Genetic Improvement of Seed Storage Proteins Using Three Variant Alleles of 7S Globulin Subunits in Soybean (*Glycine max* L.)

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Genetically fixed "7S-low lines" were developed from a cross Keburi $\times$ F₁ (Oodate No. 1 $\times$ Mo-shi-dou Gong 503) in soybean. Seven S-low lines are characterized by the lack of the α' -subunit of 7S globulin and a marked decrease of both the α - and β -subunits of 7S globulin due to the simultaneous incorporation of α' -null, α -low and β -low alleles. The 7S-low lines and ordinary varieties were examined for their respective contents of 7S and 11S globulins, total protein contents, development of protein bodies and amino acid composition in the mature seeds. The contents of 7S globulin in the 7S-low lines were only half of those in the ordinary varieties, whereas the contents of 11S globulin in the lines were about 14% higher than those in the ordinary varieties. The marked modifications in the protein composition of the seeds did not adversely affect the total protein contents as well as development and morphology of the protein bodies. A high negative correlation was found between the 7S and 11S globulin contents ($r = -0.84$, $P\{r=0\} < 0.001$), and the calculated absolute value of the slope of the regression line was slightly larger than 1.0 ($a = -1.5$). These results suggest that 11S globulin may compensate for the decrease in 7S globulin to maintain the production of total seed protein in the 7S-low lines. The mean value of the sulfur-containing amino acid contents in the seeds of four representative 7S-low lines was about 20% higher than that of four ordinary varieties.

KEY WORDS: *Glycine max*, β -conglycinin, glycinin, amino acid composition, protein body.

Introduction

The two varieties, Keburi and Mo-shi-dou Gong 503, are important genetic resources which may contribute to the improvement of the protein composition in soybean seeds. Keburi and Mo-shi-dou Gong 503 are characterized by the absence of the α' -subunit of 7S globulin (one of the major storage proteins) and low levels of both the α - and β -subunits of 7S globulin, respectively (KITAMURA and KAIZUMA, 1981). It was shown that the subunit variations resulted in higher contents of sulfur-containing amino acids in the seed proteins of the two varieties than in the ordinary varieties. The absence of the α' -subunit is controlled by a single recessive α' -null allele (KITAMURA *et al.* 1984, TSUKADA *et al.* 1986) and the marked reduction in the α - and β -subunits by respective independent single α -low and β -low alleles (TSUKADA *et al.* 1986).

On the other hand, there is a genetic polymorphism of 11S globulin (another major storage protein) which is independent of that of 7S globulin (KITAMURA *et al.* 1980, MORI *et al.* 1981.) Eleven S globulin of an ordinary variety can be classified into two types, A₅- or A₄-types, according to the presence or absence of the intermediate subunit which is formed by the A₅-subunit and the paired basic subunit of 11S globulin, respectively. The absence

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of the intermediate subunit is controlled by a single recessive allele (HARADA *et al.* 1983, KITAMURA *et al.* 1984).

To incorporate the three variant alleles for 7S globulin into one genotype, we made a cross between Keburi (A₅-type variety) and a F₁ plant, which was derived from a previous cross between Oodate No.1 (A₄-type variety) and Mo-shi-dou Gong 503 (A₅-type variety) in 1981. After the F₁ plant was grown in the next year, breeding tests were begun using the half-seed analysis to select objective genotypes. Small cotyledonary parts (abaxial side) of the individual F₂ seeds harvested from the F₁ plant were used as materials for the analysis of the 7S and 11S globulin subunit composition by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The preserved parts (antihilum side) of the F₂ seeds in which the three variant alleles were simultaneously incorporated were sown to grow the next generation and were selfed for further 4-5 generations. As a result, we eventually obtained two types of "7S-low lines" which are genetically fixed for the three variant alleles of the 7S globulin subunits and the allele producing or lacking the A₅-subunit of 11S globulin, respectively. It was shown by preliminary tests in the DAVIS disc-PAGE and successive densitometric analyses that the 7S-low lines had much lower 7S globulin contents than the ordinary varieties.

In this study, attempts were made 1) to establish the most reliable method for the quantitation of 7S and 11S globulins by use of single radial immunodiffusion with anti-7S and -11S sera, respectively, 2) to determine precisely the effects of the variant alleles on the protein composition of the seeds and 3) to confirm the increase in the sulfur-containing amino acid contents in the seeds of the 7S-low lines in comparison with those of some ordinary varieties. The results indicated that the concomitant incorporation of the three variant alleles into one genotype led to a marked reduction in the content of 7S globulin and to a simultaneous increase in the contents of 11S globulin in the lines, with the content of the sulfur-containing amino acids in the seeds being 20% higher than that of ordinary varieties.

Materials and Methods

Lines and varieties

Five lines designated A (genetically fixed for α' -null, α -low and β -low alleles and A₅-type for 11S globulin), 5 lines designated E (equivalent to A lines for three variant alleles of 7S globulin but A₄-type for 11S globulin), 20 ordinary varieties of A₅-type (having wild type of 7S globulin subunits and A₅-type for 11S globulin), 20 ordinary varieties of A₄-type (having wild type of 7S globulin subunits and A₄-type for 11S globulin), Keburi (having α' -null allele and A₅-type for 11S globulin) and Mo-shi-dou Gong 503 (having α -low and β -low alleles and A₅-type for 11S globulin) were used as materials. These 52 lines and varieties were grown and harvested at the Iwate University Experimental Farm in the summer of 1985. About 10 g of seed samples from each line or variety were ground and used for the determination of the total protein content by a NEOTEC near infrared analyzer Model 102.

Purification of 7S and 11S globulins

Seven S and 11S globulins were purified from THANH's crude 7S and 11S fractions (THANH and SHIBASAKI, 1976) by ammonium sulfate fractionation and column chromatography

using Con-A Sepharose 4B and Sephacryl S-300, essentially according to the methods of IIBUCHI and IMAHORI (1978) and KITAMURA *et al.* (1976), respectively.

Preparation of anti-7S and -11S globulin sera from rabbits

Antisera to 7S and 11S globulins were prepared by using young adult rabbits according to the method of KOSHIYAMA and FUKUSHIMA (1976). Both globulins purified from Suzuyutaka (A₄-type variety) were injected as antigens. Antisera thus obtained were stored at -80°C until use.

Single radial immunodiffusion

The contents of 7S and 11S globulin in the crude extracts from the seeds of 52 lines and varieties were determined by the single radial immunodiffusion method of MANCINI *et al.* (1965).

Fifty mg of acetone-defatted meal was extracted with 4ml of a phosphate buffer (32.5mM K₂HPO₄, 2.6mM KH₂PO₄, 0.4M NaCl, pH 7.6) for 60 minutes at room temperature, centrifuged at 15,000g for 20 minutes and the supernatant was used as 7S and 11S crude extracts. A series of standard solutions for 7S and 11S determinations were prepared from both of purified globulins. Total protein contents of the crude extracts and the standard solutions were estimated by the Biuret method (GORNALL *et al.* 1949).

Heated solution containing 150 mg of agarose in 10 ml of the extraction phosphate buffer with 0.1% NaN₃ was prepared and cooled to 45°C. Then about 200 µl of anti-7S or -11S sera were added to 10 ml of the agarose solution. After gentle mixing, the solution was immediately poured into a mold to give a 8×10×0.1cm³ size. After solidification in a water bath at about 10°C for 30 minutes, the upper glass was removed and wells 3 mm in diameter were punched out at 15 mm intervals in the agarose gel. Five µl of the crude extracts and of a series of standard solutions (7S or 11S globulin) containing 0.25, 0.50, 0.75 and 1.00 mg of protein / ml were applied to the wells. The gel was immersed in paraffin oil and incubated during diffusion at 35°C for 48 hours. After incubation, the diameter of the immunoprecipitation ring formed around each well was measured. The contents of 7S or 11S globulins of the crude extracts were calculated from the calibration curves drawn from proportional relations between the areas of immunoprecipitation rings and the given quantity of 7S or 11S globulin in the standard solutions. The contents of 7S globulin in the crude extracts of all the soybean varieties tested were determined using the purified 7S globulin from Suzuyutaka, while the contents of 11S globulin in the crude extracts of the A₄- and A₅-type varieties or lines were separately determined using the purified A₄-type 11S globulin from Suzuyutaka and the purified A₅-type 11S globulin from Nattoshoryu, respectively.

Amino acid analysis

Seed proteins (1% NH₄OH-extracts) were hydrolysed for 24 hours at 110°C in sealed evacuated tubes in an excess amount (about 600-fold volume to the protein) of 6N HCl with a constant boiling temperature. The cystine and methionine contents were determined after performic acid oxidation according to the method of HIRS (1967). The amino acid composition of the hydrolysates was determined in an ATTO amino acid analyzer MLC-203.

Light microscope observation of protein body development

Imbibed seeds with water were fixed in a 2% glutaraldehyde solution overnight at 4°C and successively in an 1% osmic acid solution for 1.5 hours at 4°C. Fixed cotyledon tissues were embedded in epoxy resin. Sections (0.5–1.0 μ m) were viewed with a light microscope after light staining with 0.25% Coomassie Brilliant Blue R-250 in 7% acetic acid.

Results

Difference in antigenicity between A₅-type and A₄-type 11S globulins

Fig. 1 shows typical single radial immunodiffusion gels of 7S(A) and 11S(B) globulins using anti-7S and -11S sera, respectively. In the course of assaying, a difference in the antigenicity between the A₅-type and A₄-type 11S globulins was detected as the area of the immunoprecipitation ring of the A₄-type 11S globulin purified from Suzuyutaka was about 20% larger than that of the A₅-type 11S globulin purified from Nattoshoryu when an equal concentration of the two antigens diffused in the gel containing the antiserum induced by the A₄-type 11S globulin (Table 1). Therefore, we used the A₄-type 11S globulin purified from Suzuyutaka and the A₅-type 11S globulin purified from Nattoshoryu as standard proteins to separately determine the contents of 11S globulin in the crude seed extracts of the A₄-type and A₅-type varieties or lines, respectively.

7S and 11S globulin contents

Fig. 2 shows the SDS-PAGE patterns of the total seed protein of six varieties, A and E

Fig. 1. Single radial immunodiffusion on the gels containing anti-7S globulin(A) and anti-11S globulin(B) sera. (A) The wells a, b, c and d were allocated to the 7S globulin standards containing 1.25, 2.50, 3.75 and 5.00 μ g of protein, respectively. The remaining wells were for crude protein solutions (0.35% protein concentration) of (e)Suzuyutaka, (f)Raiden, (g)Nattoshoryu, (h)Bonminori, (i)Keburi, (j)Mo-shi-dou Gong 503, (k)A line No.1, (l)E line No.1. (B) The wells m, n, o and p were allocated to A₄-type 11S globulin standards, and the wells q, r, s and t were allocated to A₅-type 11S globulins standards containing 1.25, 2.50, 3.75 and 5.00 μ g of proteins, respectively. The remaining wells were allocated as in the case of (A) except for the protein concentration (0.175%).

lines and the purified 7S and 11S globulins. It was confirmed by the SDS-PAGE analysis that the two lines tested had α' -null, α -low and β -low alleles. Densitometric analyses following electrophoresis indicated that the 7S-low lines had much lower 7S globulin contents and higher 11S globulin contents than the ordinary varieties (data not shown). The contents of 7S and 11S globulin of the lines and the ordinary varieties were determined by single radial immunodiffusion, as shown in Table 2. The data indicated that a significant alteration in pro-

Table 1. Difference in antigenicity between A₅-type and A₄-type of purified 11S globulins

Type of 11S globulin	Ionic strength (μ) ²⁾				
	1.0	0.8	0.5	0.3	0.1
A ₄ -type 11S (from Suzuyutaka)	100	100	100	100	100
A ₅ -type 11S (from Nattoshoryu)	77.3 ¹⁾	80.0	81.3	78.7	81.3

1) Values represent proportional areas of the immunoprecipitation rings formed by the anti-A₄-type 11S globulin sera (prepared from Suzuyutaka). Areas of the ring obtained by immunoprecipitation of the A₄-type 11S globulin were regarded as 100, and those of the A₅-type 11S globulin as relative values.

2) Ionic strengths were adjusted by adding NaCl (from 0.1 to 1.0M) to the extraction phosphate buffer (pH 7.6, μ =0.1).

Fig. 2. SDS-PAGE patterns of seed storage proteins: (1) purified A₅-type 11S globulin from Bonminori, (2) purified A₄-type 11S globulin from Suzuyutaka, (3) purified 7S globulin, (4) Suzuyutaka, (5) Raiden, (6) Nattoshoryu, (7) Bonminori, (8) Keburi, (9) Mo-shidou Gong 503, (10) A line No.1, (11) E line No.1.

Purified protein (2 mg) or soybean flour (10 mg) was suspended in 2 ml of 0.05M Tris-HCl (pH8.0) containing 0.2% SDS, 5M urea and 0.2M 2-mercaptoethanol. These protein sample solutions (about 10 μ l) were electrophoresed in a SDS-PAGE system [polyacrylamide gel with concentration gradient from 7.5%(top) to 15%(bottom) containing 5M urea] at a constant voltage of about 125 V for 5 hours. The gel stained with Coomassie Brilliant Blue R-250.

Table 2. Total and fractional protein contents of ordinary varieties and 7S-low lines

Group or variety	No. of lines tested	Total protein %	% of Total protein	
			7S	11S
7S-low lines:				
A lines (7S-low, A ₅ -type)	5	43.4 a ¹⁾	8.7 d ¹⁾	52.5 a ¹⁾
E lines (7S-low, A ₄ -type)	5	44.4 a	11.7 c	44.7 b
Ordinary variety:				
A ₅ -type variety (normal, A ₅ -type)	20	41.4 b	17.3 b	38.3 c
A ₄ -type variety (normal, A ₄ -type)	20	41.1 b	19.5 a	31.2 d
Keburi (α'-null, A ₅ -type)	—	39.4	15.7	41.8
Mo-shi-dou Gong 503 (α-low, β-low, A ₅ -type)	—	42.9	9.9	45.7

¹⁾ Different letters in a column indicate statistical significance of the differences among the mean values within a column at 5% level.

tein composition was brought about, when the three variant alleles of 7S globulin subunits were incorporated into one genotype. The contents of 7S globulin in the A and E lines were only about half of those in the ordinary varieties, whereas the contents of 11S globulin were about 14% higher than those of the ordinary varieties. Furthermore, the presence or absence of the A₅-subunit affected the contents of 11S globulin in the seeds: the A line and A₅-type varieties had higher 11S contents than the E line and A₅-type varieties, respectively. As a result, the 7S contents of the A line were the lowest, while the 11S contents were the highest among all the lines and varieties examined.

Table 2 shows that the 7S content decreased in the order of A₄-type, A₅-type, E line, A line, while the 11S content in the reverse order. The relation between the 7S and 11S contents is illustrated in Fig. 3 by plotting 52 data on a plane. There was a high negative correlation coefficient between the contents of the two globulins ($r = -0.84$, $P\{r=0\} < 0.001$). The slope value(a) of the regression line of the 7S content(x) to the 11S content(y) slightly exceeded 1.0 as absolute value ($a = -1.5$).

Total protein content

The averaged data of the total protein contents of the lines and varieties are listed in Table 2. Though in all of the four groups of lines and varieties the total protein contents in

the seeds (>40%) were high, a significant difference in the protein contents was found between the 7S-low lines and the ordinary varieties: the A and E lines had higher total protein contents than the ordinary varieties.

Microscopic observation of protein bodies

Light micrographs of transverse sections of mature cotyledon tissue are shown in Fig. 4. It was observed that many protein bodies were densely present in each of mature cotyledon cells and occupied most of the cell in the 7S-low lines (Fig. 4-C, -D) as well as in the ordinary varieties (Fig. 4-A, -B).

Amino acid composition

The amino acid composition of the seed proteins was analyzed in the representative four 7S-low lines and four ordinary varieties (Table 3). The averaged, combined contents of the two sulfur-containing amino acids (1/2 cystine + methionine) in the 7S-low lines (3.45 mol%) were 20% higher than those in the ordinary varieties (2.93 mol%). The difference was significant at 1% level.

Fig. 3. Regression of 7S globulin content on 11S globulin content. Note. ● A₄-type variety, ○ A₅-type variety, △ A line, ▲ E line, ◇ Keburi and ◆ Mo-shi-dou Gong 503
***Significant from zero at 0.1% level assuming $r=0$.

Discussion

Seven S globulin (β -conglycinin) and 11S globulin (glycinin) are the two major proteins in soybean seeds. Sulfur-containing amino acid contents of the two globulins are quite different: 11S globulin contains 2 to 3 times more cystine and methionine in unit protein than 7S globulin (KOSHIYAMA, 1968, CATSIMPOOLAS *et al.* 1971). Furthermore, it was reported by SAIO and WATANABE (1978) that the two globulins have very different functional properties in soy protein foods: the isolated 11S globulin fraction formed a much harder Tofu gel than the isolated 7S globulin fraction. These reports suggest that the genetic reduction in the 7S globulin content in the seeds may improve the nutrititional quality of the soybean protein and contribute to the production of soy protein foods with novel properties. Therefore, Keburi and Mo-shi-dou Gong 503 have been considered to be important genetic resources for improvement of the seed proteins in soybean.

The results in this study indicated that the incorporation of α' -null, α -low and β -low al-

Fig. 4. Light micrographs ($\times 400$) of cotyledon sections from mature seed of (A) Raiden, (B) Okushirome of the ordinary varieties and representative (C) A line, (D) E line of the 7S-low lines.

leles into one genotype through hybridization markedly affected the contents of 7S and 11S globulins in soybean seeds. Namely, the 7S-low lines produced only about half of 7S globulin and 14% more 11S globulin compared with the ordinary varieties. Moreover, these lines showed a considerable increase in the sulfur-containing amino acid contents though still limited

However, if the marked reduction in the content of 7S globulin were to be accompanied by a reduction in the production of total protein in the seeds, breeding by use of the three variant alleles may not be practical. Therefore, the following two characters of the seeds relating to the productivity of seed protein were analysed, i.e. the total protein contents and the development of protein bodies in mature cotyledon cells. The importance of the latter character has been pointed out in cereals carrying 'high-lysine mutant' alleles, such as opaque-2 (MUNCK and WETTSTEIN, 1976). The reduction in the prolamins fraction with a low content of lysine due to the alleles leads to the defective development of protein bodies in the mature endosperm cells, which is thought to be responsible for the reduction in the total production of protein in seeds and abnormality in the appearance of seeds. As shown in Table 2, the level of total proteins in the seeds was maintained even in the 7S-low lines in spite of the remarkable reduction in the content of 7S globulin. Microscopic observations of transverse sections prepared from mature cotyledon tissues demonstrated that the 7S-low

Table 3. Comparison of the amino acid composition of total seed proteins among representative four 7S-low lines and four ordinary varieties

Amino acid	Residues per 100 amino acid residues (mol percent)							
					A ₅ -type variety		A ₄ -type variety	
	A-1	A-2	E-1	E-2	Natto-shoryu	Bon-minori	Suzu-yutaka	Raiden
Asp	11.69	11.33	11.45	11.45	11.45	11.57	11.70	11.47
Thr	4.72	4.52	4.42	4.73	4.30	4.35	4.39	4.32
Ser	6.40	6.14	6.19	6.20	6.27	6.46	6.29	6.15
Glu	16.02	16.03	15.82	16.14	16.79	16.67	16.59	16.74
Pro	6.14	6.14	5.40	5.58	5.84	5.63	5.54	6.01
Gly	8.01	7.86	7.60	7.81	7.58	7.57	7.61	7.24
Ala	6.49	6.23	6.38	6.67	6.17	6.52	6.42	6.18
Val	5.58	5.41	5.38	5.39	5.34	5.39	5.57	5.52
1/2 Cys ¹⁾	2.05	1.95	1.94	2.15	1.69	1.66	1.86	1.61
Met ¹⁾	1.40	1.28	1.41	1.50	1.23	1.16	1.34	1.16
Ile	4.60	4.57	4.60	4.73	4.64	4.61	4.80	4.83
Leu	7.03	7.49	7.52	7.57	7.71	7.24	7.54	7.85
Tyr	2.27	2.65	2.63	2.46	2.70	2.36	2.28	2.65
Phe	3.83	4.27	4.59	4.30	4.52	4.00	4.34	4.70
His	2.22	2.10	2.12	2.01	2.22	2.23	2.29	2.14
Lys	5.14	5.63	6.40	6.01	5.42	5.50	5.53	5.51
Arg	6.36	6.36	6.31	5.36	6.11	7.12	5.97	5.95
Sulfur A.A.	3.45	3.23	3.35	3.65	2.92	2.82	3.20	2.77

¹⁾ Cystine and methionine were determined as oxidized forms, i.e. cysteic acid and methionine sulfone, respectively.

lines, regardless of A₅- or A₄-type 11S globulin, showed no apparent change in the morphology of the protein bodies compared with the ordinary varieties. In addition, the modification of the protein composition of the seeds was not associated with any abnormal traits, i.e. distorted seed appearance (e.g. wrinkling of seed), low germinability of seed, defective plant growth. Consequently it is suggested that the incorporation of the three variant alleles of 7S globulin subunits is effective and useful for developing commercial varieties with improved composition of seed proteins.

The data on the 7S and 11S globulin contents from the 52 varieties and lines examined showed that there was a significant, high negative correlation between the contents of both globulins. In addition, the slope value(a) of the regression line of the 7S globulin content(x) to the 11S globulin content(y) slightly exceeded 1.0 as absolute value (a = -1.5). This result suggests that in the 7S-low lines there is an overproduction of 11S globulin which compensates for the reduction in the production of 7S globulin associated with the three variant alleles in order to maintain the production of total seed protein at a level comparable with that of the ordinary varieties.

OSBORN and BLISS (1985) observed a similar compensatory relation between the two major storage proteins, lectin and phaseolin, in common bean seeds: phaseolin compensated for the absence of lectin in the near-isogenic lines without lectin. The mechanism that stimulates the increase in 11S globulin or phaseolin in place of the decrease in 7S globulin or lectin remains to be elucidated.

The content of the sulfur-containing amino acids in the 7S-low lines was 20% higher than that of the ordinary varieties on an average. However, the value was still too low to fulfill the requirements for animal feeds. Therefore, other novel variant alleles controlling the synthesis of 7S globulin subunits should be identified to achieve a further reduction in the content of 7S globulin along with the increase in the content of 11S globulin. The identification of α -null and β -null alleles would be desirable.

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7S グロブリンのサブユニット変異遺伝子による大豆タンパク質の改良

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毛振 $\times$ F₁ (大館1号 $\times$ 秣食豆 公503号) の交雑により自殖ホモ後代系統「7S 低下系統」が得られた。この7S 低下系統は、 α' -欠失、 α -低下、 β -低下遺伝子を合わせ持つことにより、種子タンパク質の主成分である7S グロブリンの α' -サブユニットを欠失し、 α と β の両サブユニットの生産量が大きく低下している。本研究では、これらの変異遺伝子が集積されたことによる諸形質への影響を、この7S 低下系統と普通品種とを比較し検討を行った。全種子タンパク質中の7S グロブリン含量は、7S 低下系統では普通品種の約半分まで低下していたが、一方、11S グロブリン含量は約14%増加していた (Table 2)。また、種子中の全タンパク質含量は、7S 低下系統の方が有意に高く (Table 2)、さらに、完熟子葉細胞中のプロテインボディにも、形態的な以上は見られなかった (Fig. 4)。52 の測定値から両グロブリン間の相関を調べたところ、有意に高い負の相関が認められ ($r=-0.84$, $P<0.001$)、その回帰曲線の傾きの絶対値は1よりわずかに大きかった ($a=-1.5$) (Fig. 3)。これらの結果により、 α' -欠失、 α -低下、 β -低下遺伝子を集積させた場合には、7S グロブリン生成量には大幅な低下が生じるが、11S グロブリン生成量の増加がその低下分を補い、種子中の全タンパク質の生成量を保持させると考えられた。全タンパク質のアミノ酸組成分析の結果、7S 低下系統4系統の総含硫アミノ酸含量 (半シスチン+メチオニン) の平均値は普通品種4品種の平均値の約1.2倍の値を示した (Table 3)。

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