluescript SK-. The new clone was cut with *Xba*I and *Sma*I and ligated into the *Xba*I-*Sma*I site of pBI101, designated pZP572. pZP294 (-294 bp to +91 bp), pZP114 (-114 bp to +91 bp), and pZP510 (-510 bp to +41 bp) were generated from exonuclease III deletion of pZP966, and pZP572.

Plant Transformation

The pBI101 plasmids containing the gGmPM9/GUS fusion constructs were transferred from *Escherichia coli* strain JM101 to *Agrobacterium tumefaciens* strains LBA4404 via the freeze-thaw method (Holsters et al., 1978). *Arabidopsis* transformation was carried out by vacuum infiltration (Bechtold et al., 1993). For selection of transformed *Arabidopsis*, seeds (T0) were screened on medium as described previously and supplemented with 50 μg/ml kanamycin. After 2 weeks, kanamycin-resistant seedlings (T1) were transferred to soil and grown under the same conditions as described above.

Treatment of Transgenic Plants

Transgenic *Arabidopsis* were treated with the following conditions: For NaCl and PEG treatment, seeds were surface sterilized and grown on half Murashige and Skoog plates supplemented with 100 mM NaCl or 1% PEG. For ABA treatment, fourteen-day-old seedlings grown on half Murashige and Skoog plates were transferred to half Murashige and Skoog medium supplemented with ABA (5 or 50 μM) for 24 h. For cold treatment, fourteen-day-old seedlings were placed at 4°C for 24 or 48 h. For wounding treatment, fourteen-day-old seedlings were wounded by slicing.

Fluorometric GUS Assay

Fluorometric analysis of GUS activity was carried out as described by Jefferson et al. (1987). Tissues were ground in extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM ethylenediamine tetraacetic acid (EDTA), 10 mM 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 1 mM 4-methylumbelliferyl glucuronide (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 per minute per mg fresh weight of protein. All the experiments were performed at least three times.

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. (1987). Seeds of transgenic *Arabidopsis* were cut by hand with a razor blade, and the seedlings were 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. After development, chlorophyll was removed by several applications of 95% ethanol at room temperature. Then the samples were observed and photographed under a dissecting microscope.

Results

Analysis of gGmPM9 Promoter in Mature Seeds of Transgenic Arabidopsis Plants

To assess the developmental regulation of the gGmPM9 promoter, various lengths of the promoter were fused to

the GUS reporter gene (Figure 1). The resulting clones—pZP966 (-966 bp to +91 bp), pZP572 (-572 bp to +41 bp), pZP510 (-510 bp to +41 bp), pZP294 (-294 bp to +91 bp) and pZP114 (-114 bp to +91 bp)—were transformed into *Arabidopsis* by the vacuum infiltration method (Bechtold et al., 1993). For each construct, between 11 to 33 independent lines were obtained (Table 1). Kanamycin segregation in the T1 generation was analyzed by using the Chi-square test. We collected the transformed lines with a segregation ratio of 3:1 for further analysis. The homozygous, kanamycin-resistant T2 generation was used to confirm the integration of the gGmPM9/GUS fragment by PCR and Southern blot for each construct (data not shown).

The effect of gGmPM9 promoter length on GUS expression was examined in mature, transgenic, T2 seeds by a fluorometric assay (Figure 2). The GUS activity in mature seeds of non-transformed *Arabidopsis* plants was measured, and the low value (1.79 pmol 4-MU•mg^{-1}•min^{-1}) served as a measure of the background level of enzyme activity. Dry seeds with the longest, pZP966 construct expressed the highest GUS activity. There were similar GUS activities in mature seeds transformed with pZP572 and pZP510; the GUS activity promoted by these constructs was only 23% and 25% of that promoted by the longest construct, respectively. GUS activity in transgenic seeds with pZP294 was 31% of that in pZP966 transformed seeds, and only a little higher than the seeds containing pZP572 or pZP510. The lowest GUS activity was observed in transgenic seeds containing pZP114, indicating that the fragment from -114 bp to +91 bp of gGmPM9 was sufficient to drive the GUS expression in mature seeds. These results indicated the promoter of gGmPM9 could direct the GUS reporter gene in mature seeds, and that the DNA sequence between -966 bp to -572 bp of gGmPM9 would be responsible for expressing high levels of GUS activity in mature seeds.

The gGmPM9/GUS Fusion Genes Expressed During Seed Development

To further investigate the time course of gGmPM9/GUS expression produced by each of the five constructs, the enzyme activity in the transgenic siliques was quantified fluorometrically at three-day intervals during seed development. Enzyme activity in immature, transgenic siliques with all gGmPM9/GUS constructs was very low prior to 10 DAF (days after flowering), but increased rapidly

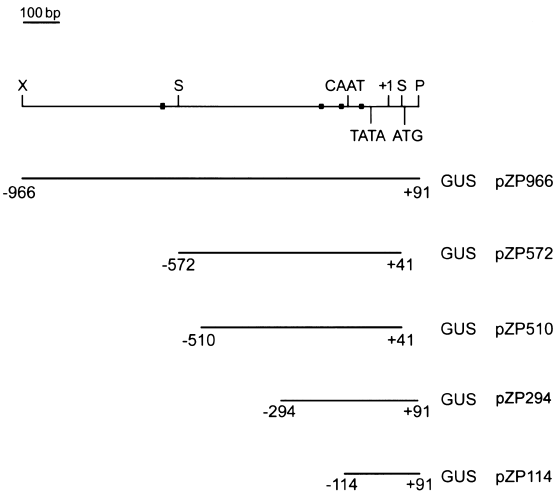

Figure 1. Schematic representation of the chimeric GmPM9/GUS constructs. The upper line provides an overview of the promoter's structure. The 5' ends of the GmPM9 promoter in these constructs were at -966 bp, -572 bp, -510 bp, -294 bp and -114 bp relative to the transcription start site. Black boxes indicate ACGT sequences at -616 bp to -613 bp, -179 bp to -176 bp, -128 bp to -125 bp, and -70 bp to -67 bp. Restriction sites are abbreviated: P, *Pvu*II; S, *Sau*3AI; X, *Xba*I.

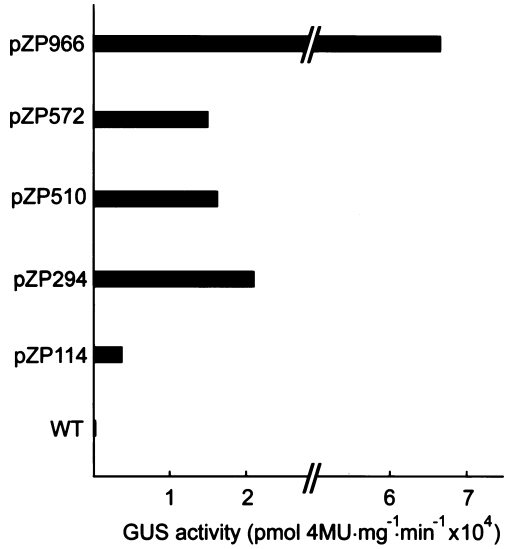

Figure 2. GUS activity in mature seeds of *Arabidopsis* transformed with five different gGmPM9/GUS fusion constructs. The activity was determined by fluorometric analysis. Values are the average GUS activity from three experiments.

Table 1. Numbers of transgenic lines and kanamycin segregation as 3:1 for each fusion clone as well as the independent lines used in the experiments.

Name of fusion clone	Numbers of transgenic line	Numbers of transgenic lines with segrtegation ration as 3:1	Transgenic line for experiment
pZP966	22	11	TAZP966-18
pZP572	19	8	TAZP572-15
pZP510	12	7	TAZP510-7
pZP294	11	6	TAZP294-1
pZP114	33	17	TAZP114-19

from 10 DAF to 13 DAF, and reached a maximum at 14 DAF (Figure 3). GUS activity in non-transformed siliques served as a control and remained relatively low throughout the experiment. Temporal regulation of the gGmPM9/GUS constructs during seed development was also assessed by histochemical staining, which yielded results similar to the patterns obtained by fluorometric assay (data not shown). The time course for the accumulation of GUS activity during seed development was basically the same for all the gGmPM9/GUS constructs in *Arabidopsis*. Thus, the promoter of gGmPM9 contained elements essential for expression during the late stages of seed development. Even the fragment from -114 bp to +91 bp of gGmPM9 was sufficient to confer this ability.

Histochemical Localization

To understand the regulation of the spatial expression pattern, histochemical assays were performed using the *Arabidopsis* plants transformed with constructs pZP966 or pZP114. Mature, transgenic seeds were hand-dissected and stained for GUS expression. Staining was observed in all part of the seeds, except the seed coat (Figure 4A). Further analysis of the distribution of GUS expression showed clear staining of the cotyledons and hypocotyls of the seedlings (Figure 4B), which remained visible even after the seedlings became adult plants (Figure 4C). At the tissue level, staining was enhanced in the vascular tissue in young seedlings. No GUS activity was detected in the leaves, stem, flowers or roots at any time during the life cycle. Non-transformed *Arabidopsis* plants did not exhibit GUS activity in mature seeds or any parts of the plant. Seeds with pZP966 were more strongly stained than seeds with other gGmPM9/GUS constructs, indicating that the former construct produced the highest levels of GUS activity; this finding concurred with the results of the fluorometric assays. Different gGmPM9/GUS constructs produced the same spatial pattern of GUS expression in *Arabidopsis* plants.

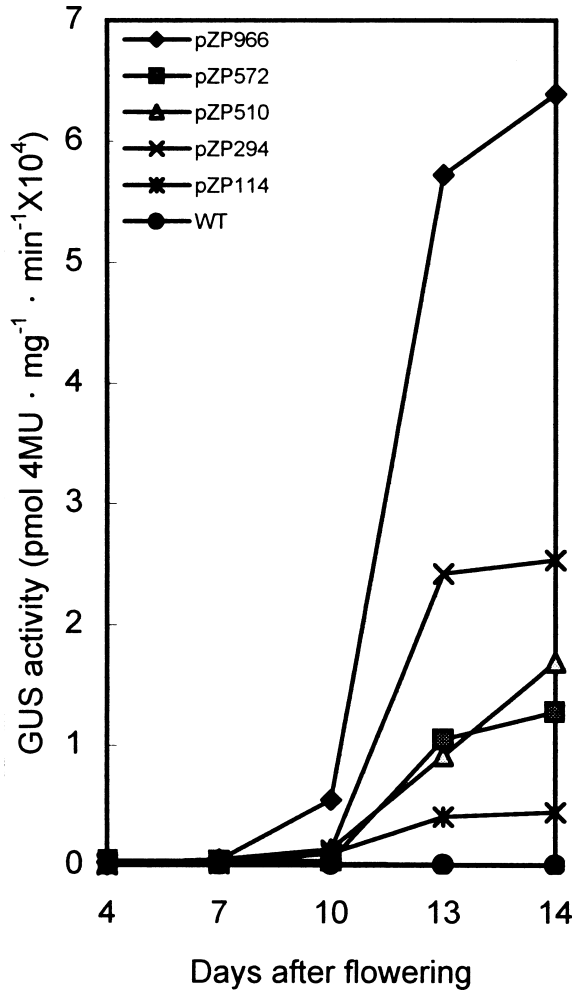

Figure 3. GUS activity during seed development. GUS activity in the extracts of siliques from transgenic plants at 4, 7, 10, 13 and 14 days after flowering was measured. Four independent transformants of each construct were used at each time point.

GUS activity was induced in the rosette of transgenic plants raised under half MS medium containing 100 mM NaCl or 1% PEG. No GUS activity was observed in the emerging leaves or roots under these treatments (Figure 4D, E). Transgenic plants grown under normal conditions displayed no enzyme activity in the rosette. ABA, cold and wounding treatments had no effect on inducible expression (data not shown). These results indicated that the promoter of gGmPM9 contains cis-acting elements that would respond to desiccation and salt stress.

Discussion

The promoter of gGmPM9 contained four ACGT sequences, which were -616 bp to -613 bp, -128 bp to -125 bp, -179 bp to -176 bp, and -70 bp to -67 bp. The last two were also G-boxes (CACGTG). Several ABREs, defined as a sequence of 8-10 base pairs with the core sequence ACGT (Niu et al., 1996; Izawa et al., 1993), have been found in ABA-inducible promoters (Guiltinan et al., 1990; Mundy et al., 1990; Pla et al., 1993). The nucleotides flanking the ACGT core might play a critical role in controlling the DNA binding specificity of bZIP (basic leucine zipper) proteins (Giraudat et al., 1994). However, the ABRE is not the only cis-element with the ACGT core sequence. ACGT elements were also found in the promoters of several plant genes, including *Arabidopsis* rubisco small subunit gene, *rbcS-1A* (Donald and Cashmore, 1990), parsley chalcone synthase gene, *Chs* (Block et al., 1990), and *Arabidopsis* alcohol dehydrogenase gene, *Adh* (McKendree et al., 1990). Furthermore, there were ACGT-containing sequences that did not function as ABREs even in ABA-inducible promoters (Busk et al., 1997; Kao et al., 1996; Straub et al., 1994). Thus, ABREs were thought to be a subset of ACGT-containing elements that were defined by function rather than flanking sequences (Busk and Pagès, 1998). Therefore, it is not surprising that the promoter of gGmPM9 was not responsive to ABA.

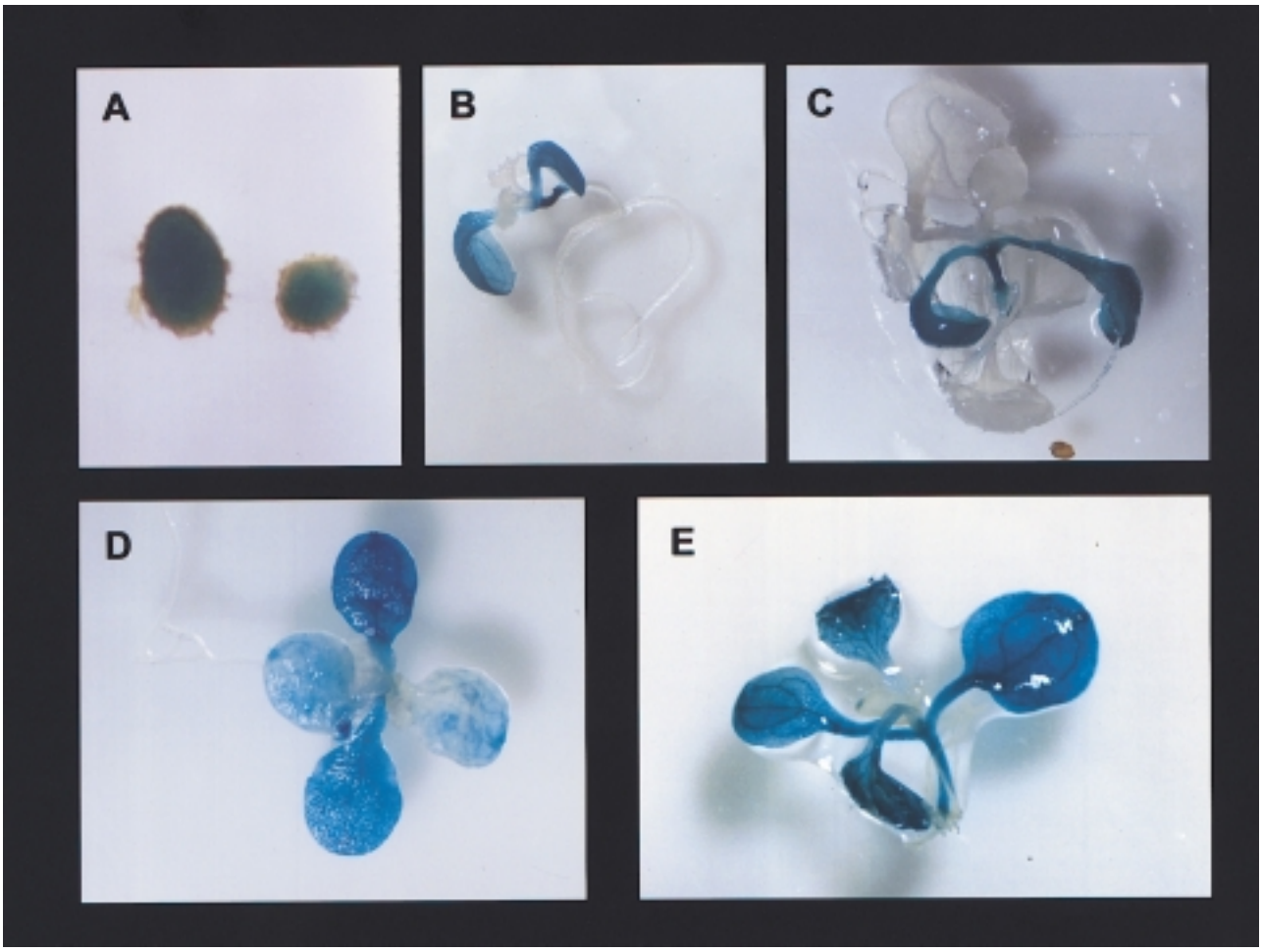

Figure 4. Histochemical localization of GUS activity in transgenic *Arabidopsis*. Transgenic seeds carrying pZP114 (A). Seven-day-old seedling carrying pZP966 (B). Fourteen-day-old plant carrying pZP966 (C). Fourteen-day-old, transgenic *Arabidopsis* carrying pZP966 were grown on medium containing 100 mM NaCl (D) or 1% PEG (E).

The region between -966 bp and -572 bp of gGmPM9 contained the ACGT element mentioned in the preceding paragraph and A/T rich sequences with an A/T content of 72%. Five motifs had 100% A/T content in this region, located at -965 bp to -952 bp, -921 bp to -909 bp, -855 bp to -842 bp, -764 bp to -740 bp and -588 bp to -573 bp. In the *β-phaseolin* gene of French bean, the A/T rich sequences in the promoter could act as general enhancers of expression, they were silent in all tissues except in the developing embryo (Bustos et al., 1989). The A/T rich elements were also important in the light-regulated photosynthesis gene, and it was suggested that they played a positive role in expression (Lam et al., 1990).

The region extending from -294 bp to -114 bp was also shown to enhance GUS activity. In addition to the G-box in this region mentioned previously, the $(CA)_n$ element was present between -172 bp and -166 bp, which was close to the G-box. These two elements were separated by only three nucleotides (CACGTGTCCCTAACAC). The $(CA)_n$ element (CNAACAC) was conserved in storage protein gene promoters (Bustos et al., 1991; Burow et al., 1992). In the promoter of storage protein gene, *napA*, from *Brassica napus*, this motif was proposed to be important for seed-specific expression (Ellerström et al., 1996); deletion of the $(CA)_n$ element decreased the transcription in seeds (Stålberg et al., 1996).

When the gGmPM9 promoter was shortened to -114 bp to +91 bp, GUS activity dropped to relatively low levels. However, the transgenic plants with pZP114 still exhibited measurable enzyme activity in the late stage of seed development, while no GUS activity was detected in other parts of transgenic plants. In the salt and drought treatments, transgenic plants with this region displayed the same inducible pattern as the transgenic plants with the longest promoter region, indicating that this region could be important to confer the abilities for spatial, temporal expression and responsive pattern. Sequence analysis of this region, indicated that it contained one G-box (CACGTG), one CAAT box and one TATA box. It was proposed that the CACGTG motif was a major cis-acting regulatory element conferring spatial and temporal control of the *β-phaseolin* gene (Kawagoe and Murai, 1992). The

pea *rbcS-3A* gene was shown to possibly be involved in the TATA box region for spatial control (Kuhlemeier et al., 1987). Another example is the *Vicia faba* non-storage seed protein gene, *usp*, in which seed specificity was mainly determined by the -68/+51 bp region containing the TATA box (Fiedler et al., 1993).

Histochemical analysis of GUS activity revealed that the promoter of gGmPM9 was induced by the salt and drought treatments, indicating that it can function in leaves of *Arabidopsis* during osmotic stress. In contrast, ABA, cold and wounding had no effect on the spatial or temporal expression. Several other genes are induced by drought and osmotic stress but not by exogenous ABA. These include clone 15a and 26g of pea (Guerrero et al., 1990), *AtDi19* and *AtDr4* of *Arabidopsis* (Gosti et al., 1995), and *RD19*, *RD21* and *RD28* of *Arabidopsis* (Yamaguchi-Shinozaki et al., 1992). Thus, it was suggested that there are ABA-independent and ABA-responsive signal transduction pathways between the initial signal for water-stress and the gene expression (Yamaguchi-Shinozaki et al., 1992). It had been suggested that the response to salt stress and exogenous ABA operated through different pathways in the *LEA B19* genes of barley (Espelund et al., 1992). We propose that the expression pattern controlled by the promoter of gGmPM9 involves the ABA-independent pathway.

Results from gGmPM9/GUS fusion gene expression in *Arabidopsis* and sequence analysis of the gGmPM9 promoter indicate multiple cis-elements in the gGmPM9 promoter and that these elements might interact, antagonistically or synergistically, to mediate the gGmPM9 promoter function. It is necessary to dissect the promoter region of gGmPM9 in greater detail and to determine the function of the putative regulatory cis-elements.

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大豆種子成熟蛋白 GmPM9 基因之調節機制

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在種子發育過程中，有一類蛋白質會大量累積於成熟後期，故被稱為 LEA (late embryogenesis abundant) 蛋白質，此類蛋白質也稱為種子成熟蛋白，在單子葉及雙子葉植物中皆已廣泛地被發表。本文利用 β-glucuronidase (GUS) 為報導基因，探討大豆 *LEA* 基因 GmPM9 之啟動子在阿拉伯芥中之調控表現。將不等長度之啟動子區域片段接上 GUS 基因，轉殖入阿拉伯芥中，得知各啟動子片段皆表現 GUS 活性於轉殖阿拉伯芥之成熟種子及子葉與上胚軸部份。對轉殖植株施以 PEG 及 NaCl 處理，可誘導 GUS 活性表現於葉片，但離層酸 (ABA) 處理則無法誘導表現。於種子發育期間，各啟動子片段皆於開花十天後開始累積GUS活性於種子中，並在開花十四天後種子成熟時達到最高值。文中數據顯示，全長之啟動子區域 (-966 bp 至 +91 bp) 可獲得最高之 GUS 表現值，而最短之啟動子片段 (-114 bp 至 +91 bp) 已足以使報導基因表現於種子中。基於上述結果，我們認為 GmPM9 之啟動子具有種子專一性，適合將其利用於研究種子專一性之課題上。

關鍵詞：β-glucuronidase (GUS)；種子成熟蛋白；啟動子；轉殖阿拉伯芥。