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The Development of a New Soybean Strain Without Kunitz Trypsin Inhibitor, Lectin, and 7S α′ Subunit Protein

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Soybean [*Glycine max* (L.) Merr.] seeds contain an average of 40% protein on a dry weight basis, but they also contain antinutritional elements such as lectin, Kunitz trypsin inhibitor (KTI), and 7S α′- subunit protein. The objective of this research was to develop a new soybean genotype with triple recessive alleles for these elements. Three parents (Gaechuck#2, PI506876, and Le-16) were used to develop the genetic population, and the presence of lectin and KTI 단백protein was detected using Western blot while 7S *α′* subunit protein was detected using SDS-PAGE. One F_3 plant strain with proper agronomical traits such as type, height, seed quality, and 100-seed weight was selected. The genotype of the developed strain is *titilelecgy1cgy1*, that is KTI, lectin, and 7S *α′* subunit protein free. The new strain has a purple flower, determinate growth habit, and light yellow pods at maturity. The seed has a buffer hilum and is yellow in color. The new strain's height was 58 cm compared to the Daewonkong cultivar at 46 cm, and its 100-seed weight was 27.1 g, smaller than the Daewonkong at 29.0 g. This is the first new soybean strain with the *titilelecgy1cgy1* genotype, and it can be used to improve yellow soybean cultivars of high quality and function.

Key words : 7S α′-subunit, Kunitz trypsin inhibitor (KTI), lectin, soybean, three proteins-free

Introduction

Soybean seed is one of the major food sources for protein, oil, carbohydrates, isoflavones, and many other nutrients to humans and animals. Soybean seed is composed of 40% protein. Demand of soybean and soybean products has increased in recent years because of high quantity and quality of soybean protein. Also, several antinutritional factors and allergenic proteins in the raw mature soybean are exist. Kunitz Trypsin Inhibitor (KTI) protein, lectin protein and 7S α′-subunit protein are main antinutrients responsible for reducing the nutritional value of unprocessed soybean.

Soybean 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 [11]. 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 [5, 16, 20]. 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 [4, 6], which was integrated in linkage group A2 (chromosome 8) of the USDA/Iowa State University soybean molecular linkage map [1].

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 [12, 13, 16]. Recently, a new soybean line with triple null recessive geno-

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types (ti/ti-le/le-p34/p34) was developed [19].

Soybean β-conglycinin (7S globulin) and glycinin (11S globulin) are the major components of storage protein in soybean. β-conglycinin consists of three subunits, α′, α, β and exhibits poorer nutritional and food processing properties than glycinin [22]. Also, β-conglycinin contains much less sulfur-containing amino acid, methionine and cysteine, than glycinin [9]. Several mutant lines affecting accumulation of β-conglycinin have been identified in soybean germplasms. Kitamura and Kaizuma [7] identified Keburi, which was characterized by the absence of the α′-subunit of β-conglycinin. Kitamura et al. [8] reported that the absence of α′-subunit were controlled by single recessive alleles, cgy_1. Wild soybean line, QT2, which lacks α′-subunit was also identified. In the QT2 line, the deficiency of α′-subunit is controlled by a single dominant gene, *Scg* [2]. *Scg* gene was inherited independently with cgy_1 gene. Studies also showed that absence of the α′-subunit in QT2 was inherited independently from the presence of lipoxygenase isozymes. Hayashi et al. [3] identified AFLP marker to be tightly linked to the gene for deficiency of the β-conglycinin. Highly negative correlation between the contents of β-conglycinin and glycinin was reported by Ogawa et al. [14]. 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 α′,α, and β-subunits of β-conglycinin.

Development of new soybean cultivar with free of KTI, lectin, 7S *a′* subunit proteins is needed to improve the nutrition values and to modify the food processing properties of soybeans. Also, this cultivar enhances the utilization of soybean in food as well as feed uses. The objective of this research was to develop new soybean genotype with triple recessive alleles (*titilelecgy1cgy1*) for KTI protein, lectin protein and 7S *a′* subunit protein. This is the first report on soybean strain with *titilelecgy1cgy1* genotype (KTI, lectin and 7S *a′* subunit protein free.

Materials and Methods

Genetic population

Three parents (Gaechuck#2, PI506876, and Le-16) were used to develop genetic population. Gaechuck#2 parent has *titiLeLeCgy1Cgy1* genotype (KTI protein absent, lectin and 7S *a′* subunit protein present), yellow seed coat and black hilum. PI506876 parent has *TiTiLeLecgy1cgy1* genotype (KTI and lectin protein present, 7S *a′* subunit protein absent), yellow seed coat and brown hilum. Le-16 parent has *TiTileleCgy1Cgy1* genotype (KTI protein present, lectin protein absent, 7S *a′* subunit protein present), greenish yellow seed coat and yellow hilum. Genotype for *Ti* (*ti*), *Le* (le), and *Cgy1* (*cgy1*) alleles of three parents is presented in Table 1. The seeds of Gaechuck#2, PI200508, and Le-16 parents were planted to cross in a greenhouse. The crosses of Gaechuck#2 (*titiLeLeCgy1Cgy1*) × PI506876 (*TiTiLeLecgy1cgy1*) and Gaechuck#2 (*titiLeLeCgy1Cgy1*) × Le-16 (*TiTileleCgy1Cgy1*) 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. Two new genotypes (*titiLeLecgy1cgy1* and *titileleCgy1 Cgy1*) were selected from the F_2 plant population. The cross of *titiLeLecgy1cgy1* x *titileleCgy1Cgy1* was 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 *titilelecgy1cgy1* genotype (KTI, lectin and 7S *a′* subunit protein free).

Determination of lectin protein by Western blot analysis

Proteins of parent, each F_2 seed, random F_3 seed and F_4 seed were separated by 10% or 12% SDS-PAGE, and transferred on 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 in-

Table 1. Genotype for *Ti* (*ti*), *Le* (le), and *Cgy1* (*cgy1*) alleles of parents used in this experiment

Parents	Trait/genotype					
	KTI protein	*Ti* (*ti*)	Lectin protein	*Le* (*le*)	7S *a′* subunit protein	*Cgy1* (*cgy1*)
Gaechuck#2	Absent	*titi*	Present	*LeLe*	Present	*Cgy1Cgy1*
PI506876	Present	*TiTi*	Present	*LeLe*	Absent	*cgy1cgy1*
Le-16	Present	*TiTi*	Absent	*lele*	Present	*Cgy1Cgy1*

cubated 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, random F_3 seed and 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 7S α'-subunit protein by SDS-PAGE

Crude proteins from parent, each F_2 seed, random F_3 seed and F_4 seed were extracted to determine the presence or absence of α'-subunit protein of β-conglycinin electrophoretically. A piece of cotyledon from parent and each F_2 seed was removed and was incubated for 30 min (at room temperature) in 1 ml Tris-HCl, pH8.0, containing 1.56%v/v β-mercaptoethanol. After centrifugation, 50 ul 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 β-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 hr. 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™, Product Code: M4038, St.Louis MO, USA) containing the 72 kDa (for α'-subunit) was used to aid recognition of samples lacking the α'-subunit of β-conglycinin protein.

Selection of *titilelecgy1cgy1* (free of KTI, lectin, and 7S α' subunit proteins) genotype

The F_2 seeds with *titilelecgy1cgy1* genotype (KTI, lectin and 7S *α'* subunit protein free) were planted to advance F_2 plant generation. F_2 plants with a proper agronomic traits were individually harvested. F_3 seeds with *titilelecgy1cgy1* 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 KTI protein free, lectin protein free, and 7S *α'* subunit protein free. Flower color, plant height, growth habit, 100-seed weight, seed coat color, and hilum color were recorded on the F_4 plant generation.

Mean values of plant height and 100-seed weight were compared by Duncan's multiple range test at the 5% level. Scheme for development of *titilelecgy1cgy1* genotype (KTI, lectin and 7S *α'* subunit protein free) is presented in Fig. 1.

Results and Discussion

F_1 seeds were obtained from the cross of *titiLeLecgy1cgy1* × *titileleCgy1Cgy1*. Genotype of F_1 seeds obtained was *titiLeleCgy1cgy1* and lectin and 7S *α'* subunit proteins were observed. F_2 seeds were harvested from F_1 hybrid plant. Both lectin protein of 120 kDa and 7S *α'* subunit protein of 72

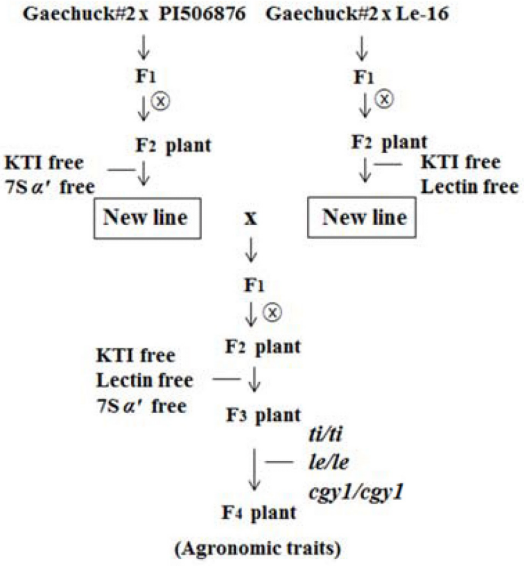

Fig. 1. Scheme for development of soybean strain with *titilelecgy1cgy1* genotype (KTI protein free, lectin protein free and 7S *α'* subunit protein free).

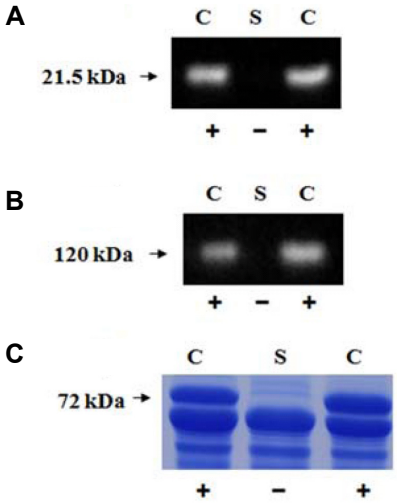

Fig. 2. Confirmation of Kunitz Trypsin Inhibitor (KTI) protein free (A), lectin protein free (B), and 7S *a'* subunit protein free (C) in the current cultivar ("Daewonkong") and new strain. C: "Daewonkomg" (*TiTiLeLeCgy1Cgy1* genotype), S: new strain (*titilelecgy1cgy1* genotype). +, -: presence and absence of KTI, lectin, and 7S *a'* subunit proteins, respectively.

kDa were segregated in the F_2 seed generation. The 3:1 segregation ratios for inheritance of lectin and 7S *a'* subunit proteins were observed in the F_2 seed generation. Among F_2 seeds obtained, 45 F_2 seeds with *cgy1cgy1* genotype (7S *a'* subunit protein free) were selected. Four F_2 seeds with *titilelecgy1cgy1* genotype (KTI, lectin and 7S *a'* subunit protein free) were selected and were planted. This result supports that absence of KTI, lectin, and 7S α'-subunit proteins was controlled by a single recessive gene [5, 8, 12, 15]. Four F_2 plants were individually harvested and two F_2 plants with a proper agronomic traits were selected. Two F_3 seed strains were planted and one F_3 plant strain with a proper agronomical traits such as plant type, plant height, seed quality, and 100-seed weight was finally selected. Random F_4 seeds were used to confirm absence for KTI, lectin and 7S *a'* subunit proteins (Fig. 2).

KTI, lectin, and 7S *a'* subunit proteins were not observed in the mature F_4 seed of new strain. However, in the seed of "Daewonkong" cultivar, KTI, lectin, and 7S *a'* subunit proteins were shown (Fig. 2). This result indicates that genotype of new strain is *titilelecgy1cgy1*. Agronomic traits such as flower color, growth habit, plant height, 100-seed weight, seed coat color, and hilum color for "Daewonkomg" (*TiTiLeLeCgy1Cgy1* genotype) and new F_4 plant strain (*titilelecgy1cgy1* genotype) are presented in Table 2.

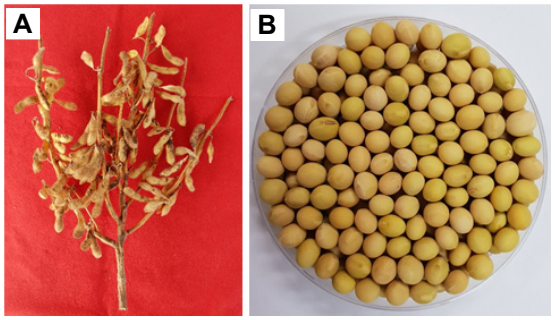

Fig. 3. Plant (A) and seed (B) of new soybean strain (*titilelecgy1cgy1* genotype) with Kunitz Trypsin Inhibitor protein free, lectin protein free, and 7S *a'* subunit protein free.

New strain has purple flower, determinate growth habit, and light yellow pods at maturity. The seed of new strain has buffer hilum color and yellow seed coat color. Plant height of new strain was 58 cm compared to the "Daewonkong" cultivar of 46 cm. The 100-seed weight of new strain was 27.1 g smaller than that of "Daewonkong" (29.0 g). Plant type harvested and seed of new strain with *titilelecgy1cgy1* genotype (KTI, lectin, and 7S *a'* subunit proteins free) is shown in Fig. 3.

KTI, lectin, and 7S *a'* subunit proteins are major antinutrients responsible for reducing the nutritional value of unprocessed soybean. Presence of these proteins in mature raw soybean seeds requires heating step to denature the activity of these antinutritional components. However, excessive heat treatment may lower amino acid availability. The genetic elimination removal of these components could be an alternative to the heat treatment. Breeding of soybean cultivar with free of KTI, lectin, and 7S *a'* subunit proteins is needed to improve the nutrition values and to modify the food processing properties of soybeans. This is the first new soybean strain with *titilelecgy1cgy1* genotype (KTI pro-

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

Cultivar/ strain	Flower color	Growth habit	Plant height (cm)	Seed weight (g/100 seed)	Seed coat color	Hilum color
"Daewonkong"	Purple	Determinate	46a	29.0a	Yellow	Yellow
New strain	Purple	Determinate	58b	27.1b	Yellow	Buffer

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

tein free, lectin protein free, and 7S *a'* subunit protein free). New strain developed in this research will be used to improve new yellow soybean cultivar with high quality and function.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Cregan, P. B., Jarvik, T., Bush, A. L., Shoemaker, R. C., Lark, K. G., Kahler, A. L., Kaya, N., VanToai, T. T., Lohnes, D. G., Chung, J. I. and Specht, J. E. 1999. An integrated genetic linkage map of the soybean. *Crop Sci.* **39,** 1464-1490.
2. Hajika, M., Takahashi, M., Sakai, S. and Matsunaga, R. 1998. Dominant inheritance of a trait lacking β-conglycinin detected in a wild soybean line. *Breed. Sci.* **48,** 383-386.
3. Hayashi, M., Nishioka, M., Kitamura, K. and Harada, K. 2000. Identification of AFLP markers tightly linked to the gene for deficiency of the 7S globulin in soybean seed and characterization of abnormal phenotypes involved in the mutation. *Breed. Sci.* **50,** 123-129.
4. Hildebrand, D. F., Orf, J. H. and Hymowitz, T. 1980. Inheritance of anacid phosphatase and its linkage with the Kunitz trypsin inhibitor seed protein of soybeans. *Crop Sci.* **20,** 83-85.
5. Hymowitz, T. and Hadley, H. H. 1972. Inheritance of a trypsin variant in seed protein of soybeans. *Crop Sci.* **12,** 197-198.
6. Kiang, Y. T. 1987. Mapping three protein loci on a soybean chromosome. *Crop Sci.* **27,** 44-46.
7. Kitamura, K. and Kaizuma, N. 1981. Mutant strains with low level of subunits of 7S globulins in soybean seed. *Japan J. Breed.* **31,** 353-359.
8. Kitamura, K., Davies, C. S. and Nielsen, N. C. 1984. Inheritance of alleles for Cgy_1 and Gy_4 storage protein genes in soybean. *Theor. Appl. Genet.* **68,** 253-257.
9. Koshiyama, I. 1968. Chemical and physical properties in soybean globulins. *Cereal Chem.* **45,** 394-404.
10. Krober, O. A. and Cartter, J. L. 1962. Quantitative interrelations of protein and nonprotein constituents of soybeans. *Crop Sci.* **2,** 171-172.
11. Kunitz, M. 1945. Crystallization of a trypsin inhibitor from soybean. *Science* **101,** 668-669.
12. Lee, K. J., Park, M. S., Sung, M. K., Kim, M. S. and Chung, J. I. 2008. Inheritance between *Le* gene and *Ti* gene in soybean (*Glycine max*L.). *Kor. J. Breed Sci.* **40,** 97-100.
13. Moraes, R. M. A., Soares, T. C. B., Colombo, L. R., Salla, M. F. S., Barros, J. G. A. and Piovesan, N. D. 2006. Assisted selection by specific DNA markers for genetic elimination of the kunitz trypsin inhibitor and lectin in soybean seeds. *Euphytica* **149,** 221-226.
14. Ogawa, T. E., Tayama, E., Kitamura, K. and Kaizuma, N. 1989. Genetic improvement of seed storage proteins using three variant alleles of 7S globulin subunits in soybean. *Japan J. Breed.* **39,** 137-147.
15. Orf, J. H., Hymowitz, T., Pull, S. P. and Pueppke, S. G. 1978. Inheritance of a soybean seed lectin. *Crop Sci.* **18,** 899-900.
16. Orf, J. H. and Hymowitz, T. 1979. Soybean linkage test between Ti and Le seed proteins. *Soybean Genet. Newslett.* **6,** 32.
17. Pull, S. P., Pueppke, S. G., Hymowitz, H. and Orf, J. H. 1978. Soybean lines lacking the 120,000 daltons seed lectin. *Science* **200,** 1277-1279.
18. Schulze, H., Saini, H. S., Huisman, J., Hessing, M., Berg, W. and Verstegen, M. W. A. 1995. Increased nitrogen secretion by inclusion of soya lectin in the diets of pigs. *J. Sci. Food Agriculture* **69,** 501-510.
19. Schmidt, M. A., Hymowitz, T. and Herman, E. M. 2015. Breeding and characterrization of soybean triple null; a stack of recessive alleles of kunitz trypsin inhibitor, soybean agglutinin, and p34 allergen nulls. *Plant Breed.* **134,** 310-315.
20. Singh, L., Wilson, C. M. and Hadley, H. H. 1969. Genetic differences in soybean trypsin inhibitors separated by disc electrophoresis. *Crop Sci.* **9,** 489-491.
21. Sung, M. K., Han, S. J., Seo, H. J., Choi, S. W., Nam, S. H. and Chung, J. I. 2014. Genotype and environment influence on raffinose and stachyose content of soybean seed. *Kor. J. Crop Sci.* **59,** 319-324.
22. Thanh, V. H. and Shibasaki, K. 1976. Heterogeneity of beta-conglycinin. *Biochim. Biophys. Acta* **469,** 326-328.

초록 : 쿠니츠트립인히비터, 렉틴 및 7S α′ 서버유닛 3가지 단백질이 없는 콩 계통의 개발

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성숙 콩[*Glycine max* (L.) Merr.] 종자는 약 40%의 단백질을 함유하고 있으며 아이소플라본, 사포닌, 루테인, 비타민등 다양한 기능성 성분을 함유하고 있다. 그러나, Kunitz Trypsin Inhibitor (KTI) 단백질, 렉틴 단백질, 7S *α′* 서버유닛 단백질이 함유되어져 있어 콩의 품질과 기능성을 저하시키고 있다. 본 연구는 콩 및 콩 제품의 품질과 기능성을 저하시키는 KTI, 렉틴 및 7S *α′* 서버유닛 3가지 단백질이 모두 유전적으로 결핍된 콩 계통(*titilelecgy1cgy1* 유전자형)을 선발하기 위하여 진행되었다. 3개의 모본(개척2호, PI506876, Le-16)을 이용한 유전집단으로부터 성숙 종자에서 KTI, 렉틴 및 7S *α′* 서버유닛 3가지 단백질이 모두 없는 *titilelecgy1cgy1* 유전자형을 가진 1개의 계통을 개발하였다. 개발된 계통은 자주색 꽃, 유한신육형, 노란종피을 가지고 있으며 초장은 58 cm로 대원콩(46 cm)보다 길었다. 백립중은 27.1 g으로 대원콩(29.0 g)보다 작았다. 본 연구를 통하여 선발된 계통은 KTI, 렉틴 및 7S *α′* 서버유닛 3가지 단백질이 모두 없는 고품질 기능성 콩 품종 육성을 위한 중간모본으로 이용될 수 있을 것으로 사료되었다.