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Impact of co-expression of maize 11 and 18 kDa δ-zeins and 27 kDa γ-zein in transgenic soybeans on protein body structure and sulfur amino acid content

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ABSTRACT

The methionine-rich seed storage proteins of maize have been expressed in transgenic plants as a means to improve the overall sulfur amino acid content of seed. Previous attempts to increase the sulfur amino acid content of soybean seeds by this approach has met with limited success. It has been shown co-expression of different class of zeins can result in their stable accumulation in transgenic plants. In this study, conventional crosses between transgenic plants individually expressing 11, 18 kDa δ-zeins and 27 kDa γ-zein were made to obtain plants that simultaneously express both the δ-zein and γ-zein. Transmission electron microscopic observation of thin-sections of transgenic soybean seeds revealed that the zeins accumulated in ER-derived protein bodies (PBs) which were found sparsely scattered in cytoplasm. The size of these PBs varied from 0.2 to 0.6 μm in soybean plants individually expressing 11, 18 kDa δ-zeins and 27 kDa γ-zein. In contrast, soybeans co-expressing the 18 kDa δ-zein and 27 kDa γ-zein the PBs was 3–4 times larger. Electron microscopic observation also revealed the sequestration of PBs inside the vacuoles where they could be subjected to degradation by vacuolar proteases. Amino acid analysis of transgenic soybean individually expressing 11, 18 kDa δ-zeins and 27 kDa γ-zein revealed only a minimal increase in the overall methionine content compared to the wild-type. In contrast, plants co-expressing 18 kDa δ-zein and 27 kDa γ-zein showed a significant increase (27%) in the methionine content compared to the control seeds.

1. Introduction

Soybean is an important legume that is widely grown in several parts of the world. In United States it is the second-largest crop only next to corn. This crop is grown in 31 states representing about 72 million acres. In 2016, approximately 4.31 billion bushels of soybeans were grown in the US valued at $40 billion (https://www.statista.com/statistics/192071/production-value-of-soybeans-for-beans-in-the-us-since-2000/). Though initially grown as a forage crop in US, it has now become a major source of protein and oil around the world. Raw soybeans are processed to separate the oil from the high-protein fiber meal. Soybean oil is predominantly used as a vegetable oil through the world. Soybean meal is the predominant protein source for animal feed, with poultry and swine industries being the main consumers of soybean meal. On account of its high protein content and balanced amino acid profile, soybean meal is touted as “gold standard” in the animal feed industry. Remarkably, about 97% of soybean produced in US is used for animal feed (https://unitedsoybean.org/article/us-soybean-meal-a-dependable-feed-ingredient). Even though soybeans are an excellent protein source, their nutritive value can be further enhanced by increasing the concentration of sulfur containing amino acids cysteine and methionine. Bioengineering approaches have been made to elevate the methionine content of seeds [1–6].

Expression of heterologous sulfur-rich seed storage proteins in transgenic soybeans have been employed as a means to improve the nutritive value of soybean [3,6–10]. Seed storage proteins that are rich in sulfur-rich amino acids have been reported in the literature [11–17]. Brazil nut 2S albumin contains18% methionine and 8% cysteine [16], and a10 kDa zein contains 22% methionine and 3% cysteine [13,14]. Even though sulfur-rich proteins have been successfully expressed in soybeans the overall impact of such attempts to elevate the sulfur amino acid content of soybean seeds have been modest at best. How-

Abbreviations: ER, endoplasmic reticulum; HRP, horseradish peroxidase; IgE, immunoglobulin E; PB, protein body; PSV, protein storage vacuole; TBS, tris-buffered saline

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ever, expression of Brazil nut 2S albumin in soybeans resulted in the doubling of the methionine content [7,18]. Unfortunately, the 2S Brazil nut albumin was identified as a potential allergen [19] and subsequently this approach was not pursued. Similar attempts have been made to express maize zeins in transgenic soybeans [8,7–10]. Expression of a maize 15 kDa zein containing 11% methionine in transgenic soybean resulted in a 12–20% increase in methionine and 15–35% increase in cysteine content compared to the control plants [8]. A methionine-rich 11 kDa δ-zein when expressed in transgenic soybeans resulted in a 1.5–1.7-fold increase in the methionine content of the alcohol-soluble protein fraction [9]. Introduction of a 27 kDa γ-zein in transgenic soybeans increased the cysteine and methionine from 26.97 to 29.33% and from 15.49 to 18.57%, respectively, compared to non-transformed control seeds [10]. However, these modest increases in the methionine content is not sufficient to meet the basic requirement for animal and human nutrition [6]. The methionine content of soybeans ranges from 1.2 to 1.4% and this amount needs to be doubled in order to meet the optimum growth and development of monogastric animals [6].

Previous studies have failed to detect the accumulation of certain maize zein seed storage proteins when expressed in transgenic plants [20,21]. For example, the 10 and 22 kDa α-zein failed to accumulate in tobacco seeds due to rapid degradation of these heterologous methionine-rich proteins [20]. It was later revealed that co-expression of γ-zein was required for α-zein accumulation in transgenic tobacco seeds [21]. Similarly, it was shown that the accumulation of α-zein in transgenic plants was stabilized by co-expression of β-Zein [22] suggesting an important role for protein–protein interactions in protein body (PB) formation. Results from co-expression of different classes of zeins in transgenic tobacco seeds demonstrates the role of the sulfur-rich β- and γ-zeins in initiating and maintaining protein body structure [23,24]. Ultrastructural investigations of transgenic tobacco seed expressing zeins have revealed that the morphology of PBs are also influenced by co-expression of different classes of zeins [23]. For example, co-expression of the δ-zein/β-zein resulted in unique structures that were not spherical like the PB that are commonly encountered in maize endosperm [21]. Even though zeins have been expressed in transgenic soybeans, only limited ultrastructural investigation on the nature of PB is available. Previously we have demonstrated the expression of the 11 kDa δ -zein gene in transgenic soybean resulted in the formation of ER-derived spherical PBs [9]. Though other classes of zeins have been expressed in transgenic soybeans no ultrastructural investigation on the PBs in these plants have been conducted. Additionally, no studies have been conducted where the different classes of zeins have been co-expressed in transgenic soybean seeds. In this study, we have generated transgenic soybeans individually expressing 11, 18 kDa δ-zeins and 27 kDa γ-zein as well soybean plants co-expressing 11 kDa and 18 kDa δ-zeins, 11 kDa δ-zein and 27 kDa γ-zein, and 18 kDa δ-zein and 27 kDa γ-zein, respectively. The results of investigation demonstrates an important role for the 27 γ-zein in influencing the PB structure and enhancing the methionine content of transgenic soybean seeds.

2. Materials and methods

2.1. Generation of transgenic soybean lines expressing the 18 kDa δ-Zein

Maize seeds accumulate two classes of methionine-rich δ-zein, 11 and 18 kDa zeins. The 11 and 18 kDa kDa δ-Zein contains 32 and 55 methionine residues representing 22 and 28% of the total amino acid of these proteins, respectively. Earlier we have expressed the 11 kDa δ-zein in transgenic soybean that resulted in a modest increase in the methionine content [9]. Since the 18 kDa δ-zein contains relatively higher methionine than the 11 kDa δ-zein, we targeted this methionine-rich zein protein for expression in soybeans. For this purpose, we first isolated the 18 kDa δ-zein cDNA from maize inbred line B73 by PCR. Nucleotide and the deduced protein sequence analysis of this cDNA revealed high homology to the previously published 18 kDa δ-zein [25]. The coding region of the 18 kDa δ-zein was cloned into a plasmid under the control of β-conglycinin α'-subunit promoter and the terminator of the potato proteinase inhibitor gene (Fig. 1A). This plasmid also includes a cassette that includes *bar* herbicide resistance gene (Fig. 1A). The 18 kDa zein construct (pZα′18 hsp) was mobilized into *Agrobacterium tumefaciens* (strain EHA105) by triparental mating [26]. Transformation of soybean (cv. Williams 82) was performed at the Plant Transformation facility, University of Missouri, employing Agrobacterium-cotyledonary node transformation utilizing glufosinate as a selective agent [27]. Seven independent transgenic events were obtained and the accumulation of the 18 kDa δ-zein was verified by PCR (Fig. 1B) and immunoblot analysis (Fig. 1C) using 18 kDa δ-zein antibodies [25]. Three independent transgenic lines (18B2, 18B5 and 18B7) that accumulated the18 kDa δ-zein at relatively at higher amounts was chosen and grown in a greenhouse for another four generations to produce T5 plants. Soybean plants were grown in 2-gallon pots containing PRO-MIX (Premier Horticulture, Quebec, Canada) medium under 16 h day length and 18/30 °C night-day temperatures. The plants were watered as needed and fertilized once every 15 days with Osmocote Plus (Scotts, Marysville, OH, USA).

2.2. Introgression of 11 and18 kDa δ-Zein and 27 kDa γ-zein in soybean

In addition to the 18 kDa δ-zein expressing transgenic soybeans generated in this study, we have earlier generated transgenic soybean plants expressing 11 kDa δ-zein and 27 kDa γ-zein [9,28]. To obtain transgenic soybean seeds co-expressing selected combinations of different classes of zeins, we made conventional crosses between transgenic plants expressing δ-zein and γ-zein. These combinations include 11 kDa δ-zein x 18 kDa δ-zein, 11 kDa δ-zein x 27 kDa γ-zein, and 18 kDa δ-zein x 27 kDa γ-zein. F1 seeds from these crosses were advanced three generations in a greenhouse. Co-expression of the desired zein combination were verified by PCR and western blot analysis.

2.3. Seed protein isolation

Twenty dry seeds collected from the transgenic and control soybean plants were ground to a fine powder using a mortar and pestle. Total

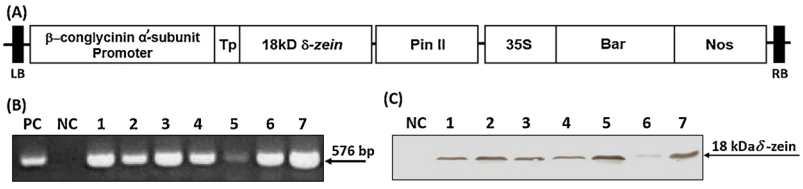

Fig. 1. Expression of 18 kDa δ-zein in transgenic soybean plants. (A) Schematic diagram of pZα′18hsp. The soybean transformation construct contains the 18 kDa δ-zein coding region under the control of the soybean β-conglycinin α′-promoter and the 3′ region of the potato proteinase inhibitor gene (Pin 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. (B) PCR confirmation of transgene. Genomic DNA isolated from young leaves of soybean (lanes 1–7) and plasmid DNA from pZα′18hsp DNA was used as a template. PC, positive control; NC, negative control (C) Immunoblot detection of 18 kDa δ-zein protein from transgenic soybean seeds. Alcohol-soluble proteins from seven transgenic soybean plants (lanes 1–7) and one non-transformed plant (NC) were resolved on a 15% SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane and incubated with antibodies specific to maize δ-zein protein. Proteins showing specific binding to the antibodies were identified using goat anti-rabbit IgG-horseradish peroxidase conjugate, followed by colorimetric detection.

soybean seed protein fraction was obtained by directly extracting 10 mg of seed powder with 1 ml of SDS-PAGE sample buffer (2% SDS, 60 mM Tris-HCl, pH 6.8, 5% β-mecaptoethanol). For zein extraction, 1 ml of 50% isopropanol and 5% β-mecaptoethanol was added to 100 mg of seed powder and placed on a vortex mixer. After vigorous shaking for 10 min at room temperature the slurry was clarified by centrifugation at 16,100*g* for 10 min. Supernatant from this step was mixed with 3 volumes of cold acetone and placed at -20 °C for several hours and centrifuged as before. The resulting pellet was dissolved in 200 µl of SDS sample extract buffer. A 10 µl aliquot of protein solution (˜50 µg) was used for electrophoresis. For comparison, total protein was also isolated from maize inbred line B73 which were kindly provided by Dr. Sherry Flint-Garcia, USDA-ARS, Columbia, MO.

2.4. Electrophoresis and immunoblot analysis

Seed proteins in SDS-sample buffer were boiled at 100 °C for 5 min and aliquots were resolved on 1-dimenisional gels utilizing Hoeffer SE-250 mini-Vertical electrophoresis apparatus (GE Healthcare, Pittsburgh, PA, USA). Zein proteins were separated with 15% SDS-PAGE and the resolved proteins were visualized by staining overnight with Coomassie Brilliant Blue.

For immunoblot analysis, the resolved proteins were transferred to a nitrocellulose membrane. Transfer efficiency was monitored by briefly staining the membrane with Ponceau S. The membrane was washed briefly with TBS (10 mM Tris-HCl, pH 7.5, 500 mM NaCl) and incubated with TBS containing 5% non-fat dry milk. Following this step, the membrane was incubated over-night with the appropriate zein antibodies. The γ-zein (obtained from Dr. Rebecca Boston, North Carolina State University) and the 11 kDa δ-zein antibodies [25] were diluted 1:10,000 in TBST (TBS with 3% non-fat dry milk containing 0.2% Tween 20). Non-specially bound antibodies were removed by washing the membrane 3 times with TBST (10 min each). Then the membrane was incubated with affinity-purified goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad Laboratories, Hercules, CA) for 1 h. Proteins that specifically bound the antibodies were detected using enhanced chemiluminescent substrate (Super Signal West Pico Kit; Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol.

2.5. Electron microscopy

Mature dry soybean seeds were imbibed in distilled for 15 min at room temperature and transferred to 1% water agar plates. The seeds were placed in a 30 °C incubator for 12 h. Imbibed seeds were sliced into 2–4 mm cubes with a double edge razor blade and fixed for 4 h in 2.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.2). After fixation, the tissues were rinsed four times in distilled water post-fixed for 1 h with 1% aqueous osmium tetroxide. Following four rinses in distilled water, the seed tissue was dehydrated in a graded acetone series and infiltrated with Spurr's resin. Resin embedded seed tissue was polymerized at 65 C oven for 48 h. Ultrathin sections of the seed tissue were cut with a diamond knife, collected on 200 mesh copper grids and stained with 0.5% uranyl acetate and 0.4% lead citrate. The grids were examined at 80 kV under JEOL 1200 EX (Tokyo, Japan) transmission electron microscope.

2.6. Amino acid analysis

For amino acid analysis, seeds from five individual transgenic soybean plants were pooled together and ground to find powder with a mortar and pestle. The accumulation of different class of zeins in these seeds were first verified by western blot analysis. Amino acid analysis of soybean seed samples were performed at the Proteomics & Metabolomics Facility, University of Nebraska-Lincoln. Amino acids were quantified using the Waters AccQ-Tag Ultra Kit on an Acquity UPLC system. Samples were run in triplicates and subjected to appropriate statistical analysis.

3. Results

3.1. Expression and accumulation of 18 kDa δ-zein in transgenic soybean seed

Transformation of soybean cultivar 'Williams 82'with the18 kDa δ-zein construct (pZα'18 hsp) by *Agrobacterium*-cotyledonary node method resulted in the regeneration of seven independent transgenic events (18B1, 18B2, 18B3, 18B4, 18B5, 18B6, and 18B7). The introduction of 18 kDa δ-zein in these transgenic events were confirmed by PCR utilizing primers designed to amplify a 576 bp of 18 kDa δ-zein. This PCR product was detected in all the seven transgenic plants but not in the non-transformed wild-type plant (Fig. 1B). The accumulation of 18 kDa δ-zein in soybean seeds were verified by immunoblot analysis using antibodies raised against the δ-zein (Fig. 1C). Immunoblot analysis of 50% isopropanol extracted seed proteins readily detected the accumulation of the18 kDa δ-zein, albeit at different levels, in all the seven transgenic events (Fig. 1C). Three transgenic events (18B2, 18B5 and 18B7) that showed high levels18 kDa δ-zein accumulation were further advanced another 4 generations to obtain T5 plants.

3.2. Co-expression of δ-zein and γ-zein in transgenic soybean seed

In addition to the the18 kDa δ-zein accumulating transgenic soybeans, we had earlier generated transgenic soybeans that accumulate the11 kDa δ-zein and 27 kDa γ-zein [9,28]. In order to co-express the different classes of zeins in the same plant, we carried out reciprocal crosses between plants individually accumulating γ-zein and δ-zein. Progeny derived from these crosses were grown in the green house and simultaneous expression of different classes of zeins were verified by immunoblot analysis. For this purpose we first isolated total seed

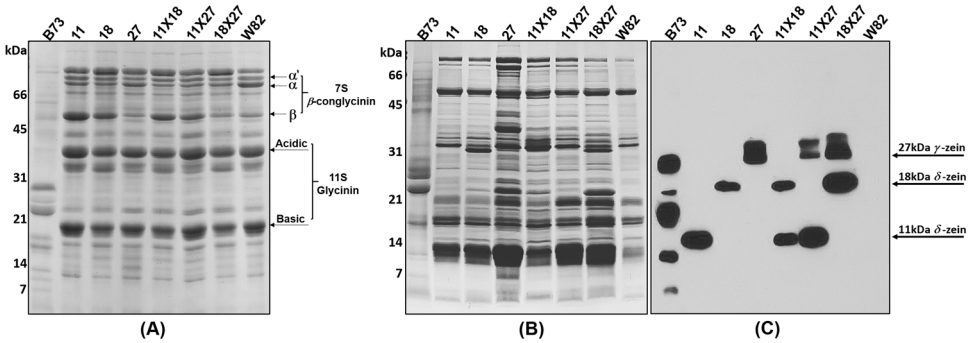

Fig. 2. Immunoblot detection of zein proteins from transgenic soybean seeds. Total seed (A) or alcohol-soluble proteins (B) from transgenic soybean seeds either individually or co-expressing the 11 kDa δ-zein, 18 kDa δ-zein or 27 kDa γ-zein proteins were resolved on a 15% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue. Alcohol-soluble proteins from transgenic soybean seeds and total seed protein from maize inbreed line B73 were subjected to immunoblot analysis employing antibodies specific to maize δ-zein and γ-zein proteins (C). Proteins showing specific binding to the antibodies were detected by chemiluminescent method. The captions on top of each gel represents the source of proteins from transgenic soybean plants expressing the different class of zeins. B73 and W82 represents maize inbreed line B73 and wild type soybean cultivar Williams 82, respectively.

proteins from transgenic and wild-type soybean plants and separated them by SDS-PAGE (Fig. 2A). The two abundant storage proteins of soybean are the 7S β-conglycinin and the 11S glycinin. The β-conglycinin are made of three subunits (76 kDa α'-, 72 kDa α-, and 52 kDa β-subunits), while the glycinin are represented by a group of proteins with approximate molecular weights of 38–40 kDa and 20–22 kDa. An examination of the SDS-PAGE profile of seed proteins of the transgenic soybeans reveals no major changes in transgenic soybeans when compared to that of the wild-type plants (Fig. 2A). However, the abundance of the 52 kDa β-subunit of β-conglycinin was found to be variable among the transgenic events. The accumulation of 52 kDa protein in plants expressing the 27 kDa γ-zein and 18 kDa δ-zein and 27 kDa γ-zein was similar to that of the wild-type soybean plants (Fig. 2A). In contrast, the other transgenic soybean events showed a higher accumulation of the 52 kDa protein when compared to that of wild-type plants (Fig. 2A). It is likely that this differences in the abundance of the 52 kDa protein is influenced by nutritional status of plants. Even though all these plants were grown under the same environmental conditions and fertilized similarly, it is possible that there are cultivar specific differences in the uptake of the nutrients by these plants. Previous studies have clearly shown that the accumulation of β-subunit of β-conglycinin is highly influenced by nutritional status of plants [29,30].

When total seed proteins were used in western blot analysis the accumulation of zeins were barely detected. This observation suggests low level accumulation of zeins in these transgenic plants. To overcome this problem, we enriched the zein fraction by isolating alcohol-soluble proteins from seeds of transgenic and wild-type soybean plants. An examination of the Coomassie Blue stained gel revealed several changes in the alcohol-soluble protein profile among the transgenic soybean plants. Though no prominent band corresponding to the 11 kDa and 27 kDa zeins could be identified, an abundant protein band corresponding to the 18 kDa zein was seen in transgenic soybean plants expressing 18 kDa δ-zein and 27 kDa γ-zein (Fig. 2B). In order to verify the accumulation of the different classes of zeins in these transgenic soybean plants we performed immunoblot analysis using pooled δ-zein and γ-zein antibodies (Fig. 2C). This analysis demonstrated successful co-expression of different classes of zeins in transgenic soybeans (Fig. 2C). As shown earlier [28], the maize γ-zein antibodies reacted against two proteins in soybean extracts with an estimated molecular weight of 27 kDa and 32 kDa (Fig. 2C). Similarly, the δ-zein antibodies reacted against an 11 kDa protein from soybean extracts. In contrast, pooled δ-zein and γ-zein antibodies recognized proteins with molecular weights of 27 kDa and 16 kDa (γ-zeins), 18 kDa and 10 kDa (δ-zein) and a 4 kDa protein from maize extract (Fig. 2C).

3.3. Transmission electron microscopic observation of zein protein bodies in transgenic soybean seed

Previously, we have shown that the 11 kDa δ-zein accumulates in protein bodies (PBs) in transgenic soybeans [9]. In order to examine the subcellular location of the 18 kDa δ-zein we conducted transmission electron microscopic observation of transgenic soybean seeds. PBs were

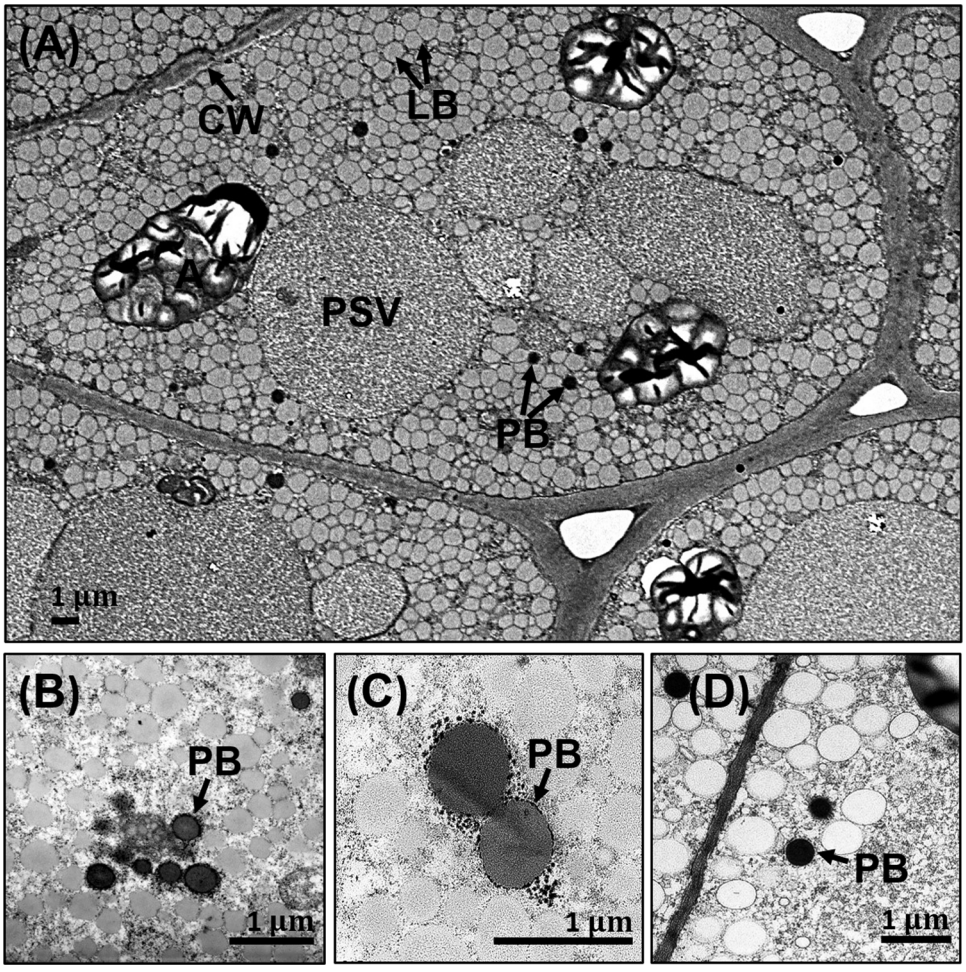

Fig. 3. Transmission electron microscopy observation of protein bodies in transgenic soybean seeds. Low magnification of view of developing transgenic soybean seed expressing the 18 kDa δ-zein (A). Several small protein bodies were found in the cell in between lipid bodies. High magnification of view of the PBs in transgenic soybean seeds accumulating the 11 kDa δ-zein (B), 18 kDa δ-zein (C) or 27 kDa γ-zein (D) proteins, respectively. Note the presence of ribosomes on the surface of the PBs. PSV, protein storage vacuole; LB, lipid bodies; PB, protein body; A, amyloplast; CW, cell wall.

observed in the cotyledons of the developing soybean seeds (Fig. 3A). In general, about 4–10 spherical PB were found in a cell in between the numerous lipid bodies (Fig. 3A). Unlike the protein storage vacuoles (PSV) that accumulate the endogenous seed storage proteins the PBs were much smaller in size (Fig. 3A). The diameter of these PBs ranged from 0.2 to 0.6 µm.

We also examined the appearance of PBs from mature soybean seeds individually accumulating the 11 kDa δ-zein, 18 kDa δ-zein, and 27 kDa γ-zein. In all three cases, the PBs were conspicuous by their spherical shape and dark appearance (Fig. 3B–D). These spherical PBs were surrounded by a distinct membrane that were studded with ribosomes (Fig. 3C). The morphology of PBs in soybean cells co-expressing the 11 kDa and 27 kDa γ-zein was also examined by transmission electron microscopy (Fig. 4). In most cases, the appearance of PB were very similar to those observed in cells expressing the 11 kDa δ-zein and 27 kDa γ-zein individually. In few instances, the central electron dense material of the PB was surrounded by a less-electron dense granular material (Fig. 4A). Zein PBs were also observed in vacuoles that were enclosed by a distinct membrane (Fig. 4B). Sequestration of several small PB were observed inside the vacuoles along with electron dense granules presumably ribosomes (Fig. 4B). Interestingly, in cells that co-expressed the 11 kDa δ-zein and 27 kDa γ-zein, these types of vacuoles were more prominent (Fig. 4C) and contained PB that showed distinct staining pattern (Fig. 4C insert). The central core of these PB were

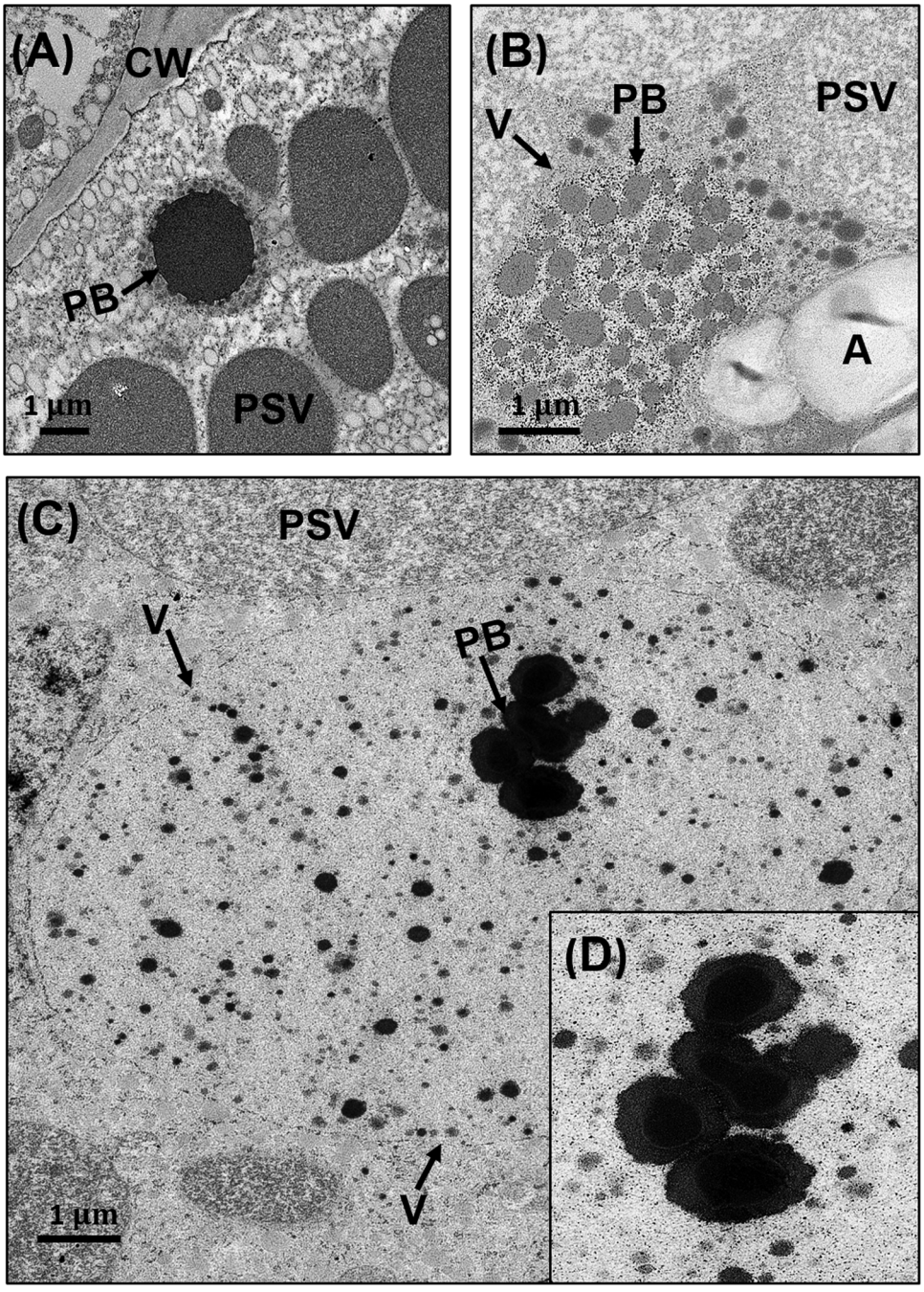

Fig. 4. Electron micrographs of protein bodies in transgenic soybean seeds co-expressing 11 kDa δ-zein and 27 kDa γ-zein proteins. (A) A protein body with a dark inclusion surrounded by a light-staining region. (B) Numerous small spherical protein bodies inside a vacuole. (C). Protein bodies revealing central dark staining core surrounded by light staining material inside a vacuole. (D) Higher magnification view of the protein body. A, amyloplast; PSV, protein storage vacuole; PB, protein body; V, vacuole; CW, cell wall.

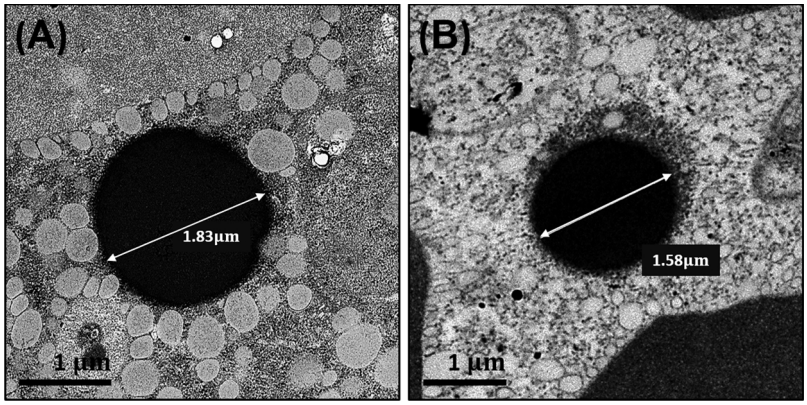

Fig. 5. (A and B) Electron micrographs of protein bodies in transgenic soybean seeds co-expressing the 18 kDa δ-zein and 27 kDa γ-zein proteins. Note the relative size of the protein bodies. PSV, protein storage vacuole; LB, lipid bodies; PB, protein body.

electron dense, while the periphery was occupied by less electron dense material (Fig. 4C insert). The appearance of these PB are similar to those that are found in the maize endosperm [31,32]. Interestingly, the co-expression of the 18 kDa δ-zein and 27 kDa γ-zein resulted in the appearance of PBs that 3 to 4 times larger than the ones observed in cells that only expressed the 11 kDa δ-zein and 27 kDa γ-zein individually (Fig. 5A and B). The large PBs were 1.5 to 1.8 µm in diameter that contained mostly electron dense material.

3.4. Impact of zein expression on the sulfur-amino acid content of transgenic soybean seed

A comparison of the amino acid profile of wild-type and transgenic soybean seeds expressing different classes of zein revealed minor changes in the overall profile (Table 1). The cysteine content of the transgenic soybeans, though slightly higher than the control seeds, was not significantly different from the control seeds (Table 1). A comparison of the total sulfur-containing amino acids (cysteine plus methionine) content revealed a marginal consistent increase in transgenic soybeans expressing the δ-zein and γ-zein either individually or in combination (Table 1). Importantly, the accumulation of zeins in soybean did not significantly elevate the overall methionine content of transgenic soybeans (Fig. 6). Even though most of the transgenic soybeans showed a slight increase in methionine content ranging from 2 to 7%, they were not statistically significant. Only transgenic soybean co-expressing 18 kDa kDa δ-zein and 27 kDa γ-zein showed a significant

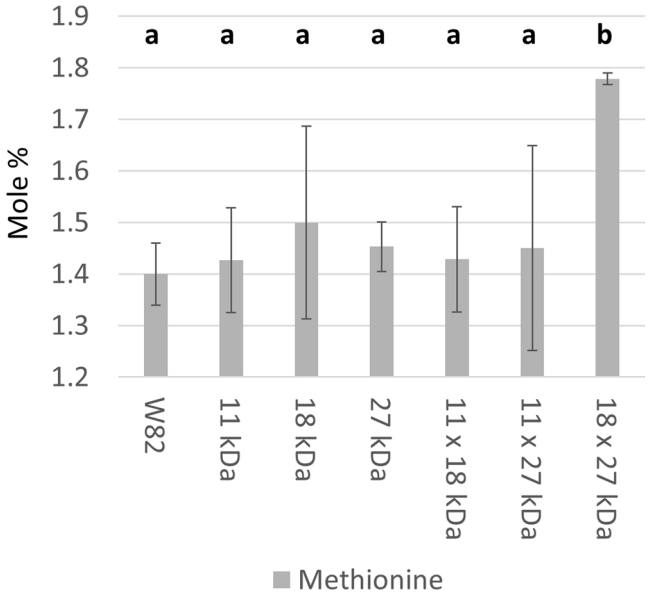

Fig. 6. Methionine content of transgenic soybean seeds. Dry seed powder from triplicate samples was analyzed by HPLC to measure the total amino acid from non-transformed (W82) and transgenic soybean plants expressing the different classes of zeins. Average values are shown ± SD. Samples with different letters are significantly different by *t*-test, (α = 0.05).

Table 1
Amino acid profile of transgenic soybean seeds expressing different classes of zeins.[a]

Amino Acid	W82	11 kDa	18 kDa	27 kDa	11 kDa x 18 kDa	11 kDa x 27 kDa	18 kDa x 27 kDa
CyA	2.20 ± 0.08a	2.38 ± 0.40a	3.04 ± 1.09a	2.63 ± 0.23a	2.39 ± 0.08a	2.70 ± 0.59a	2.27 ± 0.13a
His	2.8166 ± 0.12a	2.69 ± 0.19a	2.81 ± 0.25a	2.65 ± 0.06a	2.66 ± 0.28a	2.67 ± 0.18a	2.79 ± 0.08a
Ser	6.80 ± 0.08b	6.77 ± 0.11ab	6.76 ± 0.27ab	6.95 ± 0.07a	6.65 ± 0.09b	6.69 ± 0.21ab	6.36 ± 0.05c
Arg	6.28 ± 0.18abc	5.82 ± 0.31c	5.67 ± 0.61c	5.94 ± 0.21bc	5.93 ± 0.74bc	6.01 ± 0.61bc	6.95 ± 0.78ab
Gly	8.67 ± 0.02b	8.84 ± 0.84ab	9.95 ± 0.91a	8.21 ± 0.39b	8.88 ± 0.48ab	9.07 ± 1.02ab	8.97 ± 0.40ab
Asp	9.51 ± 0.08ab	10.14 ± 0.82ab	9.64 ± 0.57ab	10.33 ± 0.59ab	10.52 ± 0.55a	10.38 ± 0.25ab	9.96 ± 0.60ab
MetS	1.39 ± 0.06b	1.37 ± 0.05b	1.49 ± 0.18b	1.45 ± 0.04b	1.42 ± 0.10b	1.44 ± 0.19b	1.77 ± 0.01a
Glu	13.44 ± 0.20ab	13.39 ± 0.82ab	12.04 ± 0.46c	13.93 ± 0.51a	13.26 ± 0.88ab	13.32 ± 0.11ab	12.08 ± 0.78c
Thr	4.55 ± 0.01de	4.53 ± 0.06e	4.93 ± 0.17ab	4.65 ± 0.04cde	4.80 ± 0.16bc	4.73 ± 0.11 cd	5.05 ± 0.01a
Ala	5.52 ± 0.20e	5.66 ± 0.26cde	6.02 ± 0.15abc	5.58 ± 0.18de	6.10 ± 0.22ab	5.95 ± 0.06bcd	6.34 ± 0.18a
Pro	6.26 ± 0.08a	6.33 ± 0.30a	5.84 ± 0.17b	6.29 ± 0.33a	5.86 ± 0.26b	5.79 ± 0.04b	5.77 ± 0.20b
Lys	4.86 ± 0.43a	5.23 ± 0.46a	5.43 ± 0.53a	5.27 ± 0.28a	5.64 ± 0.46a	5.73 ± 0.43a	5.36 ± 0.40a
Tyr	3.54 ± 0.13a	3.26 ± 0.26a	3.40 ± 0.35a	3.30 ± 0.13a	3.37 ± 0.20a	3.13 ± 0.42a	3.61 ± 0.18a
Val	5.71 ± 0.14ab	5.84 ± 0.07ab	5.87 ± 0.18a	5.63 ± 0.09b	5.89 ± 0.07a	5.90 ± 0.15a	5.77 ± 0.17ab
Ile	5.09 ± 0.03a	4.87 ± 0.03b	4.74 ± 0.22b	4.88 ± 0.07b	4.74 ± 0.06b	4.68 ± 0.16b	4.73 ± 0.01b
Leu	8.01 ± 0.13ab	8.10 ± 0.07a	7.82 ± 0.22bcd	7.78 ± 0.08 cd	7.7380.07cde	7.61 ± 0.18de	7.54 ± 0.03e
Phe	5.29 ± 0.62a	4.71 ± 0.37ab	4.44 ± 0.63ab	4.45 ± 0.15ab	4.08 ± 0.37b	4.11 ± 0.39b	4.59 ± 0.46ab

[a] Cysteine is reported as cysteic acid (CyA) and methionine is reported as methionine sulfone (MetS). Results are the mean of three samples along with SD. Values with different letters are significantly different by *t*-test, (α = 0.05). W82, wild type cultivar.

increase (27%) in the methionine content compared to the control seeds (Fig. 6). This is most likely due to a higher accumulation of the zeins in transgenic soybean co-expressing 18 kDa kDa δ-zein and 27 kDa γ-zein. Our western blot analysis also shows a higher accumulation of the 18 kDa zein in this transgenic plant (Fig. 2B and C). Since the 18 kDa δ-zein contains relatively higher methionine (28%) than the 11 kDa δ-zein (22%) and the fact that the 18 kDa δ-zein accumulated at higher level may explain for observed increase in the methionine content of transgenic soybean plants co-expressing 18 kDa kDa δ-zein and 27 kDa γ-zein. Additionally, several other amino acids were altered in most of the transgenic soybean seeds when compared to wild-type seeds (Table 1). Proline, isoleucine, phenylalanine and serine were reduced in most of the transgenic soybean seeds. It is not clear why these amino acids are altered in transgenic seeds. It is possible that these changes could be due to alterations in the abundance of native soybean seed proteins. Even though our SDS-PAGE analysis (Fig. 2B) revealed no major changes in the protein profile, quantitate proteomic analysis may be required to detect subtle changes in the protein composition.

4. Discussion

In this study we have successfully expressed the different classes of zeins in transgenic soybeans. The expression of 11 and 18 kDa δ-zein and 27 kDa γ-zein resulted in the formation of ER-derived PBs. Even though the coding region of these zeins were fused with β-conglycinin α'-subunit signal sequences still these proteins were retained in ER-derived PBs. Our observation confirms the earlier suggestion that the zeins possess intrinsic characteristics that enables them to be retained in the lumen of ER [21]. In transgenic soybeans individually expressing the 11 and 18 kDa kDa δ-zein and 27 kDa γ-zein, the PBs were spherical and appeared similar to the ones observed in the maize endosperm. Unlike in maize endosperm, the PBs encountered in transgenic soybeans were much smaller in size. Interestingly, co-expression of the 18 kDa δ-zein and 27 kDa γ-zein resulted in the appearance of PBs that were significantly larger than those found in soybean seeds expressing either 18 kDa kDa δ-zein or 27 kDa γ-zein individually. This observation suggests a possible interaction between δ-zein and γ-zein leading to their stable accumulation of these proteins within PBs. However, a previous study employing yeast two-hybrid system failed to detect any interaction between the 27-kD γ-zein and δ-zein proteins [33]. It is possible that expression of the 27 kDa γ-zein is required for the stable accumulation of δ-zein in transgenic soybeans. An earlier study utilizing transgenic tobacco plants expressing α-zein, γ-zein, or both established that the stable accumulation of α-zein required simultaneous expression of γ-zein [21]. Our observation that simultaneous expression of the δ-zein and γ-zein results in the appearance of larger PBs indicates a role for γ-zein in stable accumulation of δ-zein in soybean seeds.

Based on previous studies it appears co-expression of γ-zein or β-zein is essential for the stable accumulation of other classes of zeins in transgenic plants [21–24]. Even though this approach can be used as an effective strategy to improve the sulfur amino acid content of legumes, this approach may not be practical. Earlier studies have been shown that the 27 kDa γ-zein is a potential allergen [28,34]. IgE from several patients allergic to corn strongly bound to the 27 kDa γ-zein [34]. In this regard it is should be pointed out that the Brazil nut 2S albumin when expressed in soybeans significantly improved the methionine content of soybean. However, later it was demonstrated that the Brazil nut 2S albumin was a major allergen [19]. Thus it becomes very critical to first assess the allergenicity of any proteins before they are introduced into important crops such as soybean.

Soybean seed proteins (7S β-conglycinin and 11S glycinin) accumulate in protein storage vacuoles (PSV). In the current study, the accumulation of zeins in PSV were not routinely observed. However, in some instances zein accumulation was observed inside vacuoles in transgenic soybeans expressing the δ-zein and γ-zein. The PBs within these vacuoles had unique appearance with a central dark region that was clearly distinguishable from the outer light staining region. In maize, the different classes of zeins are organized in distinct regions of the PBs. The α-zein and δ-zein are localized in the interior of protein bodies, whereas the β- and γ-zein are found on the exterior of the PB [31,32]. Based on this observation it is possible that the dark and light staining regions of the PBs observed in soybean seeds could represent the δ-zein and γ-zein, respectively.

Expression of methionine-rich proteins such as δ-zeins in legumes is accompanied by reduction in the endogenous sulfur-rich proteins [35,36]. However, the accumulation of methionine-rich storage proteins in legumes can be promoted by sulfur nutrition or methionine supplementation [37,38]. Earlier, we have shown a significant increase in the accumulation of the methionine-rich δ-zein in transgenic soybeans when the plants were grown in presence of high bioavailable sulfur [39]. Additionally, recent studies have shown that deregulating processes of assimilative sulfate reduction can significantly enhance the source of methionine for incorporation into zeins [40–42]. The 10-kDa δ-zein, which contains 22.5% Met and 3.9% Cys, accumulates preferentially in response to increased sulfur supply [40–42]. Based on these studies it is clear that adequate sulfur supply is essential for the accumulation of methionine-rich protein in seeds.

The impact of zein accumulation on the sulfur amino acid content of transgenic soybean have been examined in previous studies. Expression of 15 kDa zein and 27 kDa γ-zein in transgenic soybean resulted in modest increase in methionine when compared to non-transformed seeds [8,10]. In contrast, Kim and Krishnan [9] reported that the expression of the 11 kDa δ-zein gene did not affect the overall methionine content of seed flour. In in our present study, we observed only a minimal effect on the cysteine and methionine content of transgenic soybeans when compared to the wild-type cultivar. Thus, the approach to elevate the sulfur amino acid content of soybean seeds by expressing maize zeins have not been successful. Multiple factors could contribute to the failure of this approach [6]. Main among them is the low level expression of the zeins in transgenic soybeans. When compared to size and number of PSV, where the native 7S and 11S globulins accumulate, zein accumulating PBs are negligible. For significant elevation of sulfur amino acid content of soybean it will be necessary to drastically improve the accumulation of zeins to about 5–10% of the total seed protein. Co-expression of the18 kDa δ-zein or 27 kDa γ-zein appears to promote the formation of larger PBs which should lead to higher accumulation of zeins in transgenic soybean seeds. However, in these transgenic soybeans zeins were also encountered within vacuoles. It is not clear if the vacuoles accumulating the zeins represents lytic vacuoles (LV), which function as degradative organelles. The presence numerous small dark-containing inclusions within this vacuoles may indicate possible degraded products of zeins. Previous studies have expressed zeins in transgenic plants and in some instances, the expressed zeins were found to be unstable [21,23]. It was suggested that zein PBs after selective autophagy undergo degradation by vacuolar proteases [21]. Additional studies focused on zein expression, their targeting, folding and stability in transgenic soybean is required if any significant increase in sulfur amino acid content can be achieved.

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Competing interests

The authors declare that there are no competing interests.

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