J Plant Biotechnol (2024) 51:152-157
DOI:https://doi.org/10.5010/JPB.2024.51.016.152

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Development of a green soybean line with a green cotyledon and a tetra null genotype for P34, lectin, Kunitz trypsin inhibitor, and lipoxygenase proteins

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Received: 28 April 2024 / Revised: 20 May 2024 / Accepted: 20 May 2024 / Published: 23 May 2024

Abstract Soybean (*Glycine max* (L.) Merr.) varieties with a green seed coat and cotyledon have long been cultivated for their high levels of lutein, which is considered beneficial for eye health. However, mature soybean seeds also contain major antinutritional and allergenic factors, including P34, Kunitz trypsin inhibitor (KTI), lectin, and lipoxygenase proteins. The objective of this research was to develop a new soybean line with a green cotyledon and a green seed coat, and without P34, KTI, lectin, and lipoxygenase proteins. A breeding population was developed using two cultivars and three germplasms. Seventy-four F_2 seeds with a green cotyledon and a green seed coat were obtained. Twenty-four F2 seeds with a tetra null genotype (*p34p34-titi-lele-lox1 lox1lox2lox2lox3lox3*) were selected and planted in a greenhouse. One F_2 plant that exhibited suitable agronomic traits was selected. The SDS-PAGE and western blot methods were used to determine the absence (tetra null genotype) of P34, KTI, lectin, and lipoxygenase proteins in random F_3 seeds from the F_2 plant selected. The color of the cotyledon and seed coat for the new selection line was green. The stem height was 72 cm and the 100-seed weight was 21.5 g. The new line obtained in this research could be bred into a soybean variety with a green cotyledon, a green seed coat, and fewer allergenic and antinutritional properties.

Keywords P34, KTI, lectin, lipoxygenase, green, tetra null, soybean

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Introduction

A legume crop, soybean [*Glycine max (L.)* Merr. 2n = 40] has been broadly cultivated to obtain vegetable fat and protein as an ingredient in human and livestock feed throughout the world since ancient times. Genetic resources or varieties with a green cotyledon and a green seed coat are high in lutein content compared to black or yellow soybeans (Oh et al. 2019) and their effectiveness in improving the prognosis of eye disease and preventing eye diseases when consumed has been reported (Rodrigues and Shao 2004). However, mature soybean seeds with a green cotyledon and a green seed coat contain proteins including P34, Kunitz Trypsin Inhibitor (KTI), lectin, and lipoxygenase, which can reduce their processability, quality, and functionality.

P34 protein (Gly m Bd 30 K), a monomeric, insoluble, oil-body-associated glycoprotein with a molecular weight of 34 kDa consisting of 258 amino acids, has mainly been detected in cotyledons (Herman et al. 2003). Despite constituting less than 1% of total seed protein, P34 is also regarded as a potent allergen, and more than 65% of soybean allergy sufferers have been reported to react to P34 protein alone (Ogawa et al. 1993). One study reported that little or very low P34 protein content was detected in two genetic resources (PI567476 and PI 603570A) in mature soybean seeds (Joseph et al. 2006), and a four base pair insertion in the synthesis of P34 protein start codon has been reported as the cause of these variants (Bilyeu et al. 2009). The *P34(p34)* allele is located on chromosome 8, and the presence of the *p34p34* genotype indicates the absence or very low content of P34 protein in mature seeds. Lectins, which are carbohydrate-binding proteins, have a stable structure and are not easily degraded by proteases. Lectin proteins in soybean seeds bind to intestinal epithelial cells upon ingestion, causing modifications to their structure

and function and negatively affecting digestion and absorption (Pan et al. 2018; Vasconcelos and Oliveira 2004). An additional property is the capacity for agglutinating red blood cells and they can be a cause of acute symptoms including nausea, vomiting, and diarrhea after consumption of soy foods (Miyake et al. 2007). The levels of lectin protein in soybean seeds are regulated by the *Le (le)* gene located on chromosome 2 (Orf et al. 1978), and when its expression is suppressed by an insertion of a 3.5 kb *Tgm1* element in the *Le* gene, the result is the *lele* genotype, where lectin protein cannot be detected in mature seeds (Goldberg et al. 1983; Okamuro and Goldberg 1992). KTI protein, consisting of 181 amino acids with a molecular weight of 21.5 kDa (Kunitz 1945) is an inhibitor of the proteolytic enzyme trypsin, which can interfere with protein hydrolysis in the pancreas, causing development of digestive disorders, allergic reactions, and pancreatic enlargement (Rackis and Gumbmann 1981). The *Ti (ti)* gene located on chromosome 8 can determine the presence of KTI protein in soybean seed, and the seed with *titi* genotype does not contain KTI protein (Orf and Hymowitz 1979). Fermentation of soybeans can reduce the content of KTI protein and heating at 100℃ for 12-15 minutes is required for inactivation (Baker and Mustakas 1973; Vagadia et al. 2017). Oxidation of the unsaturated fatty acids linoleic acid and linolenic acid in soybeans is catalyzed by lipoxygenase proteins, which is the reason for the beany taste. Soybean seed contains three lipoxygenase isozymes (L-1, L-2, and L-3) with involvement in development of the fishy flavor (Kitamura et al. 1983). These three lipoxygenase isozymes (L-1, L-2, and L-3) are controlled by the *Lox1*, *Lox2*, and *Lox3* genes, respectively (Hildebrand and Hymowitz 1982; Kitamura et al. 1983), and when these three genes are homozygous recessive, mature seeds do not contain the lipoxygenase protein and there is no beany flavor. The *Lox2 and Lox1* genes, located on chromosome 13, are linked, and the *Lox3* gene, located on chromosome 15, is inherited independently of the *Lox2* and *Lox1* genes (Davies and Nielsen 1986).

P34, KTI, lectin, and lipoxygenase proteins in the seed of green soybean with a green cotyledon can be inactivated by high temperatures and additive treatments, however this process can lead to development of certain adverse effects, including additional costs and the depletion of essential amino acids and other useful components. Breeding of a new soybean line with a green cotyledon, a green seed coat, and without these four proteins genetically can be an effective alternative. Thus far, the studies have reported on the breeding of lines that do not contain three proteins, KTI, lectin, and P34 (Choi et al. 2022) and lines that do not contain lipoxygenase, lectin, and KTI proteins (Ly et al. 2023) in soybean seeds with a green cotyledon and a green seed coat. This research was conducted for development of a new soybean line with a green cotyledon, a green seed coat, and without P34, lectin, KTI, and lipoxygenase proteins.

Materials and Methods

Parents and breeding scheme

Two cultivars and three germplasms were used to obtain the breeding population. Color of the cotyledon and seed coat, 100-seed weight, stem height and absence or presence of P34, KTI, lectin, and lipoxygenase proteins of five parents are shown in Table 1. A breeding line with ti/p34 alleles was selected from the crossing of Gaechuck#1 (*lox2lox3*/*ti*) parent and PI567476 (*p34*) parent. From the crossing of GS146 and breeding line (*ti*/*p34*) parents, a green seed line with *ti/p34* alleles and a green seed coat was selected. The cross of the green seed line and Seonyack (*lox1lox2lox3*/*ti*) was made for selection of a green seed line with *lox1lox2lox3*/*ti*/*p34* alleles, a green seed coat, and a green cotyledon. A breeding line with *ti*/*le* alleles was selected from the cross of Gaechuck#1 (*lox2lox3*/*ti*) parent and PI548392 (*le*) parent. From the crossing of Seonyack and the breeding line (*ti*/*le*), a black seed line with *lox1 lox2lox3*/*ti*/*le* alleles, a green cotyledon, and a black seed

Table 1 Seed coat color; cotyledon color; presence or absence of lipoxygenase, Kunitz trypsin inhibitor (KTI), and lectin proteins; stem height; and 100-seed weight of five parents used in this experiment

Parent	Seed coat	Cotyledon	P34	Lectin	KTI	Lipoxygenase	Stem height (cm)	100-seed weight (g)
Gaechuck#1	Black	Green	Present	Present	Absent	2,3 absent	50	23.2
Seonyack	Black	Green	Present	Present	Absent	1,2,3 absent	62	36.5
GS146	Green	Green	Present	Present	Present	1,2,3 present	52	28.2
PI567476	Yellow	Yellow	Absent	Present	Present	1,2,3 present	116	10.8
PI548392	Black	Yellow	Present	Absent	Present	1,2,3 present	114	6.8

coat. A green seed line with *lox1lox2lox3/ti/le/p34* alleles, a green seed coat, and a green cotyledon was selected from the cross of a breeding line (*ti/le*) and green seed line (*lox1lox2lox3/ti/p34*) parents. From the cross of a black seed line (*lox1lox2lox3/ti/le*, a black seed coat, a green cotyledon) and a green seed line (*lox1lox2lox3/ti/le/p34*, a green cotyledon, a green seed coat,) parents, F_1 hybrid seeds were obtained. The F_1 seeds harvested from the female parent were planted in the greenhouse. All F_1 plants with confirmed hybridity based on morphological traits were individually harvested and bulked. F_2 seeds with a green seed coat were selected and analysis of each seed was performed for screening the *p34p34* genotype (without P34 protein). All F_2 seeds with a green seed coat and the *p34p34* genotype were planted in the greenhouse. The F_2 plants that showed superior growth were selected and harvested after maturity. Also, F_2 plant with a very uniform green color in each seed was selected. After harvesting, random F_3 seeds were used in identification of recessive genotypes (*p34p34-titi-lele-lox1lox1lox2lox2lox3lox3*) by determining the absence of P34, KTI, lectin, and lipoxygenase proteins. The breeding schemes for the tetra null genotype with a green cotyledon and a green seed coat are shown in Fig. 1.

Selection of F_2 seeds lacking P34 protein

A part of the cotyledon was excised from the each F_2 seed and parent. Protein was extracted. Protein extracted was separated by 10% or 12% SDS-PAGE and transferred onto an Immobilon-P membrane (PVDF, Millipore). The antibody was prepared and western blot analysis for detection of P34 protein was performed using a previously reported method (Koo et al. 2011). Blocking in TBS buffer containing 0.1% Tween 20, 20 mM Tris (pH 7.5), 150 mM NaCl, and 5% nonfat dried milk was performed for 2 h (Carnation, Glendale, CA), followed by incubation of the membrane with the antibody for P34 protein for 1 h. A horseradish peroxidase conjugated secondary antibody was applied to the blot for incubation after washing in TBS buffer, followed by visualization of the complex using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, United Kingdom). The absence or presence of P34 protein was determined visually. A Chi-square method was used for

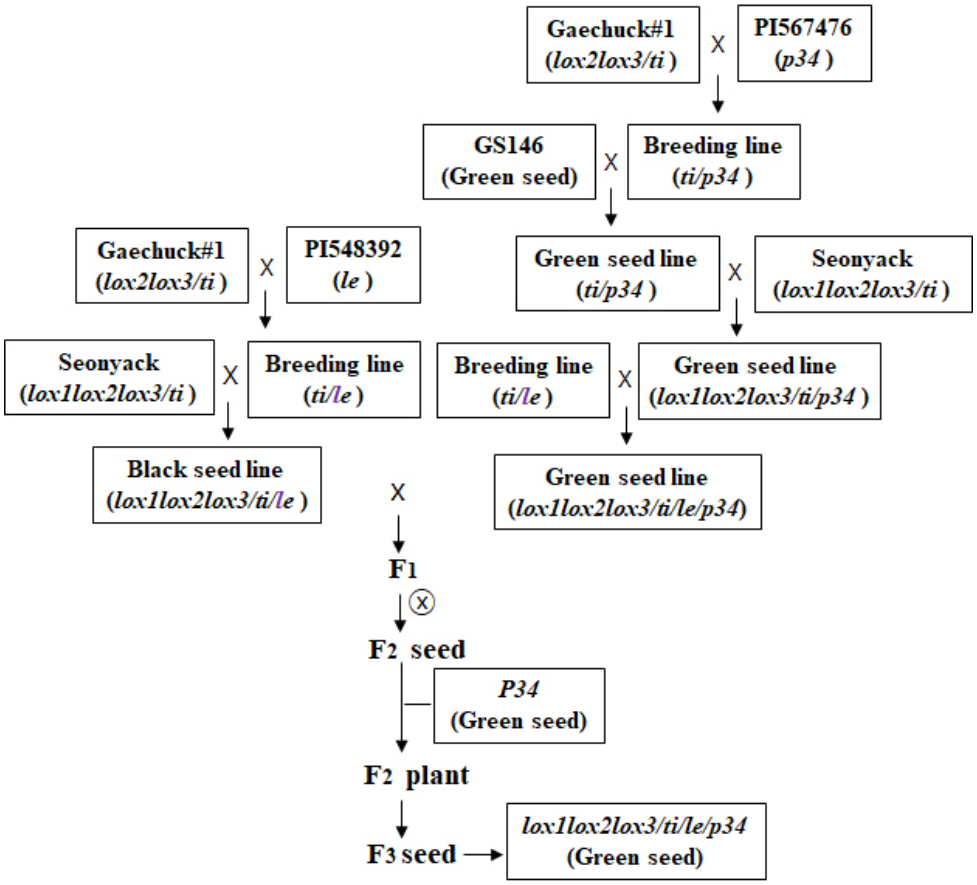

Fig. 1 Scheme for development of a new soybean line with a green seed coat, a green cotyledon, and the tetra null genotype (*p34p34-lele-titi-lox1lox1lox2lox2lox3lox3*) for P34, lectin, Kunitz trypsin inhibitor, and lipoxygenase proteins

analysis to determine the segregation ratio for determining the absence or presence of P34 protein.

Confirmation of the tetra null genotype for the new selection line

P34, lectin, KTI, and lipoxygenase proteins were detected using random F_3 seeds from the selection line, tetra null alleles (*p34p34-lele-titi-lox1lox1lox2lox2lox3lox3*). The SDS-PAGE method was used to detect the absence or presence of lipoxygenase protein (Ly et al. 2023). The Western Blot method was used to detect the absence or presence of P34, KTI, and lectin proteins (Ly et al. 2023). Cheongmiin cultivar with a green cotyledon, a green seed coat, and the *P34P34-TiTi-LeLe-Lox1Lox1Lox2Lox2Lox3Lox3* genotype (presence of P34, KTI, lectin, and lipoxygenase proteins) was used as a control cultivar. Maturing date, 100-seed weight (g), and stem height (cm) were recorded on F_3 seeds from the new selection line.

Results and Discussion

Selection of F_2 seeds without P34 protein (*p34p34* genotype)

From the crossing of a black seed line (*lox1lox2lox3*/*ti*/*le*, green cotyledon, black seed coat) and a green seed line (*lox1lox2lox3*/*ti*/*le*/*p34*, green seed coat, green cotyledon) parents, 74 F_2 seeds with a green seed coat were obtained. Analysis of individual F_2 seeds was performed to determine the absence or presence of P34 protein. In the F_2 seed generation, segregation of P34 protein of 34 kDa was observed (Fig. 2). The segregation data for P34 protein in the F_2 seed generation are shown in Table 2. Among the 74 F_2 seeds, P34 protein was detected in 60 F_2 seeds and the P34 protein was not detected in 14 F_2 seeds. To determine the absence or presence of P34 protein in the F_2 seed generation, the segregation ratio was fitted to an expected ratio of 3:1 (χ^2 = 2.95, p = 0.05 - 0.10 at α = 0.05). This data was in agreement with previous studies reporting that the absence or presence of P34 protein is controlled by a single gene (Choi et al. 2022; Han et al. 2012; Joseph et al. 2006). Among 72 F_2 seeds obtained from the cross of parents with a green cotyledon and a green seed coat, 22 F_2 seeds contained no P34 protein, which was suitable for a ratio of 3:1 (Choi et al. 2022). The segregation of a 3:1 ratio for P34 protein was observed in 479 F_2 seeds obtained from a crossing between parents with a green cotyledon and a black seed coat (Han et al 2012).

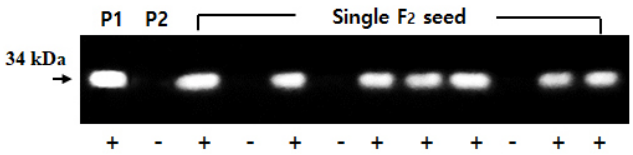

Fig. 2 Segregation of P34 protein in the parents and F_2 seeds. Arrow indicates P34 protein of 34 kDa. P1: *P34P34* genotype, P2: *p34p34* genotype. +, −: presence and absence of P34 protein

Table 2 Inheritance of the presence or absence of P34 protein in 74 F_2 seeds obtained from the cross of P1 and P2 parents

P34 Protein	Seed number		χ^2 value (3:1)	*P*
Phenotype α′-subunit	Observed	Expected	χ^2 value (3:1)	P
Present	60	55.5	2.95	0.05-0.10
Absent	14	18.5	2.95	0.05-0.10

Confirmation of the tetra null genotype and agronomic traits for the new selection line

Twenty-four F_2 seeds with *p34p34* genotype (lacking the P34 protein) were selected and planted for growing F_2 plants in the greenhouse. All F_2 seeds with a green cotyledon, a green seed coat, and the tetra null genotype (*p34p34-titi-lele-lox1lox1lox2lox2lox3lox3*) showed normal growth, flowering, and maturation (Fig. 4). These results suggest that accumulation of recessive alleles (*p34*, *ti*, *le*, and *lox1lox2lox3*) for four ingredients did not affect agronomic traits on soybean with a green cotyledon and a green seed coat. The plants with triple null alleles (*titi-lele-p34p34*) that flowered and produced seeds exhibited no overt differences in comparison with the standard Williams 82 cultivar (Schmidt et al. 2015). For each F_2 plant, evaluation of agronomic traits including maturity date, stem height, and seed quality was performed and after harvesting one F_2 plant exhibiting the most suitable traits was selected.

Random F_3 seeds harvested from an F_2 plant with a *p34p34-lele-titi-lox1lox1lox2lox2lox3lox3* genotype were used to identify the absence of P34, KTI, lectin, and lipoxygenase proteins (Fig. 3). Lipoxygenase protein was not detected in F_3 seeds from the F_2 plant selected using SDS-PAGE analysis (Fig. 3-Ⓐ). In addition, proteins of lectin, KTI, and P34 were not detected using western blot analysis (Fig. 3-Ⓑ, Ⓒ, Ⓓ). However, these four proteins were detected in the seed of the Cheongmiin (*P34P34-LeLe-TiTi-Lox1Lox1Lox2Lox2Lox3Lox3* genotype) used as a control cultivar, indicating that the absence of the four proteins in F_3 seeds for the new selection line is genetically fixed. Agronomic traits for the new selection line are shown

Table 3 Agronomic traits of the new selection line with a green seed coat, a green cotyledon, and the tetra null genotype developed in this experiment

Maturing date	Stem height (cm)	100-seed weight (g)	P34	Lectin	KTI	Lipoxygenase
Oct. 18	72	21.5	Absent	Absent	Absent	1,2,3 absent

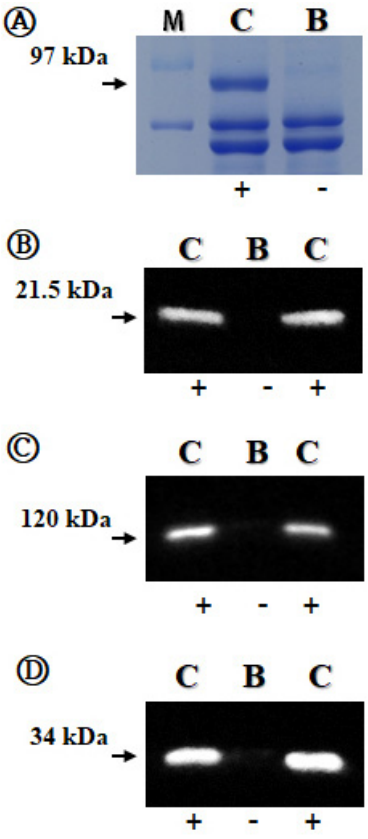

Fig. 3 Confirmation of absence of lipoxygenase (Ⓐ), Kunitz trypsin inhibitor (KTI; Ⓑ), lectin (Ⓒ), and P34 (Ⓓ) proteins in mature seeds of the new selection line with the tetra null genotype. Arrows indicate lipoxygenase protein of 97 kDa, KTI protein of 21.5 kDa, lectin protein of 120 kDa and P34 protein of 34 kDa. C: Cheongmiin (control cultivar), B: selection line. +, −: presence and absence of each protein

in Table 3.

Seeds from the selection line with a green cotyledon, a green seed coat, and the tetra null genotype for P34, KTI, lectin, and lipoxygenase proteins were sown in the greenhouse on June 22, and the maturity date was October 18. The stem height was 72 cm and the 100-seed weight was 21.5 g. The F_2 plant type harvested and F_3 seeds of the new selection line are shown in Fig. 4. This result indicates that breeding of a green soybean cultivar with fewer allergenic and antinutritional factors can be achieved through the accumulation of *p34*, *le*, *ti*, and *lox1lox2lox3* recessive alleles. Therefore, the new line obtained will be used for breeding a green soybean variety without components that

Fig. 4 Appearance of the F_2 plant and F_3 seeds with a green seed coat, a green cotyledon, and the tetra null genotype (*p34p34-lele-titi-lox1lox1lox2lox2lox3lox3*) for P34, lectin, Kunitz trypsin inhibitor, and lipoxygenase proteins

can reduce quality, processing aptitude, nutrition, and and functionality through generation advance and evaluation of agronomic traits in the field.

Conclusion

In this study, a new soybean line with a green cotyledon, a green seed coat, and without P34, KTI, lectin, and lipoxygenase proteins was developed. The 74 F_2 seeds with a green cotyledon and a green seed coat were obtained from the crossing of parents. Twenty-four F_2 seeds with the tetra null genotype (*p34p34-lele-titi-lox1lox1lox2lox2 lox3lox3*) were selected and planted in the greenhouse. Finally, one F_2 plant that exhibited suitable agronomic traits was selected and the tetra null genotype for P34, KTI, lectin, and lipoxygenase proteins was confirmed on random F_3 seeds from the F_2 plant selected. The color of the cotyledon and seed coat for the new selection line was green. The stem height was 72 cm and the 100-seed weight was 21.5 g.

Acknowledgement

This work was supported by the Basic Science Research Program (RS-2023-00242732) of the National Research Foundation of Korea (NRF).

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