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Breeding of Black Soybean with Green Cotyledon and Four Recessive Alleles for Lipoxygenase, Kunitz Trypsin Inhibitor, Lectin, and Stachyose

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Abstract: Anthocyanins from the black soybean seed coat are known to have many pharmaceutical effects. However, black soybean seed contains antinutritional factors such as lipoxygenase, Kunitz trypsin inhibitor (KTI), lectin, and stachyose. The genetic removal of these components will improve the nutritional value of black soybean seed. The objective of this research was to breed a soybean strain with the black seed coat color, green cotyledon color, and tetra recessive allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*) for lipoxygenase, KTI, lectin, and stachyose components. Eight parents were used to breed the tetra null strain. Analysis of lipoxygenase, KTI, lectin, and stachyose components in mature seeds was conducted by SDS-PAGE, Western blot, and HPLC. The soybean line with the black seed coat color, the green cotyledon color, a large seed size, and tetra recessive alleles has purple flowers, a determinate growth habit, and brown pods at maturity. The stem height of the breeding line was 52.3 cm. The 100-seed weight of the breeding line was 35.2 g and the yield (Ton/ha) was 2.50. The stachyose content of the breeding line was 3.30 g/kg. This is the first soybean strain with the black seed coat color, the green cotyledon color, a large seed size, and tetra null alleles (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*, low content of stachyose, free of lipoxygenase, KTI, and lectin proteins).

Keywords: lipoxygenase; KTI; lectin; stachyose; tetra null; black soybean

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1. Introduction

Black soybean (*Glycine max* (L.) Merr.) has been widely cultivated in Korea, China, and Japan for about 5000 years. Black soybean with green cotyledon has been known as a medicinal food by the health-promoting benefits. Anthocyanins are abundant in the seed coat of black soybean. Anthocyanins are known to have many health-promoting effects such as antioxidant effects, reduction in the risk of coronary heart disease, regulation of adhesion molecules, and protection from ischemia and reperfusion heart injury [1–3]. However, lipoxygenase protein, Kunitz trypsin inhibitor (KTI) protein, lectin protein, and stachyose components exist in the seed of black soybean. These components are mainly responsible for reducing the nutritional value of unprocessed soybeans.

Mature soybean seeds contain lipoxygenase protein, which accounts for approximately 1~2% of the total protein. Lipoxygenases can be isolated as three isoenzymatic forms and catalyze the hydroperoxidation of polyunsaturated fatty acids, such as linoleic (18:2) and linolenic (18:3) acids. Lipoxygenases play a role in the production of undesirable grassy and beany aromas and flavors in foods containing soybean. Three lipoxygenases (*Lox1*, *Lox2*, and *Lox3*) exist in mature soybean seeds [4]. Soybean strains free of *Lox1* [5,6], *Lox2* [7,8], and *Lox3* [9] have been identified, and the inheritance of the activity of each of the enzymes was studied. The absence of each enzyme is under the control of three null alleles, *lox1*, *lox2*, and *lox3*, which are inherited as simple recessive alleles. Genetic studies demonstrated that the *Lox1* and *Lox2* loci were found to be in a tight genetic linkage on chromosome 13,

with *lox1* and *lox2* mutant alleles being in the repulsion phase since they were identified in an independent germplasm [6–8]. The *Lox3* locus, on chromosome 15, segregates independently of *Lox1* and *Lox2* [7,8,10]. Extra costs are needed to inactivate lipoxygenase activity by heat at the industrial level, and soybean cultivars that are lipoxygenase-free are better accepted. A few lipoxygenase-free soybean cultivars have been developed [11].

Kunitz trypsin inhibitor (KTI) protein from soybean seeds was first isolated and crystallized in [12]. KTI protein is a small and non-glycosylated protein containing 181 amino acid residues with 21.5 kDa and strongly inhibits trypsin, thus reducing the food intake by diminishing digestion and absorption. Two soybean accessions (PI157440 and PI196168) lacking the KTI protein from the United States Department of Agriculture (USDA) Soybean Germplasm Collection have been identified [13]. Different forms of Ti^a, Ti^b, Ti^c, and Ti^d have been identified at a single locus with a codominant multiple allelic series [13–15]. Orf and Hymowitz [13] have identified a recessive allele designated *ti* that lacks soybean KTI protein. Crude protein from the *titi* genotype had a 30% to 50% reduction in trypsin inhibitor activity compared with the *TiTi* genotype. The Ti gene was found to be on chromosome 8.

Soybean lectin protein, consisting of four similar subunits that each have a molecular weight of 30 kDa, is a glycoprotein that specifically binds galactose or N-acetylgalactosamine. The concentration of soybean lectin protein ranges from about 1%~2% on seed dry mass [16]. Soybean lectin protein with 120 kDa molecular weight is a major antinutritional element and can strongly inhibit degradation by proteases under in vitro and in vivo conditions [17]. The biological activity of soybean lectin protein can be reduced by proper heating, but considerable biological activity is found after heating. This residual soybean lectin protein causes the negative effects on the nutritional quality of the soybean protein, such as the digestion and absorption of nutrients [18]. Orf et al. [19] identified that soybean seed lectin was controlled by a single gene designated *Le* (*le*) and the *lele* genotype, resulting in the lack of lectin in mature seed. The *Ti* gene was found to be on chromosome 2. Independent inheritance between *Le* and *Ti* loci was identified [13,20,21]. Triple null recessive genotypes (*ti/ti-le/le-p34/p34*) in soybeans were developed [22].

Stachyose is the primary carbohydrate in soybean seed. Content of stachyose ranges from 14 to 41 g/kg on a dry-weight basis and is environmentally stable but genotypically dependent [23,24]. Because stachyose is not readily digestible and causes flatulence or diarrhea for non-ruminant animals, stachyose is considered as an undesirable sugar in soybean seed [25]. Skoneczka et al. [26] identified that stachyose content was controlled by a single gene or a major Quantitative Trait Loci (QTL). The raffinose synthase 2 gene (*RS2*, Glyma06g18890) is the key in raffinose and stachyose biosynthesis. The *rs2* allele (containing three-bp deletions in the RS2 gene) results in low raffinose and stachyose in soybean line PI200508 [27]. The *RS2* locus is located in chromosome 6. No negative effects on traits of field emergence, seed yield, maturity, height, and fatty acid content between lines derived from PI200508 containing the reduced stachyose content and wild types were reported [28]. A heat treatment is required to reduce the activity of lipoxygenase, KTI, and lectin proteins that exist in mature raw black seeds. Additionally, this step may require energy costs as well as lower amino acid availability, and it alters the physical properties of soybean proteins. The genetic removal of these components could be an alternative to this problem. So far, only a few articles about soybean breeding in the line with the black seed coat color, the green cotyledon color, and free of both antinutritional and allergenic factors have been published. A soybean cultivar 'Gaechuck#1' that has the traits of black seed coat color, green cotyledon color, lipoxygenase-2,3-free, and KTI proteins-free was developed [11]. The genotype of 'Gaechuck#1' was *Lox1lox2lox3/Lox1lox2lox3-ti/ti*. A soybean strain with the black seed coat color, the green cotyledon color, KTI protein-free, and lectin protein-free was developed [29]. A soybean possessing a yellow seed coat color and triple recessive alleles (*ti/ti- le/le-p34/p34*) for KTI, lectin, and P34 proteins was developed [22]. The objective of this research was to breed a new black soybean line with

green cotyledon and tetra recessive alleles (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*) for lipoxygenase, KTI, lectin, and stachyose components.

2. Materials and Methods

2.1. Breeding Materials

Eight parents were used to breed a new black soybean line with green cotyledon and tetra recessive alleles. 'Seoritae' (landrace variety) was used as a parent for traits of large seed size, black seed coat color, and green cotyledon color. The PI506592 parent was used to introduce the traits for black seed coat color and large seed size into a tetra null strain. PI408251 (*lox1* allele), PI86023 (*lox2* allele), and PI417458 (*lox3* allele) parents were used for the lipoxygenase protein-free breeding line. For the breeding line without KTI and lectin proteins, PI157440 (*ti* allele) and PI548392 (*le* allele) parents were used, respectively. The PI200508 (*rs2* allele) parent was used for the breeding line with a low stachyose content. Phenotypes of eight parents for these four components, seed coat, and cotyledon are presented in Table 1.

Table 1. Phenotypes of the eight parents used in this experiment for lipoxygenase, Kunitz trypsin inhibitor (KTI), lectin, stachyose, seed coat, and cotyledon (Bold letter: recessive trait).

Cultivar/ Germplasm	Lipoxygenase			KTI	Lectin	Stachyose	Seed coat	Cotyledon
	1	2	3					
Seoritae	Present	Present	Present	Present	Present	Normal	Black	Green
PI408251	**Absent**	Present	Present	Present	Present	Normal	Black	Yellow
PI86023	Present	**Absent**	Present	Present	Present	Normal	Green	Yellow
PI417458	Present	Present	**Absent**	Present	Present	Normal	Yellow	Yellow
PI157440	Present	Present	Present	**Absent**	Present	Normal	Yellow	Yellow
PI548392	Present	Present	Present	Present	**Absent**	Normal	Black	Yellow
PI200508	Present	Present	Present	Present	Present	**Low**	Yellow	Yellow
PI506592	Present	Present	Present	Present	Present	Normal	Black	Yellow

2.2. Breeding Scheme

Recessive alleles for *lox1*, *lox2*, and *lox3* were determined by identifying absence of lipoxygenase- 1, 2, and 3 protein using SDS electrophoresis. Recessive alleles of *ti* and *le* were determined by confirming the absence of KTI and lectin proteins using the Western blot technique. The *rs2* allele, which determines the low stachyose content, was identified by examining the content of stachyose by the HPLC method. The genotype of *lox1/lox1-lox2/lox2* (lipoxygenase-1,2 protein-free) was selected from the population derived from the cross of PI408251 and PI86023. The *lox1/lox1-lox2/lox2-lox3/lox3* genotype (lipoxygenase-1,2,3 protein-free) was developed from the cross of the *lox1/lox1-lox2/lox2* genotype and PI417458. From a cross of the 'Seoritae' cultivar and the *lox1lox2lox3/lox1lox2lox3* genotype, a strain possessing the *lox1lox2lox3/lox1lox2lox3* genotype, a black seed coat, and green cotyledon was developed. A *lox1lox2lox3/lox1lox2lox3* plant with a black seed coat and cotyledon was crossed to the PI200508 parent (*rs2/rs2* genotype) to select a strain with a black seed coat, green cotyledon, lipoxygenase-free, and low content of stachyose (*lox1lox2lox3/lox1lox2lox3-rs2/rs2* genotype, lipoxygenase protein-free, and low content of stachyose). The genotype of *ti/ti-le/le* (KTI-free and lectin proteins-free) was developed from the cross of PI157440 and PI548392 parents. From a cross of the 'Seoritae' cultivar and the *ti/ti-le/le* genotype, a strain with a black seed coat, green cotyledon, and the *ti/ti-le/le* genotype was developed. The strain with a black seed coat, green cotyledon, a large seed size, and the *ti/ti-le/le* genotype was developed from the cross of a strain with a black seed coat, green cotyledon, and the *ti/ti-le/le* genotype and PI506592. A triple null genotype (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le,* free of lipoxygenase, KTI, and lectin proteins) with a black seed coat, green cotyledon, and a large seed size was developed from the cross of the *lox1lox2lox3/lox1lox2lox3* genotype (black seed coat and green cotyledon) and the

ti/ti-le/le genotype (black seed coat, green cotyledon, and large seed size). During the summer of 2016, F_1 pollinations were made between the *lox1lox2lox3/lox1lox2lox3-rs2/rs2* genotype (black seed coat and green cotyledon) and the *lox1lox2lox3/lox1lox2lox3-ti/ti-le/le* genotype (black seed coat, green cotyledon, and large seed size) to produce seeds with a tetra null allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*, low content of stachyose, and free of lipoxygenase, KTI, and lectin proteins), a black seed coat, green cotyledon, and a large seed size. F_1 seeds obtained were planted on 20 February 2017 in the greenhouse and all F_1 plants were individually harvested and bulked. A total of 92 F_2 seeds were obtained. Each seed was analyzed to screen for the genotype with a triple recessive allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le* (absence of lipoxygenase, KTI, and lectin proteins)). Among 92 F_2 seeds, 9 F_2 seeds possessing the triple null allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le*) were obtained and were planted on 10 July 2018 in the University field. Nine F_2 plants were individually harvested. Random F_3 seeds of each F_2 plant harvested were analyzed to select the *rs2rs2* genotype (low content of stachyose). Three F_2 plant strains (F_3 seed) with the tetra null genotype (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*) were selected. A random sample of 50 F_3 seeds per strain were planted on 6 July 2019 in University field. One F_3 plant strain was chosen based on plant type, maturing date, stem height, seed coat color, cotyledon color, seed quality, and seed weight. After harvesting, random F_4 seeds were used to confirm recessive genotypes (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*) for lipoxygenase, KTI, lectin, and stachyose components. The scheme for breeding of the tetra null genotype (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*) with a black seed coat and green cotyledon is presented in Figure 1.

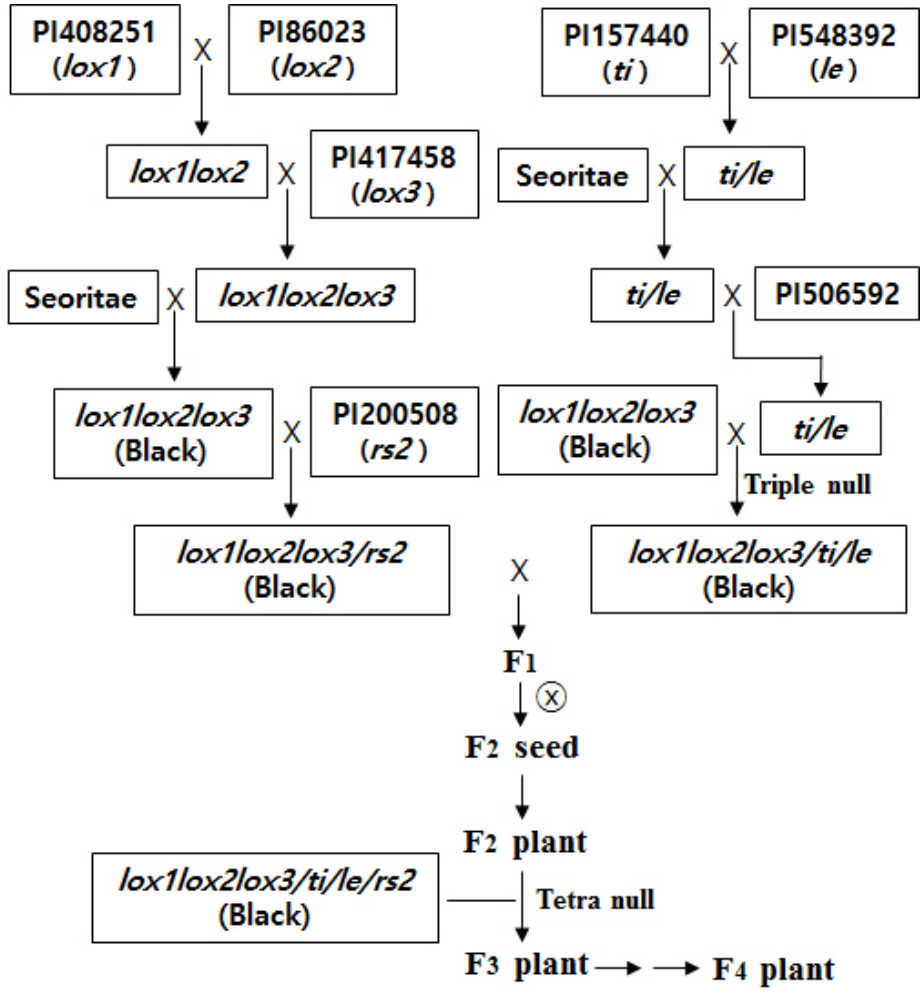

Figure 1. Scheme for development of a tetra null genotype (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*) with a black seed coat and green cotyledon using eight soybean parents.

2.3. Agricultural Traits of Tetra Null Genotype

A random 150 F_4 seeds were planted on 9 July 2020 in University field. The experimental field design was a completely randomized design with three replications. The plots were two rows 3 m long spaced 0.65 m apart within the plot. The seeding rate was 25 seeds per row. Soil type was a silty clay loam. Soil K, Ca, Mg, and Na averaged 0.46, 8.84, 2.83, and 0.28 $cmol_c/kg$, respectively. Soil pH was 6.8. Agronomic traits such as maturing date, stem height (cm), 100-seed weight (g), yield for breeding line (tetra null genotype), and check cultivar ('Chungja#3', *Lox1Lox2Lox3/Lox1Lox2Lox3-Ti/Ti-Le/Le-RS2/RS2,* black seed coat, and green cotyledon) were recorded on the F_4 plant generation. Mean values of stem height, 100-seed weight, and yield were compared by Duncan's multiple range test at the 5% level.

2.4. Determination of Lipoxygenase Protein by SDS-PAGE

Crude protein from random seeds of the check cultivar ('Chungja#3') and breeding line was obtained to identify the presence ('+') or absence ('−') of lipoxygenase protein. The fine powder samples of two materials were incubated for 30 min in 1 ml of Tris-HCl, pH 8.0, and 1.56% v/v β-mercaptoethanol. Fifty microliters (50 µl) of the supernatant through centrifugation was added to an equivalent amount of 5X sample buffer containing 1 M Tris-HCl, pH 6.8, 50% v/v glycerol, 1.96% v/v β-mercaptoethanol, and 10% w/v sodium dodecyl sulfate (SDS). The sample obtained was boiled at 97 °C for 5 min and the sample was centrifuged. Two microliters (2 µl) of the supernatant was loaded on a 12% acrylamide SDS polyacrylamide gel in electrophoresis medium gels (Owl Separation Systems Inc., Model: P9DS, Portsmouth, NH, USA). After electrophoresis at 120 V for 7 h, gels were stained. For several hours, the gels were destained in destaining solution. Protein marker (Sigma Marker, Product Code: M4038, St. Louis, MO, USA) was used to identify the presence or absence of lipoxygenase protein (97 kDa).

2.5. Determination of KTI and Lectin Protein by Western Blot Analysis

Proteins obtained from each F_2 seed, check cultivar ('Chungja#3'), and breeding line were separated by 10% or 12% SDS-PAGE and transferred onto an Immobilon-P membrane (PVDF, Millipore, Merck, Kenilwork, NJ, USA). After blocking for 2 h in TBS buffer containing 0.1% Tween 20, 20 mM Tris (pH 7.5), 150 mM NaCl, and 5% nonfat dried milk (Cell Signaling Technology, Danvers, MA, USA), the membrane was incubated with an antibody of KTI and lectin protein for 1 h. The blot was incubated with a horseradish peroxidase conjugated secondary antibody after washing in TBS buffer. Using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK), the complex was visualized. Presence or absence of KTI (21.5 kDa) and lectin (120 kDa) proteins was determined visually. The ratio of segregation for presence or absence of KTI and lectin proteins was determined by Chi-square analysis.

2.6. Stachyose Analysis

Stachyose content was determined by a High-Performance Liquid Chromatograph (HPLC) method. Each seed sample from a single F_2 plant (F_3 seed), the check cultivar ('Chungja#3'), and the breeding line was ground into powder for HPLC analysis. Two hundred milligrams (200 mg) of ground seed sample was extracted with 3 mL of acetone in a water bath for 2 h at 60 °C. The mixture was centrifuged for 5 min at 2000 rpm to remove the fat. We added 1.9 mL of double distilled H_2O to the defatted extract in a water bath for 2 h at 60 °C. After the water bath treatment, 0.1 mL of 1 M 5-sulfosalicylic acid (5-SSA) was added to the mixture and it was placed overnight at 4 °C. The mixture was centrifuged for 5 min at 3000 rpm. The supernatant was added to 0.8 mL of ddH_2O and was centrifuged for 10 min at 12,000 rpm. After centrifugation, the supernatant was filtered through a 0.2 µm membrane filter and stored at 4 °C prior to the HPLC analysis. Stachyose was analyzed using an Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany), an R1 Refractive Index Detector, and a Supelcogel 610-H-column (30cm × 7.8mm ID column,

9 µm, Supelco, Bellefonte, PA, 16823-0048 USA). The eluent solvent of 0.1% H_3PO_3 was used, and HPLC conditions were a 10 µL injection volume and a 0.6 mL/min flow rate.

3. Results

3.1. Inheritance of KTI and Lectin Proteins

KTI protein of 21.5 kDa and lectin protein of 120 kDa were segregated in the 92 F_2 seed generation (Table 2).

Table 2. Segregation for presence or absence of Kunitz trypsin inhibitor (KTI) and lectin proteins in the F_2 seed generation derived from the cross of the *lox1lox2lox3/lox1lox2lox3-Ti/Ti-Le/Le-rs2/rs2* parent and the *lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-RS2/RS2* parent.

KTI	Lectin	Number of Seeds		χ^2 Value (9:3:3:1)	*p*
		Observed	Expected		
Present	Present	45	51.75	3.13	0.5–0.1
Present	Absent	18	17.25		
Absent	Present	20	17.25		
Absent	Absent	9	5.79		

Among the 92 F_2 seeds, 63 F_2 seeds showed KTI protein and 29 F_2 seeds did not show KTI protein. Lectin protein existed in 65 F2 seeds and 27 F2 seeds did not show lectin protein. The segregation ratio for the presence or absence of KTI and lectin proteins in the F_2 seed generation was fitted to an expected 3:1 ratio (χ^2 = 2.09 for KTI and 0.93 for lectin proteins). Between KTI protein and lectin protein, the segregation ratios of 45 *Ti_Le_*: 18 *Ti_lele*: 20 *titiLe_*: 9 *titilele* were observed (χ^2 = 3.13, *p* = 0.5–0.1).

3.2. Content of Stachyose for F_2 Plants with Triple Null Allele

Stachyose content for nine F_2 plants was obtained using random F_3 seeds of each F_2 plant harvested (Table 3).

Table 3. Content of stachyose for nine F_2 plants (F_3 seeds) with the triple null allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le*).

Number of F_2 Plants	Stachyose (g/kg)	Genotype Expected
1	12.71	*RS2_*
2	13.14	*RS2_*
3	3.26	*rs2rs2*
4	12.83	*RS2_*
5	3.17	*rs2rs2*
6	3.53	*rs2rs2*
7	14.33	*RS2_*
8	13.91	*RS2_*
9	12.51	*RS2_*

The stachyose content of the nine F_2 plants was 3.17–14.33 g/kg. Three F_2 plants showed a low stachyose content of 3.26, 3.17, and 3.53 g/kg. One F_3 plant strain from three F_2 plants was chosen based on agronomical traits.

3.3. Confirmation of Tetra Null Line

Random F_5 seeds were used to confirm the absence of lipoxygenase, KTI, and lectin proteins (Figure 2).

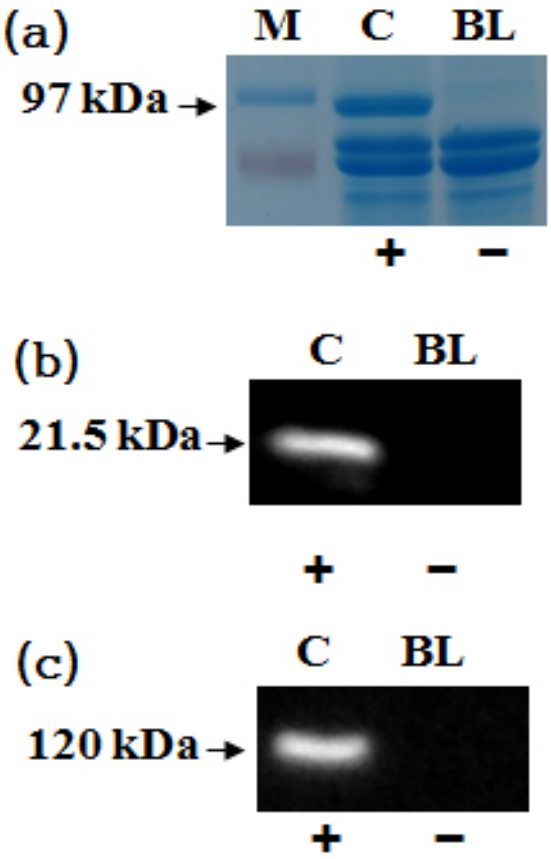

Figure 2. Determination of lipoxygenase protein (**a**), Kunitz trypsin inhibitor (KTI) protein (**b**), and lectin protein (**c**). M, marker; C, 'Chungja#3' (*Lox1Lox2Lox3/Lox1Lox2Lox3-Ti/Ti-Le/Le-RS2/RS2*); BL, tetra null line (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2*/rs2). +, −: presence and absence of lipoxygenase, KTI, and lectin proteins, respectively.

Proteins of lipoxygenase, KTI, and lectin were not observed in the mature F_5 seed of the breeding line (BL). However, these three proteins were observed in the seed of the 'Chungja#3' (*Lox1Lox2Lox3/Lox1Lox2Lox3-Ti/Ti-Le/Le-RS2/RS2*) cultivar.

3.4. Agronomic Traits of Tetra Null Line

Some agronomic traits of the breeding line are shown in Table 4.

Table 4. Agronomic performance of cultivar 'Chungja#3' and the breeding line under field conditions during 2020.

Cultivar/Breeding Line	Planting Date	Maturing Date	Stem Height (cm)	Seed Weight (g/100 Seeds)	Stachyose (g/kg)	Yield (Ton/ha)
'Chungja#3'	9 June	18 October	50.7 [a] [1]	33.3 [a]	12.64 [a]	2.10 [a]
Breeding Line	9 June	16 October	52.3 [a]	35.2 [b]	3.30 [b]	2.50 [b]

[1] Same letters in the column are not significant at the 5% significance level by Duncan's multiple range tests.

The breeding line developed in this study has purple flowers, a determinate growth habit, and brown pods at maturity. The breeding line matured on October 16, which is 2 days earlier than 'Chungja#3'. The stem height of the breeding line was 52.3 cm, while that of the check cultivar was 50.7 cm. The 100-seed weight of the breeding line was 35.2 g larger than that of 'Chungja#3' (33.3 g). The stachyose content of the breeding line was 3.30 g/kg, which was much less than the 12.64 of 'Chungja#3'. The yield of the breeding line was 2.50 Ton/ha—much higher than that of 'Chungja#3' (2.10 Ton/ha). The plant type

harvested and seeds of the tetra null strain (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*) are shown in Figure 3.

Figure 3. Appearance of the F_4 plant and F_5 seeds with the tetra null allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*), a black seed coat, and green cotyledon.

The seed of the breeding line has a black hilum and a black seed coat color. The cotyledon color of mature seeds is green.

4. Discussion

In Asia, soybean cultivars with a black seed coat, green cotyledon, and large seed size have been cultivated for a long time. Anthocyanins that are abundant in the seed coat of black soybean are known to have many pharmacological effects. Lipoxygenase, Kunitz trypsin inhibitor (KTI), lectin, and stachyose components that exist in the raw mature seeds of black soybean have been considered as antinutritional and allergenic factors [30,31]. The genetic removal of these factors is needed to modify the food processing properties and to improve the nutritional values of soybeans. Moreover, the variety of the tetra recessive allele (absence of lipoxygenase, KTI, and lectin proteins, low content of the stachyose component) enhances the utilization of soybean foods. To obtain seeds possessing the tetra null allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*), two parents (the *lox1lox2lox3/lox1lox2lox3-rs2/rs2* genotype and the *lox1lox2lox3/lox1lox2lox3-ti/ti-le/le* genotype) were developed using eight parents (Table 1, Figure 1). A total of 92 F_2 seeds were obtained from the cross of these two parents. KTI and lectin proteins were segregated in the F_2 seed generation (Table 2). The segregation ratio for the presence or absence of KTI and lectin proteins was fitted to an expected 3:1 ratio (χ^2 = 2.09 for KTI and 0.93 for lectin proteins). This result substantiates previous results that the presence or absence of KTI and lectin proteins is controlled by a single gene [13,19]. Independent inheritance between KTI protein and lectin protein was observed (χ^2 = 3.13). This result was found to be consistent with previous results, which show that both *Ti* and *Le* alleles were inherited independently [13,20,21,29]. The *Le* allele that was inherited independently with the *Ti* allele in the F_2 population consisted of 24 plants [21]. The *Ti* and *Le* alleles were inherited independently in the F_2 population with 96 plants [13]. Lee et al. [20] reported that the *Ti* and *Le* alleles were independently inherited in a 173 F_2 seed generation. Additionally, Choi et al. [29] observed that *Ti* and *Le* alleles were independently inherited in an F_2 seed generation consisting of 179 seeds. Among nine F_2 plants with the triple null allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le*), three F_2 plants showed a low stachyose content (*rs2rs2* genotype). The stachyose content of three F_2 plants was 3.17–3.53 g/kg (Table 3). This result suggests that stachyose content was controlled by a single recessive gene [26]. Three F_2 plants possessing a

triple null allele (*ti/ti-le/le-rs2/rs2*) were selected. In previous research, two F_2 seeds possessing a triple null allele (*ti/ti-le/le-p34/p34*) were selected from 150 F_2 seeds [22]. Absence of lipoxygenase, KTI, and lectin proteins in F_5 seeds of the tetra null line (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2* genotype) developed in this study was confirmed (Figure 2).

Agricultural traits of the tetra null soybean line are shown in Table 4. In spite of low content of stachyose and the absence of lipoxygenase, KTI, and lectin proteins, the tetra null soybean line germinated, grew, flowered, and reproduced normally under field conditions when compared with cultivar 'Chungja#3'. Seeds with the *lox1lox2lox3/lox1lox2lox3* genotype (free of lipoxygenase protein) have been demonstrated to develop into normal plants without defects [10]. Schmidt et al. [22] observed that plants possessing triple null alleles (*ti/ti-le/le-p34/p34*) flowered and produced seeds without any overt differences in comparison to the standard 'Williams 82' cultivar. Significant differences were observed for seed weight (g/100 seeds), content of stachyose (g/kg), and yield (Ton/ha) between the tetra null soybean line and 'Chungja#3' cultivar. These results indicate that the tetra null soybean line with a black seed coat, green cotyledon, and large seed size had no impact on these agronomic traits (Figure 3). These results suggest that accumulation of recessive alleles for the *Lox1*, *Lox2*, *Lox3*, *Ti*, *Le*, and *RS2* genes result in a soybean cultivar with significantly reduced allergy and antinutritional factors. In this study, quantitative traits such as yield, stem height, and seed weight for the tetra null line were obtained in a single location with three replications (Table 4). However, it is considered that field experiments with years and locations should be carried out for accurate evaluation in the future. For the breeding line, generation advancement should be conducted and qualitative traits like flower color, pubescence color, and pod color should be checked. Additionally, repeated experiments on quantitative traits such as maturing date, stem height, seed weight, stachyose content, protein content, oil content, and yield should be further conducted. Studies on the quality and functionality of foods made from seeds of the tetra null line should be conducted and the level of allergens present in foods must be investigated. The newly improved strain in this research will be used to develop a new soybean cultivar with a black seed coat, green cotyledon, high quality, and function.

5. Conclusions

Eight parents were used to breed a new soybean strain with a black seed coat color, a green cotyledon color, and tetra recessive alleles (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-rs2/rs2*) for lipoxygenase, KTI, lectin, and stachyose components. From segregation for KTI and lectin proteins in an F_2 seed generation, a ratio of 45 *Ti_Le_*: 18 *Ti_lele*: 20 *titiLe_*: 9 *titilele* was observed (χ^2 = 3.13, p = 0.5–0.1). Nine F_2 seeds possessing a triple null allele (*lox1lox2lox3/lox1lox2lox3-ti/ti-le/le*) were obtained. Three F_2 plants showed a low stachyose content of 3.26, 3.17, and 3.53 g/kg. One F_3 strain with proper agronomical traits was selected. Proteins of lipoxygenase, KTI, and lectin were not observed in the mature F_5 seeds of the tetra null line. The soybean line with a black seed coat, green cotyledon, large seed size, and tetra recessive allele has purple flowers, a determinate growth habit, and brown pods at maturity. The breeding line matured in October 16, which is 2 days earlier than 'Chungja#3'. The stem height of the breeding line was 52.3 cm, while that of the check cultivar was 50.7 cm. The 100-seed weight of the breeding line was 35.2 g, larger than that of 'Chungja#3' (33.3 g). The stachyose content of the breeding line was 3.30 g/kg, which was much less than the 12.64 of 'Chungja#3'. The yield of the breeding line was 2.50 Ton/ha—much higher than that of 'Chungja#3' (2.10 Ton/ha).

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