In Vitro Cell. Dev. Biol.—Plant 37:742–747, November–December 2001
© 2001 Society for In Vitro Biology
1054-5476/01 $10.00+0.00

DOI:10.1079/IVP2001205

INCREASED SULFUR AMINO ACIDS IN SOYBEAN PLANTS OVEREXPRESSING THE MAIZE 15 kDa ZEIN PROTEIN

RANDY D. DINKINS[1], M. S. SRINIVASA REDDY[1], CURTIS A. MEURER[1], BO YAN[1], HAROLD TRICK[2]*, FRANÇOISE THIBAUD-NISSEN[3]†, JOHN J. FINER[2], WAYNE A. PARROTT[3], AND GLENN B. COLLINS[1]‡

[1]*Department of Agronomy, University of Kentucky, Lexington, KY 40546-0091*
[2]*Department of Horticulture and Crop Science, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691-4096*
[3]*Department of Crop and Soil Sciences, The University of Georgia, Athens, GA 30602-7272*

(Received 21 November 2000; accepted 3 April 2001; editor K. D'Halluin)

Summary

Four transgenic soybean [*Glycine max* (L.) Merrill] lines were generated containing the maize 15 kDa zein protein gene using somatic embryogenic protocols. The zein gene was inserted behind the β-phaseolin promoter for seed-specific expression. All four lines represent separate transformation events as they were generated in different experiments at different locations. Two of the transformation events produced multiple plants, and these produced identical Southern hybridization patterns (UKY/Z1, UKY/Z2 and UKY/Z3 from the first; and OSU/Z4, OSU/Z8 and OSU/Z10 from the second). Molecular characterization revealed that multiple copies of the zein gene were present in all of the transgenic lines. Immunoblot analysis confirmed the accumulation of the zein protein product in the seeds of the UKY/Z1, UKY/Z2, UKY/Z3, OSU/Z4, OSU/Z8 and OSU/Z10 transgenic lines. However, there was no accumulation of zein protein in the UGA/Z1 line and Northern analysis confirmed that the zein transgene was silenced in this line. It was not possible to analyze the zein expression in the seeds of the UKY/Z4 line, as it was sterile. Amino acid analysis of the UKY and OSU lines confirmed that there was a 12–20% increase in methionine, and 15–35% increase in cysteine content in these lines compared to the control. There were no consistent changes in the content of the other amino acids in the transgenic lines. These results suggest that while the increase in methionine content in these lines is modest, it is possible to increase the methionine content without adversely affecting the protein composition of soybean.

Key words: plant transformation; *Glycine max*; nutritional quality; seed storage protein; methionine.

Introduction

One of the goals of plant genetic engineering has been to improve crop plants with introduced nutritional quality components for animal and human consumption as an alternative approach to conventional breeding strategies. Soybean is a major source of protein in dietary foods and animal feed, yet soybean protein is deficient in the sulfur-containing amino acids, methionine and cysteine. The major limiting sulfur amino acid is methionine, as methionine can be converted to cysteine, but animals cannot convert cysteine to methionine. Soybean methionine content is normally in the range of 1–2%, which is well below the 3–5% basic minimal requirement for human consumption (Nielsen, 1996).

Transgenic approaches to increase methionine content in crop plants have included expressing several methionine-rich 2S albumin seed storage proteins from diverse species such as Brazil nut (Altenbach et al., 1992; Saalbach et al., 1995), sunflower (Khan et al., 1996; Molvig et al., 1997), and pea (Ealing et al., 1994). Several of the methionine-rich maize zein genes have also been evaluated for modifying amino acid profiles in transgenic plants (Hoffman et al., 1987; Ueng et al., 1988; Willamson et al., 1988; Ohtani et al., 1991; Bagga et al., 1995, 1997; Coleman et al., 1996; Sharma et al., 1998; Randall et al., 2000). Most of the experiments to introduce and express high methionine protein genes have resulted in conflicting results, ranging from no differences to modest increases in overall methionine content. One of the more promising changes in protein composition resulted from the introduction of the Brazil nut 2S albumin seed storage protein gene. Unfortunately, the Brazil nut 2S seed storage protein can cause potent allergenic reactions in some individuals, thereby limiting its usefulness (Nordlee et al., 1996). Expression of other high methionine proteins have resulted in more modest gains in terms of methionine increases. Transgenic lupin plants over-expressing a sunflower albumin gene had a 15% increase in methionine in the seed. While this increase in methionine content

*Current address: Department of Plant Pathology, Kansas State University, Manhattan, KS 66506-5502, USA.

†Current address: Department of Crop Sciences, University of Illinois, Urbana, IL 61801, USA.

‡Author to whom correspondence should be addressed: Email gcollins@pop.uky.edu

in lupin still falls short of the basic animal nutritional requirements, it is useful in a crop such as lupin, which is used primarily as feed for sheep (Molvig et al., 1997).

Animal-derived genes for high methionine proteins, such as the bovine β-casein protein (Maughan et al., 1999) and an avian ovalbumin protein (Schroeder et al., 1991; Albert and Simuth, 1993), can be introduced into plants resulting in higher methionine in the seed. However, it is doubtful that consumers will accept animal-derived proteins in plant products.

The maize zein seed storage proteins are encoded by gene families that give rise to several species of proteins that are water insoluble and contain up to 37% methionine (Chui and Falco, 1995). Efforts to overexpress the high methionine 10 and 22 kDa maize α-zein proteins in tobacco were unsuccessful as the zein proteins failed to accumulate in the seed due to rapid degradation of the zein protein (Ohtani et al., 1991). In contrast, the 15 kDa zein protein is stable and accumulates in tobacco seed (Hoffman et al., 1987). Thus, the maize 15 kDa zein protein that contains 11% methionine is a good candidate for expression in species with low seed methionine content. For this reason, we sought to express the maize gene for the 15 kDa zein protein in soybean as a candidate to increase methionine content in the seed. In this report, we show that expression of the maize gene encoding the 15 kDa zein protein, driven by the seed-specific bean β-phaseolin promoter, results in an accumulation of the zein protein in the seeds and a significant increase in methionine and cysteine content in soybean seed.

Materials and Methods

Gene constructs. The plasmid vectors used for transformation are presented in Fig. 1. The zein gene-containing vector used for microprojectile bombardment (pBSPhZ) at The Ohio State University (OSU) location contained a *Bgl*II–*Hin*dIII fragment consisting of the gene for the 15 kDa zein protein, along with a portion of the zein 3′ untranslated region and a NOS 3′ terminator (Bagga et al., 1995), cloned into a BlueScript plasmid containing the bean β-phaseolin −782 bp promoter (Burow et al., 1992). The pHygr plasmid (Finer et al., 1992) containing the hygromycin phosphotransferase gene was co-bombarded allowing hygromycin B to be used as the selective agent. At The University of Georgia (UGA) and University of Kentucky (UKY) locations, an *Agrobacterium* binary plasmid vector was used for all transformations. Briefly, a *Sst*I–*Hin*dIII fragment containing the β-phaseolin promoter, the 15 kDa zein protein encoding gene, a portion of the zein 3′ untranslated region and the NOS 3′ end from the pBSPhZ plasmid above was cloned into a modified pPZP100 vector (Hajdukyiewicz et al., 1994) containing the β-glucuronidase (Jefferson, 1987) and hygromycin resistance genes (Gritz and Davies, 1983) to form pHIG/Zein (Fig. 1B). All molecular manipulations were carried out using standard procedures (Sambrook et al., 1989).

Tissue culture and transformation. Soybean somatic embryogenic cultures, initiated and proliferated on solid medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) (Finer, 1988), as modified by methods described in Trick et al. (1997), were used at all three locations. The cultivar 'Jack' was used at the UKY and OSU locations, and the line 'F173', developed from a cross between Jack and PI 417138 and selected specifically for tissue culture (Bailey et al., 1993) was used at the UGA location. *Agrobacterium*-mediated transformation protocols, as described in Yan et al. (2000), were used to introduce the transgene into soybean at the UKY location. Biolistic transformation, selection, and tissue culture protocols used at the three locations have been described in Trick et al. (1997) with modifications described in Samoylov et al. (1998a, b) for the Georgia location.

Southern analysis. Transgenic T_1 (first sexual generation from primary transgenic plants) zein plants used for Southern, immunoblotting and amino acid analysis, along with appropriate controls, were grown in the greenhouse at the UKY location. For Southern analysis, DNA was isolated from young leaves as described in Yan et al. (2000). Ten μg of DNA from each plant were digested overnight with *Hin*dIII, separated on a 0.8% agarose gel and blotted onto 'Zetaprobe' membrane (BioRad Laboratories, Hercules, CA). Hybridization was done overnight at 42°C in a formamide solution with a 710-bp *Eco*RI/*Bgl*II maize 15 kDa zein gene fragment random-primed with α^{32}P dCTP using the Prime-It© II Random Primer Labeling Kit (Stratagene, La Jolla, CA). The membrane was washed three times at room temperature in 0.1 × SSC and 0.1% SDS and exposed in a phosphorimager cassette (Molecular Dynamics, Inc., Sunnyvale, CA). The bands were quantified using the ImageQuant™ software (Molecular Dynamics, Inc.).

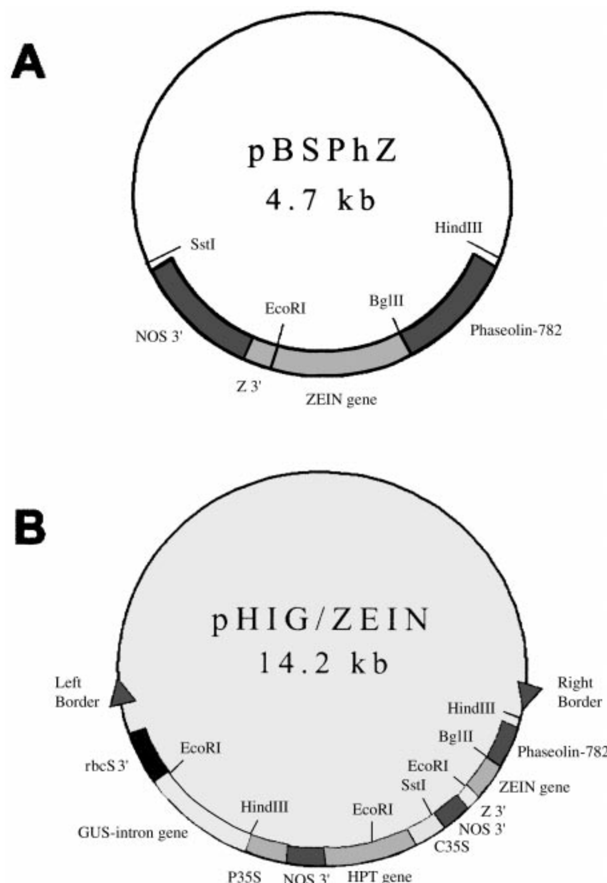

Fig. 1. The maize 15 kDa zein protein gene plasmid vectors. *A*, pBSPhZ: *Bgl*II–*Hin*dIII fragment consisting of the gene for the 15 kDa zein protein, along with a portion of the zein 3′ untranslated region and a NOS 3′ terminator (Bagga et al., 1995), cloned into a BlueScript plasmid containing the bean β-phaseolin −782 bp promoter (Burow et al., 1992). *B*, pHIG/Zein: *Sst*I–*Hin*dIII fragment of pBSPhZ containing the phaseolin-zein cassette cloned into a modified pPZP100 (Hajdukyiewicz et al., 1994) *Agrobacterium* binary plasmid vector containing the β-glucuronidase (Jefferson, 1987) and hygromycin resistance genes (Gritz and Davies, 1983).

Immunoblot analysis. Mature seeds of the different transgenic plants were homogenized in 10 m*M* sodium phosphate buffer (pH 6.8) containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 12 000 × *g* for 10 min and the supernatant was collected. The pellet from centrifugation was incubated in 70% ethanol containing 1% 2-mercaptoethanol at 65°C for 30 min with occasional shaking. The sample was then centrifuged at 12 000 × *g* for 10 min and the supernatant was collected again. The protein concentration was estimated by the BioRad protein assay method (BioRad Laboratories). For immunoblot analysis, 5 μg of phosphate buffer soluble or ethanol soluble protein was subjected to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using a 14% acrylamide gel. Fractionated proteins were

electroblotted onto a nitrocellulose membrane in 25 m*M* Tris, 192 m*M* glycine, and 5% methanol (pH 8.2). The membrane was blocked for 2 h in 5% dry milk powder in Tris-buffered saline containing 0.05% Tween 20 and then incubated in the same solution with the glycinin antisera (gift from Dr. Lila Vodkin, University of Illinois, Urbana, IL) or 15 kDa zein antisera (Bagga et al., 1995). Antigen–antibody complexes were visualized using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Amino acid analysis. A seed sample consisting of 25 mature dry seeds from each line was crushed to provide a 2 g sample that was sent to the Molecular Structure Facility (University of California-Davis) for analysis. Detection and analysis of amino acids were performed in accordance with published protocols (Cooper et al., 2000). Formic and performic acid hydrolysis reactions were conducted on sub-samples of each line (Hirs, 1967). Eluted fractions from samples were analyzed on a Beckman 6300 amino acid analyzer utilizing a sodium citrate buffer system. Amino acids were separated using ion-exchange chromatography. Sulfur-containing amino acids were extracted as methionine sulfone and cysteic acid via the performic acid protocol. These values were then normalized to the data for the remaining amino acids, which were extracted via the formic acid protocol, by calculating their presence relative to leucine and arginine extracted via perfomic acid.

Results and Discussion

A total of eight transgenic soybean plants derived from four independent transformation experiments containing the maize 15 kDa zein gene were regenerated at the UKY (UKY/Z1, UKY/Z2, UKY/Z3 and UKY/Z4), OSU (OSU/Z4, OSU/Z8 and OSU/Z10) and UGA (UGA/Z1) locations. Three of the UKY plants (UKY/Z1, UKY/Z2 and UKY/Z3) were transformed lines via the *A. tumefaciens*-mediated transformation system, and we have previously shown that these originated from a single initial transformation event (Yan et al., 2000). Since the three plants represent the same transformation event, further analysis was done on the UKY/Z1 plant and progeny only. The fourth, UKY/Z4, was obtained by particle bombardment and from a separate experiment. The three OSU plants were obtained by particle bombardment from one experiment. A Southern blot was done to confirm the presence of the zein gene in the genome and to compare lines derived from the three locations (Fig. 2). The restriction enzyme *Hin*dIII, used to digest total DNA, excises a band of 4.5 kb from the pHIG/Zein T-DNA. A single band of the correct size is found in the UKY/Z1 plant (Fig. 2). Similarly, a primary 4.5-kb band was observed in the UKY/Z4 line and the UGA/Z1 lines where the pHIG/Zein vector was used (Fig. 2). Additional bands were also observed in the two particle-gun derived lines, suggesting that rearranged, truncated or partial DNAs also integrated into the genomic DNA. The restriction enzyme *Hin*dIII cuts the DNA only once within the zein BlueScript vector, thus the multiple bands observed in the OSU lines are representative of the copy number. The three plants from OSU show the same hybridization pattern on the Southern blot indicating that the three plants are derived from the same initial transformation event.

We have previously determined that the UKY/Z1 line contains three copies of the T-DNA in tandem (Yan et al., 2000), thus we calculated the number of inserts in the other lines using the phosphorimager software ImageQuant™ (Molecular Dynamics, Inc.). The UKY/Z4 line, derived by particle bombardment using the pHIG/Zein vector, shows three bands on the Southern blot, two copies at expected 4.5 kb size, two copies of a larger (~8 kb) rearranged T-DNA and a smaller partial or truncated fragment

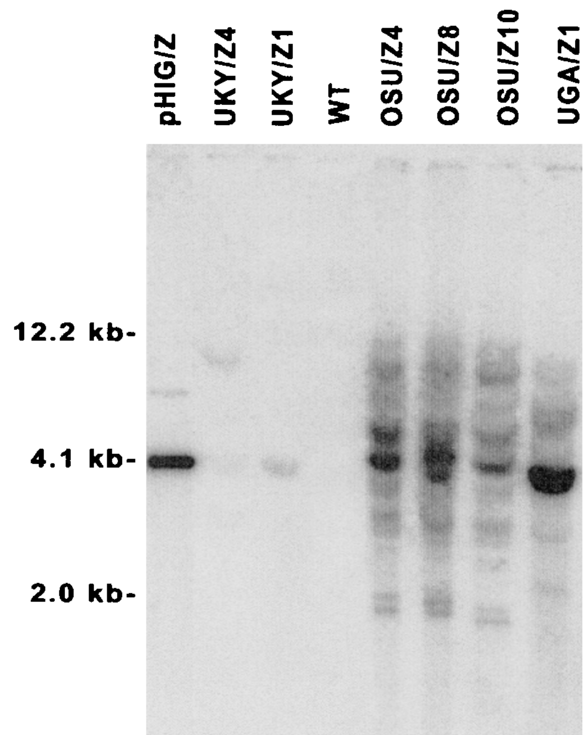

Fig. 2. Southern blot of transgenic soybean plants containing the maize 15 kDa zein protein gene. *Lanes*: (1) pHIG/Z, the pHIG/Zein plasmid cut with *Hin*dIII; (2) UKY/Z4; (3) UKY/Z1; (4) WT, non-transgenic Jack soybean cultivar; (5) OSU/Z4; (6) OSU/Z8; (7) OSU/Z10; (8) UGA/Z1. Ten micrograms of transgenic soybean genomic DNA was cut with the restriction enzyme *Hin*dIII and hybridized with an *Eco*RI/*Bgl*II fragment of the pBSPhZ plasmid containing the entire maize 15 kDa zein protein encoding region.

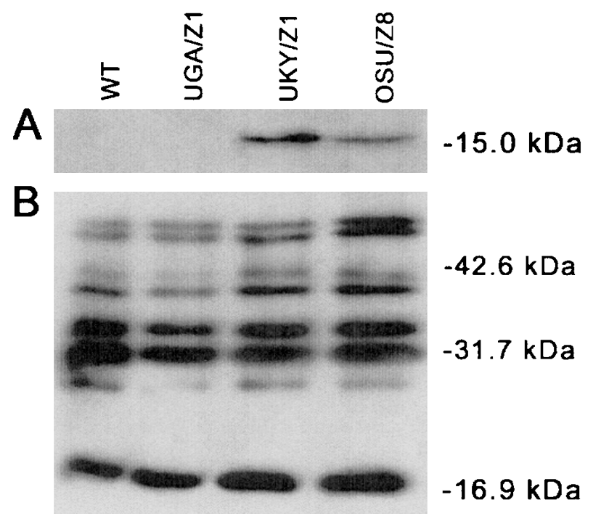

Fig. 3. Immunoblot hybridization of zein transgenic soybean plants. *A*, 15 kDa zein specific antibody (gift from S. Bagga, New Mexico State University); *B*, soybean 12 S glycinin seed storage protein polyclonal antibody (gift from L. Vodkin, University of Illinois).

(3 kb) containing the zein gene (Fig. 2). Molecular analysis has not been conducted to determine the structure of the inserts. The estimated number of copies in the UGA/Z1 line is between 100 and 110, with approximately 70 that still contain both *Hin*dIII sites in the inserted T-DNA to produce the 4.5 kb band. In addition, several bands, larger and smaller than 4.5 kb, were also observed in the UGA/Z1 line suggesting that truncation and/or re-arrangement of the bombarded T-DNA occurred in some of the inserted DNAs.

The number of copies calculated for the OSU lines is between 77 and 85. The single intense hybridization band at 4.7 kb indicates the formation of multiple tandem repeats of at least 12 plasmid copies. Insertions of multiple plasmids with some degree of concatenation are possible during microprojectile bombardment of soybean (Hadi et al., 1996). The high probability of co-transformation of multiple plasmids has been the background for co-bombardment of a plasmid containing the gene for selection along with a plasmid containing the gene of interest. Thus, it should be easier to segregate the plasmid containing the gene used for selection from the gene of interest in the genome by hybridization. However, introduction of high copy numbers of the introduced gene may result in gene silencing (Matzke et al., 1994).

Zein protein accumulation. In order to determine the accumulation of the zein protein in the seeds, an immunoblot was performed using protein samples isolated from mature seeds (Fig. 3). The results showed that zein protein accumulated in the UKY/Z1 and OSU/Z8 seeds, but not in the UGA/Z1. Analysis of the zein protein levels in seeds of UKY/Z4 line was not done, as this plant was not fertile. Immunoblots of the same seed protein samples showed that there were no differences in accumulation of the 11 S glycinin seed storage protein in the seed (Fig. 3*B*).

Zein protein accumulation correlates well with our results of zein mRNA accumulation in the seeds of the UKY and OSU lines (data not shown). Zein protein and mRNA were not detected in the UGA line suggesting that the zein transgenes were silenced in this line. Multiple inserts have a propensity to result in gene silencing due to hypermethylation (Matzke et al., 1994). Variation was also observed in the level of accumulation of the zein protein in the OSU plants (data not shown). Since these plants were all derived from a single transformation event, it is possible that gene silencing is still occurring due to the high copy number of the inserted DNA in the transformed line. Further analysis will be required to determine the extent of zein protein accumulation in future generations of these plants.

Seed amino acid analysis. Total amino acid profiles were performed on T_2 seeds derived from individual OSU and UKY lines that showed positive expression for the zein protein by immunoblot (Table 1). All of the transgenic lines had changes in methionine content greater than 10% compared to the wild-type cultivar Jack. None of the other amino acids showed any consistent differences greater than 10% compared to the control (Table 1). On average, there was a 12–20% and 15–35%, respectively, increase in the methionine and cysteine content of the transgenic-derived lines compared to the control. Since only seed from plants grown under greenhouse conditions were examined, it is not possible to determine the overall environmental variability. However, seeds from the four UKY/Z1 T_2 lines showed a similar increase in methionine and cysteine content and only minor differences in the other amino acids suggesting that the observed increase is heritable.

Although the increase in methionine and cysteine content in the transgenic soybean appears to be consistent in the transgenic lines examined, the change in the sulfur amino acid content in the transgenic plants described herein falls short of the needed increase to represent a sufficient source of methionine and cysteine in the diet (Tabe and Higgins, 1998). For this to be accomplished, the methionine content must, at least, be doubled in soybean seeds. It is possible that a higher accumulation of methionine can be found as more transgenic plants are examined. Although four original zein lines were generated, the results on zein accumulation in the seeds presented herein are based on two independent transformation events. Thus additional transgenic lines will be needed to fully determine the potential of this construct in changing the methionine content in soybean seeds.

It is possible that methionine and cysteine are in limiting supply during soybean seed development. This scenario is unlikely because changes in the methionine content of soybean seed have been shown using the Brazil nut albumin gene (Townsend and Thomas, 1994). Nitrogen application tends to result in a decrease in methionine content, as there is an increase in the β subunit of conglycinin, a subunit of the 7 S seed storage protein that is extremely poor in methionine (Pack et al., 1997). However, while direct application of methionine to whole plants during seed set

TABLE 1

TOTAL WEIGHT PERCENTAGE OF AMINO ACIDS IN SOYBEAN ZEIN TRANSGENIC LINES

Genotype[a]	ASP	THR	SER	GLX	PRO	GLY	ALA	VAL	ISO	LEU	TYR	PHE	HIS	LYS	ARG	CYST	MET
Jack Control	12.27	3.76	3.74	18.03	5.31	3.94	4.24	5.34	5.20	8.10	3.52	5.39	3.02	6.93	7.60	1.60	1.88
OSU/Z4-1	12.20	3.76	3.61	17.32	5.19	4.26	4.40	5.33	5.41	8.24	3.46	5.42	3.10	6.85	7.33	**1.97**	**2.14**
OSU/Z10-1	12.78	3.68	3.61	17.69	5.20	3.82	4.37	5.18	5.06	8.07	3.54	5.15	3.09	7.07	7.29	**2.16**	**2.24**
UKY/Z1-29	12.65	4.05	3.82	16.97	5.07	**4.40**	4.51	5.27	5.08	8.08	3.44	5.21	3.12	7.09	6.98	**2.04**	**2.21**
UKY/Z1-25	12.36	3.72	4.06	18.93	5.15	3.87	4.12	5.11	4.93	7.82	3.49	5.16	3.01	6.77	7.32	**1.91**	**2.26**
UKY/Z1-15	12.13	4.01	**4.12**	18.16	5.00	3.92	4.19	5.18	5.03	7.85	**3.96**	5.22	3.04	6.64	7.61	**1.84**	**2.11**
UKY/Z1-12	11.88	3.81	3.77	17.53	5.08	3.86	4.20	5.23	5.29	8.09	**4.17**	5.52	3.18	6.44	7.77	**1.98**	**2.20**
15 kDa zein protein	1.7	2.2	5.5	16.0	7.8	7.8	15.6	4.4	1.1	7.8	7.8	0	0	0.5	2.2	5.0	11.1

[a] Genotypes are as follows: Jack Control, field-grown plants harvested in 1997; OSU/Z4 and OSU/Z10, seeds derived from individual T_1 plants originating from OSU/Z4 and OSU/Z10; UKY/Z1-29, UKY/Z1-25, UKY/Z1-15 and UKY/Z1-12, seeds from T_1 plants derived from UKY/Z1 transgenic plant; 15 kDa zein protein, percent of each amino acid in the 15 kDa maize zein polypeptide.

Changes greater than 10% of the wild-type are presented in *bold*.

does increase the seed storage methionine content somewhat (Grabau et al., 1986), application of methionine to developing cotyledons (Holowach et al., 1984) results in no significant changes in methionine content in the seed storage proteins. These results suggest that methionine is not limiting, but that expression of the methionine-poor seed storage proteins is the major factor responsible for the low level observed. Thus, a major objective to increase the methionine content in developing soybean seed, would be to express seed storage protein genes where the protein would accumulate stably in the seed. It is possible that the bean β-phaseolin promoter is not a sufficiently 'strong' promoter compared with other soybean seed storage protein gene promoters in the developing soybean seed. The β-phaseolin promoter was used to achieve expression in the seed. However, if the observed increase in methionine content turns out to be the maximum possible with the β-phaseolin promoter, it may be necessary to use other seed-specific promoters or enhancer elements to achieve a nutritionally significant increase in methionine in the seed.

In conclusion, our results, along with previous results using the Brazil nut albumin and sunflower albumin, suggest that increase in overall methionine content in the seed is feasible for soybean using a transgenic approach. The methionine and cysteine levels observed in the present study, while greater than the wild-type control, could probably be further improved with changes in promoter and/or seed storage protein vector construction design.

Acknowledgments

The authors wish to thank Carl Redmond, Kay McAllister, Michael Hayes, Donna Tucker, Julie Essig, Holly Frantz, Ray Stevens, and Leroy Duncan for valuable technical support. This research was supported by the United Soybean Board, and the Agricultural Experiment Stations in Georgia, Kentucky, and Ohio. This paper (00-06-161) is published with the approval of the Director of the Kentucky Agricultural Experiment Station.

References

Albert, S.; Simuth, J. Construction of modified cloning system for expression of Japanese quail ovalbumin in transgenic plants. Biológia 48:689–692; 1993.

Altenbach, S. B.; Kuo, C. C.; Staraci, L. C.; Pearson, K. W.; Wainwright, C.; Georgescu, A.; Townsend, J. 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; 1992.

Bagga, S.; Adams, H.; Kemp, J. D.; Sengupta-Gopalan, C. Accumulation of 15-kilodalton zein in novel protein bodies in transgenic tobacco. Plant Physiol. 107:13–23; 1995.

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

Bailey, M. A.; Boerma, H. R.; Parrott, W. A. Genotype effects on proliferative embryogenesis and plant regeneration of soybean. In Vitro Cell. Dev. Biol. Plant 29:102–108; 1993.

Burow, M. D.; Sen, P.; Chlan, C. A.; Murai, N. Developmental control of the β-phaseolin gene requires positive, negative and temporal seed specific transcriptional regulatory elements and a negative element for stem and root expression. Plant J. 2:537–548; 1992.

Chui, C.-F.; Falco, S. C. A new methionine-rich seed storage protein from maize. Plant Physiol. 107:291; 1995.

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

Cooper, C.; Packer, N.; Williams, K. Amino acid analysis protocols. In: Methods in molecular biology, vol. 159. Totawa, NJ: Humana Press; 2000:9–30.

Ealing, P. M.; Hancock, K. R.; White, D. W. R. Expression of the pea albumin 1 gene in transgenic white clover and tobacco. Transgenic Res. 3:344–354; 1994.

Finer, J. J. Apical proliferation of embryogenic tissue of soybeans [*Glycine max* (L.) Merrill]. Plant Cell Rep. 7:238–241; 1988.

Finer, J. J.; Vain, P.; Jones, M. W.; McMullen, M. D. Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep. 11:232–238; 1992.

Grabau, L.; Blevins, D. L.; Minor, H. C. Stem infusions enhance methionine content of soybean storage protein. Plant Physiol. 82:1013–1018; 1986.

Gritz, L.; Davies, J. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25:179–188; 1983.

Hadi, M. Z.; McMullen, M. D.; Finer, J. J. Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Rep. 15:500–505; 1996.

Hajdukyiewicz, P.; Svab, Z.; Maliga, P. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. 25:989–994; 1994.

Hirs, C. H. W. Determination of cystine as cysteic acid. In: Hirs, C. H. W., ed. Methods in enzymology, vol. 11. New York: Academic Press; 1967:59–62.

Hoffman, L. M.; Donaldson, D. D.; Bookland, R.; Rashka, K.; Herman, E. M. Synthesis and protein body deposition of maize 15-kD zein in tobacco seeds. EMBO J. 6:3213–3221; 1987.

Holowack, L. P.; Thompson, J. F.; Madison, J. T. Storage protein composition of soybean cotyledons grown in vitro in media of various sulfate concentrations in the presence and absence of exogenous L-methionine. Plant Physiol. 74:584–589; 1984.

Jefferson, R. A. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5:387–405; 1987.

Khan, M. R. I.; Ceriotti, A.; Tabe, L.; Aryan, A.; McNabb, W.; Moore, A.; Craig, S.; Spencer, D.; Higgins, T. J. V. Accumulation of a sulphur rich seed albumin from sunflower in the leaves of transgenic subterranean clover (*Trifolium subterraneum* L.). Transgenic Res. 5:179–185; 1996.

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

Matzke, M.; Matzke, A. J. M.; Mittelsten-Scheid, O. Homology-dependent gene silencing in transgenic plants: epistatic silencing loci contain multiple copies of methylated transgenes. Mol. Gen. Genet. 244:219–229; 1994.

Maughan, P. J.; Philip, R.; Cho, M.-J.; Widholm, J. M.; Vodkin, L. O. Biolistic transformation, expression, and inheritance of bovine β-casein in soybean (*Glycine max*). In Vitro Cell. Dev. Biol. Plant 35:334–349; 1999.

Molvig, L.; Tabe, T. M.; Eggum, B. O.; Moore, A. E.; Craig, S.; Spencer, D.; Higgins, T. J. V. 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; 1997.

Nielsen, N. C. Soybean seed composition. In: Verma, D. P. S.; Shoemaker, R. D., eds. Soybean genetics, molecular biology and biotechnology. Wallingford, UK: CAB International; 1996:127–163.

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

Ohtani, T.; Galili, G.; Wallace, J. C.; Thompson, G. A.; Larkins, B. A. Normal and lysine-containing zeins are unstable in transgenic tobacco seeds. Plant Mol. Biol. 16:117–128; 1991.

Pack, N. C.; Imsande, J.; Shoemaker, R. C.; Shibles, R. Nutritional control of soybean seed storage protein. Crop Sci. 37:498–503; 1997.

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

Saalbach, I.; Pickardt, T.; Waddell, D. R.; Hillmer, S.; Schieder, O.; Muntz, K. The sulphur-rich Brazil nut 2S albumin is specifically formed in transgenic seeds of the grain legume *Vicia narbonensis*. Euphytica 85:181–192; 1995.

Sambrook, T.; Fritsch, E. F.; Maniatis, T. Molecular cloning – a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.

Samoylov, V. M.; Tucker, D. M.; Parrott, W. A. A liquid medium-based protocol for rapid regeneration from embryogenic soybean cultures. Plant Cell Rep. 18:49–54; 1998a.

Samoylov, V. M.; Tucker, D. M.; Parrott, W. A. Soybean [*Glycine max* (L.) Merrill] embryogenic cultures: the role of sucrose and total nitrogen content on proliferation. In Vitro Cell. Dev. Biol. Plant 34:8–13; 1998b.

Schroeder, H. E.; Khan, M. R. I.; Knibb, W. R.; Spencer, D.; Higgins, T. J. V. Expression of a chicken ovalbumin gene in three lucerne cultivars. Aust. J. Plant Physiol. 18:495–505; 1991.

Sharma, S. B.; Hancock, K. R.; Ealing, P. M.; White, D. W. R. Expression of a sulfur-rich maize seed storage protein, γ-zein, in white clover (*Trifolium repens*) to improve forage quality. Mol. Breed. 4:435–448; 1998.

Tabe, L.; Higgins, T. J. V. Engineering plant protein composition for improved nutrition. Trends Plant Sci. 3:282–286; 1998.

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

Trick, H. N.; Dinkins, R. D.; Santarem, E. R.; Di, R.; Samoylov, V.; Meurer, C. A.; Walker, D. R.; Parrott, W. A.; Finer, J. J.; Collins, G. B. Recent advances in soybean transformation. Plant Tiss. Cult. Biotechnol. 3:9–26; 1997.

Ueng, P.; Galili, G.; Sapanra, V.; Goldsbrough, P. B.; Dube, P.; Beachy, R. N.; Larkins, B. A. Expression of a maize storage protein gene in petunia plants is not restricted to seeds. Plant Physiol. 86:1281–1285; 1988.

Williamson, J. D.; Galili, G.; Larkins, B. A.; Gelvin, S. B. The synthesis of a 19 kilodalton zein protein in transgenic petunia plants. Plant Physiol. 88:1002–1007; 1988.

Yan, B.; Reddy, M. S. S.; Collins, G. B.; Dinkins, R. D. *Agrobacterium tumefaciens*-mediated transformation of soybean [*Glycine max* (L.) Merrill.] using immature zygotic cotyledon explants. Plant Cell Rep. 20:1090–1097; 2000.