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Tissue- and cellular localization of soybean (*Glycine max* L.) seed maturation protein transcripts

Ming-Der SHIH[1], Mei-Hua KAO[2], Shu-Chin LIN[2], Tzu-Fu SHU[1], Kim-Long HSIEH[1], Mei-Chu CHUNG[1], Tze-Hua TSOU[1], Yue-Ie C. HSING[1], and Jaw-Shu HSIEH[2,*]

[1]*Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan*
[2]*Department of Agronomy, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan*

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ABSTRACT. *L*ate *e*mbryogenesis *a*bundant (*LEA*) genes, with abundant expression in seeds during the late developmental stages, are associated with water loss. In the present work, we characterized the spatial expression patterns of 8 soybean *GmPM* genes, including 7 hydrophilic *LEAs* and 1 maturation protein gene, in fresh or pod-dried seeds at 35 days after flowering (DAF) by *in situ* hybridization analysis. The transcripts of hydrophilic *LEA* genes were detectable in vascular tissues of the cotyledon and embryo axis (epicotyl and radicle) in fresh seeds 35 DAF. After pod-drying, the expression of these genes was greatly increased in similar tissues and was induced in other seed tissues. A maturation protein gene, *GmPM5*, was highly expressed in mesophyll tissues but not in vascular tissues of fresh or pod-dried seeds. Hence, *LEA* genes could be be highly regulated in the conductive system with changes in water content. As well, the mRNA distribution of *GmPM5* and *GmPM8*/*10*, 2 messengers with endoplasmic reticulum signal peptides in the deduced proteins, significantly differed. The transcripts of these genes are therefore targeted towards different endoplasmic reticulum systems, which suggests that individual endoplasmic reticulum-targeting *LEA* genes may be involved in diverse translocation and subsequent protein transport systems.

Keywords: Endoplasmic reticulum; GmPM; *In situ* hybridization; Late embryogenesis abundant; Soybean; Spatial expression patterns; Vascular bundle.

INTRODUCTION

Water loss or desiccation could be an important part of the maturation process in orthodox seeds (Rosenberg and Rinne, 1986). During this process, gene expression and protein-synthesized profiles are markedly changed, which is usually associated with achieving desiccation tolerance, increased abscisic acid (ABA) content, or the capability of seed germination (Goldberg et al., 1989; Hughes and Galau, 1989; Skriver and Mundy, 1990). *LEA* genes were first identified from embryos of mature wheat (*Triticum aestivum*) (Cuming et al., 1984) and cotton (*Gossypium hirsutum*) (Galau et al., 1986). They are highly expressed during the late seed developmental stage or are induced by exogenesis ABA or environmental stress in vegetative tissues (see reviews in Dure et al., 1989; Ingram and Bartels, 1996; Cuming, 1999). *LEA* genes are not plant specific. Several *LEA* genes have been described from bacteria, fungi, or anhydrobiotic animals; examples are *Bacillus subtilis* (Mueller et al., 1992), cyanobacteria (Close and Lammers, 1993), *Tuber borchii* (Abba' et al., 2006), nematodes (Tunnacliffe and Wise, 2007), rotifers (*Adineta ricciae*) (Pouchkina-Stantcheva et al., 2007), brine shrimp (*Artemia franciscana*) (Hand et al., 2006; Wang et al., 2007), and *Polypedilum vanderplanki* (Kikawada et al., 2006). This wide distribution suggests the importance of *LEA* genes under desiccation conditions.

The deduced LEA protein sequences are classified into at least 5 groups according to their conserved motifs or sequence similarity (Shih et al., 2008). Group I to IV LEA proteins are highly hydrophilic, and the remainder are hydrophobic. Hydrophilic LEA proteins lack defined secondary structures in solution status and belong to the members of "natively unfolded proteins" (see review in Shih et al., 2008 and references). However, the detailed 3-D structures of At1g01470.1, the hydrophobic Arabidopsis D-95 type LEA protein, were determined as αβ-fold conformations by NMR spectroscopy (Singh et al., 2005). Except for group I to V LEA genes, some genes which encoded proteins lack the motifs or protein characteristics defined in other LEA proteins also express in seeds during late embryogenesis and disappear after the early hours of germination (Shih et al., 2008). Unlike typical LEA proteins, they may be carrier proteins, enzymes, fiber proteins, or plasma membrane proteins (e.g. Shen et al., 1993; Koike et al., 1997; Hsing et al., 1998; Breton et al., 2003). The overexpression of *LEA* genes was reported to improve stress tolerance of transgenic plants or recombinant cell lines. For example, transgenic

*Corresponding author: E-mail: jawar@ntu.edu.tw; Tel: +886-2-33664756; Fax: +886-2-23620879.

cereals harboring barley *HAV1* or wheat *PMA80* and *PMA1959* showed tolerance to drought or salinity stresses (Xu et al., 1996; Cheng et al., 2002). Several group II *LEA* (*dehydrin*) genes introduced into target plants also increased resistance to chilling, freezing, free radicals, or salinity (Hara et al., 2003; Houde et al., 2004; Puhakainen et al., 2004; Figueras et al., 2004). Additionally, a series of experiments with recombinant cell lines showed increased stress tolerance with the accumulation of hydrophilic LEA proteins (Imai et al., 1996; Swire-Clark and Marcotte, 1999; Zhang et al., 2000; Chakrabortee et al., 2007).

Previous work suggested that changes in seed constituents and levels of several newly synthesized proteins were similar between natural and precocious maturation (Rosenberg and Rinne, 1987). Precocious maturation was used as a model system to investigate the control of the physiological and biochemical events occurring during seed maturation (Rosenberg and Rinne1988; Hsing et al., 1990). A cDNA library was prepared from 4-day pod-dried (PD) soybean seeds at 35 days after flowering (DAF), followed by differential screening (Hsing et al., 1990; Hsing and Wu, 1992). Forty-one cDNA clones designated *Glycine max* physiological mature (*GmPM*) were sequenced, and 22 of them belonged to typical *LEA* genes. We already characterized some of these genes by studying the expression profiles under hormone or osmotic treatments, genetics, or their structural biology (Hsing and Wu, 1992; Hsing et al., 1995; Hsing et al., 1998; Lee et al., 2000; Shih et al., 2004; Tsai et al., 2008). However, information on spatial expression patterns was still missing. Here, we present the results of cellular localization analysis of these transcripts, including *GmPM11* (group I), *GmPM6/7* and *GmPM12* (group II), *GmPM2*, *GmPM8/10*, and *GmPM30* (group III), *GmPM1* (group IV), and *GmPM5* (maturation protein gene). The transcripts of most of the tested hydrophilic *LEA* genes accumulated in vascular tissues of the cotyledon and embryo axis (epicotyl and radicle). As well, the endoplasmic reticulum (ER)-targeting *LEA* genes, *GmPM5* and *GmPM8*/*10*, showed different mRNA distribution in cotyledon mesophyll cells.

MATERIALS AND METHODS

Plant materials

Cultivated soybean (cv. Shih-shih) seeds were kindly provided by Kaohsiung Agricultural Experimental Station, Pintoung, Taiwan. Plants were grown in the field under a normal day-length period. Pods at 35 DAF that were fresh or intact pods precociously matured by air-drying (PD) for 4 days were harvested as described previously (Hsing et al., 1990; Hsing and Wu, 1992).

In situ hybridization

In situ hybridization of mRNA was performed as described previously (Hsing et al., 1998). Soybean seeds at 35 DAF with or without 4-day PD treatment were harvested and immediately fixed with 4% formaldehyde in 10 mM phosphate buffered saline (pH 7.0) for 16 h at 4°C. The plant materials were washed with 0.85% NaCl solution, dehydrated in a graded ethanol series, and embedded in paraffin. Sections (6 to 8 μm thick) were cut and mounted on Vactabond-treated slides. The slides were put in a 42°C oven for 16 h and then stored at 4°C for further use. The linearized *GmPM1*, *2*, 5, *6/7*, *8*/*10*, *11*, *12*, and *30* plasmids were used as templates for the digoxigenin-labeled antisense or sense strands transcribed by T7 or T3 RNA polymerase from their corresponsive clones. Labeling with digoxigenin followed the manufacturer's instructions (Roche, Germany). Probes were hydrolysed to an average size of 150 bp at 60°C before use. Paraffin was removed from the tissue sections by incubation in a series of Histoclear solution, 100% ethanol, and ethanol with 0.85% (w/v) NaCl. Slides were treated with pronase for 4 h at 37°C, then 2 min in 0.2% glycine, 10 min in 4% formaldehyde, and 30 sec each in a series of ethanol solutions. Hybridization of the probes to the slides was performed at 50°C for 8 to 24 h, depending on the clone or tissue. After hybridization, slides were washed several times with NTE solution (500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10 mM dithiothreitol) at 37°C. Digoxigenin-labelled RNA probes were then detected by use of an antidigoxigenin alkaline-phosphate conjugate (Roche, Germany). Slides were dehydrated before drying at 50°C and observed under a microscope.

Tissue printing

The nitrocellulose membrane was soaked in 0.2 M $CaCl_2$ solution for 30 min, and dried on paper towels. Fresh cut tissue was washed in distilled water for 5 sec, and blotted onto the membrane for 5, 15, 30, 60, 90, and 120 sec. The tissue print was blocked for 30 min in phosphate-buffered saline/Tween 20 (PBST) (0.1% [w/v] Tween 20, 150 mM NaCl, 50 mM Na_2HPO_4, and 50 mM NaH_2PO_4) with 5% (w/v) defatted dry milk. The sera against GmPM5 protein (1:50,000 dilution) were used for primary labeling. The signals were then amplified by goat anti-rabbit AP-conjugate antibody (Sigma, USA) (1:5,000 dilution). After a PBST/PBS rinse, the color substrates (Roche, Germany) were used for GmPM5 protein detection.

RESULTS AND DISCUSSION

The localization of Group I *LEA* messages

Group I *LEA* genes encode proteins that contain a conserved 20-mer motif that can be unique or repeated 2 to 4 times in tandem (Espelund et al., 1992). Previous work had divided LEA I proteins into 2 subgroups according to the repeat number of the 20-mer motif. Em1-type proteins contain 2 to 4 repeats, and Em6-type proteins only one (Delseny et al., 2001). One soybean *LEA I* transcript, *GmPM11* (accession no. AF004805), which encodes an Em6-type protein, was used in the current study. The

predicted molecular mass is 9 kDa, at pI 7.5. The spatial expression patterns of *GmPM11* were analyzed in fresh or 4-day PD seeds at 35 DAF (Figure 1). In fresh seeds, *GmPM11* transcripts highly accumulate in vascular tissues of radicle but are barely detected in cotyledon vascular strands and radicle cortex (Figure 1B to E). In 4-day PD seeds, *GmPM11* mRNAs accumulate in all seed tissue, including the cotyledon and embryo axis (Figure 1F to J). Strong signals are observed in vascular tissues and in the adaxial epidermis cells of cotyledon.

Several Em6-type *LEA* genes had been studied by *in situ* hybridization. Carrot *Emb-1* mRNA was preferentially detected in the procambial meristematic region of somatic or zygotic embryos from the globular to mature torpedo stages and was greatly accumulated in cotyledons at a later stage (Wurtele et al., 1993). Sunflower *ds11* transcripts appeared to accumulate to high levels in early embryogenesis. In mature seeds, the distribution of *ds11* mRNA showed marked localization in vascular tissues of the procambium or cotyledon (Prieto-Dapena et al., 1999). Arabidopsis *AtEm6* transcripts were detected throughout the entire embryo in provascular tissues and shoot apical meristem (Vicient et al., 2000). Therefore, the *in situ* hybridization studies of other Em6-type *LEA I* genes showed a similar signal distribution in seeds or embryos similar to what we found. These results suggested that the messages of Em6-type *LEA I* genes appear from the early to middle stages of embryogenesis and reach a plateau at the late developmental stage, with preferential accumulation in vascular tissues of the procambium of the cotyledon or embryo axis.

Localization of Group II *LEA* messages

Group II LEA proteins have 3 specific, characteristic sequence "modules" (i.e., K-domain, Y-domain, and S-segment) that define this class of protein (Close, 1997). In the present study, we used these soybean *dehydrin* genes (i.e., *GmPM6*/*7* and *GmPM12*) to illustrate their spatial patterns. *GmPM6*/*7* (accession nos. M94012 and L00921) share 96% cDNA sequence identity. The deduced protein sequences of *GmPM6*/*7* belong to Y_2K-type *dehydrins*, with molecular mass of 31/32 kDa and pI 6.6/6.5, respectively. The mRNA of *GmPM6*/*7* (the 2 transcripts cannot be distinguished because of high identity) accumulates in vascular strands, mesophyll around vascular tissues, and adaxial epidermis of cotyledon, epicotyl, as well as cortex, pith, and phloem cells of radicle in fresh seed (Figure 2B to F). By contrast, no or few *GmPM6*/*7* transcript is detected in the abaxial epidermis of cotyledon (Figure 2B and C) and radicle pericycle (Figure 2D). The mRNA expression of *GmPM6*/*7* mRNA is greatly increased in the same regions in PD seeds as in fresh seeds (Figure 2G to L). The mRNA level of *GmPM6*/*7* is increased slightly in radicle pericycle and of cotyledon vascular strands after drying (Figure 2J and K).

The deduced protein sequence of *GmPM12* (accession no. AF004807) shows it to be a Y_2SK_2-type *dehydrin*. The molecular mass is 10 kDa at pI 10. In fresh seeds, no or few transcript of *GmPM12* in seeds (Figure 3B-F). In PD seeds, the expression of *GmPM12* is significantly increased in vascular tissues of the cotyledon and embryo axis (Figure 3H to K).

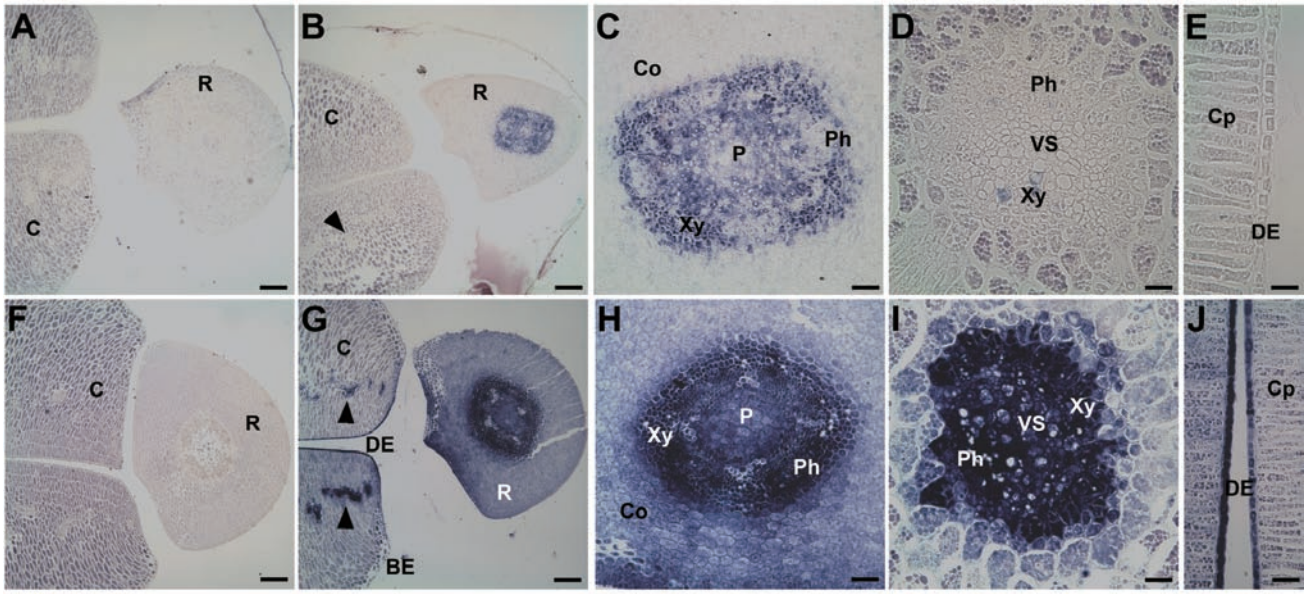

Figure 1. Localization of soybean *GmPM11* transcripts in fresh (panel A to E) or 4-day pod-dried (PD) (panel F to J) soybean seeds. *In situ* hybridization was carried out with transverse sections of seeds in this and following figures. A sense probe was used in panel A and F and an antisense probe in the remaining panels. Bar=200 μm for panel A, B, F, and G, 60 μm for panel C and H, 40 μm for panel E and J, and 20 μm for panel D, and I. Abbreviations (all the abbreviations is noted at first time): BE, abaxial epidermis; C, cotyledon; Co, cortex; Cp, palisade tissue of cotyledon; DE, adaxial epidermis; P, pith; Ph, phloem; R, radicle; VS, vascular strand; Xy, xylem. Arrowheads indicate cotyledon vascular strands.

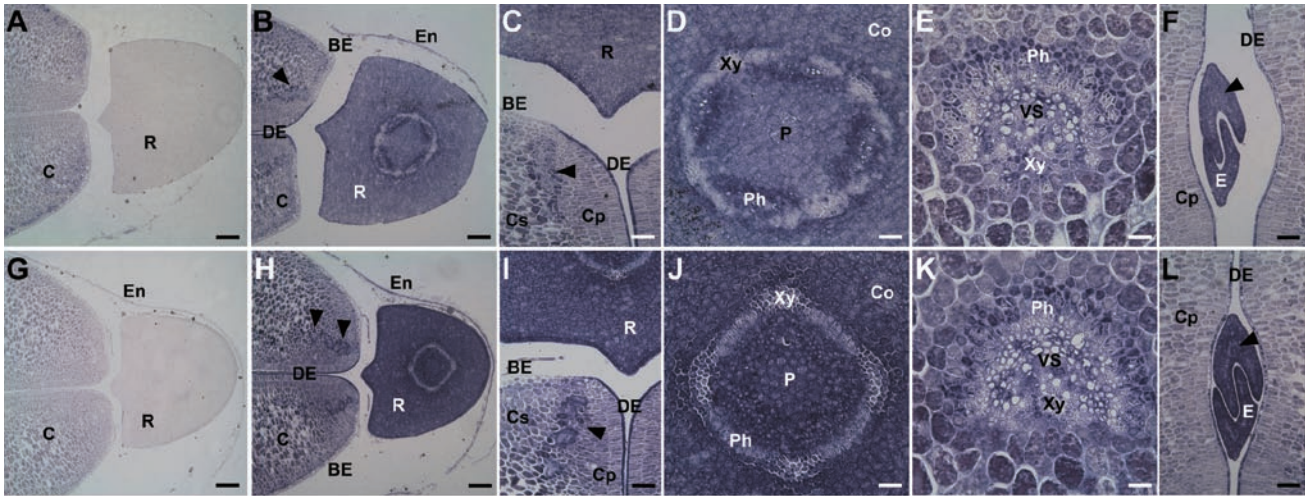

Figure 2. Localization of soybean *GmPM6/7* transcripts in fresh (panel A to F) or 4-day PD (panel G to L) soybean seeds. A sense probe was used in panels A and G and an antisense probe in the remaining panels. Bar=200 μm for panel A, B, G, and H, 80 μm for panel C, F, I, and L, 60 μm for panel D and J, 20 μm for panel E and K. Abbreviations: Cs, spongy tissue of cotyledon; E, epicotyl; En, endosperm; Pe, pericycle. Arrowheads indicate cotyledon vascular strands (panel B, C, H, I) or epicotyl procambium (panel F, L).

Several types of *dehydrins* had been studied by *in situ* hybridization. The spatial patterns of a carrot Y_2SK_2-type *dehydrin*, *Ecp40*, showed that the transcripts accumulated in embryo and endosperm in dry seeds (Kiyosue et al., 1993). The mRNA expression of maize (*Zea mays*) *Rab17*, an YSK_2-type *dehydrin*, was first detected in leaf primordia and radicle, with little expression in scutellum. During maturation, *Rab17* transcripts accumulated in the cortex, protoxylem, and metaxylem cells of radicle and leaf primordia, as well as the procambium and epidmeris cells of the scutellum (Goday et al., 1994). The rice (*Oryza sativa*) homolog of *Rab17*, *Rab21*, showed similar expression patterns, except that *Rab21* transcripts were relatively scarce in leaf primordia and the radicle (Miyoshi et al., 1999). These studies indicate that the mRNA expression of *dehydrins* is mainly in the embryo axis and vascular bundles. They may provide a protective mechanism primarily for embryos.

Localization of Group III *LEA* messages

The most significant feature of group III LEA proteins is the tandem-repeating 11-mer motifs. According to the different consensus sequences of 11-mer repeats, LEA III proteins were classified as subgroups D-7, Dc8, GmPM8, and D-29 (Shih et al., 2008). In this paper, we characterize 3 soybean *LEA III* genes. They are a Dc8 type, *GmPM2* (accession no. M80664) (Hsing et al., 1992); two GmPM8 types, *GmPM8* and *GmPM10* (accession nos. X63565 and U02966) (Hsing et al., 1995); and one D7 type, *GmPM30* (accession no. AF117884). The GmPM2 protein contains

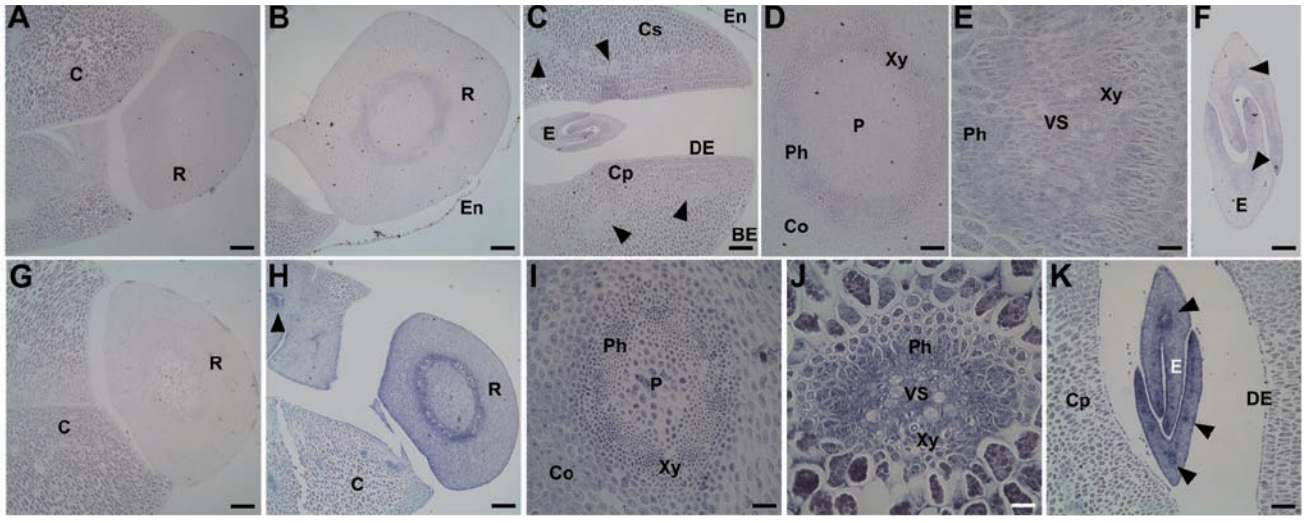

Figure 3. Localization of soybean *GmPM12* transcripts in fresh (panel A to F) or 4-day PD (panel G to K) soybean seeds. A sense probe was used in panel A and G and an antisense probe in the remaining panels. Bar=200 μm for panel A, B, C, G, and H, 80 μm for panel F and K, 60 μm for panel D and I, and 20 μm for panel E and J. Arrowheads indicate cotyledon vascular strands (panel C, H) or epicotyl procambium (panel F, K).

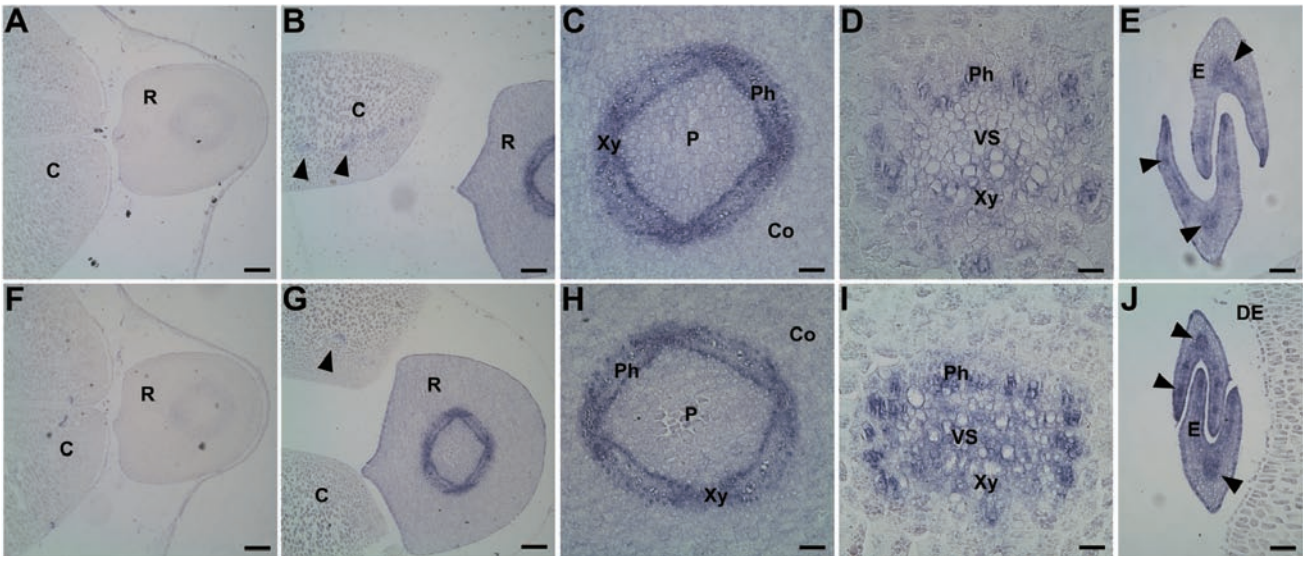

Figure 4. Localization of soybean *GmPM2* transcripts in fresh (panel A to E) or 4-day PD (panel F to J) soybean seeds. A sense probe was used in panel A and F and an antisense probe in the remaining panels. Bar=200 µm for panel A, B, F, and G, 80 µm for panel E, 60 µm for panel C, H, and J, and 20 µm for panel D and I. Arrowheads indicate epicotyl procambium (panel E, J).

20 11-mer repeats and one 36-mer motif at the carboxyl end, with molecular mass 52 kDa and pI 6.6 (Curry and Walker-Simmons, 1993). *In situ* hybridization results suggested that in fresh seeds, *GmPM2* transcripts are slightly detected in the vascular tissues of cotyledons and embryo axis (Figure 4B to E). In 4-day PD seeds, the signals of *GmPM2* transcripts are significantly enhanced in the same regions (Figure 4G to J). As well, mesophyll and epidermis of epicotyl, cortex, and endosperm also show strong signals (Figure 4G, H, and J).

GmPM8/10 share high homology in cDNA sequences (93%). The encoded proteins contain 31 and 32 similar contiguous repeats, respectively. The molecular weights are 48 and 50 kDa, at pI 7.7 and 7.1, respectively. Both proteins consist of 29-mer hydrophobic regions at the N terminus, which are removed after co-incubation with endoplasmic reticulum membranes (Hsing et al., 1995). In fresh seed, *GmPM8*/*10* transcripts (the 2 transcripts cannot be distinguished because of high identity) are detected in embryo axis, and slightly accumulated in phloem and xylem cells of vascular tissues in cotyledon (Figure 5B to F). In 4-day PD seeds, strong signals of *GmPM8*/*10* mRNA is observed in similar tissues, and the transcripts accumulate to high levels in mesophyll and the adaxial epidermis of the cotyledon (Figure 5H to L). In palisade tissue of cotyledon, all *GmPM8* transcripts aggregate around the nuclei of cells rather than being scattered around the whole cells, as for most of the other *LEA* messages (Figure 5I and J).

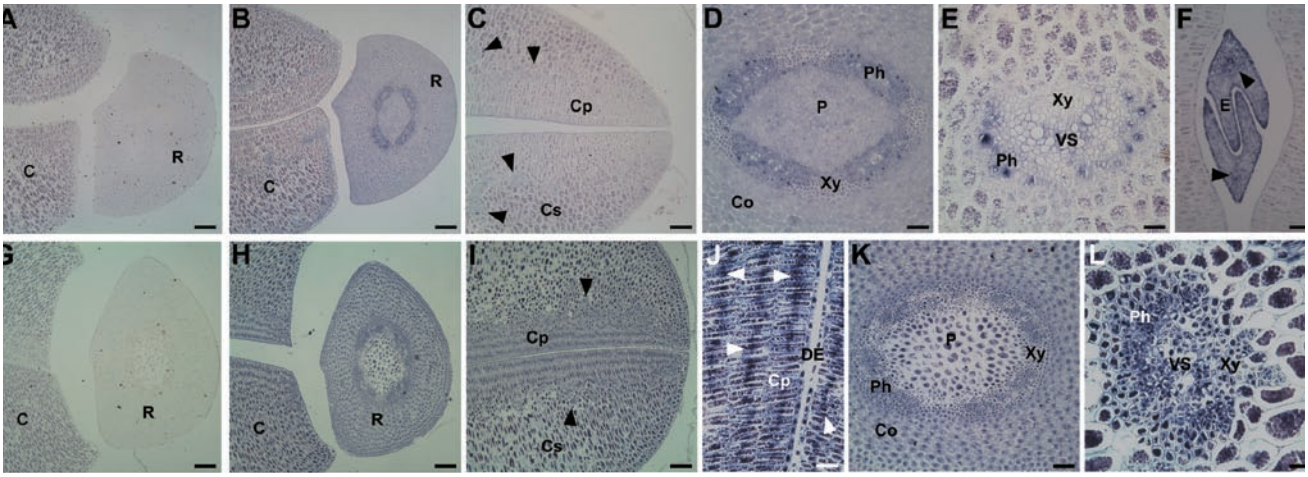

Figure 5. Localization of soybean *GmPM8/10* transcripts in fresh (panel A to F) or 4-day PD (panel G to L) soybean seeds. A sense probe was used in panel A and G, and an antisense probe in the remaining panels. Bar=200 µm for panel A to C and G to I, 80 µm for panel F, 60 µm for panel D, J, and K, and 20 µm for panels E and L. Black arrowheads indicate cotyledon vascular strands (panel C, I) or epicotyl procambium (panel F). White arrowhead indicates the aggregation of *GmPM8* transcripts in palisade tissue of cotyledon (panel J).

GmPM30 encodes a small LEA III protein, which contains five 11-mer tandem repeats. The molecular weight of GmPM30 protein is 15 kDa, at pI 9.6. In fresh seeds, the *GmPM30* mRNA accumulates to a high level in the epicotyl, as well as the phloem and xylem cells in the radicle; it is also slightly detectable in cotyledon vascular tissues (Figure 6C to G). In 4-day PD seeds, *GmPM30* transcripts significantly increase in vascular tissues of embryo axis and cotyledon. Strong signals are also observed in the cortex and epidermis cells of the radicle. In addition, *GmPM30* transcripts are slightly enhanced in the mesophyll of the cotyledon and are highly induced in adaxial epidermis cells of the cotyledon (Figure 6G-L).

Although *LEA III* genes were identified from plant and animal kingdoms, few *LEA III* genes have been studied by *in situ* hybridization. *Craterostigma plantagineum pcCAD28*, encoding a D7-type protein with eleven 11-mer repeats, is a vegetative tissue-specific gene. After dehydration treatment, *pcCAD28* transcripts were greatly increased in all cells of leaf or root tissues (Ditzer et al., 2001). However, the authors did not mention whether *pcCAD28* transcripts were detectable in dry seeds. In the present study, the transcripts of *GmPM2*, *GmPM8*/*10*, or *GmPM30* were mainly accumulated in leaf primordia and the radicle. As well, all studied *LEA III* genes were detected in vascular tissues. These results suggest that these *LEA III* subtypes may be responsive to dehydration in the embryo axis and conductive system.

Localization of Group IV *LEA* messages

Group IV *LEA* genes encode a group of proteins with small molecular weight. The proteins lack significant signature motifs or consensus sequences as do LEA I to III proteins but contain similar amino acid residue distribution along the polypeptide. They are highly hydrophilic and heat soluble (Dure, 1993; Wise, 2003). The putative open reading frame of *GmPM1* (accession no. M80666) encodes a polypeptide of 173 amino acid residues, of 17.6 kDa at 10.4 pI. *In situ* hybridization revealed that *GmPM1* transcripts accumulate in vascular tissues of embryo axis and cotyledon in fresh seed (Figure 7B to E). In 4-day PD seeds, the mRNA expression of *GmPM1* is maintained in the same region as for fresh seeds but is highly induced in vascular tissues and epidermis cells of the epicotyl (Figure 7G, H, and J) as well as xylem of cotyledon vascular strand (Figure 7I). Because the *GmPM1* transcripts are relative abundant in the radicle in both fresh and PD seeds, *GmPM1* genes may undergo tissue-specific regulation.

LEA IV genes have been isolated from Gymnosperm and Angiosperm. The expression patterns of these genes suggest that some of them, such as Arabidopsis *PAP51* (Parcy et al., 1994) and soybean *GmPM9* and *GmPM16* (Lee et al., 2000; Shih et al., 2004), are seed/embryo specific, whereas *C. plantagineum pcCC2* (Ditzer et al., 2001) is vegetative tissue specific. However, other *LEA IV* genes, such as tomato *Le25* (Kahn et al., 1993) and *Phaseolus vulgaris Pvlea4-25* (Colmenero-Flores et al., 1999), are expressed in seeds and embryos, as well as in vegetative tissues with stress treatments. Previous localization studies in our lab showed that the transcripts of another basic soybean *LEA IV*, *GmPM16*, accumulated in cotyledon mesophyll cells but not in the vascular system; they were also slightly detectable in the embryonic axis, especially in the metaxylem and pith tissues (Shih et al., 2004). Hence, these two *LEA IV* genes, *GmPM1* and *GmPM16*, have different expression patterns.

Localization of maturation protein gene messages

GmPM5 (accession no. U59425) encodes soybean seed 7S globulin, a glycolated protein, of 41 kDa at pI 8.2. Previous work showed that GmPM5 proteins underwent post-translational modification, including removal of the signal peptide and breakage into 2 polypeptides of 27 and 16 kDa that are linked by disulfide bonds and are glycolated (Hirano et al., 1992). *In situ* hybridization illustrated that *GmPM5* transcripts accumulate to extremely high levels in cotyledon mesophyll in both fresh and PD seeds (Figure 8B, C, E, G to I, and K). As well, no signal was found in all epidermal cells of cotyledon

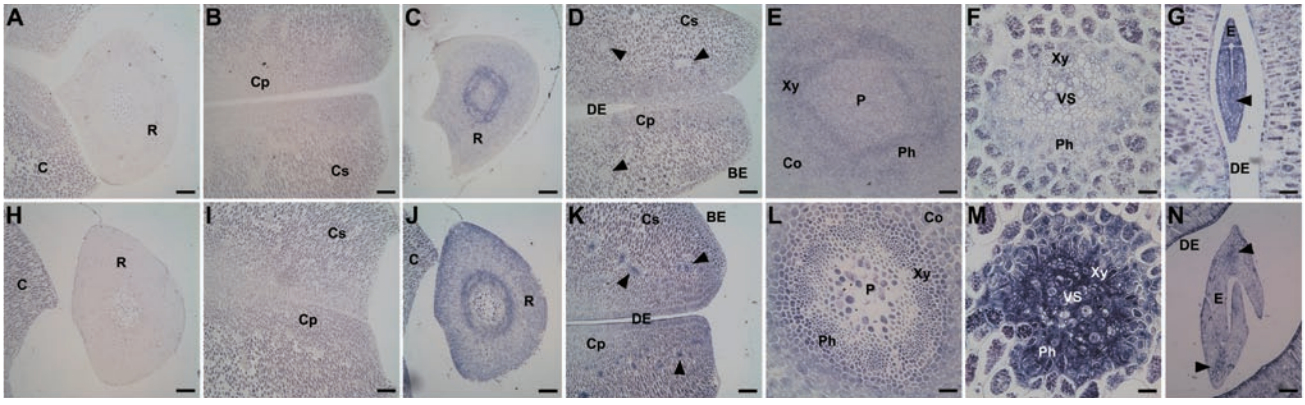

Figure 6. Localization of soybean *GmPM30* transcripts in fresh (panel A to G) or 4-day PD (panel H to N) soybean seeds. A sense probe was used in panel A, B, H, and I and an antisense probe in the remaining panels. Bar=200 μm for panel A to D and H to K, 80 μm for panel G and N, 60 μm for panel E and L, and 20 μm for panel F and M. Arrowheads indicate cotyledon vascular strands (panel D, K) or epicotyl procambium (panel G, N).

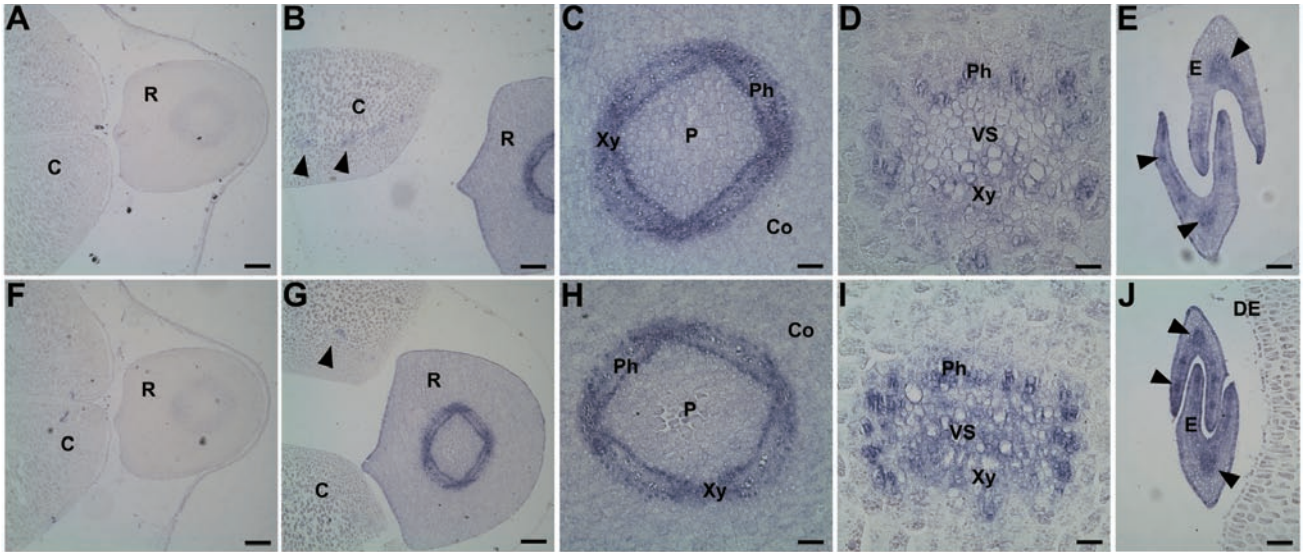

Figure 7. Localization of soybean *GmPM1* transcripts in fresh (panel A to E) or 4-day PD (panel F to J) soybean seeds. A sense probe was used in panel A and F and an antisense probe in the remaining panels. Bar=200 μm for panels A, B, F, and G, 80 μm for panel E and J, 60 μm for panel C and H, and 20 μm for panels D and I. Arrowheads indicate cotyledon vascular strands (panels B, G) or epicotyl procambium (panels E, J).

and embryo axis or in vascular tissues in either fresh or PD seeds (Figure 8D-E, I-J). Previous work demonstrated that GmPM5 proteins could be released into the media while the seeds were immersed in hot (40-60°C) water or were secreted into media from suspension-cultured cells (Hirano et al., 1992; Hsing, unpublished results). Extracellular dermal glycoprotein (EDGP), the carrot homolog of *GmPM5*, is also a secretion glycoprotein, but no post-translational cleavage was observed. In addition, the expression of *EDGP* genes was responsive to biotic or abiotic stresses (Satoh et al., 1992; Shang et al., 2005). Although these secreted glycoproteins are normally found in dermal tissues, *in situ* hybridization revealed no *GmPM5* transcript in dermal cells. Thus, protein localization analysis was performed by seed tissue printing. The immunoblotting results illustrated in Figure 9 indicate that although *GmPM5* transcripts are not present in epidermal cells, the proteins are transported and are accumulate to high level in these cells (Lane A and B). As well, the proteins do not transport into vascular bundles (Lane E and F).

CONCLUSION

The present study characterized the spatial expression patterns of 8 soybean maturation protein genes, including 7 hydrophilic *LEAs* and 1 maturation protein genes, in fresh or PD seeds at 35 DAF by *in situ* hybridization analysis.

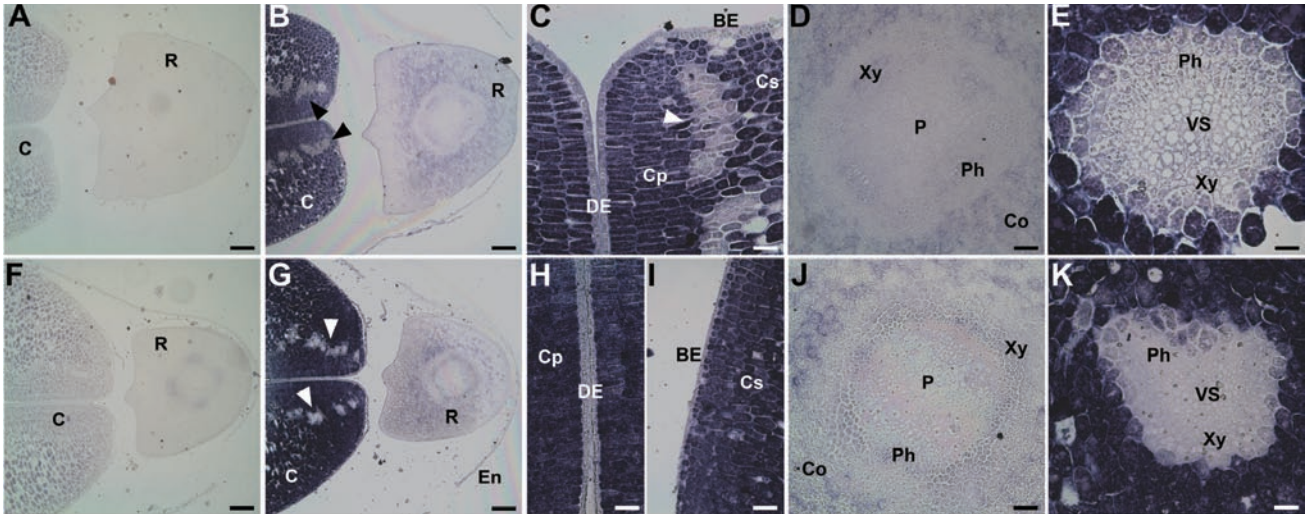

Figure 8. Localization of soybean *GmPM5* transcripts in fresh (panel A to E) or 4-day PD (panel F to K) soybean seeds. A sense probe was used in panel A and F, and an antisense probe in the remaining panels. Bar=200 μm for panel A, B, F, and G, 80 μm for panel I, 60 μm for panel C, D, H, and J, and 20 μm for panels E and K. Black or white arrowheads indicate cotyledon vascular strands (panel B, C, G).

Most of the hydrophilic *LEA* genes are expressed in vascular tissues. In fact, several LEA proteins accumulate in the conductive system of seed or vegetative tissues (e.g. Houde et al., 1995; Parra et al., 1996; Rouse et al., 1996; Danyluk et al., 1998; Arenas-Mena et al., 1999; Nylander et al., 2001; Rodrigo et al., 2004). The seed vascular system is crucial for the translocation of seed reserves from the cotyledons to the growing axis during seed germination (Garnczarska et al., 2007). Hence, the presence of LEA proteins in the vascular system of cotyledons may be required for the protection of imbibitional injury during germination. In addition, LEA proteins might also function as water attractants during the transport of water and seed reserves to sink tissue (Nylander et al., 2001; Rorat, 2006), which, as compared with other tissues, should consist of high water-uptake capacities during the early stage of imbibition (Manz et al., 2005). This phenomenon may also be involved in the protection mechanism of germination tissues in elevating the initial moisture level (Vertucci and Leopold, 1984).

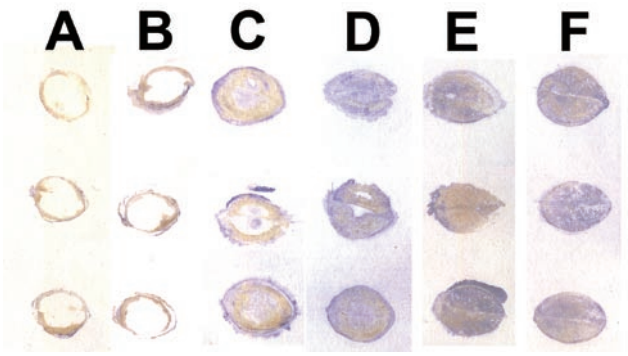

Figure 9. Localization of soybean GmPM5 proteins in 4-day PD soybean seeds by tissue printing. Protein signals of GmPM5 were detected by immunoblotting with anti-serum of GmPM5. Panel A to F indicate the blotting times 5, 15, 30, 60, 90, and 120 sec, respectively.

However, some hydrophilic *LEA* or maturation protein genes, such as *GmPM16* (Shih et al., 2004) and *GmPM5* (present study), were not detected in vascular tissues. Five tested *LEA* genes—*GmPM2*, *GmPM6*/*7*, *GmPM8*/*10*, *GmPM12*, and *GmPM30*—are expressed in adaxial but not abaxial cells of the cotyledon epidermis. Therefore, each *LEA* gene has its own tissue-specific regulation. Moreover, depending on the water status during seed maturation, seed water evaporates from the seed surface (Ishida et al., 2004; Garnczarska et al., 2007). The mesophyll or epidermis cells may sense water loss and trigger the expression of *LEA* genes. As well, the adaxial or abaxial epidermis of the cotyledon may display diverse responses to water potential by different sensitivity to osmotic stresses. The different spatial profiles of *LEA* genes reflect the large numbers of *LEA* genes in the plant genome. For example, on similarity

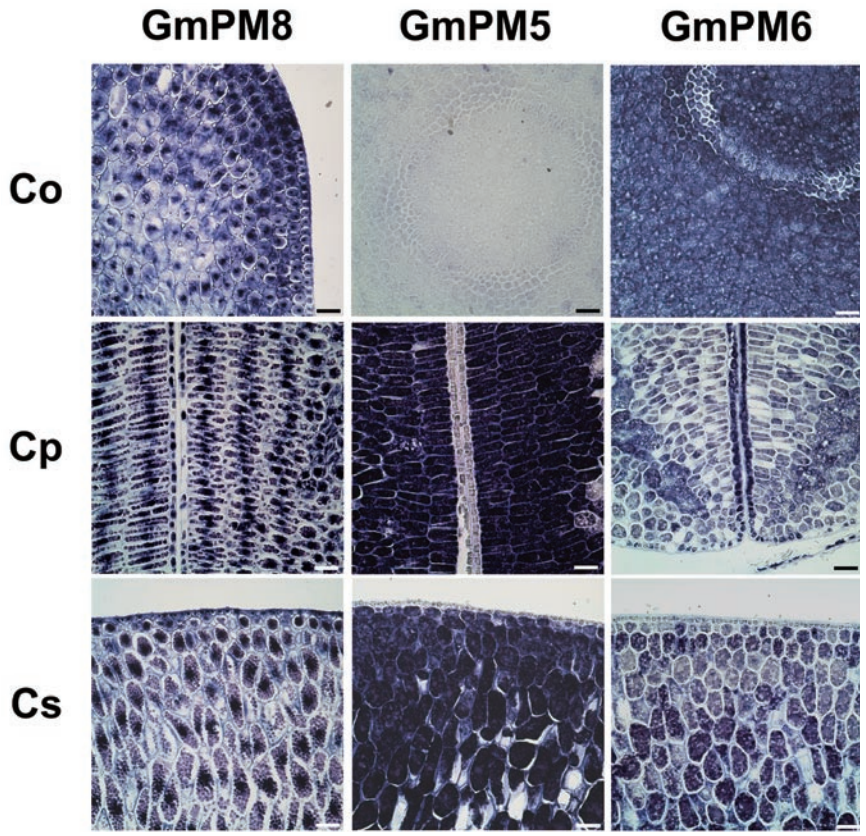

Figure 10. Detail localization of soybean *GmPM5*, *6*, and *8* transcripts in 4-day PD soybean seeds. Bar=40 μm for all panels. Abbreviations: Co, cortex; Cp, palisade tissue of cotyledon; Cs, spongy tissue of cotyledon.

search, 50 *LEA* genes were detected in the Arabidopsis genome (Bies-Etheve et al., 2008). In terms of evolution, gene redundancy and different expression patterns are good strategies for survival. Therefore, a single *LEA* gene may not provide enough protection under different stresses, whereas many *LEA* families may provide such protection.

To date, although several hundreds of LEA proteins have been identified from a wide range of species, only few of them contains an ER signal peptide. GmPM5 and GmPM8/10 proteins, both containing signal peptides, target into ER membranes (Hirano et al., 1992; Hsing et al., 1995). In the present work, these 2 transcripts showed a diverse localization pattern: *GmPM8*/*10* mRNA aggregates around nuclei whereas *GmPM5* mRNA spreads out over the whole cytoplasm (Figure 10). Association of mRNAs with different ER systems was demonstrated in animal and plant systems (Melton, 1987; Svoboda, 1991; Li et al., 1993). In rice endosperm used as a model system, the transcripts of 2 different storage protein genes, *prolamin* and *glutelin*, targeted into 2 morphologically distinct ER membranes. *Prolamin* mRNA is highly enriched on protein-body ER, which is located near the plasma membrane, whereas *glutelin* mRNA is enriched at the cisternal ER that is randomly distributed throughout the cytoplasm (Krishnan et al., 1986; Yamagata and Tanaka, 1986; Kim et al., 1993; Li et al., 1993). The localization of transcripts to distinct ER membranes might be involved in a protein-sorting network (see reviews in Okita et al., 1998; Okita and Choi, 2002; Crofts et al., 2005). Thus, the different localization of *GmPM5* and *GmPM8*/*10* mRNA provides cytological evidence that ER-targeting LEA proteins may transport into distinct organelles or secretary pathways.

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大豆種子成熟蛋白基因表現的組織與細胞定位

施明德[1] 郭美華[2] 林淑琴[2] 徐子富[1] 謝金龍[1]
鍾美珠[1] 鄒稚華[1] 邢禹依[1] 謝兆樞[2]

[1] 中央研究院 植物暨微生物學研究所
[2] 國立台灣大學 農藝學系

在種子成熟晚期大量表現的晚胚蛋白基因是與種子失去水份的時程有關。本研究將針對八種大豆的 GmPM 基因，包含七種典型與一種非典型晚胚蛋白基因，以開花後 35 天的新鮮與帶莢乾燥 4 天的種子為材料，進行原位雜交的分析。結果顯示，典型的晚胚蛋白基因會在新鮮種子的子葉與胚軸的維管束中表現。經過帶莢乾燥後，典型的晚胚蛋白基因除了在維管束中表現量會大量的增加，也會在種子的其它組織中被誘導出來。非典型的晚胚蛋白基因，GmPM5，則是在新鮮與帶莢乾燥種子的非維管束組織中大量表現。因此，晚胚蛋白基因在傳導組織的表現是受到高度調控的。此外，具有內質網訊號序列的晚胚蛋白基因，GmPM5 與 GmPM8/10 在細胞內的分佈也有明顯的差異。這結果顯示這些晚胚蛋白基因的轉錄物會進入不同的內質網系統，並影響之後蛋白的轉運方式。

關鍵詞：內質網；GmPM；原位雜交；晚胚蛋白；大豆；空間表現型式；維管束。