A New Approach to Genetic Alteration of Soybean Protein Composition and Quality

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ABSTRACT: Although soybeans produce high-quality meal, modern animal and fish production systems often require synthetic essential amino acid supplements to fortify feed rations. However, biotechnology may enable development of soybeans with naturally adequate levels of certain essential amino acids for advanced feed formulations. One approach involves genetic manipulation of glycinin (11S) and β-conglycinin (7S) contents, the principal components of soybean storage proteins. Because 11S contains more cysteine and methionine than 7S protein, a higher 11S:7S ratio could lead to beneficial changes in the nutritional quality of soybean meal. Although genotypic variation for 11S:7S may be low among soybean [*Glycine max* (L.) Merr.] germplasm, ratios ranging from 1.7–4.9 were observed among accessions of the wild ancestor of cultivated soybean (*Glycine soja* Sieb. and Zucc.). Thus, wild soybean germplasm was evaluated as a potential source of genes that govern protein synthesis that may have been lost during the domestication of *G. max*. Change in the amount of 11S protein accounts for a significant portion of the genotypic variation in protein concentration and composition among wild soybeans. Strong positive correlation exists between the 11S:7S ratio and methionine or cysteine concentration of total protein. Moderate positive associations were found for threonine or tyrosine. A moderate negative correlation was found between lysine and 11S:7S. No association was found for leucine and phenylalanine or for total essential amino acid concentration. Based on these data, *G. soja* may contain a different complement of genes that influence expression of 11S and 7S proteins than *G. max* germplasm. Thus, through interspecific hybridization, wild soybeans may be a useful genetic resource for the further improvement of protein quality in cultivated soybeans.

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Soybean accounts for approximately 62% of the 141 million metric tons (MMT) of major protein meals in world trade (1). In the United States, about 80% of the annual soybean meal production (*ca.* 29 MMT) is used in animal feeds. With continued growth in domestic poultry, swine, beef, dairy, and fish production, the demand for soybean meal is projected to increase 14% in the next 5 years. However, advances in animal and fish production systems also place greater demand on the nutritional value of feeds. Although soybeans produce a high-quality cost-effective meal, in certain applications it is necessary to supplement feed formulations with synthetic essential amino acids, such as methionine, cysteine, and lysine, to improve animal performance.

Over the years, several biotechnological approaches have been employed with varying degrees of success to enhance the level of certain essential amino acids in soy protein to meet the needs of advanced feed formulations. These efforts range from increasing the sulfur content of crop fertilizers (2–4) to transforming soybeans with genes that encode high-methionine proteins from the Brazil nut (5). Another interesting concept is genetic manipulation of the levels of glycinin (11S) and β-conglycinin (7S), which account for nearly 70% of soybean protein. This idea is based partially on observations that 11S proteins contain 3–4 times more cysteine and methionine than 7S proteins (6). Therefore, soybean meal with a higher 11S:7S (w/w) ratio should exhibit enhanced nutritional quality. Although information on genetic variation in the 11S:7S ratio among cultivated soybean varieties is limited, the range appears to be narrow, between 1.1–2.2 (7). However, ratios as high as 6.0 have been reported in a few accessions of the USDA Soybean [*Glycine max* (L.) Merr.] Germplasm Collection that exhibit allelic variations governing 7S composition (7). The low-7S trait in this germplasm was affected by mutations that reduce expression of one or more of its constituent subunits, but total seed storage protein was not changed due to an apparent compensatory increase in the amount of 11S protein (7–9).

Protein is a quantitatively inherited trait (10). Five genes reportedly encode the 11S subunits in soybean (11,12), while at least 15 genes reportedly encode the 7S subunits (13). Analyses with gene markers show that these genes may be located on 13 different loci in the soybean genome (14). Genes that govern the soybean glycinin and β-conglycinin assembly have been cloned and characterized (12,15). However, breeding methods, such as "recurrent selection," are still the most efficient means to achieve a high degree of recombination when the selected trait is governed by a multigenic system.

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However, successful conventional breeding approaches depend upon discovery of germplasm with allelic variations that influence the trait of interest (16).

Given that certain alleles, governing the expression of 11S and 7S proteins, may have been lost during the domestication of *G. max,* such putative alleles may be recovered from accessions of the ancestor of cultivated soybean, *Glycine soja* (Sieb. and Zucc.). Earlier reports have indicated significant genetic diversity in the protein composition of wild soybeans (17). The current analysis of storage proteins in wild soybeans by two-dimensional scanning densitometry confirmed that observation and demonstrated that changes in protein composition influenced essential amino acid concentration. Because interspecific hybridization is possible between wild and cultivated soybeans, findings of this investigation could lead to the discovery of alternative alleles for protein synthesis in *G. soja* germplasm that may accelerate development of new *G. max* varieties with higher quality protein.

MATERIALS AND METHODS

Forty-five Maturity Group V accessions of wild soybean (*G. soja* Sieb and Zucc.) were selected from the USDA Soybean Germplasm Collection at the National Soybean Research Laboratory (Urbana, IL). Reagents and chemicals used in this work were of analytical or high-performance liquid chromatography (HPLC) grade. Protein was extracted for 30 min at room temperature from full-fat meal in a 1:20 (wt/vol) ratio with 0.03 M Tris-HCl buffer, pH 8.0, that contained 0.01 M β-mercaptoethanol (18). The mixture was centrifuged at 10,000 × *g* for 10 min. Solubilized protein was stored at −20°C until use.

Polyacrylamide gel electrophoresis. Proteins and their subunits were separated in a Bio-Rad (Richmond, CA) Protean II vertical slab gel apparatus according to Chua (19) with the following modifications. Protein subunits were dissociated in 0.03 M Tris-HCl, pH 8.0, 2% (wt/vol) SDS, and 2% (vol/vol) β-mercaptoethanol in a boiling water bath for 10 min. Samples containing 50–100 µg protein were separated by using a linear 10–20% gradient polyacrylamide gel. Blank wells were left between samples to facilitate accurate quantitation after electrophoresis and prevent protein cross-contamination during electrophoresis. Electrophoresis was carried out at 10 mA/1.5 mm-thick gel until the tracking dye reached the bottom of the gel. Gels were stained with Coomassie brilliant blue R-250. Gels were then destained and dried with Bio-Rad GelAir Dryer.

Scanning densitometry. Dried gels were scanned with a Molecular Dynamics (Sunnyvale, CA) Personal Densitometer SI that was equipped with a HeNe laser light source. ImageQuant software for volume integration was used in data analysis to enable determination of total optical density (OD) of entire protein bands. Apparent OD of each protein was obtained by subtracting the local average background OD from the total OD of the protein bands within the same gel volume.

Total protein determination and amino acid analysis. Total protein concentration of seed was estimated from nitrogen content as determined by a modified Kjeldahl method (20). Total amino acid composition of proteins, extracted from selected *G. soja* accessions, was determined in triplicate by the method of Spackman *et al*. (21). Samples were hydrolyzed *in vacuo* with 6 N HCl that contained 0.01% phenol at 110°C for 24 h. Amino acid composition of each sample was analyzed by reverse-phase chromatography of precolumn derivatized hydrolysates (22) with a Hewlett-Packard (San Fernando, CA) Series 1050 HPLC system that was equipped with a Waters (Milford, MA) Pico-Tag column for amino acid analysis. All data were reported as means of three replications.

RESULTS AND DISCUSSION

The USDA Soybean Germplasm Collection currently has 1102 accessions of wild soybean. As determined by photoperiod response, 361 of these accessions are classified as Maturity Group V. Forty-five Maturity Group V accessions, that represent the inherent diversity in protein composition among this wild soybean germplasm, were selected for this investigation based on preliminary protein analyses by SDS-PAGE (Nelson, R.L., unpublished).

Precise documentation of genotypic variability in storage protein composition, obtained from gel electrophoresis, is critical to advances in soy-protein quality through plant breeding. Scanning densitometry has been used for almost 30 years to quantify the OD or determine the mobility of protein bands on electrophoretic gels. Frequently, one-dimensional densitometry with a single vertical density is used to determine peak areas and apparent protein composition. However, electrophoresis of proteins tends to yield uneven band distributions or wide variations in band sizes. These anomalies do not produce representative band regions in which area measurements are normally made with single-line scanning densitometry. A true representation of all protein bands is difficult, even with wide-line scanning techniques that are limited to straight lanes and do not allow absolute measurements of OD for discrete protein areas to be made. Thus, the analyses reported in this paper were performed with a two-dimensional densitometer equipped with computer-aided volume integration capability. Because volume integration measures the absolute OD of whole bands and discrete regions on a scanned image, this technique corrects for uneven distribution of material within individual bands. The authors believe such attention to detail is needed to identify useful genetic resources among soybeans, especially when the 11S:7S ratio may be a practical indicator of genes that contribute beneficial protein-quality effects in breeding populations.

To simplify presentation of these data, eight accessions were chosen to demonstrate the range of phenotypic diversity among wild soybean germplasm samples (Table 1). Significant variation in seed mass (9.3–22.6 mg/seed) and total seed protein (40.1–51.1% dry mass) was observed among the 45 accessions tested. In comparison to cultivated soybeans, the

TABLE 1
Protein Composition of Selected Wild Soybean, *Glycine soja*, Germplasm in Maturity Group V

Germplasm accession	Seed		11S Subunits		7S Subunits		Total	
	Mass (mg/seed)	Protein (% dry wt)	Acidic	Basic	α′ + α	β	11S + 7S	11S:7S (ratio)
			(% soluble protein)					
PI 339733	22.6	45.5	24.6	20.3	21.2	4.6	70.7	1.7
PI 407173	16.6	51.0	25.5	19.9	18.6	4.1	68.1	2.0
PI 407031	13.0	43.6	26.8	19.3	16.1	2.6	64.8	2.5
PI 407049	17.5	46.2	27.4	21.5	15.3	2.2	66.3	2.8
PI 504289	19.6	46.9	27.9	20.8	13.5	1.6	63.7	3.2
PI 407222	15.8	46.5	31.6	24.7	13.5	2.2	72.0	3.6
PI 407308	19.8	51.1	29.4	22.6	12.7	1.3	66.1	3.7
PI 504290	18.1	45.7	30.3	23.3	10.1	1.0	64.7	4.9
$LSD_{0.05}$	1.3	0.9	0.7	0.6	0.7	0.3	1.1	0.2
Range[a]	9.3–22.6	40.1–51.1	23.0–31.6	16.0–24.7	10.1–21.2	1.0–5.3	57.5–72.0	1.7–4.9
Mean[a]	16.5	45.9	26.9	20.7	15.2	2.7	65.5	2.7

[a]Range or mean among 45 *G. soja* accessions tested.

dry mass of these wild soybeans ranged up to about 10% of the size of common *G. max* varieties; and protein concentration was equal to or greater than the typical cultivar mean (*ca.* 40.8%, dry mass basis).

The 11S plus 7S fractions accounted for 57.5–72% of soluble protein in these 45 accessions, but no significant correlation was found between seed total protein concentration and the combined concentration of the predominate storage protein constituents. However, a high degree of variation in protein composition was evident from 11S:7S ratios among these lines (Fig. 1). Over all 45 accessions, this ratio ranged from 1.7–4.9; 1.3 ± 0.6 may be considered a normal 11S:7S ratio in cultivated soybean.

Abundance of specific protein subunits contributed to the apparent phenotypic variation in protein composition. Within the 11S fraction, the combined concentration of constituent acidic subunits ranged from 23.0–31.6%, and the combined concentration of basic subunits ranged from 16.0–24.7% of total soluble protein (Table 1). This variation may be attributed to the absence or presence of different molecular species of acidic or basic subunits. In *G. max*, 11S acidic (28–45 kDa) and basic subunits (18–22.5 kDa) usually are paired by disulfide linkages (23, 24); a similar association is assumed for 11S proteins in wild soybean. Within the 7S fraction from wild soybean accessions, the combined concentration of α plus α′ subunits ranged from 10.1–21.2%, while the β subunit ranged from 1.0–5.3% of total soluble protein. Again, these data suggested considerable variation due to the absence or presence of subunits. In *G. max*, 7S protein is formed by trimeric combinations of α′ (76 kDa), α (72 kDa), and β (53 kDa) subunits; a similar association is assumed in *G. soja*, in which the β subunit is virtually devoid of sulfur-containing amino acids (25). Considering the extreme examples among this germplasm, the high 11S:7S ratio in plant introduction PI 504290 was attributed to a greater concentration of 11S protein (acidic plus basic subunits, 53.6% of total soluble protein), and a significantly lower concentration of 7S protein (11.1% of total soluble protein), especially the β subunit. In contrast, the lowest 11S:7S ratio in PI 339733 was attributed to a lower concentration of 11S protein (acidic plus basic subunits, 44.9% of total soluble protein), and a significantly higher concentration of 7S protein (25.8% of total soluble protein). These relations were consistent over the entire group of the wild soybean accessions tested. However, variability in composition within a protein fraction also was evident. As shown in Figure 2, the ratio of acidic:basic 11S subunits declined as the 11S:7S ratio increased. This trend appeared to be linear (R^2, 0.79; slope, –0.22). No significant correlation was found between (α + α′):β 7S subunits and 11S:7S ratio. However, there was remarkable variation in 7S composition, in which (α +α′):β ranged from 2.1–12.4. These data further suggested a wide range of allelic variation for protein composition in wild soybean germplasm.

In addition to inherent differences in protein composition, differences in 11S protein content contributed a significant

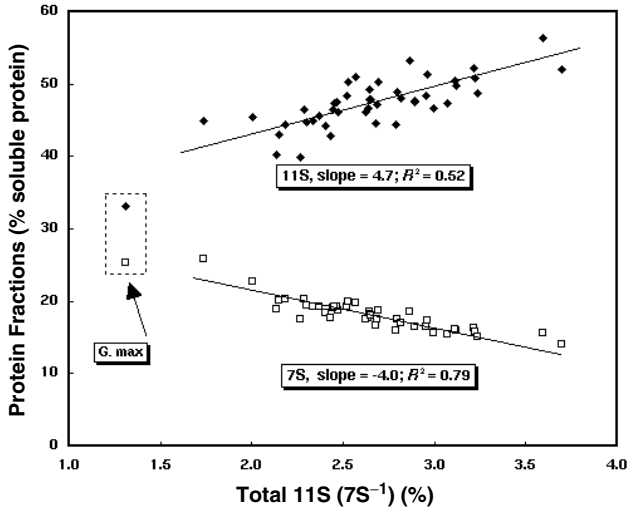

FIG. 1. Genotypic variation in 11S and 7S storage protein concentration among 45 Maturity Group V accessions of wild soybean, *Glycine soja* (Sieb. and Zucc.). The dotted rectangle represents a typical 11S and 7S protein concentration among cultivated soybeans (*Glycine max* L. Merr).

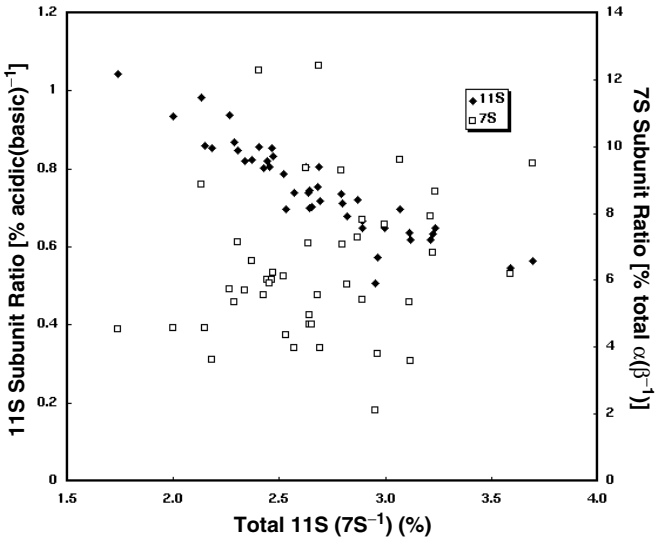

FIG. 2. Genotypic variation in expression of 11S and 7S storage protein subunits among 45 Maturity Group V accessions of wild soybean, *Glycine soja* (Sieb. and Zucc.). Regression of ratio of 11S subunits, % (acidic/basic), against % (total 11S/7S); $R^2 = 0.79$. Regression of ratio of 7S subunits,% ($\alpha + \alpha'/\beta$), against % (total 11S:7S); $R^2 = 0.11$.

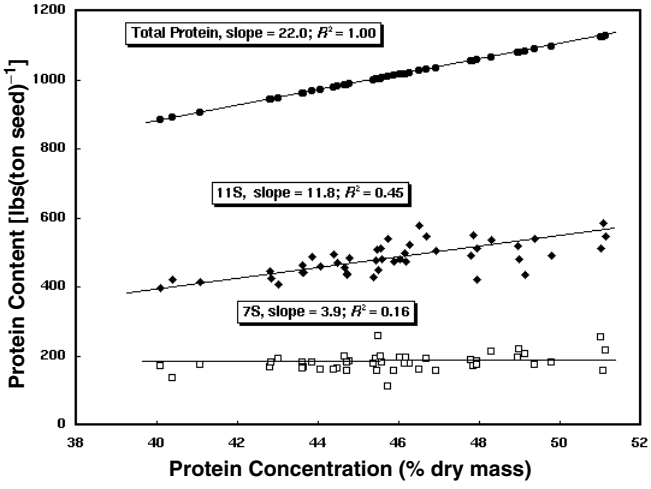

FIG. 3. Relation of 11S and 7S storage protein content to seed protein concentration among 45 Maturity Group V accessions of wild soybean, *Glycine soja* (Sieb. and Zucc.).

portion of the variation in total protein concentration among these *G. soja* accessions. When expressed on an equivalent dry seed mass basis (Fig. 3), these relations were linear, and the slope of the trend in 11S content accounted for about 54% of the increase in total seed protein content. About 45% of the variation in protein concentration among accessions was attributed to changes in the 11S content. Essentially no correlation was found between 7S levels and protein concentration. These data suggested that increased expression of the 11S fraction was associated with both elevated protein concentration and content in wild soybeans. Therefore, 11S:7S content may be a practical criterion in choosing parental lines for *G. max* × *G. soja* breeding populations in which the goal is varietal development to produce high-quality protein meal.

In order to evaluate the potential effects of altered 11S:7S ratio on nutritional quality, the amino acid composition of total seed protein was determined in the eight wild soybean accessions listed in Table 1. Regressions of 11S:7S against the concentration of individual essential amino acids for this set of germplasm are shown in Table 2. Strong positive correlations were found between 11S:7S and methionine or cysteine; moderate positive correlations were found for threonine or tyrosine; but, moderate negative correlations were found for the relations with valine, isoleucine, or lysine. Essentially no association was evident between 11S:7S and leucine, phenylalanine, or for total essential amino acid concentration. Based on this data, it appears that expression of greater amounts of 11S protein in relation to 7S protein has an overall positive effect on protein nutritional quality, especially for methionine and cysteine. Hence, genes that influence these trends in wild soybeans may exert similar effects on protein quality in the progeny of interspecific hybrids with cultivated soybeans.

In conclusion, wild soybeans have received little attention in the development of modern soybean cultivars. This is in part due to an extraordinary USDA soybean germplasm col-

TABLE 2
Effect of 11S:7S Ratio on Essential Amino Acid Concentration in Total Protein of Selected Wild Soybean Germplasm

Glycine soja Accession	11S:7S (ratio)	THR	TYR	VAL	MET	CYS	ILE	LEU	PHE	LYS	SUM	Total protein (lbs/ton)
						(% total protein)						
PI 339733	1.7	4.0	1.3	5.5	0.8	0.4	4.6	8.1	4.0	7.1	35.8	1002.2
PI 407173	2.0	4.0	1.3	5.7	0.7	0.4	4.7	8.1	3.9	6.9	35.7	1123.8
PI 407031	2.5	3.9	1.4	5.5	0.8	0.5	4.7	8.0	4.0	7.0	35.9	960.8
PI 407049	2.8	4.0	1.4	5.3	0.9	0.5	4.7	8.1	3.9	6.9	35.6	1016.7
PI 504289	3.2	4.5	1.7	4.8	1.0	0.6	4.2	8.0	4.1	6.3	35.1	1033.5
PI 407222	3.6	4.6	1.4	4.7	1.0	0.6	4.0	7.8	3.8	6.8	34.7	1024.4
PI 407308	3.7	5.0	1.9	4.6	1.1	0.7	4.0	7.6	3.8	6.0	34.6	1124.6
PI 504290	4.9	4.8	1.7	4.8	1.1	0.8	4.2	8.0	3.9	6.3	35.6	1007.2
$LSD_{0.05}$	0.2	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.3	28.8
Slope		0.3	0.2	–0.4	0.1	0.1	–0.2	–0.1	–0.0	–0.3	–0.2	
R^2		0.74	0.60	0.71	0.92	0.94	0.62	0.15	0.11	0.62	0.23	

lection that contains about 15,000 *G. max* accessions. Yet, certain breeding objectives may be slowed by difficulty in identifying germplasm with novel genetic variations for a given trait. Improvement of soybean-protein quality through altered expression of 11S and 7S proteins may be such an example. This research has demonstrated a remarkable level of phenotypic diversity in protein content and composition among a limited number of wild soybean accessions. By inference, these differences and related trends may be determined by genes that have been lost during the domestication of *G. max*. Considering that possibility, wild soybean germplasm may be a valuable genetic resource for genes that enhance the genetic complement in *G. max* to develop high-quality high-protein varieties for commercial production.

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