ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2019 Vol. 29. No. 7. 774~778 DOI : https://doi.org/10.5352/JLS.2019.29.7.774

A Stack of Recessive Alleles of Kunitz Trypsin Inhibitor, Lectin, and Stachyose in Soybean

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Received June 10, 2019 / Revised July 7, 2019 / Accepted July 7, 2019

Soybean [*Glycine max* (L.) Merr.] is one of the major food sources of protein, oil, carbohydrates, isoflavones, and other nutrients for both humans and animals. However, soybean seeds contain antinutritional factors, such as lectin protein, Kunitz Trypsin Inhibitor (KTI) protein, and stachyose. The objective of this research was to stack recessive alleles for development a triple recessive genotype, *titilelers2rs2*, with low KTI protein, lectin protein, and stachyose contents. Three parents (Gaechuck#2, PI200508, and 14G20) were used to develop the breeding population. The presence or absence of the lectin and KTI proteins was detected by western blotting. The stachyose content in mature seeds was determined by HPLC. Agronomic traits, such as plant type, plant height, maturity date, lodging, seed quality, and 100-seed weight, were evaluated for the four F_3 plant strains. One F_4 plant strain with the desired agronomical traits was selected. One new strain with the triple recessive *titilelers2rs2* genotype was developed. The plant height of the new strain was 51 cm and the 100-seed weight was 31.0 g. The new strain had a yellow seed coat and yellow hilum. The stachyose content of the new strain was 3.8 g/kg. One strain developed in this research will be used to produce improved yellow soybean cultivars that are free of lectin and KTI proteins and low in stachyose content.

Key words : Kunitz trypsin inhibitor (KTI), lectin, stachyose, soybean, triple recessive

Introduction

Soybean is the main source of protein and oil for human and animal nutrition. Soybean seed is composed of 40% protein, 20% oil, 35% carbohydrates, and 5% ash on average [9]. Demand of soybean and soybean products has increased in recent years because of high quantity and quality of soybean protein and oil. Also, there are antinutritional factors and several allergenic proteins in the raw mature soybean. Lectin protein, Kunitz Trypsin Inhibitor (KTI) protein, and stachyose are a main antinutritional factor in soybean seed.

Soybean lectin protein is a glycoprotein with a molecular weight of 120 kDa with four similar subunits [17]. 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 [18]. 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 [15]. *Ti* and *Le* loci were segregated independently [11, 13, 16]. Recently, a new soybean line with triple null recessive genotypes (ti/ti-le/le-p34/p34) was developed [19].

Kunitz Trypsin Inhibitor (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 [10]. 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^a, Ti^b, Ti^c, and Ti^d, has been reported as a codominant multiple allelic series at a single locus [6, 16, 21]. Orf and Hymowitz [16] 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 [16] 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

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[5, 8], which was integrated in linkage group A2 (chromosome 8) of the USDA/Iowa State University soybean molecular linkage map [1].

Stachyose is a major soluble sugar second to sucrose in soybean seed [12]. Stachyose are considered undesirable sugars in soybean seed, because they are not readily digestible and cause flatulence or diarrhoea for non-ruminant animals [4]. Stachyose content ranges from 14 to 41 g/kg on a dry-weight basis [7]. Stachyose content in soybean is environmentally stable but genotypically dependent [3]. Stachyose content was shown to be controlled by a single gene or a major QTL [20, 22]. The low stachyose and high sucrose phenotype in PI200508 was reported as the result of a mutation on a galactosyl transferase gene, and the low-stachyose allele was labelled as*rsm1* [22]. There were no negative agronomic characteristics between lines derived from PI200508 containing the reduced stachyose content and wild types in traits of field emergence, seed yield, maturity, height, and fatty acid content [14]. Dierking and Bilyeu [2] found that a novel allele (the deletion of a codon encoding a conserved tryptophan residue at amino acid position 331, W331 −) of a raffinose synthase gene, raffinose synthase 2 (*RS2*, Glyma06g18890) was responsible for the improved seed composition phenotype of PI200508 through complete association of the PI200508 rs2 W331− allele with the increased sucrose and decreased raffinose and stachyose phenotype. The key enzyme in raffinose and stachyose bisynthesis is *RS2*. An *rs2* enzyme containing three-bp deletions results in low raffinose and stachyose in soybean line PI200508 [22]. *RS2* locus is located in chromosome 6. Lectin protein, KTI protein, and stachyose 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 lectin and KTI protein could be an alternative to the heat treatment. Also, soybean cultivars with genetically low levels of stachyose enhance the utilization of soybean in food as well as feed uses. Development of of new soybean cultivar with lectin and KTI protein free and low levels of stachyose is needed to improve the nutrition values and to modify the food processing properties of soybeans. The objective of this research was to stack of recessive alleles (development of triple recessive genotype, *titilelers2rs2*) of KTI protein, lectin protein and stachyose.

Materials and Methods

Breeding population

Three parents (Gaechuck#2, PI200508, and 14G20) were used to develop breeding population. Gaechuck#2 parent has *titiLeLeRS2RS2* genotype (KTI protein absent, lectin protein present, normal content of stachyose), yellow seed coat and black hilum. PI200508 parent has *TiTiLeLers2rs2* genotype (KTI and lectin protein present, low content of stachyose), yellow seed coat and brown hilum. 14G20 parent has *TiTileleRS2RS2* genotype (KTI protein present, lectin protein absent, normal content of stachyose), greenish yellow seed coat and yellow hilum. Genotype for *Ti* (*ti*), *Le* (le), and *RS2* (*rs2*) alleles of three parents is presented in Table 1. The seeds of Gaechuck#2 and PI200508 parents were planted to cross in a greenhouse. The crosses of Gaechuck#2 (*titiLeLeRS2RS2*) × PI200508 (*TiTiLeLers2rs2*) were made and F_1 seeds were obtained. F_1 seeds obtained were planted in greenhouse. F_2 seeds from F_1 plant were harvested. *titiLeLers2rs2* new genotype were selected from the F_2 plant population. The cross of new genotype (*titiLeLers2rs2*) × 14G20 (*TiTileleRS2RS2*) were made and F_1 seeds were obtained. F_1 seeds obtained were planted in greenhouse. F_1 hybridity was confirmed on morphological traits. F_2 seeds were harvested. The F_2 seeds were analyzed to screen the seed with *titilele* genotype (lectin and KTI protein free).

Determination of lectin protein by Western blot analysis

Proteins of parent, each F_2 seed, and random F_4 seed were separated by 10% or 12% SDS-PAGE, and transferred on

Table 1. Genotype for *Ti* (*ti*), *Le* (le), and *RS2* (*rs2*) alleles of parents used in this experiment

Parents	Trait/genotype					
	KTI protein	*Ti* (*ti*)	Lectin protein	*Le* (*le*)	Stachyose content	*RS2* (*rs2*)
Gaechuck#2	Absent	*titi*	Present	*LeLe*	Normal	*RS2RS2*
PI200508	Present	*TiTi*	Present	*LeLe*	Low	*rs2rs2*
14G20	Present	*TiTi*	Absent	*lele*	Normal	*RS2RS2*

to Immobilon-P membrane (PVDF, Millipore). After blocking for 2 hr in TBS buffer [20 mM Tris (pH7.5), 150 mM NaCl, and 0.1% Tween 20] with 5% nonfat dried milk (Carnation, Glendale, CA) at room temperature, the membrane were incubated with lectin antibody for 1 hr. 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.

Determination of KTI protein by Western blot analysis

Proteins of parent, each F_2 seed, and random F_4 seed were separated by 10% or 12% SDS-PAGE, and transferred onto Immobilon-Pmembrane (PVDF, Millipore). After blocking for 2 hr in TBS buffer [20 mM Tris (pH7.5), 150 mM NaCl, and 0.1% Tween 20] with 5% nonfat dried milk (Carnation, Glendale, CA) at room temperature, the membrane were incubated with KTI antibody for 1 hr. 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.

Determination of stachyose content

Randon seeds of parent, F_3 seeds, and F_4 seeds were used for stachyose analysis. Each sample was ground to fine powder. The fine powder sample was used for stachyose extraction and quantified by HPLC as described by Sung et al. (2014). Stachyose content was expressed as g/kg in dry weight after moisture correction.

Selection of triple recessive *titilelers2rs2* genotype

The F_2 seeds with *titilele* genotype (lectin and KTI protein free) were planted to advance F_2 plant generation. F_2 plants with a proper agronomic traits were individually harvested. Randon F_3 seeds of each F_2 plants harvested were analyzed to select the *rs2rs2* genotype (low content of stachyose). F_3 seeds with triple recessive *titilelers2rs2* genotype were planted to F_3 plant generation. F_3 plants with a proper agronomic traits were individually harvested. Random F_4 seeds from F_3 plants harvested were used to confirm lectin protein free, KTI protein free, and low content of stachyose. Plant height, 100-seed weight, seed coat color, hilum color, and content of stachyose were recorded on the F_4 plant generation. Mean values of plant height, 100-seed weight, and content of stachyose were compared by Duncan's multiple range test at the 5% level. Scheme for development of new soybean strain with triple recessive *titilelers2rs2* genotype is presented in Fig. 1.

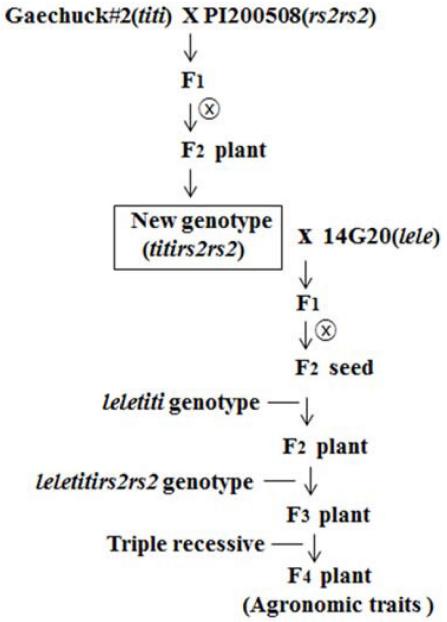

Fig. 1. Scheme for development of new soybean strain with Kunitz Trypsin Inhibitor protein free, lectin protein free, and low content of stachyose (triple recessive genotype, *titilelers2rs2*).

Results and Discussion

A total 250 F_2 seeds from the cross of new genotype (*titiLeLers2rs2*) and 14G20 parent (*TiTileleRS2RS2*) were obtained. Seventeen F_2 seeds with *titilele* genotype (lectin and KTI protein free) were selected. These results supported the previous reports [6, 11, 15]. Seventeen F_2 seeds were planted and fifteen F_2 plants were individually harvested. Among the 15 lines obtained, four lines with low stachyose content were selected. This result supports that stachyose content was controlled by a single recessive gene [20, 22]. Four F_3 seed strains were planted. Agronomic traits such as plant type, plant height, maturity date, lodging, seed quality, and 100-seed weight were evaluated on the four F_3 plant strains. One F_3 plant strain with a proper agronomical traits were selected and harvested. Random F_4 seeds were used to confirm absence for both lectin and KTI proteins (Fig. 2).

Lectin and KTI proteins were not observed in the mature

Table 2. Agronomic traits of parents and new strain developed in this experiment

Parent/strain	Plant height (cm)	Seed weight (g/100 seed)	Stachyose content (g/kg)	Seed coat color	Hilum color
Gaechuck#2	54a	24.4a	13.5a	Yellow	Black
PI200508	75b	13.1b	3.2b	Yellow	Brown
14G20	45a	34.5c	14.5a	Greenish Yellow	Yellow
New strain	51a	31.0d	3.8b	Yellow	Yellow

a - d: Different letters in the column are significantly different by DMRT at 5%.

F_4 seed of new strain. F_4 seeds were planted. Plant height, 100-seed weight, stachyose content, seed coat color, and hilum color were recorded on F_4 plant strain. Agronomic trait and stachyose content for three parents and new strain are presented in Table 2.

Plant height of new strain was 51 cm and 100-seed weight was 31.0 g. New strain has yellow seed coat and yellow hilum. Stachyose contents (g/kg) of Gaechuck#2, PI200508, 14G20 parents were 13.5, 3.2 and 14.5, respectively. Stachyose contents (g/kg) of new strain was 3.8. This result indicates that genotype of new strain is *rs2rs2*. Plant type harvested and seed of new strain with triple recessive *titilelers2rs2* genotype is shown in Fig. 3.

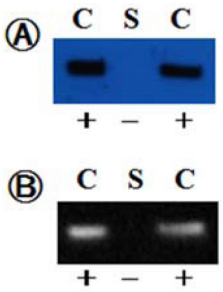

Fig. 2. Confirmation of lectin protein free (A) and Kunitz trypsin inhibitor (KTI) protein free (B) in the general cultivar and new strain. C: cultivar, S: new strain (*titilelers2rs2* genotype). +, -: presence and absence of lectin and KTI proteins, respectively.

Fig. 3. Plant (left) and seed (right) of new soybean strain (triple recessive genotype, *titilelers2rs2*) with Kunitz Trypsin Inhibitor protein free, lectin protein free, and low content of stachyose.

One strain developed in this research will be used to improve new yellow soybean cultivar with lectin prtoein free, KTI protein free, and low content of stachyose.

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018 R1D1A1B07045483).

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초록 : 콩에서 쿠니츠트립인히비터, 렉틴 및 스타키오스에 대한 열성 유전자의 집적

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성숙 콩[*Glycine max* (L.) Merr.] 종자에는 단백질, 지방, 탄수화물의 3대 영양소와 아이소플라본 등 다양한 기능성 성분이 함유되어 있다. 그러나, Kunitz Trypsin Inhibitor (KTI) 단백질, 렉틴 단백질, 난소화성 올리고당인 stachyose 성분이 함유되어져 있어 품질과 기능성을 저하시키고 있다. 본 연구는 콩 및 콩 제품의 품질과 기능성을 저하시키는 KTI 및 렉틴 단백질과 stachyose의 성분 함량과 관련된 유전자들이 모두 열성으로 작용하는 콩 계통(triple recessive genotype)을 선발하기 위하여 진행되었다. 3개의 모본(개척2호, PI200508, 14G20)을 이용한 육종집단으로부터 성숙 종자에서KTI 및 렉틴 단백질이 없으면서 stachyose의 함량이 일반콩보다 현저히 적은 triple recessive 유전자형(*titilelers2rs2*) 개체를 선발하였다. 선발된 계통의 초장은 51 cm 정도였으며 백립중은 31.0 g으로 대립이었으며 종피색 및 제색은 노란색이었다. Stachyose의 함량(g/kg)은 일반콩(13.5 g/kg)보다 훨씬 낮았다(3.8 g/kg). 본 연구를 통하여 선발된 계통은 KTI 단백질과 렉틴 단백질이 동시에 없으며 stachyose의 함량이 낮은 다양한 콩 품종 육성을 위한 중간모본으로 이용될 수 있을 것으로 사료되었다.