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Alteration of Seed Storage Protein Composition in Soybean [*Glycine max* (L.) Merrill] Mutant Lines Induced by γ-Irradiation Mutagenesis

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Supporting Information

ABSTRACT: This study investigated the alteration of seed storage proteins in soybean mutants induced by γ-irradiation. Five soybean cultivars and four landraces were irradiated with 250 Gy of γ rays to induce variability. The seed storage protein profiles of 414 genetic fixed mutants (M_{12}−M_{20}) having excellent agricultural traits were analyzed by SDS-PAGE. Among the 414 mutants, 58 were identifed as lacking lipoxygenase, 89 lacking the α' subunit, 113 lacking the α subunit, and 40 with an altered β subunit. One hundred and forty-nine mutants lacked the A_3 subunit of glycinin. Fifty-four mutants showed higher trypsin inhibitor (TIA) activity, whereas 139 showed lower TIA activity compared to their original cultivars. The selected mutants with low amounts of antinutritional factors such as trypsin inhibitor, lipoxygenase, and α subunit will constitute genetic resources for improving soybean protein quality.

KEYWORDS: γ ray, mutation, SDS-PAGE, seed storage protein, soybean

INTRODUCTION

Soybean is an important plant protein source for human nutrition and animal feed due to its high protein content and quality. Two major storage proteins, 7S and 11S, account for about 70−80% of the total storage proteins in soybean seed.[1] Although neither protein is particularly abundant in sulfur amino acids, 11S glycinin is superior in its content of cysteine and methionine.[2] β-Conglycinin (7S globulin) is a trimeric glycoprotein consisting of three types of subunits, α, α', and β, of which only the α subunit of β-conglycinin has allergenic reactivity.[1,3] Glycinin (11S globulin) consists of five subunits, G1, G2, G3, G4, and G5, of which G1 and G2 are allergens.[4] The 11S and 7S contents have been reported to vary depending on the soybean variety and environment.[5]

The 11S/7S ratio can be used as an indicator of protein quality in seed storage proteins; a greater 11S/7S ratio implies better nutritional quality.[6] The composition of the 7S and 11S globulin subunits varies between soybean varieties and legume species. It was previously reported that the gelation force of 7S globulin is mainly due to hydrogen bonding, whereas that of 11S globulin is mediated by disulfide and hydrogen bonding. Disulfide bonding in 7S globulin is limited because it contains only 2−3 cysteine groups, but 11S globulin contains 6−37 sulfhydryl and disulfide groups per mole of protein.[7] Several induced mutants with altered soybean 7S storage protein subunits have been reported. Mutant lines lacking α, α', $(\alpha + \alpha')$, $(\alpha + \beta)$, or $(\alpha + \alpha' + \beta)$ subunits or with reduced levels of α and β subunits have already also been reported.[8,9]

γ-Irradiation affects proteins by causing conformational changes, oxidation of amino acids, rupture of covalent bonds, and formation of protein free radicals.[10] Chemical changes in proteins caused by γ-irradiation include fragmentation, cross-linking, aggregation, and oxidation by oxygen radicals generated upon radiolysis of water.[11] The hydroxyl and superoxide anion radicals generated by radiation of film-forming solution could modify molecular protein films by covalent cross-linkages formed in protein solution after irradiation.[12]

The objectives of this study were to identify the changes of seed storage proteins in soybean mutant lines induced by γ-irradiation mutagenesis and to select the excellent soybean mutant lines with superior characteristics for use as food or feed. To induce the soybean mutants, we irradiated 4 soybean landraces and 5 cultivars with 250 Gy of γ rays and selected 414 genetically fixed lines (M_{12}−M_{20}) with excellent agricultural traits from 1989 to 2007. For seed storage protein profiling, we studied the protein profiles of soybean mutant lines using SDS-PAGE.

MATERIALS AND METHODS

Plant Material and Mutagenesis. One thousand seeds each of the four soybean landraces (LS1, LS2, LS3, and LS4) and five cultivars [Bangsa (BS), Baekwoon (BW), Hwanggum (HG), Paldal (PD), and Suwon115 (SW115)] were irradiated with 250 Gy of γ rays generated using a ^{60}Co γ-irradiator (150 TBq of capacity; ACEL, Ontario, Canada) at the Korea Atomic Energy Research Institute (KAERI). The irradiated seeds and control were sown at the Radiation Breeding Research Farm. M_1 plants were harvested in bulk. M_2 plants were sown the next year, harvested individually, and carried forward to the M_3 generation as plant-to-row progeny were produced. We selected mutant lines with excellent agricultural characteristics, including yield, disease resistance, and tolerance of environmental stress, during the M_3−M_{20} generations. Finally, we selected 414 genetically fixed mutant lines and evaluated

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Table 1. Diversity of Agricultural Traits and KTi Characters among Four Soybean Landraces and Five Cultivars

line	seed coat color[a]	stem color[b]	flower color[b]	day to 50% flowering[c] (day)	maturity[c] (days)	100 seed weight[c] (g)	KTi activity[c] (TIU)	KTi type
LS1	Bl	P	P	67.3 ± 11.2	145.7 ± 13.8	33.9 ± 1.7	18.4 ± 0.6 a	a
LS2	Bl	P	P	56.0 ± 11.0	131.3 ± 17.7	29.1 ± 5.8	20.5 ± 0.9	a
LS3	Bl	P	P	68.5 ± 14.5	136.0 ± 17.9	7.3 ± 1.5	20.9 ± 0.4	a
LS4	Bl	P	P	62.3 ± 9.0	138.0 ± 15.6	35.4 ± 3.5	19.4 ± 0.4	a
BS	Y	P	P	61.5 ± 9.3	139.2 ± 12.3	16.0 ± 1.5	21.7 ± 0.3	a
BW	Y	P	P	60.3 ± 9.6	139.3 ± 17.2	20.8 ± 2.3	20.1 ± 0.4	a
HG	Y	P	P	54.3 ± 13.2	128.8 ± 20.0	27.7 ± 2.5	20.5 ± 0.4	a
PD	Y	P	P	47.3 ± 5.5	119.5 ± 13.4	15.5 ± 2.5	19.2 ± 0.4	a
SW115	Y	P	P	62.8 ± 9.3	132.2 ± 16.2	14.2 ± 3.0	17.5 ± 1.4	b
LSD						5.5	1.1	

[a] Bl, black; Y, yellow. [b] P, purple. [c] Mean ± SD.

morphological traits, including seed coat color, stem color, flower color, day to 50% flowering, maturity, and 100 seed weight.

Protein Profile Studies. Total crude proteins were extracted from 100 mg of dry seeds in 5 mL of 100 mM Tris-HCl buffer (pH 8.0) containing 20 mM $CaCl_2$. The suspension was centrifuged at 15000 rpm for 30 min at 4 °C. The clear supernatant was used for SDS-PAGE. For SDS-PAGE, 50 μL of the total crude protein was added to an equivalent amount of 5× SDS sample buffer (10% SDS, 50% glycerol, 1.96% β-mercaptoethanol, 0.002% bromophenol blue, and 1 M Tris-HCl, pH 6.8). The samples were held in boiling water for 5 min and then centrifuged for 5 min. Supernatants containing total seed protein were used for SDS-PAGE and nondenaturing PAGE. Ten microliters of the supernatant was used for 12% SDS-PAGE in a Mini-PROTEAN 3 Cell (Bio-Rad, Hercules, CA). After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue (CBB) prepared in destaining solution (acetic acid/methanol/water, 1:4.5:4.5 v/v), and the gel was destained in the same solution.

Trypsin Inhibitor Assay. Crude protein extracts were precipitated from 1 g of soybean dry seeds in 5 mL of 100 mM Tris-HCl buffer (pH 8.2) containing 20 mM $CaCl_2$. The suspension was centrifuged at 15000 rpm for 30 min at 4 °C. The clear supernatant was collected and used for trypsin inhibitor assay. The suspension was then centrifuged at 15000 rpm for 30 min, followed by collection of the clear supernatant and 100-fold dilution with trypsin inhibitor solution (TIS). Specifically, 200 μL of the trypsin solution (4 mg of trypsin in 200 mL of 1 mM HCl) was added to 200 μL of TIS, followed by preincubation for 10 min at 37 °C. Next, 500 μL of the substrate solution (80 mg of benzoyl-DL-arginine-*p*-nitroanilide in 2 mL of dimethyl sulfoxide diluted to 200 mL with 100 mM Tris-HCl (pH 8.2) containing 20 mM $CaCl_2$) was added, after which the samples were incubated at 37 °C for 10 min. The reaction was terminated by the addition of 100 μL of 30% acetic acid. The absorbance of the total reaction solution was measured at 410 nm.

RESULTS

Change in Agricultural Traits and Kunitz Trypsin Inhibitor (KTi) Activity between the Soybean Mutant Lines. The agricultural traits and KTi activity of the four soybean landraces (LS1, LS2, LS3, and LS4) and five cultivars (BS, BW, HG, PD, and SW115) were evaluated. Nine soybean genotypes showed differences in seed coat color, 100 seed weight, KTi activity, and KTi type (Table 1). The four landraces had black (Bl) seed coat color, whereas the five cultivars had yellow (Y). The 100 seed weight ranged from 7.3 g (LS3) to 35.4 g (LS4). KTi activity ranged from 17.5 to 21.7 trypsin inhibitor units (TIU) for SW115 and BS, respectively. Among the nine soybean genotypes, one cultivar, SW115, was of Tib type, whereas the other soybeans were of Tia type.

The 414 mutant lines derived from the 4 landraces and 5 cultivars showed normal plant growth, although they showed altered agricultural traits compared to each of their original soybean genotypes. Among the 414 mutant lines, those derived from the 5 soybean cultivars had a higher mutation frequency than those from the 4 landraces (Table 2). Especially, the mutant lines derived from cv. Bangsa (BS) showed the highest mutation frequency among the 5 cultivars. The 100 seed weight had the highest mutation frequency (31.6%) among the mutant lines. For KTi activity, 59 mutants from the 4 landraces were not significantly changed, whereas 193 of the 355 mutant lines from the 5 soybean cultivars did show significant changes (53.4%). Among the 5 soybean cultivars, cv. Bangsa (BS) had the mutant lines with the largest changes in KTi activity (64.3%). Of the 193 total soybean mutant lines with altered KTi activity, 139 showed decreases, whereas 54 showed increases in KTi activity compared to their original cultivars. The 98 mutant lines derived from cv. BS, BW, and PD showed lower KTi activity than their original cultivars, whereas the 4 mutant lines derived from SW115 showed higher activity. In the 91 mutant lines derived from cv. HG, 50 showed lower KTi activity and 41 mutant lines showed higher KTi activity than their original cultivars.

Change in Agricultural Traits and Kunitz Trypsin Inhibitor Activity between the Soybean Mutant Lines Derived from Four Landraces. Among the 17 soybean mutant lines derived from LS1, 2 mutant lines (LS1-15 and LS1-17) were altered in seed coat color from black to dark brown, whereas 2 (LS1-1-10 and LS1-15) showed smaller 100 seed weight (Supplemental Table 1, Supporting Information). KTi activity was not significantly changed in any of the 17 soybean mutant lines. The 10 soybean mutant lines derived from LS2 did not show alterations in morphological traits and KTi activity (Supplemental Table 2, Supporting Information). Thirteen soybean mutant lines derived from LS3 showed only one altered morphological trait, the 100 seed weight. The 100 seed weight of one mutant line, LS3-10, was about 2.3 times (17.1 g) higher compared to that of its original cultivar (7.3 g) (Supplemental Table 3, Supporting Information). Among the 19 soybean mutant lines derived from LS4, 4 (LS4-3, LS4-4, LS4-5, and LS4-6) had altered seed coat color. Specifically, two mutant lines (LS4-4 and LS4-5) were changed from black to yellow (Supplemental Table 4, Supporting Information). LS4-6 was changed from black to brown and LS4-3 from black to black-striped brown. Two mutant lines (LS4-3 and LS4-4) were also

Table 2. Number of Soybean Mutant Lines with Altered Agricultural Traits and KTi Activity

		no. of soybean mutant lines						
							KTi activity[a]	
original landrace or cultivar	no. of mutant lines	seed coat color	flower color	stem color	day to 50% flowering	100 seed weight	I	D
landrace								
LS1	17	2				2		
LS2	10							
LS3	13					1		
LS4	19	4	2	2				
subtotal	59	6	2	2		3		
cultivar								
BS	143		93	101		113		92
BW	6		4	4		4		2
HG	165						50	41
PD	27	11	2	2	11	9		4
SW115	14	1				2	4	
subtotal	355	12	99	107	11	128	54	139
total	414	18	101	109	11	131	193	

[a] I, increase in KTi activity; D, decrease in KTi activity.

changed in flower color and stem color from purple to white and from purple to green, respectively. KTi activity was not significantly changed in the 59 soybean mutant lines derived from the 4 soybean landraces.

Change in Agricultural Traits and Kunitz Trypsin Inhibitor Activity between the Soybean Mutant Lines Derived from Five Soybean Cultivars. Among the five soybean cultivars, cv. Bangsa (BS) showed the most mutation in morphological traits (Table 2). Ninety-three mutant lines were altered in flower color from purple to white. One hundred and one mutant lines were altered in stem color from purple to green (Supplemental Table 5, Supporting Information). One hundred and thirteen mutant lines were altered in 100 seed weight. Among the 113 mutant lines with altered 100 seed weight, 112 were reduced in weight and 1 was increased. Ninety-two mutant lines showed low-level KTi activity compared to their original cultivar, BS. Four mutant lines (BW-1, BW-7-1, BW-7-1-1, and BW-7-2) of a total of six derived from cv. Baekwon (BW) showed changes from purple to white and from purple to green in flower color and stem color, respectively (Supplemental Table 6, Supporting Information). Four mutant lines (BW-1, BW-4, BW-6, and BW-7-2) were altered in 100 seed weight. Specifically, three mutant lines (BW-1, BW-6, and BW-7−2) were decreased in seed weight, whereas BW-6 was increased. KTi activity was significantly decreased in the two mutant lines BW-1 and BW-7-2. One hundred and sixty-five soybean mutant lines derived from cv. Hwanggum (HG) did not show changes in morphological traits (Supplemental Table 7, Supporting Information). Ninety-one mutant lines showed significant changes in KTi activity. Among them, 41 mutant lines had higher KTi activity compared to their original cultivar, whereas 50 were decreased. Among the 27 soybean mutant lines derived from cv. Paldal (PD), 11 were altered in seed coat color (Supplemental Table 8, Supporting Information). Six mutant lines (PD-21, PD-22, PD-23, PD-24, PD-25, and PD-26) changed from yellow to black and five (PD-1, PD-5-4, PD-5-12-1, PD-5-12-11, and PD-16) from yellow to striped yellow. Two of the mutant lines (PD-5-4 and PD-13) showed changes from purple to white and from purple to green in flower color and stem color, respectively. Eleven mutant lines showed late day to 50% flowering, and 10 mutant lines showed heavier 100 seed weight compared to their original cultivar, PD. KTi activity was significantly decreased in four mutant lines (PD-5-10, PD-6, PD-11, and PD-19). Among the 14 soybean mutant lines derived from cv. Suwon115, SW115-5-1 and SW115-9-1-1 were altered in 100 seed weight (Supplemental Table 9, Supporting Information). SW115-5-1 was increased in 100 seed weight, whereas SW115-9-1-1 was decreased compared to their original cultivar, SW115. SW115-5-1 also changed its seed coat color from yellow to black. KTi activity was significantly increased in four mutant lines (SW115-9-1-1, SW115-10, SW115-11-2, and SW115-24).

Protein Profiling among the 414 Soybean Mutant Lines. Profiles of soybean seed storage protein among the 414 soybean mutant lines and their original landraces or cultivars were analyzed by SDS-PAGE. The crude soybean extracts contained many proteins covering a wide range of molecular masses, but the electrophoretic patterns of total seed protein subunits showed similarities among the four soybean landraces and five cultivars (Figure 1A). Panels B, C, and D, respectively, of Figure 1 show examples of the SDS-PAGE profiles obtained from the seed storage protein of the mutant lines derived from LS3, BS, and HG. Among the 414 mutant lines, the A_3 subunit had the highest mutation rate (36.0%), whereas the β subunit had the lowest (9.2%) (Figure 2). Alteration of the lipoxygenase band was observed in 58 mutant lines (14.0%). Three subunits (α', α, and β) of 7S globulin were changed by 21.5, 27.3, and 9.7%, respectively. However, in 11S globulin, only the A_3 subunit was changed (36.0%).

Protein Profiling among the Soybean Mutant Lines Derived from Four Landraces. Among the 59 mutant lines derived from the 4 soybean landraces, 5 mutant lines (LS2-1, LS2-2, LS4-7, LS4-9, and LS4-10) derived from LS2 and LS4 were characterized by a low level of lipoxygenase compared to their original landraces (Table 3). The α subunit of 7S showed the highest

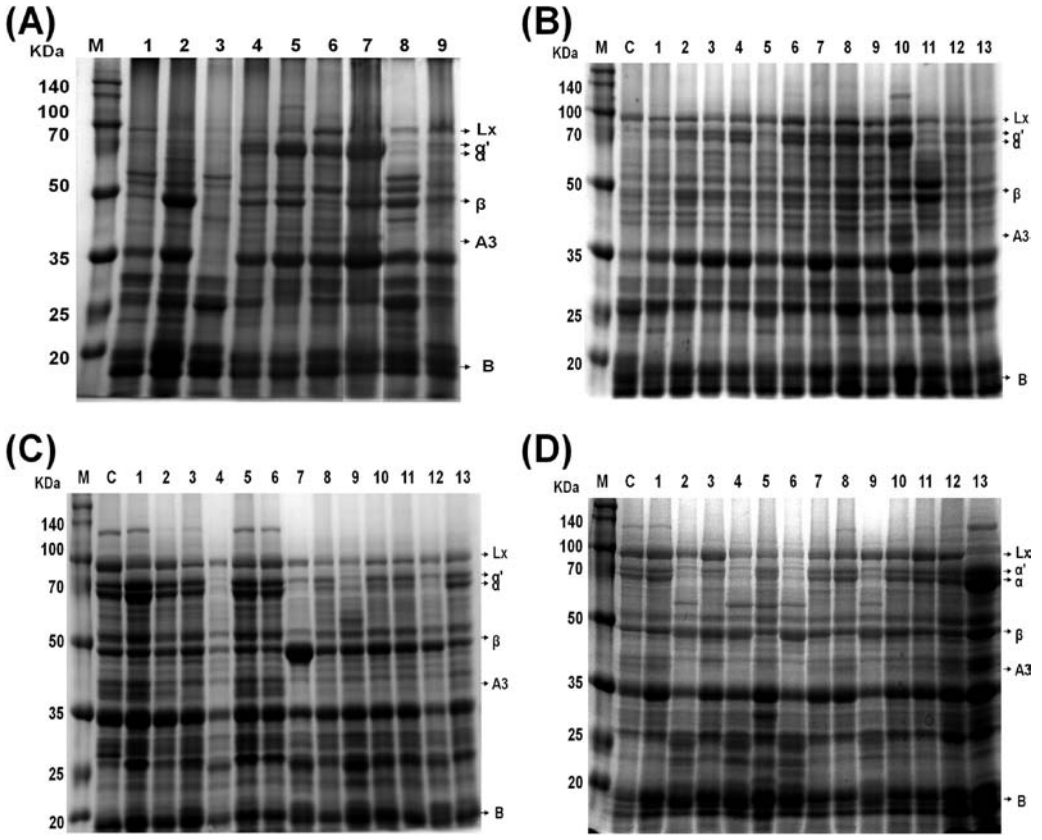

Figure 1. Protein profiles of soybean mutants derived from four soybean landraces and five cultivars by 12% SDS-PAGE. (A) Protein profiling of the four soybean landraces and five cultivars. Lanes: M, molecular weight marker; 1, LS1; 2, LS2; 3, LS3; 4, LS4; 5, BS; 6, BW; 7, HG; 8, PD; 9, SW115. (B) Protein profiling of landrace LS3 and the mutant lines derived from LS3. Lanes: M, molecular weight marker; 1, LS3; 2−13, LS3-1−LS3-13. (C) Protein profiling of cv. BS and the mutant lines derived from BS. Lanes: M, molecular weight marker; 1, BS; 2−13, BS1−BS13. (D) Protein profiling of cv. HG and the mutant lines derived from HG. Lanes: M, molecular weight marker; 1, HG; 2−13, HG40−HG52.

mutation rate (47.5%) compared to the other proteins. All mutant lines derived from LS3 showed an altered α subunit. The average number of changed protein bands in the mutant lines derived from the four soybean landraces was 1.2 per mutant, with LS3 having the highest (average 1.6) and LS2 the lowest (0.6). In the three subunits of 7S, the average number of changed protein bands was 0.7 per mutant.

Protein Profiling among the Soybean Mutant Lines Derived from Five Cultivars. Among the 355 mutant lines derived from the 5 soybean cultivars, 128 mutant soybean lines showed the highest frequency of altered A_3 subunit (36.0%) (Table 3). Lipoxygenase was altered in 53 mutant lines derived from three cultivars, BS, HG, and PD. Seventy-six lacking the α' subunit, 85 lacking the α subunit, and 36 with an altered β subunit (7S) were identified in SDS-PAGE. The average number of changed protein bands in the mutant lines derived from the five soybean cultivars was 1.12 per mutant. BS had the highest average (average 1.3) and BW the lowest (0.6). In the three subunits of 7S, the average number of changed protein bands was 0.5 per mutant.

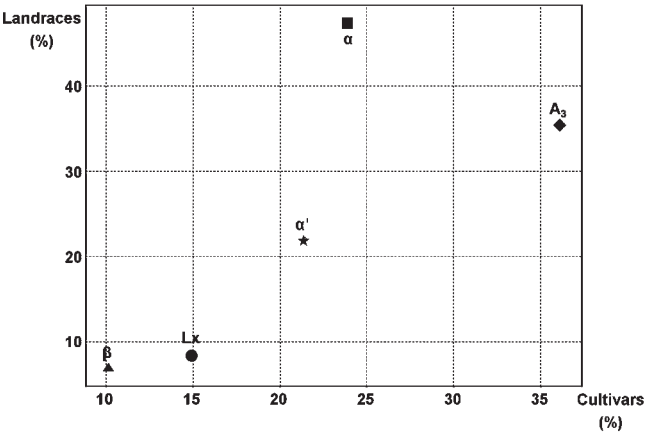

Figure 2. Percentages of changed proteins in the 414 mutant lines derived from the 4 soybean landraces and 5 cultivars.

DISCUSSION

In the various compositions of soybean seed storage protein, the α subunit of β-conglycinin is one of the major allergen materials causing soybean allergy disease, which is of atopic dermatitis.[13] Also, the α' and β subunits of β-conglycinin are considered to be potential food allergens.[14] In addition, the β subunit of β-conglycinin is not a desirable soybean protein subunit because it contains no methionine residues.[15] Therefore, by producing a recombinant β-conglycinin subunit with suppressed expression of the β subunit of soybean β-conglycinin, it should be possible to improve the quality of soybean.[16] In previous studies, several mutants of 7S globulin subunits were obtained.[8,9,13,17] Manjaya et al.[9] reported that the mutants M-17 and M-231 can be

Table 3. Number of Soybean Mutant Lines That Changed Protein Patterns

		no. of changed protein patterns[a]					av of changed protein bands	
line	no. of mutant lines	Lx	α'	α	β	A_3	7S	total
landraces								
LS1	17		5	6	1	7	0.7	1.1
LS2	10	2	2	1	1	1	0.4	0.6
LS3	13		1	13			1.0	1.0
LS4	19	3	5	8	2	13	0.8	1.6
subtotal	59	5	13	28	4	21	0.7	1.2
cultivars								
BS	143	23	44	52	7	62	0.7	1.3
BW	6		1	1		2	0.3	0.6
HG	165	27	25	26	22	54	0.4	0.9
PD	27	3	3	3	4	7	0.4	0.7
SW115	14		3	3	1	3	0.5	0.7
subtotal	355	53	76	85	34	128	0.5	1.0
total	414	58	89	113	38	149	0.5	1.1

[a] Lx, lipoxygenase; α', α' subunit; α, α subunit; β, β subunit; A_3, A_3 subunit.

identified by the lack of α' and α subunits of β-conglycinin and by increased levels of β subunits of β-conglycinin. In our study, mutant lines derived from soybean cultivars or landraces showed various changes of β-conglycinin. One hundred and thirteen mutant lines showed low or null band intensity of the α subunit. Eighty nine and 38 mutant lines showed changes of the α' and β subunit, respectively. These soybean mutant lines with changed β-conglycinin could be used as a food material with low levels of the soybean allergen. Especially, the 38 mutant lines with changed β subunit will help to breed soybeans containing higher sulfur amino acids.

In this study, 149 mutant lines were characterized by the lack of the A_3 subunit of glycinin. Khatib et al.[18] reported that the A_3 subunit has fewer cysteine residues than the other acidic subunits, which would affect its ability to form intermolecular disulfide linkages during gelation of the protein and contribute to the development of an elastic network. Poysa et al.[19] reported that the A_3 subunit played the major role in contributing to tofu firmness, regardless of coagulant. We considered that 149 mutant lines without the A_3 subunit were not suitable for making some foods such as tofu.

Soybean contains certain antinutritional factors such as trypsin inhibitors, lipoxygenase, phytic acid, saponins, and lectins, all of which exert a negative impact on the nutritional quality of protein.[20] The two major undesirable components of soybean, lipoxygenase and trypsin inhibitors, limit its wider utilization.[21] Lipoxygenase is considered to be an antinutritional factor due to its adverse effects on unsaturated fatty acids and flavor. This enzyme mediates the oxidation of linoleic and linolenic acids and consequently causes off-flavor in soybean food products and beverages.[22] Trypsin inhibitor is an antinutritional factor that affects protein digestibility.[23] Although it is heat-labile, heat treatment also insolubilizes the much-valued proteins[24] and, more importantly, can cause loss of amino acids in soy protein.[25] Hence, development of cultivars with low or null contents of lipoxygenase and trypsin inhibitors will help improve the nutritional quality of soybean. In our study, 27 mutant lines showed low-intensity or null lipoxygenase bands by SDS-PAGE. Furthermore, the BS100 mutant line showed the lowest trypsin inhibitor activity (12.3 TIU), and 143 mutant lines showed lower trypsin inhibitor activity than their original cultivar. These mutant lines with altered lipoxygenase or trypsin inhibitor activities could constitute a useful genetic stock for breeding low or null lipoxygenease and trypsin inhibitor lines.

Mutants with high oil content,[26] high protein content,[27] altered fatty acid composition,[28] altered soybean globulins,[29] high sulfur amino acid composition,[29] null lipoxygenase content,[30] and low lectin content[31] have been reported in soybean. In the present study, we identified mutant lines with various characteristics such as low trypsin inhibitor activity, low or null lipoxygenase content, and altered α', α, and β subunits of β-conglycinin. Thus, these mutant lines can be useful for the breeding of soybean varieties fit for consumption. Development of soybean lines with a lower content of antinutritional factors will promote the use of soy products in daily diet. Therefore, the selected mutant lines can be used to improve soybean protein quality, thereby reducing financial burden on the soybean industry for processing soybean meal.

■ ASSOCIATED CONTENT

Supporting Information. Soybean mutant lines used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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