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**Original Paper**

# Maturation of somatic embryos as a model for soybean seed development

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**Abstract** Soybean somatic embryos have attracted attention both as a model of zygotic embryos and as explants for the generation of stable transgenic plants. We have now characterized the maturation of soybean somatic embryos in detail by examining both the accumulation of the major seed storage proteins $\beta$-conglycinin and glycinin as well as changes in cellular organization. Protein storage vacuoles and oil bodies, which are the main depositories of seed storage reserves, formed within cells during the maturation of somatic embryos. The seed storage proteins were gradually synthesized and accumulated in the protein storage vacuoles in a manner similar to that apparent in seeds. The $\alpha$ and $\alpha'$ subunits of $\beta$-conglycinin were detected earlier than the $\beta$ subunit of this protein and glycinin. In addition, The $\alpha$ and $\alpha'$ subunits of $\beta$-conglycinin accumulated in both the cotyledons and the hypocotyl of somatic embryos, whereas the $\beta$ subunit of $\beta$-conglycinin and glycinin accumulated only in the cotyledons. These temporal and spatial characteristics of storage protein production in maturating somatic embryos are similar to those in developing seeds, although the maturation of somatic embryos ceases prematurely without attainment of the final stages of development. Our findings suggest that somatic embryos are suitable for verification of seed-specific traits such as the biosynthesis of seed components.

**Key words:** Seed development, somatic embryogenesis, soybean (*Glycine max* [L.] Merr.), storage protein.

Soybean [*Glycine max* (L.) Merr.] is a staple source of vegetable protein and oil for human food, animal feed, and industry, given that the seed contains large amounts of high quality protein (~40% by weight) and oil (~20% by weight). Other components of the seed are also of substantial economic importance for use in a wide range of industrial, pharmaceutical, food, and agricultural products. In addition, consumption of food containing soybean or soybean constituents has been suggested to provide protection against several chronic diseases (Birt et al. 2004). Such features render soybean one of the most economically important grain crops.

The genomic era is now under way for soybean as for other model plants. In addition to efforts to sequence the entire genome, several resources including an expressed sequence tag (EST) database and cDNA microarrays have been developed (Stacey et al. 2004). These resources will provide a foundation for the application of functional genomics approaches such as systemic RNA interference and TILLING (targeting induced local lesions in genomes) (Nagamatsu et al. 2007; Lightfoot 2008). Elucidation of biosynthetic pathways for seed components and their regulation at the molecular level should provide important information for improvement of seed quality by genetic engineering or marker-assisted selection. However, it is not efficient or practical to screen zygotic embryos for the effects of the large number of genes implicated in seed quality because of the long life cycle of soybean.

Somatic embryos of soybean are induced from immature cotyledons cultured on medium containing a moderately high concentration of synthetic auxin, either 2,4-dichlorophenoxyacetic acid or $\alpha$-naphthaleneacetic acid. These embryos are able to generate proliferative embryogenic cultures, and whole plants can be recovered from them through differentiation, maturation, and germination (Lazzeri et al. 1985; Ranch et al. 1985; Finer and Nagasawa 1988; Parrott et al. 1988). Immature somatic embryos can be maintained with regenerative capacity for more than a year, with differentiation and maturation being readily induced when required (Finer and Nagasawa 1988; Parrott et al. 1988; Walker and Parrott 2001). Moreover, immature somatic embryos of soybean have been subjected to transformation (Finer and Mcmullen 1991; Sato et al. 1993; Ko et al. 2006), and, in the case of that mediated by particle bombardment, transgenic embryos can be generated within 3 months after the introduction of exogenous genes (Finer and

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Abbreviations: BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PSV, protein storage vacuole; RT, reverse transcription; $\alpha$-TIP, $\alpha$–tonoplast intrinsic protein.
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Mcmullen 1991; Khalafalla et al. 2005). Transgenic immature somatic embryos can also be maintained in culture, and homogeneous masses of such embryos can be readily and repeatedly subjected to induction of differentiation and maturation. Somatic embryos have therefore been used to screen for transgene expression and to examine transgene effects before recovery of whole plants (Cahoon et al. 1999; Cahoon et al. 2000; Cahoon et al. 2002; Herman et al. 2003; Chen et al. 2006; Kajikawa et al. 2008). In addition to the use of somatic embryos as a provisional screening system, we hypothesized that such embryos could be substituted for seeds for validation of gene function in the synthesis of seed components. Mature somatic embryos of soybean have been found to contain seed storage proteins (Komatsuda et al. 1992; Stejskal and Griga 1995), and the fatty acid composition of such embryos is similar to that of seeds (Shoemaker and Hammond 1988; Dahmer et al. 1991). However, relatively little information has been available regarding common features of somatic embryos and seeds.

We have now investigated in detail the maturation process of somatic embryos in comparison with that of seeds, especially with regard to cellular organization and the accumulation of seed storage proteins, the latter of which is one of the most prominent events during seed development. Our results provide insight into the potential advantages of the use of somatic embryos for the study of gene function in the biosynthesis of seed components.

## Materials and methods

### Plant material and culture conditions

Induction, proliferation and maturation of soybean somatic embryos were performed as previously described (Kita et al. 2007). Briefly, somatic embryos were induced from immature cotyledons of soybean (cv. Jack) cultured on MSD40 medium (Finer and Nagasawa 1988) for 4 weeks at 25°C under cool white fluorescent light (5–10 $\mu$mol m$^{-2}$ s$^{-1}$; 23-h-light, 1-h-dark cycle). Induced somatic embryos were maintained in FN Lite liquid (FNL) medium (Samoylov et al. 1998) on a rotary shaker at 100 rpm at 25°C under cool white fluorescent light (12.1 $\mu$mo m$^{-2}$ s$^{-1}$). Somatic embryos (~20 mg) were matured on a rotary shaker at 100 rpm in histodifferentiation and maturation medium, FNL0S3S3 liquid medium (Walker and Parrott 2001), at 25°C under white fluorescent light (12.1 $\mu$mol m$^{-2}$ s$^{-1}$; 23-h-light, 1-h-dark cycle). For characterization of the maturation process, somatic embryos were collected every 5 days, flash-frozen in liquid nitrogen, and stored at −80°C until analysis of protein or RNA content. Alternatively, the embryos were fixed in glutaraldehyde immediately after sampling for microscopy.

### RT-PCR analysis

Total RNA was isolated from somatic embryos or developing soybean seeds at mid to late maturation stage with the use of a NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany). For synthesis of first-strand cDNA, 1 $\mu$g of total RNA was subjected to reverse transcription (RT) with an oligo(dT)$_{20}$ primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) in a total volume of 40 $\mu$l. The resulting cDNA (1 $\mu$l) was subjected to polymerase chain reaction (PCR) analysis with various sets of gene-specific primers (Table 1).

### Protein extraction

Proteins were extracted from frozen somatic embryos, cotyledons or hypocotyls excised from somatic embryos, or developing seeds at 4°C with 1 ml of protein extraction buffer [55 mM Tris-HCl (pH 8.0), 0.22% SDS, 5.5 M urea, 2.2% 2-mercaptoethanol, 2.75% (v/v) protease inhibitor cocktail for plants (Sigma-Aldrich, St. Louis, MO)] per 100 mg of fresh tissue weight. The mixtures were boiled for 5 min and then centrifuged at 17,800×g for 10 min at 4°C, and the supernatants were used for analysis. Protein concentration was estimated with the use of a Protein Assay kit (Bio-Rad, Richmond, CA), with bovine serum albumin (BSA) as a standard. For analysis of the protein composition of dry seeds, total protein was extracted from seed flour with SDS sample buffer [50 mM Tris-HCl (pH 8.0), 0.2% SDS, 5 M urea, 2% 2-mercaptoethanol] at a ratio of 1 ml per 100 mg. The mixture was centrifuged at 17,800×g for 10 min at room temperature, and the resulting supernatant was used for analysis. For analysis of $\alpha$-tonoplast intrinsic protein ($\alpha$-TIP) accumulation, total protein was extracted from the ground powder of lyophilized samples with

Table 1. Primers and conditions for RT-PCR.

| Gene | GenBank accession no. | Forward primer (5′→3′) | Reverse primer (5′→3′) | Annealing temperature (°C) | Expected product size (bp) |
|---|---|---|---|---|---|
| $\alpha$ | X17698 | CCAAACACAACAAGTGTCTCCAG | GGGAATGGGAATTGACGTTCATCTTG | 55 | 370 |
| $\alpha'$ | M13759 | CACAAGCAGGAAAAGCACCAAGGA | TCCCCTTGTTGCTGCCCC | 60 | 662 |
| $\beta$ | S44893 | GAGAATAACCCTTTCTACTTTAGAAGC | AAGTTTGATTATTTTGAGATTCTGGTGGTC | 55 | 357 |
| Gy1 | X15121 | GCCTCAACAACCTCAACAAA | ACCCTTGCACTGTGGCTTCT | 60 | 539 |
| Gy2 | X15122 | GCCGCAAGAATCTCAGCAAC | TTCTTGCTGTGGCTTCCTCA | 60 | 487 |
| Gy3 | X15123 | CACATTTGAAGAGCCTCAACAAAAAGGA | GCAATGTTTGTCTTTCTCGTCACAATC | 55 | 544 |
| Gy4 | X52863 | ACCCAGAAGACCTAGACAAG | GGACCCCAATTTCCTTCATA | 60 | 561 |
| Gy5 | AB003680 | GACCAAGCAGGCCCGAACAA | GCCGGAGTTTCCTTGATACT | 60 | 560 |
| Actin1 | J01298 | GACGCTGAGGATATTCAACC | AGAAATCTGTGAGGTCACGA | 50 | 565 |

The amplification protocol comprised 30 cycles of 94°C for 1 min, the annealing temperature shown for 1 min, and 72°C for 1 min.

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4% SDS sample buffer [50 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 2% 2-mercaptoethanol, 2% protease inhibitor cocktail for plants] at a ratio of 1 ml per 100 mg of dry weight. The mixture was centrifuged at 17,800×$g$ for 10 min at room temperature, and the resulting supernatant was used for analysis.

### SDS-PAGE and immunoblot analysis

Samples were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) on a 10.5% gel, which was then either stained with Coomassie brilliant blue or processed for immunoblot analysis. Immunoblot analysis was performed with rabbit polyclonal antisera to the $\alpha$ (unpublished), $\alpha'$ (Nishizawa et al. 2003), or $\beta$ (Mori et al., 2004) subunits of $\beta$-conglycinin, to glycinin (Teraishi et al. 2001), or to $\alpha$-TIP (Johnson et al. 1989). Immune complexes were detected with horseradish peroxidase–onjugated antibodies to rabbit immunoglobulin G (Cappel, West Chester, PA).

### Microscopy

Immature somatic embryos and cotyledons excised from mature somatic embryos were vacuum-infiltrated for 30 min with a fixative consisting of 1.5% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). They were then sectioned at a thickness of <1 mm, fixed for an additional 2 h in freshly prepared fixative, washed with phosphate buffer, dehydrated in a graded series of ethanol solutions, and embedded either in epoxy resin (Quetol-812; Nisshin EM, Tokyo, Japan) for transmission electron microscopy or in LR White (London Resin, Reading, UK) for light microscopy and immuno–electron microscopy. For light microscopy, sections (1 $\mu$m thickness) were stained with paragon stain. For transmission electron microscopy, ultrathin sections (50 nm thickness) were stained with 4% uranyl acetate and lead citrate. For immuno–electron microscopy, ultrathin sections were treated with blocking solution [5% BSA in phosphate-buffered saline (PBS)] for 30 min at room temperature and then incubated for 1 h at room temperature with antisera to the $\alpha'$ subunit of $\beta$-conglycinin (1 : 500) or to glycinin (1 : 1000) in diluting solution (1% BSA and 0.25% Tween 20 in PBS). The sections were then washed with diluting solution and incubated for 30 min at room temperature with colloidal gold (15 nm)–conjugated antibodies to rabbit immunoglobulin G (GE Healthcare, Piscataway, NJ) diluted 1 : 25 in diluting solution. For detection of $\alpha$-TIP, sections were blocked with 2% dried skim milk for 30 min at room temperature before incubation for 45 min on ice in a solution containing 25% antiserum to $\alpha$-TIP, 0.5% dried skim milk, and 50% (v/v) soybean leaf tissue ground in water (Melroy and Herman 1991). The sections were then washed consecutively with PBS containing 0.25% Tween 20 and with diluting solution before incubation for 30 min at room temperature with colloidal gold–conjugated secondary antibodies. All sections for immuno–electron microscopy were finally washed consecutively with PBS and distilled water, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (model H-7000; Hitachi, Tokyo, Japan).

## Results and discussion

### Histodifferentiation and maturation of somatic embryos

Somatic embryos organize on the adaxial surface of immature cotyledons of soybean (cv. Jack) during incubation on MSD40 medium for 4 weeks. They proliferate during subsequent incubation in FNL medium containing synthetic auxin. If the medium is maintained fresh, no further embryological maturation is apparent, regardless of the duration of culture (Finer and Nagasawa 1988; Samoylov et al. 1998). After transfer to histodifferentiation and maturation medium, FNL0S3S3 liquid medium, somatic embryos gradually differentiate, eventually attaining germination competency, and their morphology changes as shown in Figure 1. Globular somatic embryos grow to cotyledonary somatic embryos during the culture.

### Temporal regulation of storage protein expression during somatic embryo maturation

In developing soybean seeds, accumulation of the $\alpha$ and

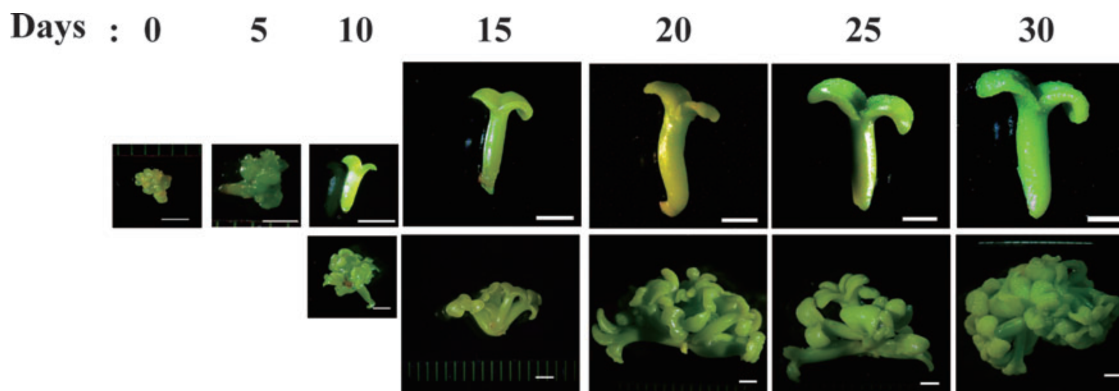


Figure 1. Morphological changes of somatic embryos during maturation in histodifferentiation and maturation medium. Somatic embryos were transferred to FNL0S3S3 medium and incubated for the indicated times. The panels for 0 and 5 days and the bottom panels for 10 to 30 days after induction of maturation show raspberry- or cauliflower-like clumps of somatic embryos. A single somatic embryo excised from each clump is shown in the top panels for 10 to 30 days. Scale bars, 2 mm.

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$\alpha'$ subunits of $\beta$-conglycinin as well as expression of the respective genes are apparent earlier than the corresponding changes for the $\beta$ subunit of $\beta$-conglycinin and glycinin (Meinke et al. 1981; Ladin et al. 1987). To compare the maturation process between somatic embryos and zygotic embryos, we first examined the expression profiles of the major storage proteins $\beta$-conglycinin and glycinin. RT-PCR analysis revealed that the mRNAs for the $\alpha$ and $\alpha'$ subunits of $\beta$-conglycinin were produced early during the maturation of somatic embryos, whereas those for the $\beta$ subunit of this protein and for the Gy1 to Gy5 subunits of glycinin were

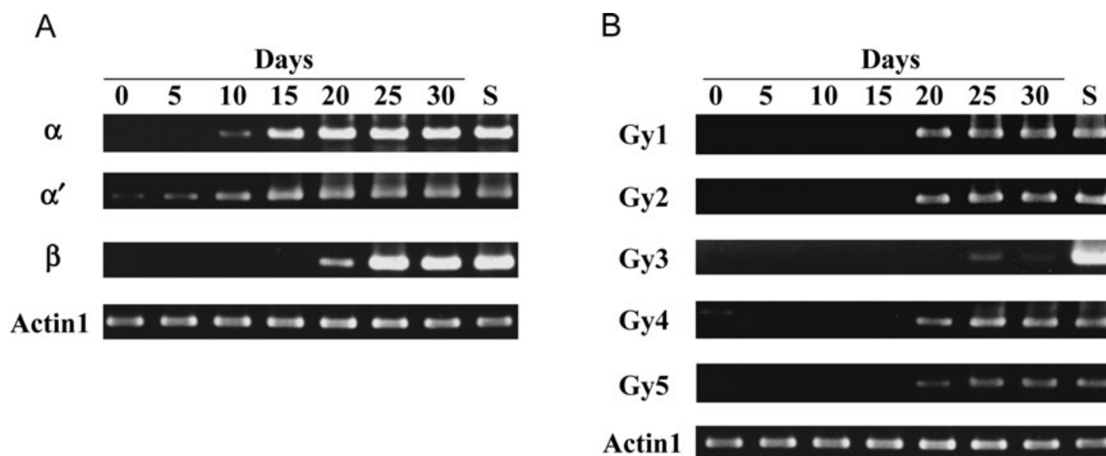


Figure 2. Expression of genes for seed storage proteins during somatic embryo maturation. The expression of the genes for subunits of $\beta$-conglycinin (A) and glycinin (B) was analyzed by RT-PCR at the indicated times after transfer of somatic embryos to FNL0S3S3 medium. Expression of the gene for Actin1 was examined as an internal control. The expression of each gene in developing soybean seeds (lanes S) was also examined.

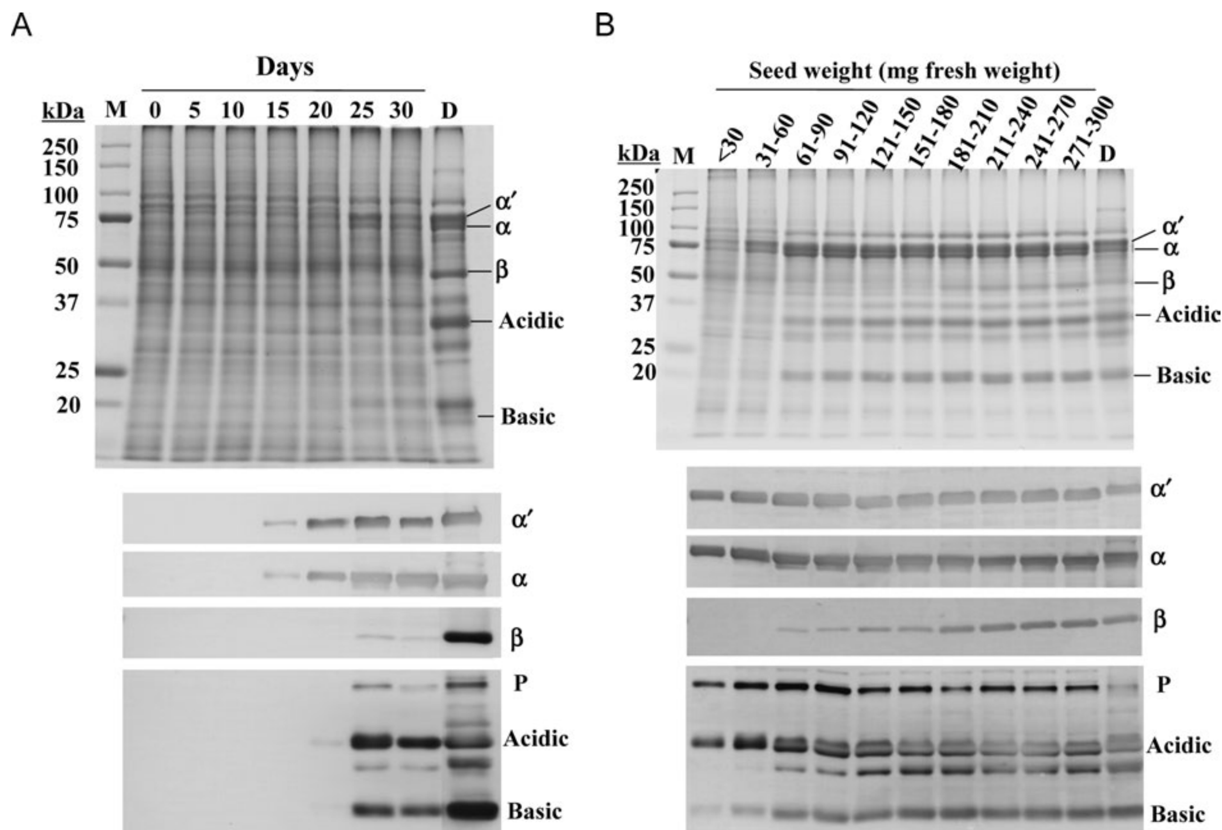


Figure 3. Accumulation of seed storage proteins during somatic embryo maturation. The accumulation of the major storage proteins was examined by SDS-PAGE (top panels) and immunoblot analysis (bottom panels) either at the indicated times after transfer of somatic embryos to FNL0S3S3 medium (A) or for developing seeds of the indicated fresh weight (B). Dry seeds were also examined (lanes D). All lanes in all gels were loaded with 16 $\mu$g of protein extract. The molecular mass of marker proteins (lanes M) is indicated on the left. The positions of $\alpha'$, $\alpha$, and $\beta$ subunits of $\beta$-conglycinin as well as of the proprotein (P) and acidic and basic chains of glycinin are indicated on the right of each panel.

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not apparent until 20 days after the induction of maturation (Figure 2). With the use of microarray analysis, Thibaud-Nissen et al. (2003) detected $\beta$-conglycinin and glycinin mRNAs in soybean somatic embryos at the globular stage, although this analysis was performed with somatic embryos not cultured in histodifferentiation and maturation medium, with maturation beginning automatically in association with the depletion of 2,4-dichlorophenoxyacetic acid. We have also now shown that the mRNA for the $\alpha'$ subunit of $\beta$-conglycinin is present in immature (globular) somatic embryos. In zygotic embryos, $\beta$-conglycinin genes are not expressed until the cotyledonary stage (Perez-Grau and Goldberg 1989). This discrepancy may represent a difference between somatic and zygotic embryos at the globular stage. However, mRNAs for the $\alpha$ and $\beta$ subunits of $\beta$-conglycinin and for all subunits of glycinin were not detected in somatic embryos until after cotyledon emergence in the present study, consistent with the developmental process in seeds.

SDS-PAGE and immunoblot analyses revealed that the overall profile of storage protein expression changed during the maturation of somatic embryos (Figure 3A). Both $\beta$-conglycinin and glycinin were detected in developing embryos, with accumulation of the $\alpha$ and $\alpha'$ subunits of $\beta$-conglycinin beginning earlier than that of the $\beta$ subunit of $\beta$-conglycinin and glycinin, as is the case in developing seeds (Figure 3B). The levels of seed storage proteins in somatic embryos were previously found to be constant during maturation, with the $\beta$ subunit of $\beta$-conglycinin (Dahmer et al. 1992) or glycinin (Stejskal and Griga 1995) not being detected even in mature somatic embryos. However, in the present study, storage protein composition of somatic embryos and the amounts of these proteins changed during maturation. Expression of storage protein genes and accumulation of the encoded proteins, characteristic events of seed development, thus occur in developing somatic embryos, with the relative time courses of these events resembling those apparent in developing seeds.

### *Spatial regulation of storage protein expression during maturation of somatic embryos*

We next examined the distribution of storage proteins

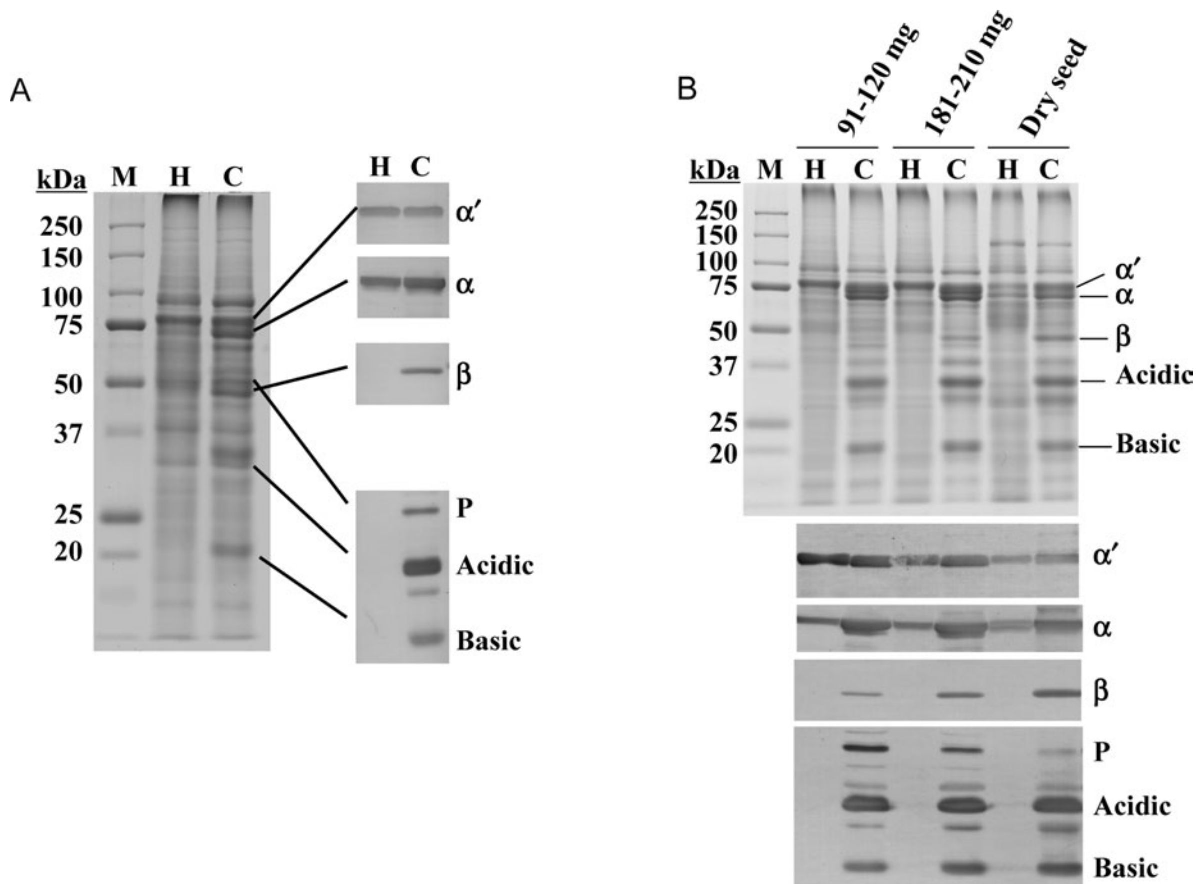


Figure 4. Tissue specificity of the accumulation of storage proteins in somatic embryos. The accumulation of storage proteins in the hypocotyl (lanes H) and cotyledons (lanes C) both of mature somatic embryos 25 days after the induction of maturation (A) and of seeds of the indicated fresh weights or dry seeds (B) was examined by SDS-PAGE [left panel in (A), upper panel in (B)] and by immunoblot analysis with antisera specific for the indicated subunits of $\beta$-conglycinin or for glycinin [right panels in (A), lower panels in (B)]. All lanes were loaded with 16 $\mu$g of protein extract. The molecular mass of marker proteins (lanes M) is indicated on the left. The positions of $\alpha'$, $\alpha$, and $\beta$ subunits of $\beta$-conglycinin as well as of the proprotein (P) and acidic and basic chains of glycinin are indicated on the right.

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among the cotyledons and hypocotyl of mature somatic embryos (day 25). The $\alpha$ and $\alpha'$ subunits of $\beta$-conglycinin were detected not only in cotyledons but also in the hypocotyl, whereas the $\beta$ subunit of $\beta$-conglycinin and glycinin were detected only in cotyledons (Figure 4A). Such spatial regulation of the accumulation of seed storage proteins is also observed in the cotyledons and hypocotyl during seed development (Figure 4B) (Meinke et al. 1981; Ladin et al. 1987). These results thus suggested that maturation of somatic embryos is regulated in a manner similar to that of seed development with regard to the tissue specificity of storage protein accumulation.

### *Changes in cellular organization during maturation of somatic embryos*

Marked changes in cell morphology accompany the development of soybean seeds as well as the seeds of other plant species. In the early phase of seed development, cells of soybean cotyledons show characteristics of undifferentiated plant cells, including the presence of a single large vacuole. Concomitant with seed development, multiple protein storage vacuoles (PSVs) and oil bodies form within the cells (Melroy and Herman 1991; Mori et al. 2004). The PSVs store various proteins including $\beta$-conglycinin and glycinin, and they are distinct from other types of vacuole, such as lytic vacuoles, that are largely restricted to vegetative organs (Neuhaus and Rogers 1998). $\alpha$–Tonoplast intrinsic protein ($\alpha$-TIP) is a specific aquaporin of PSVs and accumulates during seed development (Melroy and Herman 1991; Neuhaus and Rogers 1998).

We examined the morphology of cells in soybean somatic embryos by light and electron microscopy. In immature somatic embryos, each cell was found to contain one large vacuole but no oil bodies (Figure 5A). In contrast, in cotyledons of mature somatic embryos, there were oil bodies (Figure 6B, arrows) and multiple vacuoles containing electron-dense aggregates (Figure 5B, Figure 6B-D). Immunoblot analysis revealed the presence of $\alpha$-TIP in mature somatic embryos as well as in seeds, although it was virtually undetectable in immature somatic embryos as well as in leaves (Figure 6A). In addition, $\alpha$-TIP was detected in the tonoplast of vacuoles in cotyledons of mature somatic embryos by immuno–electron microscopy (Figure 6B), indicating that these vacuoles are indeed PSVs. Furthermore, antibodies to $\alpha'$ subunit of $\beta$-conglycinin and glycinin specifically bound to the electron-dense aggregates in the PSVs, indicating $\beta$-conglycinin and glycinin accumulate in the PSVs of somatic embryos (Figure 6C, D). The cellular changes that accompany the maturation of somatic embryos thus resemble those associated with seed development. However, the electron density of PSVs, which reflects the accumulation of storage proteins, was not as pronounced in mature somatic embryos at day 25 (Figure 5B, Figure 6B–D) as in seeds near maturity (Melroy and Herman 1991; Mori et al. 2004), and it did not substantially increase further during additional incubation of the somatic embryos (data not shown). These findings are consistent with the results of our SDS-PAGE analysis (Figure 3, Figure 4) showing

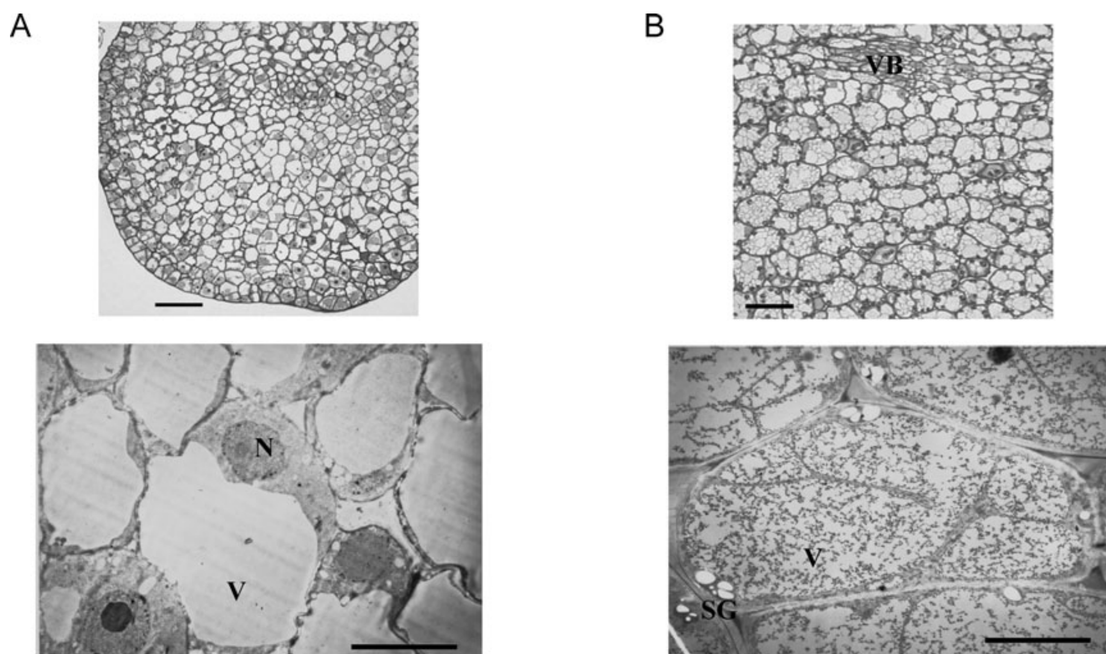


Figure 5. Morphological changes of cells during somatic embryo maturation. Immature somatic embryos (A) and cotyledons of mature somatic embryos (B) obtained 5 and 25 days after induction of maturation, respectively, were examined by light microscopy (top panels) and electron microscopy (bottom panels). N, nucleus; SG, starch granule; V, vacuole; VB, vascular bundle. Bars, 50 $\mu$m (top panels) or 10 $\mu$m (bottom panels).

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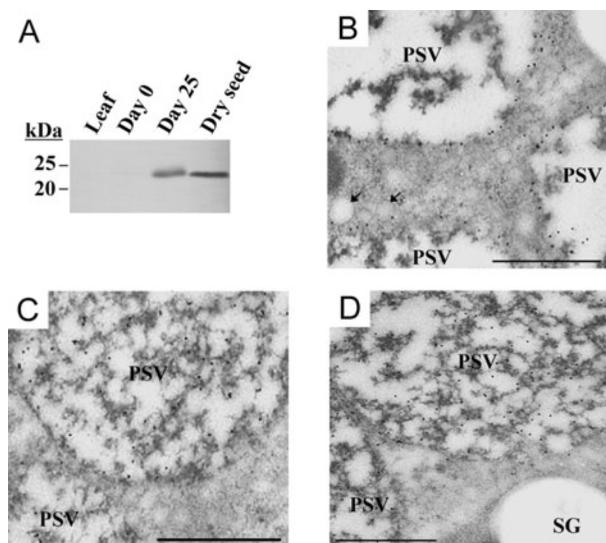


Figure 6. Accumulation of seed storage proteins in the PSVs of mature somatic embryos. (A) Immunoblot analysis of $\alpha$-TIP in immature (day 0) and mature (day 25 after the induction of maturation) somatic embryos. As negative and positive controls, the accumulation of $\alpha$-TIP in leaves and dry seeds, respectively, was also examined. All lanes were loaded with 20 $\mu$g of protein extract. (B–D) Immuno–electron microscopic localization of $\alpha$-TIP (B) in the tonoplast as well as that of the $\alpha'$ subunit of $\beta$-conglycinin (C) and glycinin (D) in PSVs of cotyledons of mature somatic embryos (25 days after induction of maturation). Arrows, oil bodies; SG, starch granule. Bars, 1 $\mu$m.

that the protein profiles of mature somatic embryos (days 25 and 30) corresponded to those of developing seeds (91–150 mg) rather than to those of mature dry seeds. The similarity of somatic embryos to seeds may thus be lost at the late stages of maturation. However, transcriptome and proteome analyses of developing soybean seeds have indicated that many, but not all, genes involved in the production of seed components, including storage reserves and secondary metabolites, are expressed from an early stage of seed development (Hajduch et al. 2005; Dhaubhadel et al. 2007). Transcripts of such genes were also detected by microarray analysis of somatic embryos (Thibaud-Nissen et al. 2003). Soybean somatic embryos therefore appear to be a suitable experimental model with which to study the synthesis of seed components.

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