Accumulation of triple recessive alleles for three antinutritional proteins in soybean with black seed coat and green cotyledon

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Abstract The black seed coat of soybeans contain anthocyanins which promote health. However, mature soybean seeds contain anti-nutritional factors like lipoxigenase, lectin and Kunitz Trypsin Inhibitor (KTI) proteins. Furthermore, these seeds can be used only after the genetic elimination of these proteins. Therefore, the objective of this study was to develop novel soybean genotypes with black seed coat and triple recessive alleles (*lx1lx1lx2lx2lx3lx3*, *titilele*) for lipoxigenase, lectin, and KTI proteins. From a cross of parent1 (*lx1lx2lx3/lx1lx2lx3*, *ti/ti*, *Le/Le*) and parent2 (*lx1lx2lx3/lx1lx2lx3*, *Ti/Ti*, *le/le*), 132 F₂ seeds were obtained. A 3:1 segregation ratio was observed during F₂ seed generation for the inheritance of lectin and KTI proteins. Between a cross of the *Le* and *Ti* genes, the observed independent inheritance ratio in the F₂ seed generation was 9: 3 : 3 : 1 (69 *Le_Ti_* : 32 *leleTi_* : 22 *Le_titi* : 9 *leletiti*) ($\chi^2=2.87$, $P=0.5-0.1$). From nine F₂ seeds with triple recessive alleles (*lx1lx1lx2lx2lx3lx3*, *titilele* genotype), one novel strain possessing black seed coat, and free of lipoxigenase, lectin and KTI proteins, was selected. The seed coat color of the new strain was black and the cotyledon color of the mature seed was green. The weight of 100 seeds belonging to the new strain was 35.4 g. This black soybean strain with *lx1lx1lx2lx2lx3lx3*, *titilele* genotype is a novel strain free of lipoxigenase, lectin, and KTI proteins.

Keywords Lipoxigenase, Kunitz Trypsin Inhibitor, lectin, *lx1lx1lx2lx2lx3lx3* *titilele* genotype

Introduction

Soybean [*Glycine max* (L.) Merr.] protein is excellent nutritional factors and is widely used for human and animal feed in the world. In seed coat of black soybean, anthocyanins are especially abundant (Choung et al. 2001). Anthocyanins from soybean with black seed coat are known to have many pharmaceutical effect. Health-promoting effects such as reduction in the risk of coronary heart disease, regulation of adhesion molecules, protection from reperfusion, and potential antioxidant effects were reported (Burns et al. 2000; Kim et al. 2006). However, a few antinutritional factors such as lipoxigenase, lectin, and Kunitz trypsin inhibitor (KTI) proteins in raw mature soybean seed with black seed coat are present. These components reduce the nutritional value.

Lipoxigenases are a class of enzymes that catalyze the hydroperoxidation of polyunsaturated fatty acids such as the linoleic and linolenic acids. Lipoxigenase proteins constitute about 1 ~ 2% of the total protein. End-products from lipoxigenase activity are converted to many volatile compounds, which are responsible for the beany flavor in soybean products. Many researchers (Davies and Nielsen, 1986; Hildebrand and Hymowitz, 1981; Kitamura et al. 1983) have reported on the heredity and genetic elimination of lipoxigenase protein. Single dominant genes (*Lx1*, *Lx2* and *Lx3*) control lipoxigenase protein and recessive alleles (*lx1*, *lx2*, *lx3*) are responsible for absence of lipoxigenase protein in mature seed. Kobayashi et al. (1995) reported that soybean seeds with lipoxigenase free are better accepted due to production of very low levels of hexanal compounds. Macleod and Ames (1988) reported that extra costs need to inactivate lipoxigenase activity by heat at industrial level and the solubility and functionality of proteins was adversely affected. Breeding of soybean cultivar with lipoxigenase free through genetic elimination is the key to get rid of the beany flavour. So far, several cultivars with lipoxigenase free have been

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developed (Chung, 2009; Kim et al. 1997).

Kunitz (1945) isolated and crystallized Kunitz Trypsin Inhibitor (KTI) protein from soybean seeds for the first time. KTI protein is a small and non-glycosylated protein possessing 181 amino acid residues with 21.5 kDa. Trypsin is strongly inhibited by KTI protein and food intake is reduced by diminishing digestion and absorption. Four forms of Ti^a , Ti^b , Ti^c , and Ti^d have been reported at a single locus with a codominant multiple allelic series (Orf and Hymowitz, 1979; Hymowitz and Hadley, 1972; Sing et al. 1969). The fifth form as a recessive allele designated ti does not exhibit a soybean KTI protein (Orf and Hymowitz, 1979). Crude protein from $titi$ genotype soybean had a 30 to 50% reduction in trypsin inhibitor activity compared with ‘Amsoy 71’ that has the $TiTi$ genotype. The Ti gene has been located on chromosome 8 (molecular linkage group A2) of the USDA/Iowa State University soybean linkage map (Cregan et al. 1999; Hildebrand et al. 1980; Kiang, 1987).

Soybean agglutinin (lectin) protein is a major antinutritional element and can strongly endure degradation by proteases under in vitro and in vivo conditions. Soybean lectin protein is a glycoprotein that specifically binds galactose or N-acetylgalactosamine. Molecular weight of soybean lectin protein is 120 kDa (Pull et al. 1978). Soybean lectin protein contains four subunits that each have a molecular weight of 30 kDa. The concentration of lectins in soybean seed was ranged 1~2% on seed dry mass (George et al. 2008). By proper heating, the biological activity of soybean lectin protein can be reduced. However, considerable quantity is found after heating. The nutritional quality of the soybean protein was affected negatively by this residual soybean lectin and the digestion and absorption of nutrients was decreased (Schulze et al. 1995). Soybean seed lectin was controlled by a single gene designated Le (le) and $lele$ genotype results in the lack of lectin in mature seed (Orf et al. 1978). Several researchers observed that Le and Ti loci were independently inherited (Lee et al. 2008; Moraes et al. 2006; Orf and Hymowitz, 1979). The soybean line with triple null recessive genotypes ($ti/ti-le/le-p34/p34$) was developed (Schmidt et al. 2015).

Presence of lipoxigenase, lectin, and KTI proteins in

mature raw soybean seeds requires heating step to reduce the activity of these antinutritional components. But, excessive heat treatment may diminish amino acid availability. The genetic elimination of these factors could be an alternative to the heat treatment. New black soybean cultivars with free of lipoxigenase, lectin, and KTI proteins improve the nutrition values and food processing properties of soybeans. This cultivar enhances the utilization of soybean in food as well as feed uses. The objective of this study was to improve new black soybean genotype with green cotyledon and triple recessive alleles ($lx1lx1lx2lx2lx3lx3titi$) for lipoxigenase, lectin, and KTI proteins. This is the first report on black soybean line with green cotyledon and $lx1lx1lx2lx2lx3lx3titi$ genotype (free of lipoxigenase, lectin, and KTI proteins).

Materials and Methods

Genetic population

Four parents (“Gaechuck#1”, “Jinpum#2”, 12N1, and Le-16) were used to create genetic population. Genotype of “Gaechuck#1” is $Lx1Lx1lx2lx2lx3lx3titiLeLe$ (lipoxigenase-2,3 and KTI proteins free and lectin protein present). “Jinpum#2” has $lx1lx1lx2lx2lx3lx3TiTiLeLe$ genotype (lipoxigenase-1,2,3 protein free, KTI and lectin proteins present). 12N1 parent has $lx1lx1lx2lx2lx3lx3TiTiLeLe$ genotype (lipoxigenase-1,2,3 protein free, KTI and lectin proteins present). Le-16 parent has $Lx1Lx1Lx2Lx2Lx3Lx3TiTiLeLe$ genotype (absence of lectin protein and presence of lipoxigenase-1,2,3 and KTI proteins). Color of seed coat, presence or absence for lipoxigenase, lectin, and KTI proteins of four parents are presented in Table 1.

F_1 seeds were obtained from cross of “Gaechuck#1” and “Jinpum#2” parents and were planted in the greenhouse. F_2 seeds were harvested from F_1 hybrid plants. From F_2 seeds, new parent1 with $lx1lx1lx2lx2lx3lx3titiLeLe$ genotype (lipoxigenase-1,2,3 and KTI proteins free, lectin protein present) was developed. From the cross of 12N1 and Le-16 parents, the F_1 seeds were obtained. F_1 seeds obtained were planted in the greenhouse. F_2 seeds from F_1 hybrid plants

Table 1 Seed coat color, presence or absence of lipoxigenase, lectin, and Kunitz Trypsin Inhibitor (KTI) proteins in four parent strains

Parents	Seed coat color	Lipoxigenase	KTI	Lectin
Gaechuck#1	Black	2,3 absence	Absence	Presence
Jinpum#2	Yellow	1,2,3 absence	Presence	Presence
12N1	Black	1,2,3 absence	Presence	Presence
Le-16	Yellow	1,2,3 presence	Presence	Absence

were obtained. From F_2 seeds, new parent2 with $lx1lx1lx2lx2lx3lx3titilele$ genotype (lipoxygenase-1,2,3 and lectin proteins free, KTI protein present) was developed. From the cross of new parent1 and new parent2, F_1 seeds were obtained and were planted in greenhouse. F_2 seeds were harvested from F_1 plants. The F_2 seeds harvested from F_1 hybrid plants were used to screen the seed with $lx1lx1lx2lx2lx3lx3titilele$ genotype (lipoxygenase, lectin, and KTI proteins free).

Identification of lipoxygenase protein by SDS-PAGE

Total proteins from the parents, individual F_2 seed, and random F_4 seeds were obtained to identify the presence ('+') or absence ('-') of lipoxygenase protein. A part of cotyledon from the parent, each F_2 seed, and random F_4 seed was removed and was incubated for 30 min in 1 ml Tris-HCl, pH 8.0 and 1.56% v/v β -mercaptoethanol. Through centrifugation, 50 μ l of the supernatant was added to an equivalent amount of 5X sample buffer containing 1M Tris-HCl, pH 6.8, 50% v/v glycerol, 1.96% v/v β -mercaptoethanol, and 10% w/v sodium dodecyl sulfate (SDS). Sample obtained was boiled at 97°C for 5 min and sample was centrifuged. 2 μ l of the supernatant was loaded on a 12% acrylamide SDS polyacrylamide gel electrophoresis medium gels in Owl Separation Systems Inc (Model: P9DS, Portsmouth, NH USA). After electrophoresis for 7 hrs at 120 V, 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).

Identification of lectin and KTI proteins by western blot analysis

Total proteins obtained from parental seeds, individual F_2 seed, and random F_4 seeds were separated by 10% or 12% SDS-PAGE, and transferred onto Immobilon-P membrane (PVDF, Millipore). After blocking for 2 hr 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 were incubated with antibody of KTI and lectin protein for 1 hr. The blot was incubated with a horseradish peroxidase conjugated secondary antibody after washing in TBS buffer. Using enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK), the complex was visualized. Presence or absence of KTI and lectin protein was determined visually. The ratio of segre-

Table 2 Heredity pattern for the presence or absence of lectin and KTI proteins in the F₂ seed generation

Seed protein		Number of seed		χ^2 value (9:3:3:1)	P
KTI	Lectin	Observed	Expected		
+	+	69	74.25	2.87	0.5 - 0.1
+	-	32	24.75		
-	+	22	24.75		
-	-	9	8.25		

+: presence, -: absence

Fig. 2 Verification of the absence of lipoxigenase protein (A), Kunitz Trypsin Inhibitor (KTI) protein (B), and lectin protein (C) in the current cultivar (“Chungja#3”) and new strain. C: “Chungja#3” (*Lx1Lx1Lx2Lx2Lx3Lx3, TiTiLeLe* genotype), S: new strain (*Lx1Lx1Lx2Lx2Lx3Lx3, titilele* genotype). +, -: presence or absence of lipoxigenase, lectin, and KTI proteins, respectively

Lx1Lx1Lx2Lx2Lx3Lx3TiTiLeLe and KTI and lectin proteins were observed. Lectin protein of 120 kDa and KTI protein of 21.5 kDa were segregated in the F₂ seed generation. The data of segregation ratio for presence or absence at lectin and KTI proteins are presented in Table 2.

From 132 F₂ seeds obtained, KTI protein was observed in 101 F₂ seeds and was not observed in 31 F₂ seeds. The segregation ratio of 3:1 was observed in the F₂ seed generation for inheritance of KTI protein ($\chi^2=0.16$, $P=0.9 - 0.5$). From 132 F₂ seeds obtained, lectin was observed in 91 F₂ seeds and was not observed in 41 F₂ seeds. For inheritance of lectin protein, the 3:1 segregation ratio was observed in the F₂ seed generation ($\chi^2=2.59$, $P=0.5 - 0.1$). Between *Le* allele and *Ti* allele, segregation ratio of 9 : 3 : 3 : 1 (69 *Le Ti* : 32 *lele Ti* : 22 *Le titi* : 9 *leletiti*) was observed ($\chi^2=2.87$, $P=0.5 - 0.1$) in the F₂ seed generation. Nine F₂ seeds with triple recessive alleles (*Lx1Lx1Lx2Lx2Lx3Lx3titilele*) were planted and one seed was not germinated. Each F₂ plant with black seed coat color and green cotyledon color was harvested. Total four F₂ plants were selected. Random F₃ seeds of each F₂ plant strain were

Fig. 3 Physical appearance of F₅ seeds possessing triple recessive alleles (*Lx1Lx1Lx2Lx2Lx3Lx3, titilele*) expressing black seed coat and green cotyledon, with the absence of lipoxigenase, lectin, and KTI proteins

planted to advance F₃ plant generation. One F₃ plant line possessing a proper agronomical traits among four strains was selected and was harvested. Random F₄ seeds were used to identify the free for lipoxigenase, lectin, and KTI proteins (Fig. 2).

The absence for lipoxigenase, lectin, and KTI proteins was confirmed in protein extracted from random F₄ seeds of new strain. However, lipoxigenase, lectin, and KTI proteins were observed in the seed of “Chungja#3” (*Lx1Lx1Lx2Lx2Lx3Lx3TiTiLeLe* genotype) cultivar (Fig. 2). Color of seed coat, hilum, and cotyledon was recorded on F₅ seed. Seed weight (g/100 seeds) was recorded on the F₄ plant generation. F₅ seeds harvested from F₄ plant strain with triple null alleles (*Lx1Lx1Lx2Lx2Lx3Lx3titilele*) are shown in Figure 3. Color of seed coat for new strain was black and color of cotyledon in mature seed was green. The 100-seed weight (g) of new strain was 35.4.

Discussion

Soybean seeds contain 40% protein, 20% oil, 30% carbohydrate, anthocyanin, saponin, and many other nutrients to human food and animal feed. By high quantity and quality of soybean protein, demand of soybean and soybean products has increased in recent years. However, a few antinutritional factors and allergenic proteins are exist in the raw mature soybean. Lipoxigenase protein, lectin protein, and Kunitz Trypsin Inhibitor (KTI) protein are major antinutrients affecting in reducing functional or nutritional value of unprocessed soybean. To denature the activity of these antinutritional components, heating step is necessary. However, excessive heat process may lower amino acid availability of soybean and soybean products. The genetic elimination of these antinutritional components could be

an alternative to the severe heat process. From the cross of new parent1 (*lx1lx2lx3/lx1lx2lx3, ti/ti, Le/Le*) and new parent2 (*lx1lx2lx3/lx1lx2lx3, Ti/Ti, le/le*), 132 F₂ seeds were obtained to develop a new soybean line with black seed coat color, green cotyledon color, and triple recessive alleles for lipoxygenase, lectin, and KTI proteins. The ratios of segregation for presence and absence of lectin and KTI proteins are presented in Table 2. A 3:1 segregation ratio was observed ($\chi^2=0.16$, $P=0.9 - 0.5$) for the presence or absence of KTI protein in the F₂ seed generation. Many researchers observed that the presence or absence of KTI protein is controlled by a single gene (Choi et al. 2016; Eun et al. 2012; Kim et al. 2006; Orf and Hymowitz, 1979). Also, a 3:1 segregation ratio was observed ($\chi^2=2.59$, $P=0.5 - 0.1$) for the presence or absence of lectin protein in the F₂ seed generation. This result substantiate previous observations that lectin protein is controlled by a single gene (Choi et al. 2016; Orf and Hymowitz, 1979; Sung et al. 2013).

Between *Le* gene and *Ti* gene, segregation ratio of 9 : 3 : 3 : 1 (69 *Le_Ti_* : 32 *lele Ti_* : 22 *Le_titi* : 9 *leletiti*) was observed ($\chi^2=2.87$, $P=0.5 - 0.1$) in the F₂ seed generation. This result agreed with previous papers that both *Ti* and *Le* alleles were independently inherited (Choi et al. 2016; Lee et al. 2008; Moraes et al. 2006; Orf and Hymowitz, 1979). Independent inheritance of *Ti* and *Le* loci was observed in F₂ population consisted with 24 plants (Moraes et al. 2006). Orf and Hymowitz (1979) reported that *Le* and *Ti* alleles were inherited independently by using F₂ population with 96 plants. Lee et al. (2008) reported that *Ti* and *Le* alleles were independently inherited in 173 F₂ seed generation. Also, Choi et al. (2016) observed that *Le* and *Ti* alleles were independently inherited in F₂ seed generation consisted with 179 seeds.

Nine F₂ seeds with triple recessive alleles (*lx1lx1lx2lx2lx3lx3titilele* genotype) from 132 F₂ seeds were selected. One F₃ plant line possessing a proper agronomical traits among four strains was obtained. Random F₄ seeds were used to identify the presence or absence for lipoxygenase, lectin, and KTI proteins (Fig. 2). The absence for lipoxygenase, lectin, and KTI proteins was confirmed at the mature F₄ seeds of new strain. However, in the seed of “Chungja#3” (*Lx1Lx1Lx2Lx2Lx3Lx3 TiTiLeLe* genotype) cultivar, lipoxygenase, KTI, and lectin proteins were observed (Fig. 2). F₅ seeds with triple recessive alleles (*lx1lx1lx2lx2lx3lx3titilele*) are shown in Figure 3. Color of seed coat for new strain was black and color of cotyledon was green in mature seed. The 100-seed weight (g) for new strain was 35.4. This is the first new black soybean

strain with *lx1lx1lx2lx2lx3lx3titilele* genotype (absence of lipoxygenase, lectin, and KTI proteins). The strain improved newly in this study will be used to develop new soybean cultivar with black seed coat, green cotyledon, lipoxygenase protein free, KTI protein free, lectin protein free, and high quality.

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