Breeding of Black Soybean Line with *ti* and *le* Allele

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ABSTRACT Soybean is an economically important leguminous seed crop for feed and food products that are rich in seed protein, oil and saccharides. Also, black soybean has been known as medicinal food. However, Kunitz trypsin inhibitor (KTI) and lectin proteins are a main antinutritional factor in mature black soybean seed. The genetic removal of the KTI and lectin proteins will improve the nutritional value of black soybean seed. The objective of this research was to develop a new black soybean lines with double recessive allele for both KTI and lectin protein. A total of 179 F₂ seeds were obtained from the cross of 'Gaechuck#1' (*Le/Le, ti/ti* genotype) and 'LE-16' (*le/le, Ti/Ti* genotype) parents. The segregation ratios for the presence or absence of KTI and lectin proteins in the F₂ seed generation were well fitted to an expected 3:1 ratio. The segregation ratios of 9:3:3:1 (113 *Ti_Le_*:28 *Ti_lele*:30 *titiLe_*:8 *titilele*) between *Ti* gene and *Le* gene in F₂ seed generation were observed ($\chi^2=3.71$, $P=0.5-0.1$). From eight F₂ seeds with double recessive allele (*ti/ti, le/le* genotype), one new strain (*titilele*, both KTI, and lectin protein free) was developed. New strain has black seed coat, black hilum and green cotyledon in mature seed and 100-seed weight was 19.5 g. This strain will be helpful in breeding program for selecting black soybean line with lacking both KTI and lectin protein.

Keywords Kunitz trypsin inhibitor, Lectin, *ti*, *le*, Recessive allele, Soybean

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is the main source of protein and oil for human and animal nutrition. From the ancient time, black soybean has been consumed in Korea, China, and Japan. Black soybean has been known as medicinal food because of the presence of its antioxidant compound, especially anthocyanins in their seed coat. Anthocyanins are abundant in the epidermis palisade layer of black soybean seed coats. Three main anthocyanins (cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside) were characterized in black soybean seed coats (Taylor 1976; Choung *et al.* 2001). Previous reports have demonstrated the health-promoting benefits of anthocyanins, which include antioxidant effects, reduction in the risk of coronary heart disease, regulation of adhesion molecules, and protection from ischemia and reperfusion heart injury (Burns *et al.* 2000; Kim *et al.* 2006). In addition, it has been reported that the anthocyanins or their

components affect hyperglycemia and insulin secretion in a diabetic animal model (Jayaprakasam *et al.* 2005; Sasaki *et al.* 2007). Also, several antinutritional factors present in raw mature black soybean seeds. Kunitz trypsin inhibitor (KTI) and lectin proteins of mature black soybean seed reduce the nutritional value.

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). The KTI protein strongly inhibits trypsin, thus reducing food intake by diminishing digestion and absorption. In United States Department of Agriculture (USDA) germplasm collection, two soybean accessions (PI157440 and PI196168) have been identified which lacks KTI protein (Orf and Hymowitz 1979a). Five electrophoretic forms of KTI have been discovered. The genetic control of four forms, *Ti^a*, *Ti^b*, *Ti^c*, and *Ti^d*, has been reported as a codominant multiple allelic series at a single locus (Singh *et al.* 1969; Hymowitz and Hadley 1972; Orf

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and Hymowitz 1979a). Orf and Hymowitz (1979a) also found out that the fifth form does not exhibit a soybean trypsin inhibitor-A2 band and is inherited as a recessive allele designated as *ti*. 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 (Cregan *et al.* 1999).

Soybean lectin protein is a glycoprotein with a molecular weight of 120 kDa with four similar subunits (Pull *et al.* 1978). The soybean lectin is able to link to carbohydrate chains found in glycoproteins and glycolipids and present a strong affinity for N-acetyl-D-galactosamine and to a lower extent for D-galactose. This lectin-carbohydrate interaction will consequently result into a changed morphology of the intestinal epithelium, as well as a decrease in the digestion and absorption of nutrients (Schulze *et al.* 1995). The presence of seed lectin is controlled by a single dominant gene designated *Le* and the homozygous recessive *lele* results in the lack of lectin (Orf *et al.* 1978). *Ti* and *Le* loci were segregated independently (Orf and Hymowitz 1979b; Moraes *et al.* 2006; Lee *et al.* 2008). Recently, a new soybean line with triple null recessive genotypes (*ti/ti-le/le-p34/p34*) was developed (Schmidt *et al.* 2015).

KTI and lectin proteins 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. The objective of this research was to develop a new black soybean lines with double recessive allele for both KTI and lectin protein.

MATERIALS AND METHODS

Breeding population

Two parents were used to develop breeding population. ‘Gaechuck#1’ parent has *Le/Le* genotype (lectin protein present) and *ti/ti* gene (KTI protein absent). ‘LE-16’ parent

has *le/le* genotype (lectin protein absent) and *Ti/Ti* genotype (KTI protein present). The F_1 seeds were obtained from the cross of ‘Gaechuck#1’ and ‘LE-16’ parents. F_1 seeds were planted and F_1 hybridity was confirmed on morphological traits in the greenhouse. F_2 seeds derived from F_1 hybrid plants were harvested. All F_2 seeds harvested were analyzed for segregation and selection for KTI and lectin proteins.

Determination of Kunitz trypsin inhibitor protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)

Crude protein from the parents and individual F_2 seed was extracted to determine the presence (+) or absence (–) of KTI protein electrophoretically. A piece of cotyledon from the parents and individual F_2 seed was removed and was incubated for 30 minutes (at room temperature) in 1 ml Tris-HCl, pH 8.0, containing 1.56% v/v β -mercaptoethanol. After centrifugation, 50 μ l of the supernatant was added to an equivalent amount of 5X sample buffer (10% w/v SDS, 50% v/v glycerol, 1.96% v/v β -mercaptoethanol, 1 M Tris-HCl, pH 6.8). The samples were boiled at 97°C for 5 minutes and then centrifuged. Two microliters of the supernatant was 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 hours. 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 MarkerTM, product code: M4038; Sigma, St. Louis, MO, USA) containing the 21.5 kDa (for KTI protein) was used to aid recognition of samples lacking the KTI protein. χ^2 analysis was used to test the segregation ratio for KTI protein.

Determination of lectin protein by western blot analysis

Proteins of parent and each F_2 seed were separated by 10% or 12% SDS-PAGE, and transferred onto Immobilon-P membrane (PVDF, Millipore). After blocking for 2 hours in TBS buffer (20 mM Tris [pH 7.5], 150 mM NaCl, and

0.1% Tween 20) with 5% nonfat dried milk (Carnation, Glendale, CA, USA) at room temperature, the membrane were incubated with lectin antibody for 1 hour. 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, Buckinghamshire, UK). The thickness of band was then determined visually. χ^2 analysis was used to test the segregation ratio for lectin protein.

Development of double recessive genotype and seed traits

The F_2 seeds with double null allele (*ti/ti*, *le/le* genotype) were planted to advance F_2 plant generation. F_2 plants were individually harvested. Random F_3 seeds from each F_2 plant harvested were used to confirm absence for both KTI and lectin proteins. F_3 seeds with double recessive genotype (*ti/ti*, *le/le*) were planted to F_3 plant generation. One strain with a proper agronomic traits was selected. Seed coat color, hilum color, cotyledon color, and 100-seed weight (g) were recorded on the F_4 seeds harvested.

Fig. 1. Segregation of the Kunitz trypsin inhibitor (KTI) protein in the parents and F_2 seeds. S: KTI protein, P1: 'LE-16', P2: 'Gaechuck#1'. +, -: presence and absence of KTI protein, respectively.

Table 1. Segregation for the presence or absence of the Kunitz trypsin inhibitor (KTI) protein in F_2 seed generation.

KTI protein	No. of F_2 seed		χ^2 value (3:1)	P-value
	Observed	Expected		
+	141	134.25	1.36	0.5-0.1
-	38	44.75	2.24	0.10-0.20

+, -: presence (+) or absence (-) of KTI protein.

RESULTS

A total of 179 F_2 seeds were obtained from the crosses of 'Gaechuck#1' and 'LE-16' parents. F_1 seeds produced KTI and lectin proteins. A part of the SDS-PAGE pattern for KTI protein that appeared in the parents and F_2 seed generation is shown in Fig. 1.

KTI protein of 21.5 kDa was segregated in F_2 seed generation. Segregation data for presence and absence of KTI protein are presented in Table 1.

Among 179 F_2 seeds harvested, 141 F_2 seeds showed KTI protein and 38 F_2 seeds did not show KTI protein. The segregation ratio for the presence or absence of KTI protein in the F_2 seed generation was well fitted to an expected 3:1 ratio ($\chi^2=1.36$, $P=0.5-0.01$).

Segregation pattern for lectin protein that appeared in the parents and F_2 seed generation is shown in Fig. 2.

Segregation data for presence and absence of lectin protein are presented in Table 2.

Among 179 F_2 seeds, 143 F_2 seeds showed lectin protein and 36 F_2 seeds did not show lectin protein. The segregation ratio for the presence or absence of lectin protein in the F_2 seed generation was well fitted to an expected 3:1 ratio ($\chi^2=2.28$, $P=0.5-0.01$).

Fig. 2. Segregation of lectin protein in the parents and F_2 seeds. S: lectin protein, P1: 'Gaechuck#1', P2: 'LE-16'. +, -: presence and absence of lectin protein, respectively.

Table 2. Segregation for the presence or absence of the lectin protein in F_2 seed generation.

Lectin protein	No. of F_2 seed		χ^2 value (3:1)	P-value
	Observed	Expected		
+	143	134.25	2.28	0.5-0.1
-	36	44.75		

+, -: presence (+) or absence (-) of lectin protein.

Table 3. Segregation for the presence or absence of Kunitz trypsin inhibitor (KTI) and lectin proteins in F₂ seed generation.

Seed protein		Seed number		χ^2 value (9:3:3:1)	P-value
KTI	Lectin	Observed	Expected		
+	+	113	100.69	3.71	0.5-0.1
+	-	28	33.56		
-	+	30	33.56		
-	-	8	11.19		

+: presence, -: absence.

Fig. 3. Appearance of F₅ seed (double recessive allele, *ti/ti*, *le/le* genotype) with Kunitz trypsin inhibitor and lectin protein free, black seed coat and green cotyledon.

The segregation data for KTI and lectin proteins in the F₂ seed generation are presented in Table 3. The segregation ratios of 9:3:3:1 (113 *Ti_Le_*:28 *Ti_lele*:30 *titiLe_*:8 *titilele*) between *Ti* gene and *Le* gene in F₂ seed generation were observed ($\chi^2=3.71$, $P=0.5-0.1$).

Eight F₂ seeds with double recessive allele (*ti/ti*, *le/le* genotype) were planted and six seeds were germinated. Six F₂ plants were individually harvested. Random F₃ seeds from each F₂ plant were used to confirm absence for both KTI and lectin proteins. Among six F₃ plant lines, one line with a proper agronomical traits were selected and were advanced to F₄ plant generation. F₅ seeds (*ti/ti*, *le/le* genotype) are shown in Fig. 3. Seed coat and hilum color were black. Cotyledon color in mature seed was green and 100-seed weight was 19.5 g.

DISCUSSION

Soybean seed contains nutritional components such as protein, oil, carbohydrate, isoflavone, saponin, and anthocyanin as well as several antinutrients responsible for reducing the nutritional value of unprocessed soybean. The antinutrients include trypsin inhibitor, and lectin proteins. KTI and lectin proteins limit the utilization of raw soybeans as direct feed requiring a heating step to denature the activity that imposes facility and energy costs as well as altering the physical properties of the soybean proteins. The genetic removal of KTI and lectin proteins could be an alternative to the heat treatment. A total of 179 F₂ seeds were obtained to develop a new black soybean lines with double recessive allele for both KTI and lectin proteins from the crosses of 'Gaechuck#1' and 'LE-16' parents. KTI protein in F₂ seed generation was segregated (Fig. 1, Table 1). The segregation ratio for the presence or absence of KTI protein in the F₂ seed generation was well fitted to an expected 3:1 ratio ($\chi^2=1.36$, $P=0.5-0.01$). This result shows that the absence of the KTI protein is controlled by a single recessive gene. Previous studies have shown that KTI protein is inherited as a single recessive allele (Orf and Hymowitz, 1979a; Kim *et al.* 2006; Eun *et al.* 2012). Also, lectin protein in F₂ seed generation was segregated (Fig. 2, Table 2). The segregation ratio for the presence or absence of lectin protein in the F₂ seed generation was well fitted to an expected 3:1 ratio ($\chi^2=2.28$, $P=0.5-0.01$). These results substantiate earlier reports that *Le* locus is inherited as a single gene (Orf and Hymowitz 1979b; Sung *et al.* 2013).

The segregation data for both KTI and lectin protein in the F₂ seed generation were presented in Table 3. The segregation ratios of 9:3:3:1 (113 *Ti_Le_*:28 *Ti_lele*:

30 *ttiLe_8 titilele*) between *Ti* gene and *Le* gene in F₂ seed generation were observed ($\chi^2=3.71$, $P=0.5-0.1$). This data showed that *Ti* gene was inherited independently with the *Le* gene in soybean. These results substantiate earlier reports that *Le* and *Ti* loci are segregate independently (Orf and Hymowitz 1979b; Moraes *et al.* 2006; Lee *et al.* 2008). Moraes *et al.* (2006) reported that *Ti* and *Le* loci were segregated independently using only 24 F₂ individuals. Orf and Hymowitz (1979b) observed that *Ti* and *Le* genes were not linked using F₂ population with 96 individuals. Lee *et al.* (2008) were observed independent inheritance between *Ti* gene and *Le* gene from the 173 F₂ seeds. From eight F₂ seeds with double recessive allele (*ti/ti*, *le/le* genotype), one new strain was selected. Seed coat and hilum color of F₅ seed (*ti/ti*, *le/le* genotype) strain was black and cotyledon color in mature seed was green (Fig. 3). Genotype of new strain for *Ti* and *Le* loci was *titilele* (both KTI and lectin protein free) and 100-seed weight was 19.5 g. This strain will be helpful in breeding program for selecting black soybean line with lacking both KTI and lectin protein in mature seed.

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