Plant Biotechnology Journal (2004) **2**, pp. 199–210 doi: 10.1111/j.1467-7652.2004.00063.x

Expression of an 11 kDa methionine-rich delta-zein in transgenic soybean results in the formation of two types of novel protein bodies in transitional cells situated between the vascular tissue and storage parenchyma cells

Won-Seok Kim[1] and Hari B. Krishnan[2,*]

[1]*Department of Agronomy, University of Missouri, Columbia, MO 65211, USA*
[2]*Plant Genetics Research Unit, United States Department of Agriculture/Agricultural Research Service, 108W Curtis Hall, University of Missouri, Columbia, MO 65211, USA*

Received 6 August 2003;
revised 27 October 2003;
accepted 18 November 2003.
**Correspondence* (fax 573-884-7850;
e-mail KrishnanH@missouri.edu)

Summary

Soybean (*Glycine max* (L.) Merr.) is an important protein source in human diets and animal feeds. The sulphur content of soybean seed proteins, however, is not optimal for ration formulations. Thus, increasing the methionine and cysteine content of soybean seed proteins would enhance the nutritional quality of this widely utilized legume. We have earlier reported the isolation of an 11 kDa δ-zein protein rich in methionine from the endosperm of the maize (*Zea mays* L.) inbred line W23a1 [Kim, W.-S. and Krishnan, H.B. (2003) Allelic variation and differential expression of methionine-rich-δ-zeins in maize inbred lines B73 and W23a1. *Planta*, **217**, 66–74]. Using *Agrobacterium*-mediated transformation, a construct consisting of the coding region of the cloned δ-zein gene under regulation of the β-conglycinin α′-promoter was introduced into the soybean genome. The 11 kDa δ-zein gene was expressed in the seeds of transgenic soybeans, although low-level expression was also detected in the leaves. *In situ* hybridization indicated that the 11 kDa δ-zein mRNA was expressed predominantly in transitional cells located between the vascular tissue and storage parenchyma cells. Immunohistochemistry of developing transgenic soybeans revealed that the accumulation of the 11 kDa δ-zein occurred primarily in these transitional cells. Expression of the 11 kDa δ-zein gene in transgenic soybean resulted in the formation of two endoplasmic reticulum-derived protein bodies that were designated as either spherical or complex. Immunocytochemical localization demonstrated that both the spherical and complex protein bodies accumulated the 11 kDa δ-zein. Although expression of the 11 kDa δ-zein gene elevated the methionine content of the alcohol-soluble protein fraction 1.5–1.7-fold above that of the non-transgenic line, the overall methionine content of seed flour was not increased. Our results suggest that the confined expression of the 11 kDa δ-zein gene in transitional cells could be limiting the increase in methionine content in transgenic soybean seeds.

Keywords: methionine, nutritional quality, protein bodies, soybean, zein.

Introduction

Seeds synthesize and accumulate macromolecules, such as oils, starch and proteins, which are utilized during germination and early plant development. These compounds are an important nutritional resource for both humans and animals. A preponderance of cultivated crops produce seed which is deficient, with respect to monogastric diets and rations, in one or more of the essential amino acids. Cereal seeds are deficient in lysine, tryptophan and threonine, whereas legume seeds contain inadequate amounts of methionine and cysteine (Shewry *et al.*, 1995). The optimal growth and development of humans and animals, consuming a grain-based diet, requires supplementation with synthetic essential amino acids.

Soybean is an important legume, providing 67% of the protein meal and 31% of the vegetable oil consumed worldwide (www.unitedsoybean.org). Glycinin (11S) and β-conglycinin (7S) account for 70% of the total seed protein of soybean (Nielsen, 1996; Krishnan, 2000). Although glycinins are relatively rich in methionine, the companion storage protein, β-conglycinin, is essentially devoid of this amino acid (Nielsen, 1985). The overall content of methionine and cysteine in soybean seed protein is not optimal for the formulation of poultry or swine rations. To circumvent this deficiency, the animal industry supplements the soybean-based rations with synthetic methionine, at an estimated cost of 100 million dollars annually (Imsande, 2001).

Molecular techniques have been employed to improve the concentration of sulphur-containing amino acids in both legume and non-legume plant seed proteins. One widely used approach has been to express the sulphur-rich 2S albumin gene from Brazil nut (*Bertholletia excelsa*) or sunflower (*Helianthus annuus*) in target plants. The 2S albumin has been successfully expressed in *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*) and canola (*Brassica napus*), with a concomitant increase in the methionine contents in transgenic plants (Clercq *et al*., 1990; Altenbach *et al*., 1992; Tu *et al*., 1998). A similar approach has also been used to improve the sulphur-containing amino acid content of legume crops (Müntz *et al*., 1998). Saalbach *et al*. (1995) have successfully expressed the sulphur-rich 2S albumin in narbon bean (*Vicia narbonensis*). The 2S albumin accumulated to approximately 5% of the total seed protein in transgenic plants, resulting in a two-fold increase in the methionine content of seed proteins (Müntz *et al*., 1998). Using 2S albumin gene from sunflower, Higgins and associates have engineered lupin (*Lupinus angustifolius*) that contains twice the methionine content when compared with non-transgenic lupin (Molvig *et al*., 1997). This increase in methionine enhanced the nutritive value of transgenic lupin seeds for rats and sheep (Molvig *et al*., 1997; White *et al*., 2000). However, the methionine and cysteine content of transgenic lupin was still not optimal for animal nutrition (Tabe and Droux, 2002).

In an attempt to increase the sulphur-containing amino acid content of soybean seeds, 2S albumin from Brazil nut was expressed in soybeans (Townsend and Thomas, 1994). In one transgenic line, the 2S albumin accounted for 10% of the total seed protein. This expression level was calculated to double the methionine content in transgenic plants, but an increase of only 40–50% was observed (Townsend and Thomas, 1994). It was also noted that these transgenic soybeans accumulated lower amounts of protease inhibitors (Beach *et al*., 1995), which are rich in sulphur-containing amino acids. As the 2S Brazil nut albumin has been identified as a potential allergen (Nordlee *et al*., 1996), its use to enhance the methionine content of legume seed proteins is compromised (Streit *et al*., 2001). A similar approach was used to express a 15 kDa zein in soybean, which resulted in a 12–20% increase in methionine content (Dinkins *et al*., 2001). These studies demonstrate the feasibility of increasing the methionine content of soybean by the expression of heterologous seed proteins rich in methionine.

The β-conglycinin α′-subunit promoter is tissue-specific and is temporally regulated (Beachy *et al*., 1985; Chen *et al*., 1989; Chamberland *et al*., 1992). It is expressed in both the axis and cotyledons of developing soybean seeds (Ladin *et al*., 1987). The β-conglycinin α′-subunit has been expressed in transgenic plants and was reported to be regulated in a similar manner as in soybean (Chen *et al*., 1989; Nishizawa *et al*., 2003). Expression was detected in transgenic seeds during mid to late stages of seed development (Chen *et al*., 1989). However, the expression pattern of this promoter in different cell types within the seed has not been investigated. Regulatory information for the tissue-specific expression of the β-conglycinin α′-subunit has been localized to DNA sequences approximately 250 bp upstream of the transcription start site (Allen *et al*., 1989; Chen *et al*., 1989). In this study, the gene encoding the methionine-rich 11 kDa δ-zein was placed under the control of the β-conglycinin α′-subunit promoter and introduced into the soybean genome with the objective of improving the sulphur-containing amino acid content of soybean seed protein. Seed-specific expression of the 11 kDa δ-zein in transgenic soybeans resulted in a 1.5–1.7-fold increase in the methionine content of the alcohol-soluble protein fraction. The 11 kDa δ-zein accumulated in two novel endoplasmic reticulum (ER)-derived protein bodies in transitional cells located between the vascular tissue and storage parenchyma cells. The accumulation of the 11 kDa δ-zein in these unique storage bodies suggests a putative method of producing and sequestering transgenic proteins.

Results

Expression and accumulation of 11 kDa δ-zein in transgenic soybean

A gene encoding a novel 11 kDa δ-zein protein has been cloned from the maize inbred line W23a1 (Kim and Krishnan, 2003). Methionyl and cysteinyl residues combined account for 25% of the total amino acids of this protein. A plant transformation vector was constructed in which the coding

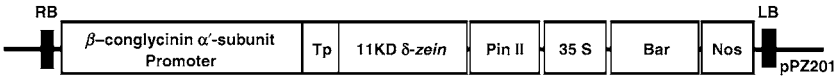

Figure 1 Schematic diagram of pZα′11hsp used for transformation of soybean. The construct contains the 11 kDa δ-zein coding region under the control of the soybean β-conglycinin α′-promoter and the 3′ region of the potato proteinase inhibitor gene (*Pin* II), together with a gene expression cassette that includes the cauliflower mosaic virus 35S promoter, the *bar*-coding region and the 3′ region of the nopaline synthase gene (*nos*). Tp, soybean β-conglycinin β-subunit transit peptide.

region of the 11 kDa δ-zein gene was placed under the control of the soybean β-conglycinin α′-promoter (Schuler *et al*., 1982) and potato protease inhibitor II terminator sequences. This construct was ligated into the expression cassette containing the phosphinothricin acetyl transferase gene (*bar*) under control of the 35S promoter and nopaline synthase terminator. The resulting plasmid was designated pZα′11hsp (Figure 1). Six independent transgenic lines (A4, A8, A9, A11, A13 and A14) of soybean, expressing the 11 kDa δ-zein gene, were obtained by *Agrobacterium tumefaciens*-mediated transformation (Hinchee *et al*., 1988; Zhang *et al*., 1999). Expression of the 11 kDa δ-zein gene in these transgenic soybean lines was verified by Northern blot analysis. When nylon filters containing total RNA from developing soybean seeds were probed with the α^{32}P-labelled coding region of the 11 kDa δ-zein gene, hybridization signals were detected in each transgenic line (Figure 2). The probe hybridized to a 350 bp transcript, which approximates the transcript size observed in W23a1 developing maize seeds (Figure 2). The 11 kDa δ-zein gene probe was specific to the transgenic soybean lines and did not detect transcripts in RNA extracts from non-transformed soybean plants. Accumulation of the transgenic protein was examined by Western blot analysis (Figure 3A). Antibodies raised against purified maize δ-zein (Kim and Krishnan, 2003) reacted specifically to a low-molecular-weight protein from the transgenic soybean seed extracts. Although the 11 kDa δ-zein was observed in all six transgenic lines, there was considerable variation in the accumulation of

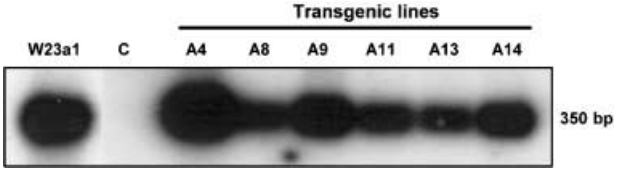

Figure 2 Expression of the 11 kDa δ-zein in transgenic soybean lines. Total RNA (5 μg) from developing (35–50 days after flowering) transgenic soybean seeds was fractionated on a denaturating formaldehyde-agarose gel and transferred to a Hybond-N+ membrane. The blot was probed with the 11 kDa δ-zein coding region. Total RNA from developing maize (W23a1) seeds was also included in the blot. The probe hybridized to a 0.35 kB RNA transcript in all the tested transgenic soybean lines. Note the absence of RNA transcript in the non-transgenic control (C).

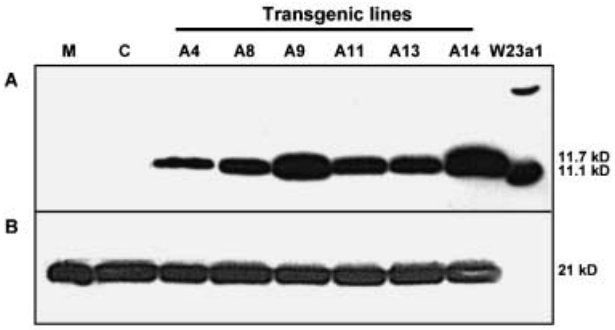

Figure 3 Accumulation of 11 kDa δ-zein protein in transgenic soybean lines. Total seed proteins from developing (35–50 days after flowering) soybean seeds from six independent transgenic lines were fractionated in duplicate gels by 15% sodium dodecylsulphate polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. The membrane was probed with the maize δ-zein antibodies (panel A) or soybean Kunitz trypsin inhibitor antibodies (panel B). Note the variation in the accumulation of 11 kDa δ-zein among the different transgenic lines, in contrast with the uniform accumulation of the Kunitz trypsin inhibitor.

the protein. The δ-zein antibodies did not recognize any seed proteins extracted from control soybean plants. An earlier study has shown that the expression of methionine-rich 2S albumin from Brazil nut resulted in the reduction of Kunitz trypsin inhibitor (Beach *et al*., 1995). Western blot analysis revealed that the accumulation of the inhibitor was not repressed in these transgenic soybean lines (Figure 3B).

11 kDa δ-zein in transgenic soybean shows organ-specific expression

The organ-specific expression of the 11 kDa δ-zein gene was examined by Northern blot analysis using total RNA isolated from different organs of transgenic soybean plants (Figure 4). A strong signal was detected in developing seeds, indicating that the 11 kDa δ-zein gene is expressed predominantly in the soybean seeds (Figure 4). Although the 11 kDa δ-zein mRNA was not detected in roots, stem, hypocotyl or flower, a faint signal was detected in leaf tissue (Figure 4). To determine whether the transgenic soybean leaves accumulated the 11 kDa δ-zein, Western blot analysis was performed on total leaf protein. Enzyme-linked detection using secondary antibodies conjugated

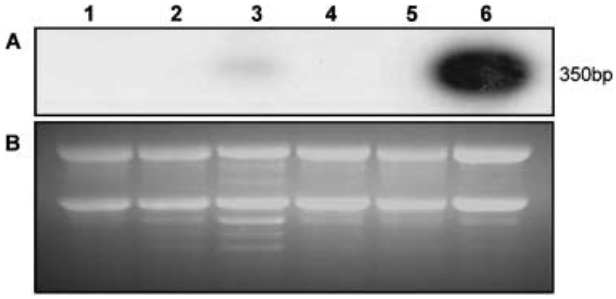

Figure 4 Organ-specific expression of 11 kDa δ-zein in transgenic soybeans. Total RNA (5 μg) from roots (lane 1), hypocotyls (lane 2), leaves (lane 3), stems (lane 4), flower (lane 5) and seeds (lane 6) was subjected to Northern blot analysis. The coding region of the 11 kDa δ-zein was used as probe. Note the weak hybridization signal in the leaf samples (lane 3) and that the size of the RNA transcript is similar to that detected in the seed (lane 6). The ethidium bromide-stained gel picture shows uniform loading of RNA samples (panel B).

to horseradish peroxidase (HRP) failed to detect the accumulation of the 11 kDa δ-zein in the leaves of the transgenic soybean plant. The more sensitive chemiluminescent system (Pierce, Rockford, IL, USA) detected the 11 kDa δ-zein in leaves, albeit at extremely low levels (data not shown).

11 kDa δ-zein in transgenic soybean retains its alcohol solubility

A common feature of zeins is that they are soluble in aqueous alcohol (Esen, 1986). To ascertain whether the 11 kDa δ-zein expressed in transgenic soybeans retained alcohol solubility, seed proteins were separated into salt-soluble globulin, alcohol-soluble prolamin and total seed protein fractions. These fractions were resolved by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to Western blot analysis (Figure 5). The δ-zein antibodies recognized 11 kDa and 18 kDa polypeptides from the total protein and prolamin fraction, respectively, of W23a1 maize seeds. The antibody also detected an 11 kDa polypeptide from the total protein and prolamin fraction from the transgenic soybean seed extract (Figure 5). Antibodies failed to detect the δ-zein in the globulin fraction isolated from either maize or transgenic soybean, demonstrating that the 11 kDa δ-zein fractionates similarly in seed extracts from maize and transgenic soybean plants.

Western blot analysis of dry seed flour from six different transgenic events indicated that two lines, A11 and A13, accumulated a higher amount of δ-zein. Amino acid analysis of the alcohol-extractable protein fraction from these two transgenic lines revealed 1.5–1.7 times more methionine in transgenic lines than in non-transformed plants (Table 1). To ascertain whether the increased methionine content of the prolamin fraction was also reflected in the total seed flour, we determined the amino acid profile of the whole soybean seed. The methionine contents in the seed flour of transgenic line A11 and control soybean were 2.02 ± 0.06% and 2.03 ± 0.02%, respectively, indicating that there was no significant difference in methionine content.

11 kDa δ-zein is processed accurately in transgenic soybean

The construct used for the transformation of soybean contained the 26 amino acid signal sequence of the β-conglycinin β-subunit coupled to the coding region of the mature

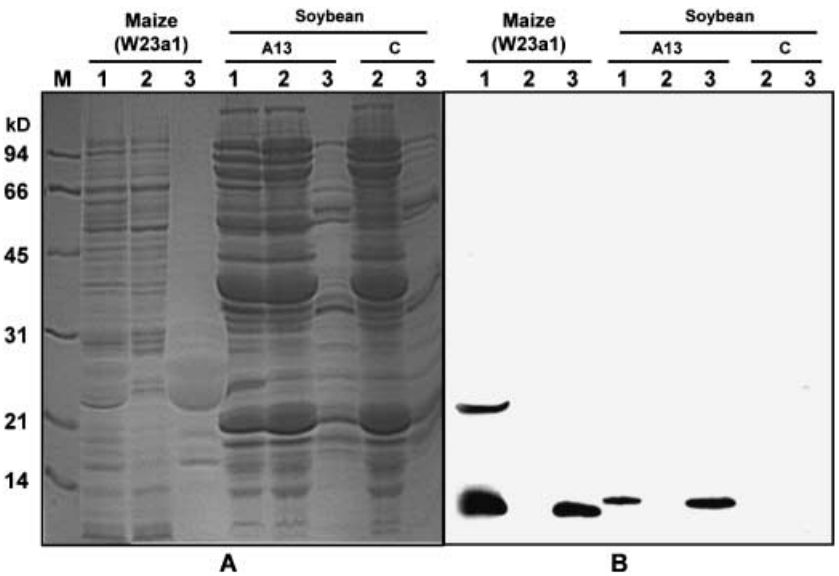

Figure 5 The 11 kDa δ-zein in transgenic soybean is soluble in aqueous alcohol. Total, salt-soluble and alcohol-soluble protein fractions isolated from mature dried seeds of maize, transgenic soybean line A13 and non-transgenic soybean control were fractionated by sodium dodecylsulphate polyacrylamide gel electrophoresis and either stained with Coomassie Brilliant Blue (panel A) or transferred to a nitrocellulose membrane for immunodetection analysis with δ-zein-specific antibodies (panel B). The 11 kDa δ-zein detected in the transgenic soybean seeds is slightly larger than the protein detected in the maize W23a1 inbred line. Lane 1, total protein; lane 2, salt-soluble fraction; lane 3, alcohol-soluble fraction. Note that the 11 kDa δ-zein protein is absent in the salt-soluble fraction. The sizes of the molecular weight markers in kDa are also shown.

Table 1 Amino acid composition of isopropanol-extracted proteins from soybean. Data are the means of three different preparations of prolamin fractions

Amino acid	Control	A11*	A13*
Aspartic acid	14.40a	13.56a	13.64a
Threonine	8.00a	7.75a	7.85a
Glutamic acid	19.17ab	18.48b	20.25a
Proline	6.62a	7.04a	6.86a
Glycine	6.29a	5.96b	6.31a
Alanine	7.40a	7.28a	7.56a
Cysteine	5.41a	5.22a	4.30b
Valine	5.26b	5.46b	5.92a
Methionine	2.52c	4.36a	3.74b
Isoleucine	4.36b	4.48ab	4.69a
Leucine	8.99b	9.77a	9.55a

Means showing the same letter within each row are not significantly different at $P \leq 0.05$.

*Transgenic soybean lines expressing 11 kDa δ-zein.

11 kDa δ-zein (Figure 1). During the cloning process, an *Nde*I site was inserted between the signal sequence and the 11 kDa δ-zein coding region, which added two amino acid residues to the mature 11 kDa δ-zein protein. Immunoblot analysis showed that the molecular weight of δ-zein expressed in transgenic soybean was slightly larger than that of the protein expressed in maize (Figure 5). A comparison of the NH_2-terminal amino acid sequence deduced from cDNA sequences with that of the mature β-subunit of β-conglycinin revealed that the NH_2-terminal signal sequence is cleaved after a serine residue (Figure 6; Tierney *et al*., 1987). To examine the precise cleavage site, the NH_2-terminal sequence of the 11 kDa δ-zein expressed in the transgenic soybean was determined. The first seven amino acid residues of the 11 kDa δ-zein were determined to be LKVHMTH (Figure 6). This observation indicates that 11 kDa δ-zein was processed correctly in transgenic soybean and results in the synthesis of an 11 kDa δ-zein containing a lysine residue, which is not normally found in the zeins.

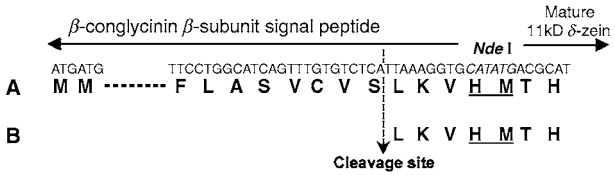

Figure 6 The 11 kDa δ-zein protein is correctly processed in the transgenic soybean. The nucleotide sequence and the deduced amino acid sequence near the junction of the NH_2-terminal signal sequence and the coding region of the 11 kDa δ-zein are shown (panel A). Creation of an *Nde*I site (underlined) at the junction region resulted in the addition of two amino acid residues to the mature protein. The signal sequence cleavage site is shown with an arrow. The NH_2-terminal sequence of the purified 11 kDa zein protein from dried mature transgenic soybean seeds is also shown (panel B).

Expression of 11 kDa δ-zein mRNA is restricted to specific cell types in transgenic soybean

The distribution of the 11 kDa δ-zein mRNA in developing transgenic soybean seeds was determined by *in situ* hybridization. Thin slices of soybean seed hybridized with ^{35}S-labelled antisense probe and viewed under bright-field microscopy revealed that the 11 kDa δ-zein mRNA was not evenly distributed in the cotyledon (Figure 7). The hybridization signal was primarily found in cells proximal to the vascular bundles. The position of the hybridizing cells in the cotyledons resembles that of paraveinal mesophyll (PVM) cells that occur in soybean leaves (Fisher, 1967; Franceschi and Giaquinta, 1983). When viewed by dark-field microscopy, the white/silver grains in the autoradiogram were clearly seen in cells neighbouring the vascular strand (Figure 7). Thin sections of soybean seeds hybridized with sense-strand probe gave no specific hybridization signal (Figure 7). A strong signal was seen at the hilum with both the sense-strand and antisense probe, indicating that this hybridization signal was non-specific (Figure 7).

Expression of 11 kDa δ-zein results in the formation of novel protein bodies

As the distribution of the 11 kDa δ-zein mRNA is restricted to specific cells in the cotyledons of the transgenic soybeans, we examined whether the accumulation of the protein also exhibited a similar spatial distribution. Immunohistochemical staining of paraffin-embedded soybean seeds indicated that the δ-zein was predominantly localized in cells surrounding the vascular bundle (Figure 8). This distribution pattern was similar to the 11 kDa δ-zein mRNA expression pattern (Figure 7). Thin sections incubated with preimmune serum revealed no reaction with the proteins accumulating in these cells (Figure 8). In developing maize seeds, different classes of zeins are deposited into ER-derived protein bodies (Herman and Larkins, 1999) and are localized in a specific manner within the protein bodies (Lending *et al*., 1988). The α- and δ-zeins form the core of the protein bodies, while the β- and γ-zeins occur at the periphery (Lending and Larkins, 1989). Soybeans accumulate storage proteins within specialized protein storage vacuoles (PSVs) (Chrispeels and Herman, 2000). ER-derived protein bodies are not known to occur in developing soybean seed. To determine if the 11 kDa δ-zein expressed in transgenic soybean accumulated in ER-derived

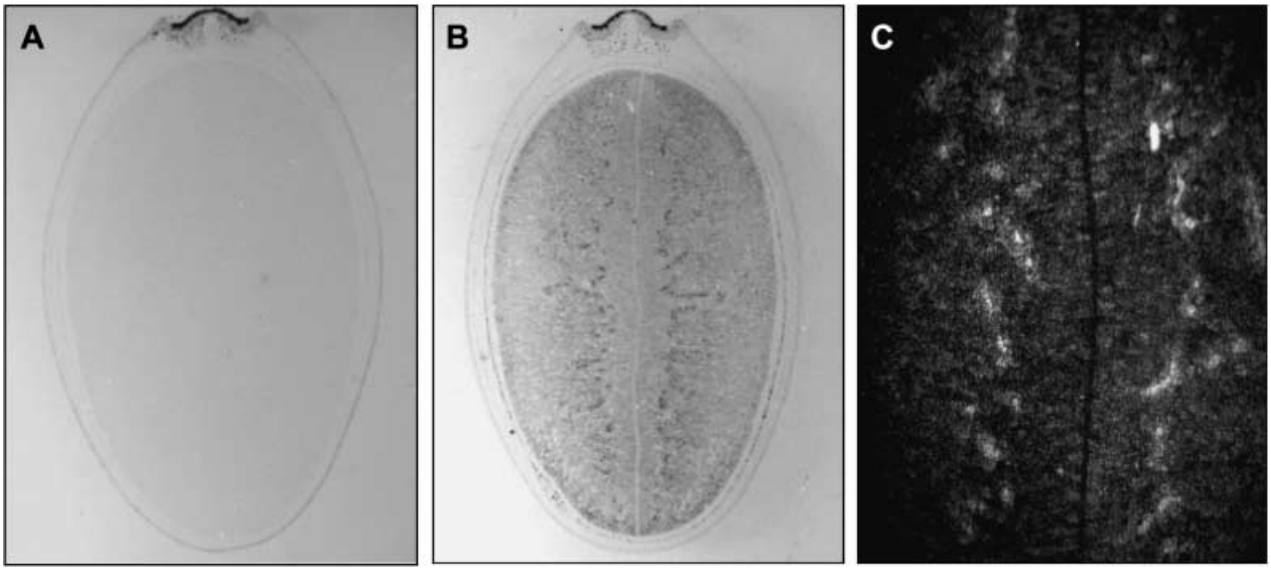

Figure 7 Spatial distribution of 11 kDa δ-zein mRNA in transgenic soybean seeds. Light micrographs of paraffin-embedded developing (35 days after flowering) soybean seed sections hybridized with 11 kDa δ-zein sense strand RNA viewed by bright-field microscope (panel A) or antisense strand viewed by bright-field (panel B) and dark-field (panel C) microscope. Note that the silver grains predominantly occur in cells near the vascular tissue (panel C).

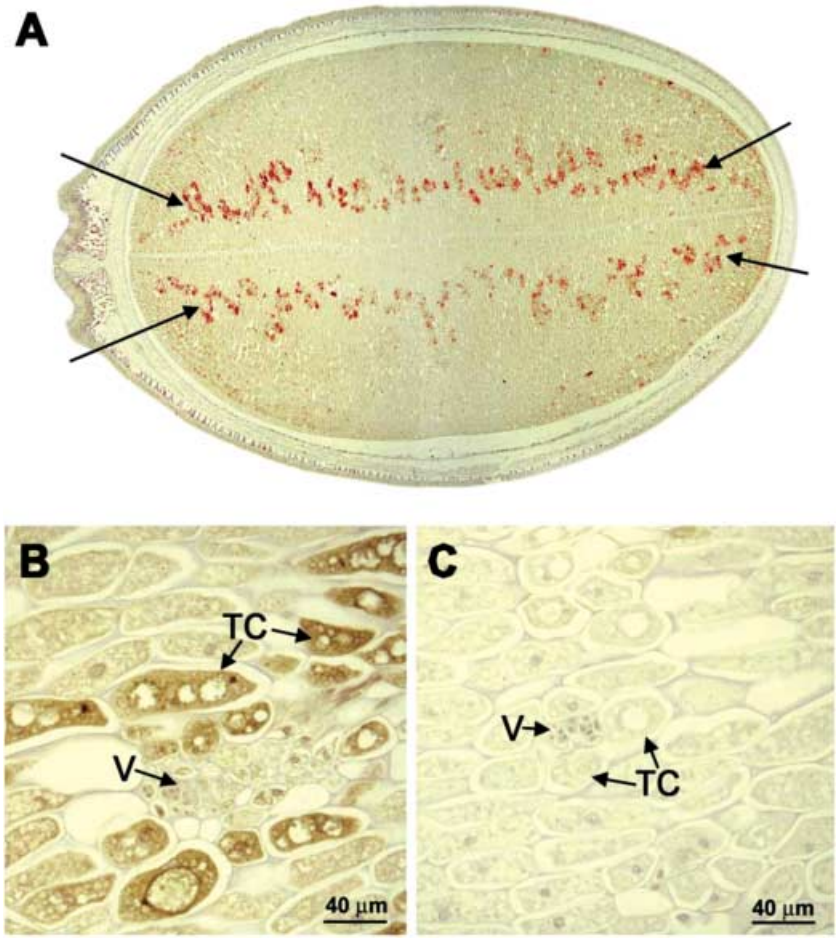

Figure 8 Immunocytochemical analysis of the spatial distribution of the 11 kDa δ-zein protein in developing (35 days after flowering) transgenic soybean seeds. Photomicrographs of a longitudinal section of a transgenic soybean seed immunostained with maize δ-zein antibody and counter-stained with haematoxylin. Note the accumulation of the 11 kDa δ-zein protein in cells around the vascular tissue (panels A and B). Sections treated with preimmune serum reveal no immunostaining in the cells around the vascular tissue (panel C). The arrows in panel A denote the transitional cell layer. TC, transitional cell; V, vascular bundle.

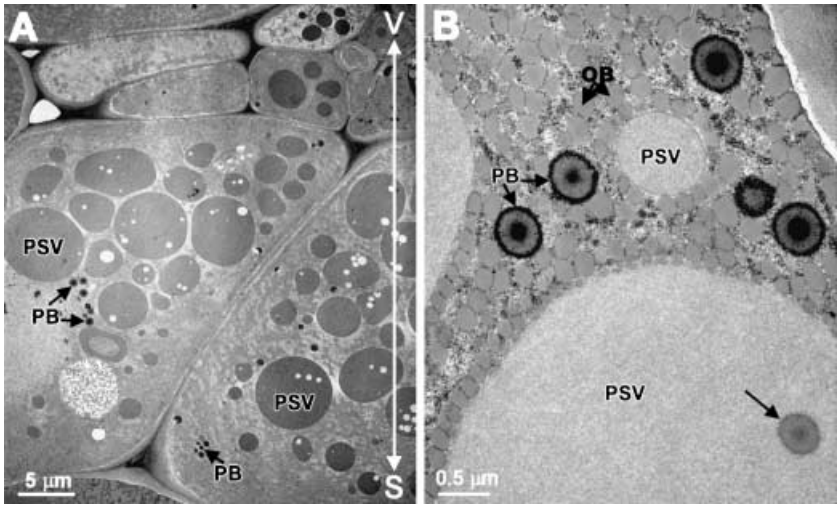

Figure 9 Novel protein bodies in transgenic soybean seeds. Low magnification view of a transgenic soybean cotyledon (35 days after flowering) reveals the presence of dark-staining spherical protein bodies in transitional cells (panel A). The long arrow indicates the location of the vascular cells (V) and the storage parenchyma cells (S). The endoplasmic reticulum-derived spherical protein bodies exhibit a dark-staining central core and a light-staining periphery (panel B). Note the presence of a spherical protein body within the protein storage vacuole (PSV). OB, oil bodies; PB, protein body.

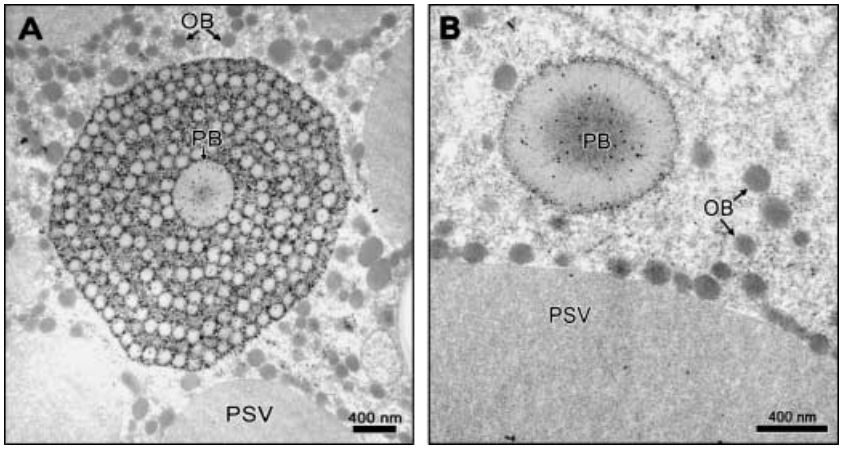

Figure 10 Immunogold localization of the 11 kDa δ-zein in complex (panel A) and spherical (panel B) protein bodies. The complex protein body contains a centrally located protein body that is surrounded by small protein vesicles that are arranged in concentric layers (panel A). Thin sections of transgenic soybean were labelled with δ-zein antibodies and protein A-gold particles. Note the specific deposition of gold particles on both types of protein bodies. OB, oil bodies; PB, protein body; PSV, protein storage vacuole.

protein bodies or PSVs, we conducted ultrastructural analysis of transgenic soybean seeds. Low magnification electron microscopy of thin sections of transgenic soybean seeds revealed that storage parenchyma cells were the predominant cell type in the cotyledons. Cells representing vascular tissue were also seen. Cells near the vascular tissue were smaller than storage parenchyma cells (Figure 9A). These types of cells have been observed in jack-bean cotyledons and were designated transitional cells (Herman and Shannon, 1984). Transitional cells in transgenic soybean seeds contained both PSVs and novel electron-dense spherical protein bodies (Figure 9B). Spherical protein bodies were surrounded by ER-derived membrane containing ribosomes. The centres of these protein bodies were highly osmiophilic and were surrounded by a light-staining uniform protein matrix (Figure 9B). Spherical protein bodies were generally found in the cytosol surrounded by lipid bodies. In rare cases, the spherical protein bodies were also found within the PSVs (Figure 9B). These protein bodies generally were not seen in storage parenchyma cells that were distal from the vascular tissue. Interestingly, we observed a second type, which was morphologically distinct from the first, and designated it as a complex protein body (Figure 10A). This unique structure appears to be an aggregate of several uniformly sized small protein bodies, arranged in a concentric circle surrounding a large central protein body. Each protein body was surrounded by an osmiophilic membrane. Unlike the spherical protein bodies, the complex protein bodies were found infrequently. Neither type of protein body was observed in control non-transformed soybean seed. To demonstrate that the novel ER-derived protein bodies in the transgenic soybean seeds accumulated the 11 kDa δ-zein, we performed immunocytochemical localization studies. When thin sections were incubated with δ-zein antibodies and protein A gold particles, specific labelling was seen on both types of novel protein body (Figure 10A,B). No labelling of the PSVs or other organelles was observed.

Discussion

The accumulation of the 11 kDa δ-zein in transgenic soybean resulted in a 1.5–1.7-fold increase in the methionine content of the alcohol-soluble protein fraction. This increase, however, did not improve the overall methionine content of soybean flour, as the 11 kDa δ-zein accounted for less than 0.5% of the total protein. A substantial increase in the accumulation of the 11 kDa δ-zein may be necessary to elevate the methionine content. Earlier, Brazil nut 2S albumin was expressed in transgenic soybeans, accounting for 10% of the salt-soluble seed protein, which resulted in a 30% increase in methionine content (Townsend and Thomas, 1994). Accumulation of the 2S seed albumin, however, was countered by a reduction in trypsin and chymotrypsin inhibitors, proteins rich in sulphur-containing amino acids (Beach *et al*., 1995). The increase in the methionine content in transgenic soybeans may be limited by the paucity of sulphur-containing amino acids in soybean seeds. Consequently, increasing the availability of methionine and cysteine during seed fill by manipulating synthesis and transport are essential considerations for the improvement of soybean protein quality.

The accumulation of the 11 kDa δ-zein in transgenic soybeans resulted in the formation of two morphologically distinct protein bodies, designated as spherical and complex. These protein bodies, easily distinguishable from PSVs due to their osmiophilic nature, were found in the midst of oil bodies. Rarely, the ER-derived spherical protein bodies were found inside the PSVs. A similar phenomenon has been reported to occur in transgenic tobacco seeds expressing the γ-zein gene (Coleman *et al*., 1996). An autophagous process may be involved in the engulfment of the ER-derived protein bodies by the PSVs (Herman and Larkins, 1999). Ribosomes on the periphery of the spherical bodies were indicative of an ER origin and their morphology is analogous to that found in maize endosperm (Lending *et al*., 1988). Complex protein bodies, which were also observed in 11 kDa δ-zein transformed soybeans, have a well-defined symmetry. At the centre lies a spherical protein body, surrounded by concentric layers of small protein vesicles. Similar structures, termed precursor-accumulating vesicles, occur in maturing pumpkin seed (Hara-Nishimura *et al*., 1998). ER-derived protein bodies have also been noted in other transgenic plants accumulating zeins (Bagga *et al*., 1995, 1997; Randall *et al*., 2000). As in maize seed, it appears that ER-derived protein bodies in transgenic plants are formed due to self-assembly and aggregation of the zeins within the lumen of the rough ER (Herman and Larkins, 1999).

In tobacco, expression of a modified 10 kDa zein resulted in the formation of spherical and aggregate protein bodies (Randall *et al*., 2000). When native zein signal sequences were retained, only spherical protein bodies were observed in transgenic tobacco. However, the introduction of three additional amino acids to the NH_2-terminal signal sequences resulted in the formation of aggregate protein bodies (Randall *et al*., 2000). Even though we modified the NH_2-terminal signal sequence of the 11 kDa δ-zein, the spherical protein bodies remained the most prevalent 11 kDa δ-zein storage structure. Thus, it appears that, in addition to NH_2-terminal signal sequences, other factors could be involved in the formation of complex or aggregate protein bodies. For example, sequence-mediated gene silencing of the α-subunits of β-conglycinin in transgenic soybeans resulted in the formation of ER-derived protein bodies (Kinney *et al*., 2001). Normal storage protein trafficking was affected in these transgenic soybeans, resulting in the accumulation of proglycinin, which leads to the formation of ER-derived protein bodies (Kinney *et al*., 2001).

The reason for the restricted accumulation of the 11 kDa δ-zein mRNA in cells closer to the vascular bundles is unclear. To our knowledge, the β-conglycinin α′-subunit promoter and β-conglycinin β-subunit signal peptide combination have not been used to express proteins in transgenic plants. Although the β-conglycinin α′-subunit promoter has been used to express reporter genes in transgenic plants (Chen *et al*., 1989; Chamberland *et al*., 1992), the spatial expression of the introduced gene has not been investigated. Immunological studies have shown that the β-conglycinin accumulates throughout the embryo (Krishnan, 2002), indicating that the β-conglycinin α′-subunit promoter is expressed in most cells of the embryo. The restricted expression pattern of the 11 kDa δ-zein mRNA seen in this study may be unique to the 11 kDa zein. We have created a new construct in which the 11 kDa zein coding region has been replaced by green fluorescent protein (GFP), and are in the process of generating transgenic soybean plants. If the GFP mRNA expression pattern is similar to that of the 11 kDa zein, it would suggest that sequences upstream of the coding region are responsible for the localized expression. Clearly, the molecular mechanism that dictates the restricted pattern of 11 kDa δ-zein mRNA accumulation in transgenic soybean needs further investigation.

The spatial distribution of the ER-derived protein bodies in transgenic soybeans expressing the 11 kDa δ-zein is notable. These protein bodies occur in transitional cells located between the vascular tissue and storage parenchyma cells. The occurrence of transitional cells was first reported in jack-bean cotyledons (Herman and Shannon, 1984). Although protein bodies occurring in the transitional cells and storage parenchyma

are structurally indistinguishable, immunocytochemistry has shown differences in the types of proteins they store (Herman and Shannon, 1984). The PVM layer, a specialized cell layer analogous to the transitional cells, occurs in soybean leaves. The PVM layer accumulates vegetative storage proteins that are involved in the temporary storage of nitrogen. This layer functions in the transfer of assimilates from the photosynthetically active tissue to minor veins for subsequent translocation (Fisher, 1967; Franceschi and Giaquinta, 1983). Similarly, the transitional cells could serve as a temporary repository for methionine synthesized in either the seed or other tissues. Synthesis of the methionine-rich 11 kDa δ-zein increases the demand for sulphur-containing amino acids. The availability of methionine is presumably greater in the cells nearer the vascular strand than in those distal to the vascular tissue. This gradient in methionine concentration could favour the accumulation of the 11 kDa δ-zein in cells proximal to the vascular tissue. To ascertain whether the availability of methionine is limiting the accumulation of the 11 kDa δ-zein, we are currently conducting studies in which transgenic soybeans are grown in the presence of exogenously applied methionine.

Experimental procedures

Plasmid construction

The α′-promoter region (–828 to –1) of β-conglycinin (Schuler *et al*., 1982) and the β-subunit signal peptide region (+1 to +78) of β-conglycinin (Tierney *et al*., 1987) were amplified from genomic DNA of soybean (cv. Peking) by polymerase chain reaction (PCR). The primers utilized for the amplification of the α′-promoter region were: 5′-AACC**CTCGAG**GGCAAAAACA-TTTAATAC-3′ and 5′-GGTT**GGATCC**AGTATATCTTAAATTC-3′; *Xho*I and *BamH*I sites, indicated by bold letters, were created in these two primers. The primers used for amplification of the β-conglycinin β-subunit signal peptide region were: 5′-AACC**GGATCC**ATGATGAGAGTGCGG-3′ and 5′-GGTT-**CATATG**CACCTTTAATGAGAC-3′. *BamH*I and *Nde*I sites, indicated by bold letters, were created in these two primers for facilitation of cloning. Each amplified fragment was individually inserted into pGEM-T easy vector (Promega, Madison, WI, USA). Utilizing unique restriction sites created by PCR, both the α′-subunit promoter and β-subunit signal sequences were cloned into an intermediate vector (pBintM). Isolation of the 11 kDa δ-zein gene has been described previously (Kim and Krishnan, 2003). The mature protein coding region of the 11 kDa δ-zein protein was recovered by digestion with *Nde*I and *Not*I and inserted into corresponding sites in the intermediate vector, resulting in pBintMα′Tp. Introduction of the *Nde*I restriction site added two amino acids, histidine and methionine, to the N-terminal of the mature coding sequence. The insert from this intermediate vector (pBintMα′Tp11) was digested with *XhoI*/*Xba*I and cloned into the corresponding sites of pZP201, resulting in pZα′11hsp (Figure 1). This plasmid consisted of the α′-promoter, β-conglycinin signal peptide, 11 kDa δ-zein gene coding region, the 3′ region of the potato proteinase inhibitor gene (*Pin* II), together with the cassette containing the cauliflower mosaic virus 35S promoter, the *bar*-coding region and the 3′-region of the nopaline synthase gene (*nos*). Transfer of pZα′11hsp into *Agrobacterium tumefaciens* (strain EHA105) was performed by triparental mating (Friedman *et al*., 1982).

Production of transgenic soybean lines

Transformation of soybean (cv. Williams 82) was performed by *Agrobacterium*-cotyledonary node transformation (Hinchee *et al*., 1988) utilizing glufosinate as a selective agent (Zhang *et al*., 1999). Regenerated transgenic soybean plants were screened for tolerance to herbicide Liberty by a leaf-painting assay as described earlier (Zhang *et al*., 1999). The presence of the 11 kDa δ-zein gene in glufosinate-resistant plants was confirmed by PCR and Southern blot analysis. Transgenic T1 plants were used in subsequent analysis.

RNA isolation and RNA gel blot analysis

Total RNA was extracted from leaves, roots, hypocotyls, stems, flowers and seeds using Trizol reagent (Invitrogen, Grand Island, NY, USA) following the manufacturer's protocol. The roots and hypocotyls were harvested from 6-day-old soybean plants, while the stem, leaves and flowers were collected from 60-day-old plants. Developing seeds used for RNA analysis were collected at 35 days after flowering, unless otherwise stated. RNA was quantified by measuring the A_{260}/A_{280} ratio using a spectrophotometer. RNA (5 μg) was fractionated in a 1.3% agarose-formaldehyde gel, transferred to a Hybond-N+ membrane and then fixed by UV cross-linking. The coding region of the 11 kDa δ-zein was amplified by PCR and purified from an agarose gel using Ultrafree-DA columns (Millipore Corporation, Bedford, MA, USA). The insert was labelled with [^{32}P] dCTP using the Ladderman labelling kit (PanVera Corporation, Madison, WI, USA). Prehybridization was performed for 6 h at 65 °C in 7% SDS, 191 mM Na_2HPO_4, 58 mM NaH_2PO4, 1% bovine serum albumin (BSA) and 100 μg/mL denatured salmon sperm DNA. Hybridization was performed overnight using the same buffer at 65 °C. The

membranes were washed at high stringency with 0.5 × SCC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate) and 0.1% SDS at 65 °C. Hybridization signals were detected by autoradiography with an intensifying screen at –80 °C. The sizes of the hybridizing transcripts were estimated with reference to RNA molecular weight markers.

Protein isolation and immunoblot analysis

Total soybean seed proteins were obtained by extracting 10 mg of dry seed powder with 1 mL of SDS sample buffer (2% SDS, 60 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol), followed by boiling at 100 °C for 5 min. The sample was centrifuged for 10 min at 22 150 ***g*** and the clear supernatant was treated as the total seed protein fraction. The globulin fraction was obtained by extracting 100 mg of seed powder with 1 mL of extraction buffer (100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl, 0.1 mM phenylmethylsulphonyl fluoride) for 60 min in a 30 °C orbital shaker. The mixture was centrifuged for 10 min at 22 150 ***g*** and the supernatant was designated as the globulin fraction. The pellet was extracted with 1 mL 60% isopropanol containing 1% β-mercaptoethanol for 60 min in a 30 °C shaker. The slurry was centrifuged, three volumes of acetone were added to the supernatant, and it was incubated at –20 °C for 10–12 h. Precipitated proteins were recovered by centrifugation for 10 min at 22 150 ***g***. The resulting pellet was re-suspended in SDS sample buffer and designated as the alcohol-soluble prolamin fraction. Seed proteins were resolved by SDS-PAGE (Laemmli, 1970) and visualized by staining with Coomassie Brilliant Blue. Immunoblot analysis was carried out as described earlier (Kim and Krishnan, 2003). Proteins were transferred to a nitrocellulose membrane and incubated with δ-zein antibodies that had been diluted 1 : 3000 in TBS (10 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 5% non-fat dry milk. After several washes with TBST (TBS containing 0.3% Tween 20), the nitrocellulose was incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate. Immunoreactive polypeptides were detected either with HRP colour development reagent (4-chloro-1-naphthol, Bio-Rad Laboratories, Richmond, CA, USA) or with an enhanced chemiluminescent substrate (Super Signal West Pico Trial Kit, Pierce), according to the manufacturer's instructions.

Amino acid analysis

Amino acid analysis was performed at the University of Missouri Agriculture Experiment Station Chemical Laboratories. Dry seeds of transgenic soybean and untransformed soybean lines, which were grown under identical conditions in a greenhouse, were ground to a fine powder and subjected to hydrolysis for 16 h at 115 °C with 6.0 mM HCl. For the quantification of methionine and cysteine, duplicate samples were oxidized with performic acid prior to acid hydrolysis. Amino acids were separated on a Beckman 6300 Amino Acid Analyser (Beckman Instruments, Fullerton, CA, USA) equipped with a high-performance, cation exchange resin column.

Protein sequencing

The alcohol-soluble protein fraction from transgenic soybean was resolved by SDS-PAGE and then electrophoretically transferred to a PVDF (polyvinylidene flouride) membrane. After brief subjection to Coomassie Brilliant Blue, the membrane was destained with 50% methanol and 10% acetic acid. The immobilized 11 kDa δ-zein on the PVDF membrane was excised and rinsed with distilled water. The amino-terminal sequence was analysed on an Applied Biosystems Protein Sequencer at the University of Nebraska Proteomics/Protein Core Facility.

In situ hybridization

The *in situ* hybridization procedure was performed according to Barker *et al*. (1988). Histology dishes were baked overnight to ensure RNase-free conditions. Deparaffinized sections were treated with Proteinase K (20 μg/mL), washed with phosphate-buffered saline and incubated in acetic anhydride solution. Sections were dehydrated in a graded ethanol series and allowed to dry in a dust-free area. ^{35}S-labelled sense and antisense probes were synthesized *in vitro* using T7 and SP6 promoters. Following 2 h of prehybridization, the sections were hybridized overnight at 50 °C. After treatment with RNase A (10 μg/mL), the sections were washed extensively, coated with Kodak NTB-2 emulsion and exposed to X-ray film at 4 °C for 1 week. Sections were counter-stained with haematoxylin and viewed under an Olympus photomicroscope fitted with both dark- and bright-field condensers.

Immunostaining of paraffin sections

Developing (35 days after flowering) soybean seeds were separated into cotyledonary halves and immediately fixed in 50% ethyl alcohol, 5% glacial acetic acid and 10% formaldehyde for 24 h at 4 °C. The seed tissue was dehydrated sequentially in a graded ethanol/xylene series and infiltrated with paraffin. Sections were cut with a microtome and collected on poly-L-lysine-coated slides. Following the removal of paraffin with xylene, the sections were processed for

immunostaining. To inactivate endogenous peroxidase activity, the deparaffinized sections were first treated with methanol/hydrogen peroxide. After incubating the sections with 5% goat serum and 1% BSA, they were incubated for 20 min with 1 : 500 diluted δ-zein antiserum (Kim and Krishnan, 2003). Following this step, the sections were treated sequentially with biotinylated linker, streptavidin conjugated to horseradish peroxidase and substrate-chromogen solution, according to the manufacturer's recommendation (DAKO Corporation, Carpinteria, CA, USA). The sections were counter-stained with haematoxylin and observed with bright-field optics.

Electron microscopic immunocytochemistry

Developing soybean cotyledons (35 days after flowering) were cut into 2 mm pieces and fixed in 2.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.2. Fixation was carried out at room temperature for 4 h. After several washes in phosphate buffer, the tissue was post-fixed in 1% aqueous osmium tetroxide. The tissue was dehydrated in a graded acetone series and embedded in Spurrs resin (Polysciences, Inc. Warrington, PA, USA). Ultrathin sections were cut with a diamond knife on an ultramicrotome and collected on uncoated 200-mesh nickel grids. Immunocytochemistry was performed essentially as described earlier (Krishnan *et al*., 1986). Thin sections were reacted with δ-zein antibodies diluted 1 : 200 in TBS containing 1% BSA (Kim and Krishnan, 2003). Sections were incubated with 10 nm anti-rabbit IgG colloidal gold particles (Sigma Chemical Co., St. Louis, MO, USA) diluted 1 : 10 in a solution consisting of 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20 with 1% BSA. The sections were stained with 1% aqueous uranyl acetate and were examined with a JEOL 1200 EX (Tokyo, Japan) transmission electron microscope at 80 kV.

Acknowledgements

We thank Dr Zhanyuan Zhang at the University of Missouri for the production of transgenic soybean lines and Dr Gautam Sarath at the University of Nebraska for providing the NH_2-terminal amino acid analysis. We also thank Dr Larry Darrah and John Bennett for critical reading of the manuscript.

References

Allen, R.D., Bernier, F., Lessard, P.A. and Beachy, R.N. (1989) Nuclear factors interact with a soybean beta-conglycinin enhancer. *Plant Cell*, **1**, 623–631.

Altenbach, S., Kuo, C.-C., Staraci, L.C., Pearson, K.W., Wainwright, C., Georgescu, A. and Townsend, J. (1992) Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine. *Plant Mol. Biol*. **18**, 235–245.

Bagga, S., Adams, H.P., Kemp, J.D. and Sengupta-Gopalan, C. (1995) Accumulation of the 15-kD zein in novel protein bodies in transgenic tobacco. *Plant Physiol*. **107**, 13–23.

Bagga, S., Adams, H.P., Rodriguez, F.D., Kemp, J.D. and Sengupta-Gopalan, C. (1997) Coexpression of the maize δ-zein and β-zein genes results in stable accumulation of δ-zein in endoplasmic reticulum-derived protein bodies formed by β-zein. *Plant Cell*, **9**, 1683–1696.

Barker, S.J., Harada, J.J. and Goldberg, R.B. (1988) Cellular localization of soybean storage protein mRNA in transformed tobacco seeds. *Proc. Natl. Acad. Sci. USA*, **85**, 458–462.

Beach, L., Orman, B., Townsend, J. and Thomas, L. (1995) *Reduction of Endogenous Seed Protein Levels in Plants*. U.S. Patent 9503784.

Beachy, R.N., Chen, Z.-L., Horsch, R.B., Rogers, S.G., Hoffmann, N.J. and Fraley, R.T. (1985) Accumulation and assembly of soybean β-conglycinin in seeds of transformed petunia plants. *EMBO J*. **4**, 3047–3053.

Chamberland, S., Daigle, N. and Bernier, F. (1992) The legumin boxes and the 3′ part of a soybean beta-conglycinin promoter are involved in seed gene expression in transgenic tobacco plants. *Plant Mol. Biol*. **19**, 937–949.

Chen, Z.L., Naito, S., Nakamura, I. and Beachy, R.N. (1989) Regulated expression of genes encoding soybean beta-conglycinins in transgenic plants. *Dev. Genet*. **10**, 112–122.

Chrispeels, M.J. and Herman, E.M. (2000) Endoplasmic reticulum-derived compartments function in storage and as mediators of vacuolar remodeling via a new type of organelle, precursor protease vesicles (PPV). *Plant Physiol*. **123**, 1227–1234.

Clercq, A.D., Vandewiele, M., Damme, J.V., Guerche, P., Montagu, M.V., Vandekerckhove, J. and Krebbers, E. (1990) Stable accumulation of modified 2S albumin seed storage proteins with higher methionine contents in transgenic plants. *Plant Physiol*. **94**, 970–979.

Coleman, C.E., Herman, E.M., Takasaki, K. and Larkins, B.A. (1996) The maize γ-zein sequesters α-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. *Plant Cell*, **8**, 2335–2345.

Dinkins, R.D., Reddy, M.S., Meurer, C.A., Yan, B., Trick, H., Thibaud-Nissen, F., Finer, J.J., Parrott, W.A. and Collins, G.B. (2001) Increased sulfur amino acids in soybean plants overexpressing the maize 15 kDa zein protein. *In Vitro Cell. Dev. Biol.-Plant*, **37**, 742–747.

Esen, A. (1986) Separation of alcohol-soluble proteins (zeins) from maize into three fractions by differential solubility. *Plant Physiol*. **80**, 623–627.

Fisher, D.B. (1967) An unusual layer of cells in the mesophyll of the soybean leaf. *Bot. Gaz*. **128**, 215–221.

Franceschi, V.R. and Giaquinta, R.T. (1983) The paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. I. Ultrastructure and histochemistry during vegetative development. *Planta*, **157**, 411–421.

Friedman, A.M., Long, S.R., Brown, S.E., Buikema, W.J. and Ausubel, F.M. (1982) Construction of broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene*, **18**, 289–296.

Hara-Nishimura, I., Shimada, T., Hatano, K., Takeuchi, Y. and Nishimura, M. (1998) Transport of storage proteins to protein

storage vacuoles is mediated by large precursor-accumulating vesicles. *Plant Cell*, **10**, 825–836.

Herman, E.M. and Larkins, B.A. (1999) Protein storage bodies and vacuoles. *Plant Cell*, **11**, 601–613.

Herman, E.M. and Shannon, L.M. (1984) Immunocytochemical localization of concanavalin A in developing jack-bean cotyledons. *Planta*, **161**, 97–104.

Hinchee, M.A.W., Connor-Ward, D.V., Newell, C.A., McDonnell, R.E., Sato, S.J., Gasser, C.S., Fischhoff, D.A., Re, D.B., Fraley, R.T. and Horsch, R.B. (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. *Bio/Technology*, **6**, 915–922.

Imsande, J. (2001) Selection of soybean mutants with increased concentrations of seed methionine and cysteine. *Crop Sci.* **41**, 510–515.

Kim, W.S. and Krishnan, H.B. (2003) Allelic variation and differential expression of methionine-rich δ-zeins in maize inbred lines B73 and W23a1. *Planta*, **217**, 66–74.

Kinney, A.J., Jung, R. and Herman, E.M. (2001) Cosuppression of the α-subunits of β-conglycinin in transgenic soybean seeds induces the formation of endoplasmic reticulum-derived protein bodies. *Plant Cell*, **13**, 1165–1178.

Krishnan, H.B. (2000) Biochemistry and molecular biology of soybean seed storage proteins. *J. New Seeds*, **2**, 1–25.

Krishnan, H.B. (2002) Biochemical and ultrastructural trends in proteolysis of the β-subunit of 7S protein in the cotyledons during germination of soybean seeds. *Korean J. Crop Sci*. **47**, 85–94.

Krishnan, H.B., Franceschi, V.R. and Okita, T.W. (1986) Immunochemical studies on the role of the Golgi complex in protein body formation in rice seeds. *Planta*, **169**, 471–480.

Ladin, B.F., Tierney, M.L., Meinke, D.W., Hosangadi, P., Veith, M. and Beachy, R.N. (1987) Developmental regulation of β-conglycinin in soybean axes and cotyledons. *Plant Physiol*. **84**, 35–41.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lending, C.R., Kriz, A.L., Larkins, B.A. and Bracker, C.E. (1988) Structure of maize protein bodies and immunocytochemical localization of zeins. *Protoplasma*, **143**, 51–62.

Lending, C.R. and Larkins, B.A. (1989) Changes in the zein composition of protein bodies during maize endosperm development. *Plant Cell*, **1**, 1011–1023.

Molvig, L., Tabe, L.M., Eggum, B.O., Moore, A.E., Graig, S., Spencer, D. and Higgins, J.V. (1997) Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. *Proc. Natl. Acad. Sci. USA*, **94**, 8393–8398.

Müntz, K., Christov, V., Saalbach, G., Saalbach, I., Waddell, D., Pickardt, T., Schieder, O. and Wustenhagen, T. (1998) Genetic engineering for high methionine grain legumes. *Nahrung*, **42**, 125–127.

Nielsen, N.C. (1985) The structure and complexity of the 11S polypeptides in soybeans. *J. Am. Oil Chem. Soc*. **62**, 1680–1686.

Nielsen, N.C. (1996) Soybean seed composition. In: *Soybean: Genetics, Molecular Biology and Biotechnology* (Verma, D.P.S. and Shoemaker, R.C., eds), pp. 127–163. Wallingford: CAB.

Nishizawa, K., Maruyama, N., Satoh, R., Fuchikami, Y., Higasa, T. and Utsumi, S. (2003) A C-terminal sequence of soybean β-conglycinin α′ subunit acts as a vacuolar sorting determinant in seed cells. *Plant J*. **34**, 647–659.

Nordlee, J.A., Taylor, S.L., Townsend, J.A., Thomas, L.A. and Bush, R.K. (1996) Identification of a Brazil-nut allergen in transgenic soybeans. *N. Engl. J. Med*. **334**, 688–692.

Randall, J., Bagga, S., Adams, H. and Kemp, J.D. (2000) A modified 10 kD zein protein produces two morphologically distinct protein bodies in transgenic tobacco. *Plant Sci*. **150**, 21–28.

Saalbach, I., Waddell, D., Pickardt, T., Schieder, O. and Müntz, K. (1995) Stable expression of the sulphur-rich 2S albumin gene in transgenic *Vicia narbonensis* increases the methionine content of seeds. *J. Plant Physiol*. **145**, 674–681.

Schuler, M.A., Schmitt, E.S. and Beachy, R.N. (1982) Closely related families of genes code for the α and α′ subunits of the soybean 7S storage protein complex. *Nucl. Acids Res*. **10**, 8245–8261.

Shewry, P.R., Napier, J.A. and Tatham, A.S. (1995) Seed storage proteins: structures and biosynthesis. *Plant Cell*, **7**, 945–956.

Streit, L.G., Beach, L., Register, J.C., III, Jung, R. and Fehr, W.R. (2001) Association of the Brazil nut protein gene and Kunitz trypsin inhibitor alleles with soybean protease inhibitor activity and agronomic traits. *Crop Sci*. **41**, 1757–1760.

Tabe, L.M. and Droux, M. (2002) Limits to sulfur accumulation in transgenic lupin seeds expressing a foreign sulfur-rich protein. *Plant Physiol*. **128**, 1137–1148.

Tierney, M.L., Bray, E.A., Allen, R.D., Ma, Y., Drong, R.F., Slightom, J. and Beachy, R.N. (1987) Isolation and characterization of a genomic clone encoding the β-subunit of β-conglycinin. *Planta*, **172**, 356–363.

Townsend, J.A. and Thomas, L.A. (1994) Factors which influence the *Agrobacterium*-mediated transformation of soybean. *J. Cell Biochem. Suppl*. **18A**, 78.

Tu, H.M., Godfrey, L.W. and Sun, S.S.M. (1998) Expression of the Brazil nut methionine-rich protein and mutants with increased methionine in transgenic potato. *Plant Mol. Biol*. **37**, 829–838.

White, C.L., Tabe, L.M., Dove, H., Hamblin, J., Young, P., Phillips, N., Taylor, R., Gulati, S., Ashes, J. and Higgins, T.J.V. (2000) Increased efficiency of wool growth and live weight gain in Merino sheep fed transgenic lupin seed containing sunflower albumin. *J. Sci. Food Agric*. **81**, 147–154.

Zhang, Z., Xing, A., Staswick, P. and Clemente, T.E. (1999) The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean. *Plant Cell Tissue Organ Cult*. **56**, 37–46.