Breeding of Penta Null Soybean [Glycine max (L.) Merr.] for Five Antinutritional and Allergenic Components of Lipoxygenase, KTI, Lectin, 7S α′ Subunit, and Stachyose

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INTRODUCTION

Soybean [Glycine max (L.) Merr.] is a major leguminous crop that has been cultivated for thousands of years. Soybean seeds are an important source of protein, oil, carbohydrates, isoflavones, and many other nutrients for human food and animal feed. Generally, soybean seeds contain about 40% protein, 20% oil, 30% carbohydrates, and various health functional ingredients. However, there are also antinutritional and harmful immunological components...
such as lipoxygenase protein, Kunitz trypsin inhibitor (KTI) protein, 7S α’ subunit protein, and stachyose in mature soybean seeds.

Lipoxygenase protein causes undesirable grassy and beany flavors in foods containing soybean due to the oxidation of polyunsaturated fatty acids. There are three lipoxygenases (Lox1, Lox2, and Lox3) in mature seeds. Previous studies demonstrated that the absence of each enzyme is under the control of three null alleles, lox1, lox2, and lox3, which are inherited as simple recessive alleles (Hildebrand and Hymowitz, 1982; Kitamura et al., 1983; Davies and Nielsen, 1986).

Lipoxygenase-free genotypes are accepted better due to the production of very low levels of hexanal compounds (Kobayashi et al., 1995). The development of lipoxygenase-free genotypes through genetic elimination is the key to removing the beany flavor. Several lipoxygenase protein-free cultivars have been improved (Kim et al., 1997, 2015; Chung, 2014). Recently, lipoxygenase-free mutants were obtained using a pooled CRISPR-Cas9 system (Wang et al., 2020). Soybean Kunitz trypsin inhibitor (KTI) protein, which was isolated and crystallized by Kunitz (1945), is a small and non-glycosylated protein possessing 181 amino acid residues in a lack of lectin in mature seed (Orf et al., 1978). The single gene designated (Katsuya et al., 2007). Soybean seed lectin is controlled by coat color and penta null recessive alleles (Hildebrand and Hymowitz, 1979). Crude protein from the allele designated ti (Medic et al., 2014). The three subunits of β-conglycinin (7S), α, α’, and β are dominated by genes Cgy1, Cgy2, and Cgy3, respectively (Davies et al., 1985). Among these three genes, a line with the homozygous recessive Cgy1 gene cannot produce the 7S α’ subunit protein in mature seed (Kitamura et al., 1984). Cgy1 gene was found to be on chromosome 10. A soybean experimental line (BSH-3) that is 7S α’ subunit protein free was developed by crossing the mutant donor line “HS99B” with the Chinese cultivar “Dongnong47” (Song et al., 2018).

Raffinose and stachyose are considered anti-nutritional factors because humans cannot digest them after absorption (Hata et al., 1991). Stachyose is the primary carbohydrate in soybean seed. Stachyose content ranges from 14 to 41 g/kg on a dry weight basis and is environmentally stable but genetically dependent (Hymowitz et al., 1972). The amount of stachyose was controlled by a single gene or by a major quantitative trait locus (QTL; Škonečzka et al., 2009). The raffinose synthase 2 gene is a pathway for raffinose and stachyose biosynthesis. Soybean line PI200508 with a homozygous recessive genotype (rs2R) showed low raffinose and stachyose content (Dierking et al., 2008). R52 locus was found to be located on chromosome 6. Two SSR markers, Sat_293 (LG-K/chr9) and Satt281 (LG-C2/chr6), were identified for stachyose in two F2 populations (Jha et al., 2022).

Because of these anti-nutritional factors and allergens that exist in raw soybean seeds, heat treatment or other methods are needed to eliminate or reduce these components and secure the efficiency of nutrient absorption and food safety. But these treatments cause some changes and reduce soybean quality (Chen et al., 2019). Also, heat inactivation of the lipoxygenase at an industrial level not only incurs extra cost but also affects the solubility and functionality of proteins (MacLeod and Ames, 1988). Antigenic proteins remain in soybean food even after heat treatment and fermentation (Wilson et al., 2008). Genetic removal of lipoxygenase, KTI, lectin, 7S α’ subunit, and stachyose components that exist in mature soybean seed would be the best method for the soybean food industry. Only a few papers on soybean free of these anti-nutritional and allergenic components have been published. A soybean line with a triple null recessive genotype (ti/ti-le/le-p34/p34) for KTI, lectin, and P34 proteins was developed (Schmidt et al., 2015). A soybean line with a tetra null recessive genotype (lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-cgy1/cgy1) for lipoxygenase, KTI, lectin, and 7S α’ subunit proteins was also developed (Choi et al., 2021). So far, a soybean line with a penta null recessive genotype for anti-nutritional and allergenic factors has not been developed. Therefore, the objectives of this research were to breed a new soybean line with a yellow seed coat color and penta null recessive alleles (lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-cgy1/cgy1-rs2R) for lipoxygenase, KTI, lectin, 7S α’ subunit, and stachyose components and to evaluate agronomic traits for a breeding line with penta null alleles.
MATERIALS AND METHODS

Breeding Materials

Seven germplasms were used to improve the new soybean strain with the penta null genotype for five components. The presence and absence of four proteins, stachyose content, seed coat, 100-seed weight (g), and origin of the seven germplasms used in this study are presented in Table 1. Three breeding lines (lox1lox1/lox2lox2/lox3lox3 genotype—lipoxygenase protein free; lox1lox1/lox2lox2/lox3lox3-α′ subunit genotype—lipoxygenase and 7S α′ subunit proteins free; lox1lox1/lox2lox2/lox3lox3-α′ allele—lipoygenase, KTI, lectin, and 7S α′ subunit proteins free, Choi et al., 2021) and one germplasm (PI200508) were used to create a genetic population. The PI200508 parent has an rs2rs2 genotype with low stachyose content (Dierking and Bilyeu, 2008). Three breeding lines and one germplasm have a yellow seed coat color in mature seeds.

Breeding Scheme

The parent with the lox1lox2lox3/lox1lox2lox3 genotype was crossed with the PI200508 parent with the rs2rs2 genotype to select a seed with a homozygous lox1lox2lox3/lox1lox2lox3-α′ genotype. The plants were grown in the greenhouse to produce seeds possessing penta null alleles (lox1lox2lox3/lox1lox2lox3-α′). After harvesting, random F5 seeds were used to confirm recessive genotypes (lox1lox2lox3/lox1lox2lox3-α′) by observing the absence of lipoxygenase, KTI, lectin, and 7S α′ subunit proteins. Low stachyose content (rs2rs2 genotype) for the strain developed was confirmed by HPLC (Sung et al., 2014). The scheme for the improvement of penta null lines (lox1lox2lox3/lox1lox2lox3-α′) is presented in Figure 1.

Determination of Lipoxygenase and 7S α′ Subunit Proteins by SDS-PAGE

Crude protein from the random F5 seeds of the breeding line and random seeds of the cultivar (“Daewon”) was obtained to identify the presence (“+”) or absence (“−”) of lipoxygenase and 7S α′ subunit proteins. Lipoxygenase and 7S α′ subunit proteins were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to Fling and Gregerson (1986). The cultivar (“Daewonkong”) was used as a control for the presence of lipoxygenase and 7S α′ subunit proteins (lox1lox2lox3/lox1lox2lox3-Cgly1/Cgly1 genotype). Fine powder samples of the two materials were incubated for

| Germplasm name | Lipoygenase | KTI | Lectin | 7S α′ subunit | Stachyose | Seed coat | 100-seed weight (g) | Origin |
|----------------|-------------|-----|--------|---------------|-----------|-----------|-------------------|--------|
| PI408251       | Absent      | Present | Present | Present | Present | Present | Normal | Black | 6.1 | Korea |
| PI88023        | Present     | Absent | Present | Present | Present | Present | Present | Normal | Green | 16.8 | Japan |
| PI417458       | Present     | Present | Absent  | Present | Present | Present | Absent | Yellow | 11.8 | Japan |
| PI200508       | Present     | Present | Absent  | Present | Present | Present | Present | Normal | Yellow | 14.5 | Korea |
| PI506876       | Present     | Present | Present | Present | Absent  | Present | Normal | Black | 6.7 | United States |
| PI157440       | Present     | Present | Present | Absent  | Present | Present | Present | Normal | Yellow | 15.1 | Japan |
| T102           | Present     | Present | Present | Present | Present | Present | Present | Normal | Black | 6.7 | United States |

TABLE 1 | Seed coat, 100-seed weight, stachyose content, origin, presence or absence of lipoxygenase, Kunitz trypsin inhibitor (KTI), lectin and 7S α′ subunit for seven germplasms.
30 min in 1 ml of Tris–HCl, pH 8.0, and 1.56% v/v β-mercaptoethanol. About 50 μl of the supernatant collected through centrifugation was added to an equivalent amount of 5× 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 centrifuged. About 2 μl of the supernatant was loaded on a 12% acrylamide SDS polyacrylamide gel electrophoresis medium from Owl Separation Systems Inc. (model: P9DS, Portsmouth, NH, United States). After electrophoresis at 120 V for 7 h, the gel was stained. The gel was then destained in destaining solution for several hours. A protein marker (Sigma Marker, Product Code: M4038) was used to identify the presence or absence of lipoxygenase protein (97 kDa) and 7S α′ subunit protein (72 kDa).

**Determination of KTI and Lectin Protein by Western Blot Analysis**

Proteins obtained from the parental seed, each F2 seed, and random F5 seeds of the breeding line and cultivar (“Daewon”) were separated by 10% or 12% SDS-PAGE and transferred onto an Immobilon-P membrane (PVDF, Millipore). Western blot analysis for the KTI protein was performed as previously described (Krishnan et al., 2000; Krishnan, 2001). Preparation of the antibody and western blot for lectin protein analysis was performed according to a previous method (Vodkin and Raikhel, 1986). The cultivar (“Daewonkong”) was used as a control for the presence of KTI and lectin proteins (TTI-LeLe genotype). 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 (Carnation, Glendale, CA), the membrane was incubated with the 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. The complex was then visualized using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, United Kingdom). The presence or absence of KTI and lectin proteins was determined visually. In F2 seed generation, the ratio of segregation for the presence or absence of KTI and lectin proteins was determined by Chi-square analysis.

**Determination of rs2rs2 Genotype and Stachyose Component**

Young leaves from each of the 10 F2 plants possessing tetra null alleles (lox1lox2lox3/lox1lox2lox3-ti/ti-le/le-cgy1/cgy1-rs2rs2) for lipoxygenase, KTI, lectin, 7S α′ subunit and stachyose components.
CGTGGAGCAGGTATGTGGG-3’, Reverse:5’-GGCACCA GTCTAACT CCG TTAC-3’) were designed according to previous results (Dierking and Bilyeu, 2008). PCR for the genotype assay was carried out in a PTC-200 thermocycler (MJ Research/ Bio-Rad, Hercules, CA, United States) with the following conditions: 95°C for 5 min followed by 29 cycles of 95°C for 20 s, 65°C for 20 s, 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were electrophoresed in 2.5% 0.5x TBE agarose gels and were stained with EtBr. Gels were photographed under transmitted UV light. Stachyose content was analyzed for the segregation of KTI and lectin proteins. The KTI protein of 21.5 kDa and lectin protein of 120 kDa were segregated in the F2 seed generation (Figure 2). The segregation data for KTI and lectin proteins in the F2 seed generation are presented in Table 2.

### Agronomic Traits of Penta Null Genotype

First, 360 random F2 seeds of the breeding line and seeds of a cultivar (“Daewonkong”) as a control were planted in the university field on 15 July 2021. The experimental field had a completely randomized design with three replications. The plots included four rows 3-m long spaced 0.65 m apart. The seeding rate was 30 seeds per row. The soil type was a silty clay loam. Soil K, Ca, Mg, and Na averaged 0.46, 8.84, 2.83, and 0.28 cmolc/kg, respectively. Soil pH was 6.8. Agronomic traits such as maturation date, stem height, number of pods per plant, number of seeds per plant, 100-seed weight, stachyose content, and yield were recorded for the F2 plant generation of the breeding line (penta null genotype). The mean values of stem height, number of pods per plant, number of seeds per plant, 100-seed weight, stachyose content, and yield were compared by Duncan’s multiple range test at the 5% level.

### RESULTS

#### Selection of F2 Seeds With KTI and Lectin Proteins Free

A total of 172 F2 seeds were obtained from the cross of the lox1lox2lox3/lox1lox2lox3-rs2/rs2-cgy1/cgy1 parent and lox1lox2lox3/lox1lox2lox3-ti/ti-le-le-cgy1/cgy1 parent. Each seed was analyzed for the segregation of KTI and lectin proteins. KTI protein of 21.5 kDa and lectin protein of 120 kDa were segregated in the F2 seed generation (Figure 2). The segregation data for KTI and lectin proteins in the F2 seed generation are presented in Table 2.

Among the 172 F2 seeds, 138 F2 seeds showed KTI protein and 34 F2 seeds did not show KTI protein. Lectin protein existed in 126 F2 seeds, and 46 F2 seeds did not show lectin protein. The segregation ratio for the presence or absence of KTI and lectin proteins in the F2 seed generation was fitted to an expected 3:1 ratio (\( \chi^2 = 2.51 \) for KTI and 0.28 for lectin proteins). Between KTI protein and lectin protein, the segregation ratios of 102 Ti_Le_: 36 Ti_le: 24 titiLe_: 10 titile were observed (\( \chi^2 = 2.883, p = 0.5–0.1 \)). Ten F2 seeds possessing tetra null alleles (lox1lox2lox3/lox1lox2lox3-ti/ti-le-le-cgy1/cgy1) were selected and planted to select the plant in the greenhouse with the rs2rs2 genotype based on the DNA marker.

#### Selection of F2 Plants With rs2rs2 Genotype Using DNA Marker

The rs2 allele-specific DNA marker showed segregation according to the individual plant in the F2 plant population consisting of 10 plants with the tetra null genotype (lox1lox2lox3/lox1lox2lox3-ti/ti-le-le-cgy1/cgy1). Among 10 F2 plants, eight showed a band (Rs2_genotype) and two showed no band (Figure 3). Thus, two F2 plants were identified as having the rs2rs2 genotype. At maturity, two F2 plants identified

![FIGURE 2](https://www.frontiersin.org) Segregation of KTI (A) and lectin (B) proteins in the parents and F2 seeds. Arrows indicate the KTI protein of 21.5 kDa and lectin protein of 120 kDa. P1: lox1lox2lox3/lox1lox2lox3-Ti/Ti-Le/Le-cgy1/cgy1-rs2/rs2 genotype, P2: lox1lox2lox3/lox1lox2lox3-ti/ti-le-le-cgy1/cgy1-Rs2/Rs2 genotype. +, - : presence and absence of KTI and lectin proteins.
As having the penta null genotype (lox1lox2lox3/lox1lox2lox3-\textit{ti}/\textit{ti}-\textit{cgy1}/\textit{cgy1}) were harvested separately. The \( F_3 \) seeds obtained were used to advance \( F_5 \) plant generation.

**Confirmation of Penta Null Line**

Random \( F_2 \) seeds for the breeding line were used to confirm the absence of lipoxygenase, KTI, lectin, and 7S \( \alpha' \) subunit proteins (Figure 4). Proteins of lipoxygenase and 7S \( \alpha' \) subunit were not observed in the mature \( F_2 \) seed of the breeding line by using SDS-PAGE analysis. Also, proteins of KTI and lectin were not observed by using western blot analysis. However, these four proteins were observed in the seed of the “Daewonkong” \((\text{Lox1Lox2Lox3 }/\text{Lox1Lox2Lox3-}\text{Ti}/\text{Ti-Le}/\text{Le-Cgy1/Cgy1-Rs2/Rs2})\) cultivar.

**Agronomic Traits of Penta Null Line**

The breeding line has purple flowers, tawny pubescence, a determinate growth habit, and light yellow pods at maturity. Some quantitative traits of the breeding line are shown in Table 3. The breeding line matured on 16 October, which was 3 days later than “Daewonkong.” The stem height of the breeding line was 53.0 cm compared to the cultivar, “Daewonkong,” at 48.0 cm. The number of pods per plant and seeds per plant for the breeding line was 52 and 84, respectively. The stachyose content of the breeding line was 2.9 g/kg, which was much lower than 12.7 g/kg of the cultivar, “Daewonkong.” The 100-seed weight of the breeding line was 31.1 g, a little higher than that of “Daewonkong” (29.5 g). The yield of the breeding line was 2.80 t/ha, which was slightly higher than that of “Daewonkong” (2.78 t/ha). The plant type harvested and seeds of the breeding line with the penta null genotype \((\text{lox1lox2lox3/lox1lox2lox3-}\text{ti}/\text{ti}-\text{le-}\text{cgy1/cgy1-}\text{rs2/rs2})\) are shown in Figure 5. The seed of the breeding line has a yellow hilum and yellow seed coat color. The cotyledon color of the mature seed is yellow.

**DISCUSSION**

Demand for soybean and soybean products has increased in recent years because soybean is an excellent source of protein, oil, carbohydrates, and many other bioactive ingredients for humans. However, the lipoxygenase, KTI, lectin, 7S \( \alpha' \) subunit, and stachyose components that exist in the raw mature seeds of soybean are considered antinutritional and allergenic factors.

**Table 2** Segregation for the presence (+) or absence (−) of Kunitz trypsin inhibitor (KTI) and lectin proteins in \( F_2 \) seed generation derived from the cross of a \( \text{lox1lox2lox3/lox1lox2lox3-}\text{rs2/rs2-}\text{cgy1/cgy1} \) parent and \( \text{lox1lox2lox3/lox1lox2lox3-}\text{ti/le-cgy1/cgy1} \) parent.

| KTI  | Lectin | Seed number | \( \chi^2 \) value (9:3:3:1) | \( p \)-value |
|------|--------|-------------|-----------------------------|--------------|
| Present | Present | 102          | 96.75                       | 2.883        | 0.5–0.1 |
| Present | Absent  | 36           | 32.25                       |              |          |
| Absent  | Present | 24           | 32.25                       |              |          |
| Absent  | Absent  | 10           | 10.75                       |              |          |

**Figure 3** Segregation of DNA marker based on \( \text{Rs2} \) allele in parents and 10 \( F_2 \) individual plants with the tetra null genotype \((\text{Lox1Lox2Lox3-}\text{Ti/Lox1Lox2Lox3-}\text{Lox1Lox2Lox3-}\text{ti/Lox1Lox2Lox3-}\text{ti}-\text{cgy1/cgy1-Rs2/Rs2} )\) are harvested separately. The \( F_3 \) plant—4 and 9; \( F_5 \) plant—4 and 9.
TABLE 3 | Quantitative characteristics of the cultivar (“Daewonkong”) and breeding line strain in field conditions during 2021.

| Cultivar/breeding line | Planting date | Maturing date | Stem height (cm) | NP/P | NS/P | Stachyose (g/kg) | SW (g) | Yield (Ton/ha) |
|------------------------|---------------|---------------|------------------|------|------|-----------------|--------|--------------|
| “Daewonkong”           | June 15       | October 13    | 48.0*            | 55*  | 86*  | 12.7*           | 29.5*  | 2.78*        |
| Breeding line          | June 15       | October 16    | 53.0*            | 52*  | 84*  | 2.9*            | 31.0*  | 2.80*        |

NP/P, number of pods per plant; NS/P, number of seeds per plant; SW, 100-seed weight. The same letters in the column are not significant at the 5% significance level by DMRT.

Figure 5 | Appearance of F2 plants and F2 seeds possessing penta null alleles (loxllox2loxl/loxllox2loxl-t/le-le-cgy1/cgy1rs2/rs2).

(Hata et al., 1991; Liener, 1995; Robinson et al., 1995; Katsuya et al., 2007; Krishnan et al., 2009). Heat treatment or other methods are needed to eliminate or reduce these components and secure the efficiency of nutrient absorption and food safety. But these treatments cause some changes and reduce soybean quality (Chen et al., 2019). Genetic removal or reduction of antinutritional and allergic factors such as lipoxygenase, KTI, 7S α′ subunit, and stachyose components that exist in mature soybean seeds is needed to improve the nutritional values for the soybean food industry. Also, a cultivar with the penta null genotype (loxllox2loxl2/loxl2/loxl3-t/ti-le-le-cgy1/cgy1rs2/rs2) for lipoxygenase, KTI, lectin, 7S α′ subunit, and stachyose components enhances the utilization of soybean in food as well as feed. A total of 172 F2 seeds were obtained from the cross of a loxllox2loxl3/loxllox2loxl3-3rs22-cgy1/cgy1 parent and loxllox2loxl3 /loxllox2loxl3-t/ti-le-le-cgy1/cgy1 parent to produce seeds possessing penta null alleles (loxllox2loxl3/loxllox2loxl3-t/ti-le-le-cgy1/cgy1rs2/rs2). KTI and lectin proteins were segregated in the F2 seed generation (Figure 2; Table 2). The segregation ratio for the presence or absence of KTI and lectin proteins was fitted to an expected 3:1 ratio ($\chi^2 = 2.51$ for KTI and 0.28 for lectin protein). This result substantiates previous observations that the presence or absence of KTI and lectin proteins is controlled by a single gene (Orf et al., 1978; Orf and Hymowitz, 1979). Segregation ratios of independent inheritance between KTI protein and lectin protein were observed ($\chi^2 = 2.883$, $p = 0.5–0.1$). This result was consistent with previous studies reporting that both Ti and Le alleles were independently inherited (Orf and Hymowitz, 1979; Moraes et al., 2006; Lee et al., 2008; Choi et al., 2021). Ti allele was independently inherited with the Le allele in the F2 population consisting of 24 plants (Moraes et al., 2006). Orf and Hymowitz (1979) observed that Le and Ti alleles were inherited independently by the F2 population with 96 plants. Lee et al. (2008) observed that Ti and Le alleles were independently inherited in 173 F2 seeds. Also, Choi et al. (2021) reported that Le and Ti alleles were independently inherited in F2 seed generation consisting of 210 seeds. Ten F2 seeds possessing tetra null alleles (loxllox2loxl3/loxllox2loxl3-t/ti-le-le-cgy1/cgy1) were obtained (Table 2). In a previous study, two F2 seeds possessing triple null alleles (ti-ti-le-le-p34/p34) were obtained from 150 F2 seeds (Schmidt et al., 2015). Also, three F2 seeds possessing tetra null alleles (loxllox2loxl3/loxllox2loxl3-t/ti-le-le-cgy1/cgy1) were obtained from 210 F2 seeds (Choi et al., 2021). Among the 10 F2 plants with the tetra null genotype (loxllox2loxl3/loxllox2loxl3-t/ti-le-le-cgy1/cgy1), only two F2 plants were selected by DNA marker (Figure 3). Its genotype was found to be rs2rs2, and it had low stachyose content compared to the normal cultivar with the Rs2Rs2 genotype. The result that two individuals had the rs2rs2 genotype in the F2 population of 10 individuals was consistent with the results of a previous study (Skoneczka et al., 2009; Yang et al., 2014). The absence of lipoxygenase, KTI, lectin, and 7S α′ subunit proteins was confirmed in random F2 seeds for the breeding line possessing the penta null genotype (loxllox2loxl3/loxllox2loxl3-t/ti-le-le-cgy1/cgy1rs2rs2). However, in the “Daewonkong” (Loxllox2loxl3 /Loxllox2loxl3-Ti/Le-Le-Cgy1/Cgy1-Rs2/Rs2) cultivar, these four proteins were observed (Figure 4). For the rs2 allele, the rs2rs2 genotype of the breeding line was confirmed by analysis of stachyose content using random F3 seeds (Table 3). The stachyose content of the breeding line was 2.9 g/kg, which was much lower than 12.7 g/kg of the check cultivar, “Daewon” (Rs2Rs2 genotype). 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 (Neus et al., 2005). Song et al. (2018) reported that BSH-3 seeds with 7S α′ subunit protein-free accumulated high levels of free amino acids as compared with normal seeds, particularly arginine, and the amounts of several essential amino acids were significantly elevated in BSH-3 seeds. Rani et al. (2020) developed KTI-free soybean with normal protein content. Normal-protein KTI-free RILs were significantly higher in both acidic and basic subunit of glycisin and β-subunit of β-conglycinin fraction compared to low-protein KTI-free RILs. Kumar et al. (2021) developed 21 F6 KTI-free lines exhibiting 100-fresh seed weight >45 g, sucrose content >7%, and morphologically similar to vegetable soybean. Moisture
content and pod yield of KTI-free lines at R6 stage were 64%–74.1% and 7.0–9.5 t/ha−1, respectively. Genetic removal of lipoxygenase-2 improved the speed of emergence of sprouts and the length of the sprouts and sprouting, thereby enhancing the suitability of beans for sprouting. Genetic removal of the KTI gene did not have a significant effect on sprouting attributes, though it enhanced BBI concentration and improved protein digestibility (Kumar et al., 2022).

Quantitative traits of the breeding line with the penta null genotype are shown in Table 3. In spite of the absence of antinutritional and harmful immunological components such as lipoxygenase, KTI, lectin, 7S α′ subunit, and stachyose that exist in the mature soybean seed, the breeding line germinated, grew, flowered, and reproduced normally in the greenhouse and under field conditions when compared to the cultivar “Daewonkong” (Figure 5). Schmidt et al. (2015) reported that plants possessing triple null alleles (ti/ti-le-le-p34/p34) flowered and produced seeds without any overt differences in comparison with the standard “Williams 82” cultivar. No significant differences were observed for stem height, number of pods per plant, number of seeds per plant, 100-seed weight, and yield between the breeding line with the penta null genotype and the “Daewonkong” cultivar. These results indicate that the penta null soybean line had no impact on these agronomic traits. These results suggest that stacking of recessive alleles for Lox1, Lox2, Lox3, Ti, Le, Cgy1, and Rs2 genes results in a soybean cultivar with significantly reduced antinutritional and allergenic ingredients. This is the first soybean strain with the penta null (lox1lox2lox3/lox1lox2lox3-ti/ti-le-cgy1/cgy1-rs2/rs2) genotype (free of lipoxygenase, KTI, lectin, and 7S α′ subunit proteins, and low stachyose content). Since mature soybean seeds have more than 20 kinds of allergens, it is considered that some allergens are also present in the seeds of the breeding line with the penta null genotype obtained in this study. However, when compared to common soybean seeds, it seems that allergens are greatly reduced. The breeding line obtained through this study could be used as a valuable parent for the improvement of soybean cultivars that do not contain several antinutritional properties in mature seeds.

CONCLUSION

Lipoxygenase, KTI, lectin, 7S α′ subunit, and stachyose components that exist in mature soybean seeds are considered antinutritional and allergenic factors. The objective of this study was to breed a new soybean strain with a penta null genotype (lox1lox2lox3/lox1lox2lox3-ti/ti-le-cgy1/cgy1-rs2/rs2) for these five components. A total of 172 F2 seeds were obtained from the cross of a lox1lox2lox3/lox1lox2lox3-rs2-cgy1/cgy1 parent and lox1lox2lox3/lox1lox2lox3-ti/ti-le-cgy1/cgy1 parent. Ten F2 seeds possessing tetra null alleles (lox1lox2lox3/lox1lox2lox3-ti/ti-le-cgy1/cgy1) were selected and two F3 plants with the rs2/rs2 genotype (low stachyose content) were developed. One F3 strain with proper agronomical traits was selected. Proteins of lipoxygenase, KTI, lectin, and 7S α′ subunit were not observed in the random mature F3 seeds of the breeding line with the penta null genotype. The penta null soybean line has purple flowers, a determinate growth habit, light yellow pod, yellow seed coat color, and yellow hilum. The breeding line matured on October 16. The stem height of the breeding line was 53.0 cm. The number of pods per plant and seeds per plant for the breeding line was 52 and 84, respectively. The stachyose content of the breeding line was 2.9 g/kg, which was much lower than 12.7 g/kg of the cultivar, “Daewonkong” (Rs2Rs2 genotype). The 100-seed weight of the breeding line was 31.1 g. The yield of the breeding line was 2.80 t/ha.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SC, SL, JL, HO, SK, NK, and JC were involved in the experimental design, crossing, protein analysis, planting, harvesting, and data collection and interpretation as well as write-up of this research. All authors contributed to the article and approved the submitted version.

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