

# The development of new soybean strain with *ti* and *cgy<sub>1</sub>* recessive allele

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**Abstract** Soybean [*Glycine max* (L.) Merr.] seed is an important dietary source of protein, oil, carbohydrate, isoflavone and other various nutrients for humans and animals. However, there are anti-nutritional factors in the raw mature soybeans. Kunitz trypsin inhibitor (KTI) protein and stachyose are the main anti-nutritional factors in soybean seed. The  $\alpha'$ -subunit of  $\beta$ -conglycinin protein exhibit poor nutritional and food processing properties. The genetic removal of the KTI and  $\alpha'$ -subunit proteins will improve the nutritional value of the soybean seed. The objective of this research was to develop a new soybean strain with KTI and  $\alpha'$ -subunit protein free (*titicgy<sub>1</sub>cgy<sub>1</sub>* genotype) and proper agronomic traits. A breeding population was developed from the cross of the BL-1 and 15G1 parents. A total of 168 F<sub>2</sub> seeds from the cross of the BL-1 and 15G1 parents were obtained. The segregation ratios of 9: 3: 3: 1 (104 *Ti\_Cgy<sub>1</sub>\_*: 30 *Ti\_cgy<sub>1</sub>cgy<sub>1</sub>*: 21 *cgy<sub>1</sub>cgy<sub>1</sub>Ti\_*: 13 *titicgy<sub>1</sub>cgy<sub>1</sub>*) between the *Ti* and *Cgy<sub>1</sub>* genes in the F<sub>2</sub> seeds were observed ( $\chi^2= 5.12$ ,  $P=0.5-0.10$ ). Two F<sub>4</sub> plant strains with proper agronomical traits and *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype (free of both KTI and  $\alpha'$ -subunit protein) were selected and harvested. 2 strains (S1 and S2) had yellow seed coats and hilum. The plant height of the S1 strain was 65 centimeters. The 100-seed weight was 29.2 g. The plant height of the S2 strain was 66 centimeters and 100-seed weight was 26.2 g. The two strains selected in this research will be used to improve the new cultivar that will be free of the KTI and  $\alpha'$ -subunit proteins.

**Keywords** Kunitz trypsin inhibitor (KTI),  $\alpha'$ -subunit, breeding lines, soybean

## Introduction

Soybean [*Glycine max* (L.) Merr.] seeds contain protein, carbohydrate, oil, vitamins, minerals, and many functional components. Also, there are antinutritional factors and several allergenic proteins in the raw mature soybean. Kunitz trypsin inhibitor (KTI) protein of mature soybean seed is a main antinutritional factor in soybean seed. KTI protein is a small and non-glycosylated protein containing 181 amino acid residues with 21.5 kDa. KTI protein was first isolated and crystallized from soybean seeds by Kunitz (1945). Kunitz trypsin inhibitor protein strongly inhibits trypsin, thus reducing food intake by diminishing digestion and absorption. Five electrophoretic forms of KTI have been discovered. The genetic control of four forms, *Ti<sup>a</sup>*, *Ti<sup>b</sup>*, *Ti<sup>c</sup>*, and *Ti<sup>d</sup>*, has been reported as a codominant multiple allelic series at a single locus (Sing et al. 1969; Hymowitz and Hadley 1972; Orf and Hymowitz 1979). Orf and Hymowitz (1979) found that the fifth form does not exhibit a soybean trypsin inhibitor-A2 band and is inherited as a recessive allele designated *ti*. Orf and Hymowitz (1979) identified two soybean accessions (PI157440 and PI196168) which lacks KTI protein from USDA germplasm collection. The *Ti* locus has been located on linkage group 9 in the classical linkage map of soybean (Hildebrand et al. 1980; Kiang 1987), which was integrated in linkage group A2 (chromosome 8) of the USDA/Iowa State University soybean molecular linkage map (Creagan et al. 1999).

$\beta$ -conglycinin (7S globulin) and glycinin (11S globulin) are the major components of storage protein in soybean.  $\beta$ -conglycinin consists of three subunits,  $\alpha'$ ,  $\alpha$ ,  $\beta$  (Thanh and Shibasaki 1976) and exhibits poorer nutritional and food processing properties than glycinin. Also,  $\beta$ -conglycinin contains much less sulfur-containing amino acid, methionine and cysteine, than glycinin (Koshiyama 1968). Several mutant lines affecting accumulation of  $\beta$ -conglycinin

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**Table 1** Seed coat color, hilum color and genotype for *Cgy1* (*cgyl*) and *Ti* (*ti*) alleles of the two parents used in this experiment

Parents	Trait/genotype					
	Seed coat color	Hilum color	KTI protein	<i>Ti</i> ( <i>ti</i> )	$\alpha'$ -subunit protein	<i>Cgy1</i> ( <i>cgyl</i> )
BL-1	Yellow	Yellow	Absent	<i>titi</i>	Present	<i>Cgy1Cgy1</i>
15G1	Yellow	Yellow	Present	<i>TiTt</i>	Absent	<i>cgylcgyl</i>

have been identified in soybean germplasms. Kitamura and Kaizuma (1981) identified Keburi, which was characterized by the absence of the  $\alpha'$ -subunit of  $\beta$ -conglycinin. Kitamura et al. (1984) reported that the absence of  $\alpha'$ -subunit were controlled by single recessive alleles, *cgyl*. Wild soybean line, QT2, which lacks  $\alpha'$ -subunit was also identified. In the QT2 line, the deficiency of  $\alpha'$ -subunit is controlled by a single dominant gene, *Scg* (Hajika et al. 1998). *Scg* gene was inherited independently with *cgyl* gene. Studies also showed that absence of the  $\alpha'$ -subunit in QT2 was inherited independently from the presence of lipoxigenase isozymes. Hayashi et al. (2000) identified AFLP marker to be tightly linked to the gene for deficiency of the  $\beta$ -conglycinin. Highly negative correlation between the contents of  $\beta$ -conglycinin and glycinin was reported by Ogawa et al. (1989). Breeding of soybean containing large amount of glycinin compared with current varieties is possible by selecting strain of soybean that does not exhibit or lacks  $\alpha'$ ,  $\alpha$ , and  $\beta$ -subunits of  $\beta$ -conglycinin. KTI and  $\alpha'$  protein limit the utilization of raw soybeans as direct feed requiring a heating step to denature the activity and energy costs as well as altering the physical properties of the soybean proteins. Also excessive heat treatment may lower amino acid availability. The genetic elimination of these factors could be an alternative to the heat treatment. Development of new soybean cultivar with both KTI and  $\alpha'$ -subunit protein free is needed to improve the nutrition values and to modify the food processing properties of soybeans. The objective of this research was to develop a new soybean strains with KTI and  $\alpha'$ -subunit protein free (*titicgylcgyl* genotype) and proper agronomic traits.

## Materials and Methods

### Breeding population

Two parents were used to develop breeding population. BL-1 parent has *Cgy1* gene ( $\alpha'$ -subunit of  $\beta$ -conglycinin protein present, *Cgy1Cgy1* genotype) and *ti* gene (KTI protein absent, *titi* genotype). 15G1 parent has *cgyl* gene ( $\alpha'$ -subunit of  $\beta$ -conglycinin protein absent, *cgylcgyl* genotype)

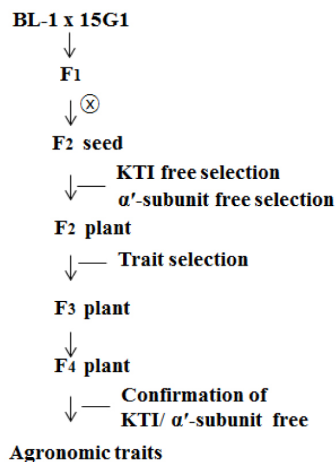
and *Ti* gene (KTI protein present, *TiTt* genotype). Seed traits and genotypes for *Cgy1*(*cgyl*) and *Ti*(*ti*) alleles of two parents are presented in Table 1. The seeds of BL-1 and 15G1 parents were planted to cross in a greenhouse. The crosses of BL-1 (*Cgy1Cgy1titi*) x 15G1 (*cgylcgylTiTt*) were made and F<sub>1</sub> seeds were obtained. F<sub>1</sub> seeds obtained were planted in greenhouse. F<sub>1</sub> hybridity was confirmed on morphological traits. F<sub>2</sub> seeds from F<sub>1</sub> plant confirmed were harvested. The F<sub>2</sub> seeds were analyzed to screen the seed with *cgylcgyltiti* genotype (KTI and  $\alpha'$ -subunit protein free).

### Determination of $\alpha'$ -subunit protein by SDS-PAGE

Crude protein from parent and each F<sub>2</sub> seeds extracted to determine the presence or absence of  $\alpha'$ -subunit protein of  $\beta$ -conglycinin electrophoretically. A piece of cotyledon from parent and each F<sub>2</sub> seed was removed and was incubated for 30 min (at room temperature) in 1 ml Tris-HCl, pH8.0, containing 1.56% v/v  $\beta$ -mercaptoethanol. After centrifugation, 50  $\mu$ l of the supernatant was added to an equivalent amount of 5X sample buffer [10% w/v sodium dodecyl sulfate (SDS), 50% v/v glycerol, 1.96% v/v  $\beta$ -mercaptoethanol, 1M Tris-HCl, pH 6.8]. The samples were boiled at 97°C for 5 min and then centrifuged. Two microliters of the supernatant were loaded on a 12% acrylamide SDS polyacrylamide gel electrophoresis (SDS-PAGE) medium gels in Owl Separation Systems Inc (Model:P9DS, Portsmouth, NH, USA). Electrophoresis was performed at 120 V for 7 hrs. Gels were stained overnight in an aqueous solution of 0.25 g Coomassie blue R250, 10% acetic acid, and 45% methanol. The gels were then destained with destaining solution (5% acetic acid, 14% methanol) for several hours. A Wide-Range SDS-PAGE molecular mass standard (Sigma Marker<sup>TM</sup>, Product Code: M4038, St.Louis MO, USA) containing the 72kDa (for  $\alpha'$ -subunit) was used to aid recognition of samples lacking the  $\alpha'$ -subunit of  $\beta$ -conglycinin protein.

### Determination of KTI protein by western blot analysis

Proteins of parent and each F<sub>2</sub> seed were separated by 10%

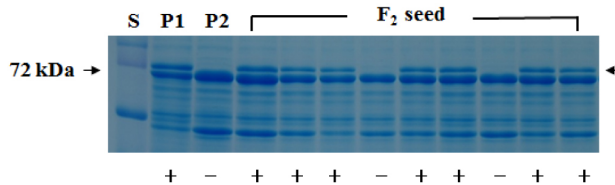


**Fig. 1** Scheme for development of new soybean strains with *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype (KTI and  $\alpha'$ -subunit protein free)

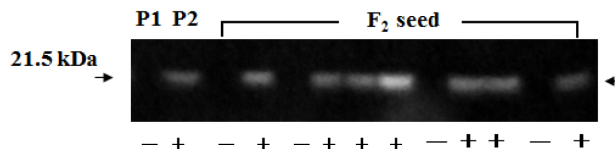
or 12% SDS-PAGE, and transferred onto Immobilon-Pmembrane (PVDF, Millipore). After blocking for 2hr in TBS buffer [20mM Tris(pH7.5), 150mM NaCl, and 0.1% Tween20] with 5% nonfat dried milk (Carnation, Glendale, CA) at room temperature, the membrane were incubated with KTI antibody for 1hr. After washing in TBS buffer three times, the blot was incubated with a horseradish peroxidase conjugated secondary antibody, and the complex was visualized using an enhanced chemiluminescence kit (Amersham, Bucking-hamshire, UK). The thickness of band was then determined visually.

Selection of *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype (KTI and  $\alpha'$ -subunit protein free)

The F<sub>2</sub> seeds with *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype (KTI and  $\alpha'$ -subunit protein free) were planted to advance F<sub>2</sub> plant generation. F<sub>2</sub> plants with a proper agronomic traits were individually harvested. Two strains with a proper agronomical traits were advanced to F<sub>4</sub> plant generation. Random F<sub>5</sub> seeds from each F<sub>4</sub> plant harvested were used to confirm absence for both KTI and  $\alpha'$ -subunit proteins. Plant height and 100-seed weight was recorded on the F<sub>5</sub> plant generation. Mean values of plant height and 100-seed weight were



**Fig. 2** Segregation of  $\alpha'$ -subunit protein in the parents and F<sub>2</sub> seeds. Arrow is the  $\alpha'$ -subunit protein band. S: marker, P1: BL-1, P2: 15G1. +, -: presence and absence of  $\alpha'$ -subunit protein, respectively



**Fig. 3** Segregation of the Kunitz trypsin inhibitor (KTI) protein in the parents and F<sub>2</sub> seeds. Arrow is the KTI protein band. P1: BL-1, P2: 15G1. +, -: presence and absence of the KTI protein, respectively

compared by Duncan's multiple range test at the 5% level. Scheme for development of new soybean strain with *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype is presented in Figure 1.

## Results and Discussion

A total 168 F<sub>2</sub> seeds from the cross of BL-1 and 15G1 parents were obtained and analyzed for the segregation of Kunitz trypsin inhibitor (KTI) protein and  $\alpha'$ -subunit protein of  $\beta$ -conglycinin. A part of the SDS-PAGE pattern for  $\alpha'$ -subunit protein that appeared in the parents and F<sub>2</sub> seed generation is shown in Figure 2.

Bands for  $\alpha'$ -subunit of  $\beta$ -conglycinin were segregated in F<sub>2</sub> seeds. Segregation pattern for Kunitz trypsin inhibitor (KTI) protein that appeared in the parents and F<sub>2</sub> seed generation is shown in Figure 3.

The segregation data for KTI protein and  $\alpha'$ -subunit protein of  $\beta$ -conglycinin protein in the F<sub>2</sub> seed generation are presented in Table 2.

The segregation ratios of 9 : 3 : 3 : 1 (104 *Ti\_Cgy<sub>1</sub>* : 30

**Table 2** Segregation for the presence or absence of Kunitz trypsin inhibitor (KTI) and  $\alpha'$ -subunit proteins in F<sub>2</sub> seed generation

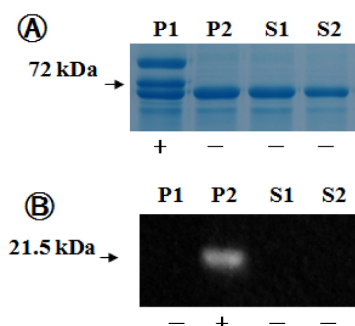
Seed protein		Seed number		$\chi^2$ value (9:3:3:1)	P
KTI	$\alpha'$ -subunit	Observed	Expected		
+	+	104	94.5	5.12	0.5 - 0.1
+	-	30	31.5		
-	+	21	31.5		
-	-	13	10.5		

+, -: presence and absence of protein.

**Table 3** Agronomic traits of parents and two new strains selected in this experiment

Parent/ strain	Plant height (cm)	Seed weight (g/100 seed)	Seed coat color	Hilum color
BL-1	63a <sup>2</sup>	24.5a	Yellow	Yellow
15G1	75b	32.1b	Yellow	Yellow
S1	65a	29.2b	Yellow	Yellow
S2	66a	26.1a	Yellow	Yellow

<sup>2</sup>Different letters in the column are significantly different by DMRT at 5%.

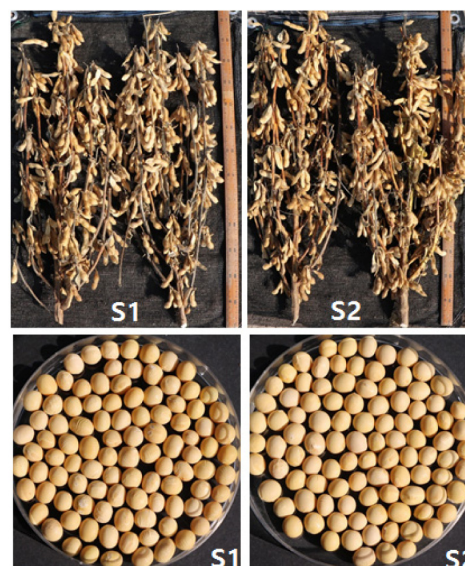


**Fig. 4** Confirmation of the  $\alpha'$ -subunit protein free (A) and Kunitz trypsin inhibitor (KTI) protein free (B) in the parents and new strains. Arrows are the  $\alpha'$ -subunit band (72 kDa) and KTI protein band (21.5 kDa). P1: BL-1, P2: 15G1, S1, S2: new strain (*titicgy<sub>1</sub>cgy<sub>1</sub>* genotype). +, -: presence and absence of  $\alpha'$ -subunit and KTI proteins, respectively

*Ti<sub>1</sub>cgy<sub>1</sub>cgy<sub>1</sub>*; 21 *cgy<sub>1</sub>cgy<sub>1</sub>Ti<sub>1</sub>*; 13 *titicgy<sub>1</sub>cgy<sub>1</sub>*) between *Ti* gene and *Cgy<sub>1</sub>* gene in F<sub>2</sub> seeds were observed ( $\chi^2 = 5.12$ ,  $P = 0.5-0.10$ ). This data showed that *Ti* gene was inherited independently with the *Cgy<sub>1</sub>* gene in soybean. These results supported the previous reports (Kitamura et al. 1984; Orf and Hymowitz 1979; Sung et al. 2010). Only thirteen F<sub>2</sub> seeds with recessive allele *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype were planted and Ten F<sub>2</sub> plants were individually harvested. Agronomic traits such as plant type, plant height, maturity date, lodging, seed quality, and 100-seed weight were evaluated on the ten F<sub>3</sub> plant strains with *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype. Two F<sub>4</sub> plant strains with a proper agronomical traits were selected and harvested. Random F<sub>5</sub> seeds from each F<sub>4</sub> plant strain were used to confirm absence for both KTI and  $\alpha'$ -subunit proteins (Fig. 4).

Kunitz trypsin inhibitor (KTI) and  $\alpha'$ -subunit proteins were not observed in the mature F<sub>5</sub> seed of two new strains. Agronomic traits of two parents and two new strains with *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype are presented in Table 3.

Two strains (S1 and S2) have yellow seed coat and hilum. Plant height of S1 strain was 65 cm compared to the parents of 63 ~ 75 cm. The 100-seed weight of S1 strain was 29.2 g compared to the parents of 24.5 ~ 32.1 g. Plant height of S2 strain was 66 cm compared to the



**Fig. 5** Plants (up) and seeds (down) of the two new strains (S1 and S2) with *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype

parents of 63 ~ 75 cm. The 100-seed weight of S1 strain was 26.1 g compared to the parents of 24.5 ~ 32.1 g. Plants and seeds of two new strains with *titicgy<sub>1</sub>cgy<sub>1</sub>* genotype are shown in Figure 5.

Two strains selected in this research will be used to improve new cultivar with free of KTI and  $\alpha'$ -subunit proteins.

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