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Identification and Confirmation of SSR Marker Tightly Linked to the *Ti* Locus in Soybean [*Glycine max* (L.) Merr.]

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1. Introduction

Soybean [*Glycine max* (L.) Merr.,] is considered a high quality source of oil and protein for food and feed. However, the several antinutritional factors (lipxygenase, trypsin inhibitor, lectin, and P34 allergen protein) present in raw mature soybean seeds. Soybean Kunitz trypsin inhibitor (KTI) protein has been proposed as one of the major antinutritional factor (Westfall and Hauge, 1948). KTI protein is a small, monomeric and non-glycosylated protein containing 181 amino acid residues. This 21.5 kDa non-glycosylated protein was first isolated and crystallized from soybean seeds by Kunitz (1945). KTI protein can cause the induction of pancreatic enzyme hypersecretion and a fast stimulation of pancreas growth, which is histologically described as pancreatic hypertrophy and hyperplasia (Liencer, 1995). Also, KTI may cause unfavorable physiological effects (Vasconcelos et al., 2001) and decrease weight gain in animals (Palacios et al., 2004). Proper heat processing is required to destroy KTI protein. However, excessive heat treatment may lower amino acid availability. The genetic removal of the KTI protein will improve the nutritional value of soybean. From the USDA germplasm collection, two soybean accessions (PI157440 and PI196168) lacking the KTI protein have been identified (Orf and Hymowitz, 1979). Based on the availability of soybean null lines lacking the KTI protein, it was suggested that KTI protein is not essential for soybean growth or development. Five electrophoretic forms of KTI have been discovered. The genetic control of four forms, *Ti^a*, *Ti^b*, *Ti^c*, and *Ti^d*, has been reported as a codominant multiple allelic series at a single locus (Singh et al., 1969; Hymowitz and Hadley, 1972; Orf and Hymowitz, 1979). Orf and Hymowitz (1979) found that the fifth form does not exhibit a soybean trypsin inhibitor-A2 band and is inherited as a recessive allele designated *ti*. Studies of amino acid and nucleotide sequences of polymorphic variants of KTI have revealed that there is a large sequence differences in nine amino acid residues between *Ti^a* and *Ti^b* (Song et al., 1993; Wang et al., 2004). Each *Ti^c*, *Ti^d* and *Ti^e* differ by only one amino acid from *Ti^a* type and *Ti^f* differs by one amino acid from *Ti^b* type (Wang et al., 2004). The *Ti* locus has been located on linkage group 9 in the classical linkage map of soybean (Hildebrand et al., 1980; Kiang, 1987), which is integrated in molecular linkage map A2 (chromosome number 8) of the USDA/Iowa State University soybean molecular linkage map (Cregan et al., 1999).

DNA markers have become fundamental tools for research involving soybean improvement programs. Microsatellites or simple sequence repeat (SSR) markers are highly polymorphic, abundant, and distributed throughout the genome (Cregan et al., 1999). With the development and public release of SSR primers, SSR markers have become available on molecular soybean linkage group (Cregan et al., 1999). Molecular markers tightly linked to desired genes are a valuable tool to detect genotypes of interest, saving time and resources. Marker assisted selection (MAS) using DNA markers instead of phenotypic assays reduces cost and increases the precision and efficiency of subsequent selection steps applied in breeding. To date, detection of the KTI protein free genotypes has been based on SDS-PAGE gel electrophoresis analysis of crude protein from mature seeds, however, with this method, test samples are restricted to proteins from mature soybean seeds. This is a time-consuming process, which is not possible in the early seedling stages of the corresponding population. SSR markers tightly linked to the *Ti* locus were identified and confirmed in soybean populations for marker assisted selection. If a marker linked to the *Ti* locus can be confirmed, then selection for KTI protein free genotypes might be performed at early seedling stages with relative ease.

2. Identification of SSR marker

2.1 Plant genotypes

Soybean genotype C242 (clark derived near isogenic line) has the *ti* allele and lacks a soybean kunitz trypsin inhibitor. C242 was a generous gift from J. Specht, professor of Agronomy, University of Nebraska-Lincoln, USA. Cultivar Jinpungkong2 and Clark has kunitz trypsin inhibitor protein band (*TiTi*). Two mapping populations were developed. Population 1 was derived from a cross between cultivar Jinpungkong2 and C242. Population 2 was made from a mating between cultivar Clark and C242. The F_1 plants from two populations were grown in the greenhouse to produce F_2 seeds.

2.2 Determination of kunitz trypsin inhibitor genotype

98 F_2 seed from F_1 plants for population 1 and 243 F_2 seed from F_1 plants for population 2 was analysed electrophoretically to determine the presence (SKTI-⁺) or absence (SKTI-^{null}) of kunitz trypsin inhibitor. A piece of cotyledon from each F_2 seed was removed and the remaining embryo germinated to given a F_2 mapping population. The separated cotyledon tissue was incubated for 30 min (room temperature) in 1 ml Tris-HCl, pH 8.0, containing 1.56 % v/v β -mercaptoethanol. After centrifugation, 50 μ l of the supernatant were added to an equivalent amount of 5X sample buffer [10% w/v sodium dodecyl sulphate (SDS), 50% v/v glycerol, 1.96% v/v β -mercaptoethanol, 1 M Tris-HCl, pH 6.8]. The samples were boiling at 97°C for 5 min and then centrifuged. Two microlitre of the supernatant were used for electrophoresis on 12% acrylamide SDS polyacrylamide gel electrophoresis (SDS-PAGE) medium gels in Owl Separation Systems. Inc(Model: P9DS, Portsmouth, NH USA). Electrophoresis was practiced at 120 V for 7 hr. Gels were stained overnight in an aqueous solution of 0.25 g coomassie brilliant blue R250, 10% acetic acid, 45% methanol and destaining solution (5% acetic acid, 14% methanol) for several hours. A Wide-Range SDS-PAGE molecular mass standard (Sigma Marker™, Product Code : M4038) containing the 21.5kDa soybean trypsin inhibitor protein, was used to aid recognition of samples lacking the kunitz trypsin inhibitor.

2.3 DNA extraction and DNA marker analysis

F₂ seeds tested for kunitz trypsin inhibitor protein were planted in the field on May, 2004. Young leaves were collected from the 94 individual F₂ plants germinated among 98 F₂ seeds and parent plants in population 1. In population 2, random 97 F₂ seeds among 243 F₂ seeds were planted in the greenhouse on April, 2005. Young leaves were collected from the 94 individual F₂ plants. Genomic DNA was extracted from finely ground leaf tissue by means of a modified CTAB procedure (Saghai Maroof et al., 1984). For the analysis of random amplified polymorphic DNA (RAPD) markers, One-thousand 10-mer oligonucleotide primers were obtained from Operon Technologies (Alameda, U.S.A). For the analysis of simple sequence repeat (SSR) marker, total 35 SSR primers were selected from the A2 soybean molecular linkage map (Cregan et al., 1999) that contains *Ti* locus. Satt primers selected were synthesized by Bioneer, Inc. (Korea). For the analysis of amplified fragment length polymorphic (AFLP) markers, 342 primer sets were used. Amplification and electrophoresis for RAPD, SSR, and AFLP markers was performed as described by Kim et al., (2003). Based on the results of F₁ seed genotype for kunitz trypsin inhibitor, the present and absent bulk populations from F₂ plant population were made (Michelmore et al., 1991). The present and absent bulk population contained twenty F₂ individuals each, which were selected on the basis of the kunitz trypsin inhibitor protein electrophoresis, respectively. RAPD, SSR, and AFLP markers were used in population 1. Only the markers linked in population 1 including *Ti* locus were used in population 2.

2.4 Genetic linkage analysis

Primers that distinguished the bulks and the parents were tested on the entire F₂ population. Marker (RAPD, AFLP, and SSR) data obtained from 94 F₂ progenies of population 1 and 2 were used to construct genetic linkage map including *Ti* locus using the computer program MAPMAKER v. 3.0 (Lander et al., 1987). Markers were assigned to group using the "Group" command, with a LOD score of 4.0 and maximum recombination distance of 50 cM. Once markers were assigned to a given linkage group, the most linkage marker order within the group was determined using the "Compare" command. Marker orders within each linkage group were ascertained by use of "Ripple" command. Map distance (cM) were computed using the Kosambi (Kosambi, 1944) mapping function.

2.5 Detection of Satt228 marker

The banding patterns of kunitz trypsin inhibit protein (SKTI) that appeared in the parents and F₂ seeds from the cross between cultivar Jinpungkong2 and C242 (population 1) are shown in Figure 1. Jinpungkong2 parent had band in 21.5 KDa position and the band was segregated in F₂ seeds. The observed data for population 1 were 72 seeds with SKTI protein band and 26 seeds with no SKTI protein band ($\chi^2=0.12$, $P=0.70-0.80$). For population 2, the observed data were 185 seeds with SKTI protein band and 58 seeds with no SKTI protein band ($\chi^2=0.17$, $P=0.70-0.80$). These observations fit the expected 3 : 1 ratio for the presence or absence of the SKTI protein band. Earlier studies have shown that the null phenotype of SKTI is inherited as a recessive allele designated *ti* (Orf and Hymowitz, 1979). The segregation ratios of 3 : 1 observed in the F₂ seed (population 1 and 2) and the Chi-square values strongly suggest that kunitz trypsin inhibitor protein band is controlled by a single recessive gene.

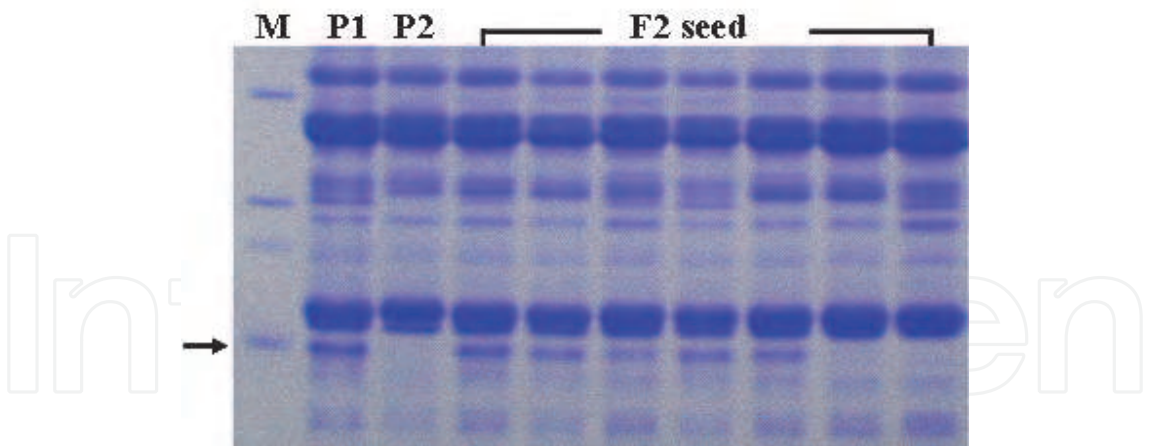


Fig. 1. Polyacrylamide gels of protein extracted from parents and F₂ seeds. P1 (Jinpumkong2) and P2 (C242) are parents and arrow points to the Kunitz trypsin inhibitor band (21.5 KDa).

Of the 1,000 RAPD primers tested on two parents of population 1 (Jinpumkong2 and C242), approximately 12 % (124) primers produced polymorphic DNA fragment differences between the parents. Only 35 primers were identified as being polymorphic between bulked DNA samples with SKTI protein and bulked DNA samples with no SKTI protein. Of those 35 primers, only 16 also exhibited polymorphism between parents. Among 342 primer sets of AFLP analysis, only 10 primers were shown polymorphism between parents. Three SSR primers (Satt409, Satt228 and Satt429) among 35 primers selected were shown polymorphism between parents. Total 48 marks (35 RAPD, 10 AFLP, and 3 SSR) were used to obtain segregation data from 94 F₂ individuals of population 1. Figure 2 represents some example of segregating DNA fragment for SSR markers (Satt228) in parents, bulked samples and F₂ population.

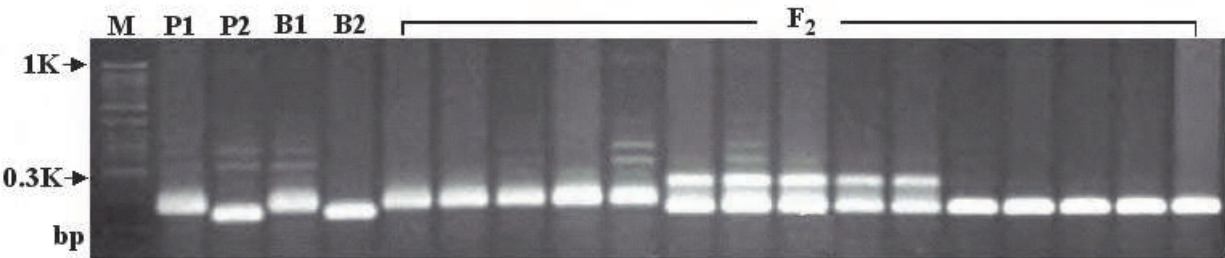


Fig. 2. Patterns of segregating DNA fragment for SSR primer Satt228 in parents, bulked samples, and F₂ population. P1 is Jinpumkong2 (*TiTi*) and P2 is C242 (*titi*). B1 is bulked of present kunitz trypsin inhibitor protein individuals and B2 is bulked of absent individuals.

A genetic map was constructed from the 48 segregating DNA markers and *Ti* locus. A total 11 DNA markers (4 RAPD, 4 AFLP, and 3 SSR) and *Ti* locus was found to be genetically linked in population 1. Three SSR markers, Satt409, Satt228, and Satt429 linked with *Ti* locus within 10 cM (Figure 3). Satt228 marker was very tightly linked with *Ti* locus at 0 cM. Three SSR markers linked with *Ti* locus in population 1 were applied in population 2. Only two SSR markers, Satt228 and Satt409 were linked with *Ti* locus. Satt228 marker was linked with *Ti* locus in 3.7 cM distance (Figure 3). The order and map distances of SSR markers and *Ti* locus differed between populations 1 and 2.

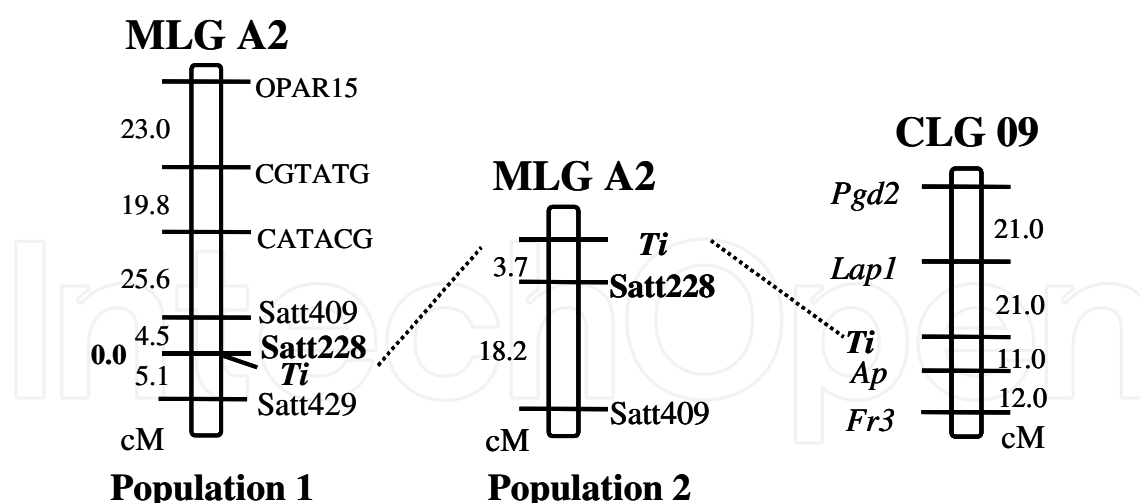


Fig. 3. Molecular linkage map A2 (Cregan et al., 1999) of *Ti* locus defined using population 1 and population 2. Population 1 was derived from the cross of Jinpungkong2 (*TiTi*) and C242 (*titi*). Population 2 was derived from the cross of Clark (*TiTi*) and C242 (*titi*). Map was constructed using MAPMAKER/EXP (LOD 4.0 maximum distance 50 cM). Marker loci names are on the right and kosambi map distances are on the left. CLG09 is the classical linkage group 9 (Hildebrand et al., 1980; Kiang 1987).

3. Confirmation of Satt228 marker

3.1 Screening of *titi* genotype using Satt228 marker

Only two genotypes (PI 157440 and PI 196168) and two near isogenic lines (C242, W60) have been known as soybean genotypes with lacking Kunitz trypsin inhibitor protein (*titi* genotype). C242 is a near isogenic line derived from cultivar 'Clark' and W60 is a near isogenic line derived from cultivar 'William'.

Satt228 marker very tightly linked to *Ti* locus at distance of 0 cM was used to screen germplasms with *titi* genotype (Kunitz trypsin inhibitor protein absent) for marker confirmation and testing the possibility of marker-assisted selection (MAS). Amplification patterns obtained from Satt228 marker using genomic DNA of four soybean strains (PI157440, PI196168, W60, and C242) with *titi* genotype (Kunitz trypsin inhibitor protein absent) and three cultivars ('Jinpungkong2', 'Clark', and 'William') with *TiTi* genotype (Kunitz trypsin inhibitor protein present) are shown in Figure 4-1A. Also, polyacrylamide gel banding patterns of protein extracted from random 10 seeds of these seven germplasms used are shown in Figure 4-1B. *TiTi* genotypes ('Jinpungkong2', 'Clark', and 'William') had allele1, however, *titi* genotypes (PI196168, C242, W60 and PI157440) had allele2 in the result of PCR by Satt228 marker (Figure 4-1A). *TiTi* genotypes ('Jinpungkong2', 'Clark', and 'William') had 21.5 kDa band that indicates Kunitz trypsin inhibitor protein, however *titi* genotypes (PI196168, C242, W60 and PI157440) did not have the band in of protein gel electrophoresis from the mature seed (Figure 4-1B). From the comparison of gel electrophoresis for Kunitz trypsin inhibitor protein (Figure 4-1B) and banding pattern amplified by Satt228 marker from the genomic DNA (Figure 4-1A), there was a strong agreement between protein band (21.5 kDa) for Kunitz trypsin inhibitor protein and banding pattern by Satt228 marker. All *TiTi* genotypes ('Jinpungkong2', 'Clark', and 'William') which shown 21.5 kDa protein band in protein electrophoresis of mature seed had

the allele1 amplified by Satt228 marker from the genomic DNA. However, all *titi* genotypes (PI196168, C242, W60 and PI157440) which shown no 21.5 kDa protein band in electrophoresis of mature seed had allele2 amplified by Satt228 marker from the genomic DNA.

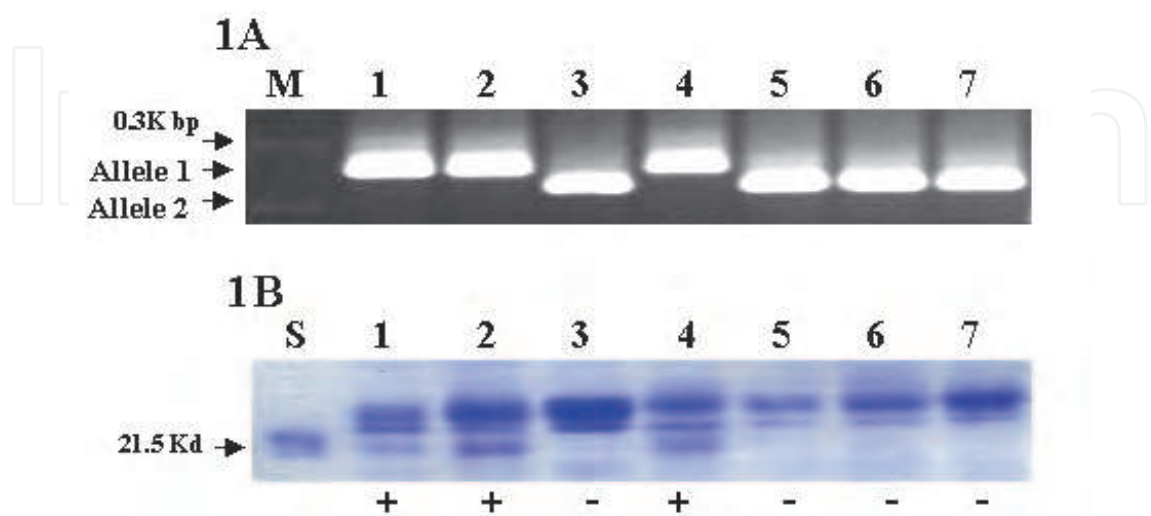


Fig. 4. Pattern of genomic DNA amplification by Satt228 marker using leaf tissue of germplasms (1A) and pattern of polyacrylamide protein gel electrophoresis extracted from 10 random seeds harvestes (1B). M; molecular marker, S; Kunitz trypsin inhibitor protein (Sigma, product number: T6522). 1:Jinpumkong2(*TiTi*), 2:Clark (*TiTi*), 3:PI196168 (*titi*), 4: William (*TiTi*), 5: C242 (*titi*), 6: W60 (*titi*), 7: PI157440 (*titi*). +: present of KTI protein and -: absent of KTI protein.

Moraes et al. (2006) reported specific DNA marker designed to detect the absence of SKTI protein. For markers to be most useful in breeding programs, they should reveal polymorphism in different genetic backgrounds, which is referred to as marker validation (Sharp et al., 2001). Specific DNA marker designed to detect the absence of SKTI protein reported by Moraes et al. (2006) was not valid between germplasms of *TiTi* (SKTI protein present) and *titi* (SKTI protein absent) genotype used in this study. No polymorphism was observed among germplasms used. However, Cosegregation between allele of Satt228 marker and presence or absence of SKTI protein in several soybean germplasms of *TiTi* and *titi* genotypes was observed (Figure 4-1A and 1B). This results indicate that selection of germplasms or lines with lacking Kunitz trypsin inhibitor protein is possible by Satt228 marker analysis.

3.2 Confirmation of Satt228 marker in four different soybean populations

Cosegregation between Satt228 marker and *Ti* locus was confirmed in four different populations. Two cultivars (Jinpumkong2, Hannamkong) and two landraces (GS06, 20M183) have Kunitz trypsin inhibitor protein (*TiTi* genotype) in their mature seeds. The C242 parent is a clark-derived near isogenic line and does not have Kunitz trypsin inhibitor protein (*titi* genotype) in the mature seeds. Four different populations were developed. Four female parents (Jinpumkong2, Hannamkong, GS06, 20M183) and one male parent C242 were crossed in the greenhouse in June 2002. F₁ seeds from the cross of Jinpumkong2 x C242, Hannamkong x C242, GS06 x C242, and 20M183 x C242 were obtained and planted in

the greenhouse. F₂ seeds per each cross were harvested from several F₁ plants in November 2002. All F₂ seeds per each cross were planted in the field in May 2003. F₂ plants per each cross were harvested individually. Random F₃ seeds from individual F₂ plant per each cross were tested by SDS-PAGE protein analysis to detect Kunitz trypsin inhibitor protein. Individual F₂ plants (F₃ seeds) with free Kunitz trypsin inhibitor protein (*titi* genotype) per each cross were planted in the greenhouse and harvested individually in June 2004. Random F₄ seeds from individual F₃ plant harvested per each cross were planted in the field in June 2004. At maturity, F₄ plants (F₅ seeds) were harvested individually per each cross in November 2004. Random F₅ seeds from individual F₄ plant harvested per each cross were planted in the field in May 2005. Five parents and individual F₅ plants per each cross were used to confirm the SSR marker tightly linked to *Ti* locus. Agronomical traits except for the Kunitz trypsin inhibitor protein were not considered in each generation. The pedigree for the development of the four populations lacking the Kunitz trypsin inhibitor protein is summarized in Figure 5.

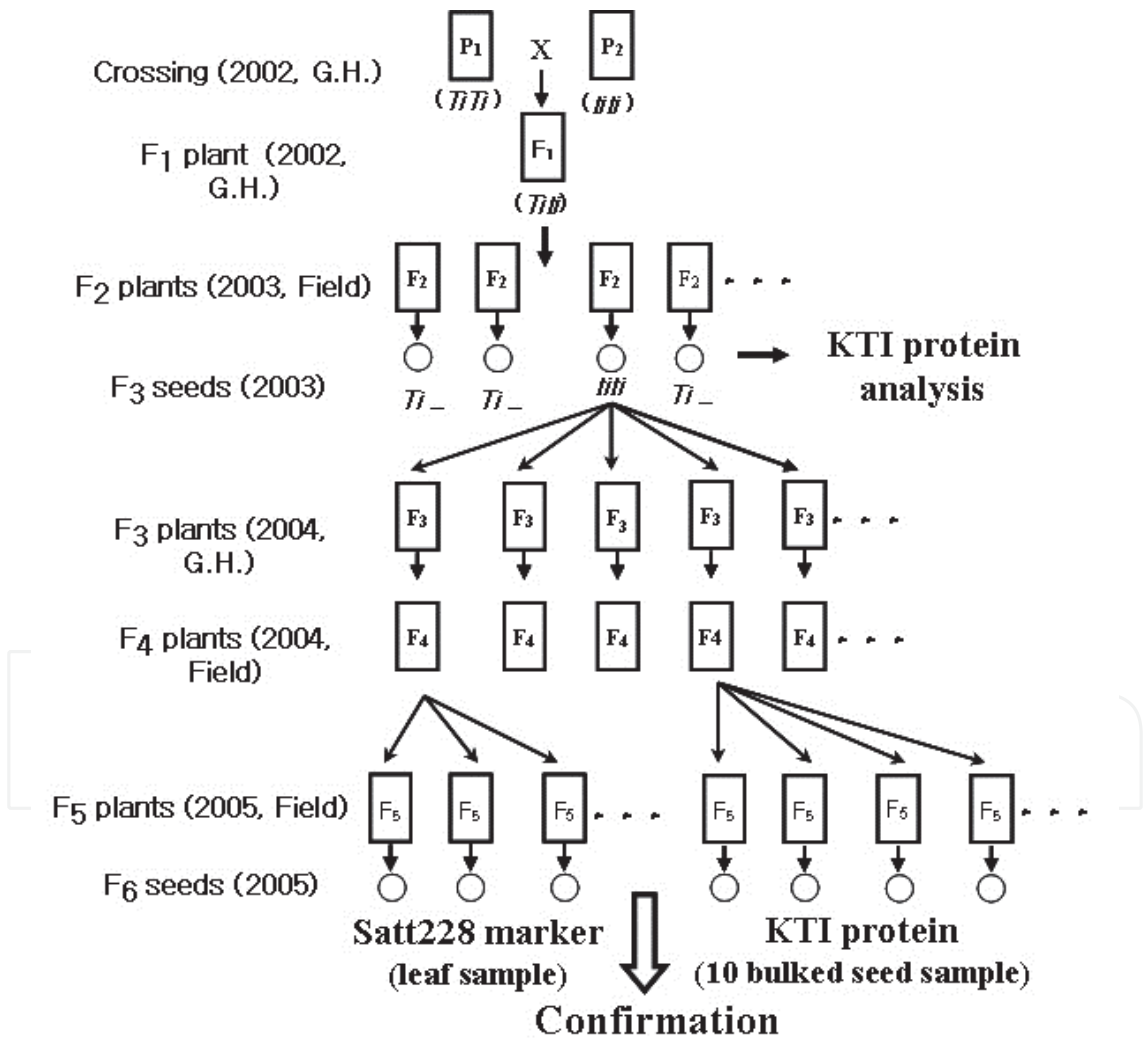


Fig. 5. The pedigree of the four population development to confirm cosegregation between marker Satt228 and the *Ti* locus. F₂ plants lacking the KTI protein from each population were selected and advanced to the next generation. All F₅ plants have the *titi* genotype (lacking Kunitz trypsin inhibitor protein). G.H is greenhouse.

Segregation patterns of genomic DNA amplification by the Satt228 marker using young leaf tissue of four parents and several individual F₅ plants (A, C, E, G) and patterns of polyacrylamide protein gel using protein extracted from 10 random seeds of four parents and individual F₆ harvested (B, D, F, H) are shown in Figure 6. The bands amplified by the Satt228 marker are clearly detecting the AA and BB genotypes. The seed protein band by the SDS-PAGE is a little different in color density according to each population, staining time of Coomassie blue and protein content. However, the detection of the presence or absence of the Kunitz trypsin inhibitor protein was very clear because the Kunitz trypsin inhibitor protein is controlled by single gene and is not influenced by environment.

Satt228 marker analysis was conducted on the genomic DNA of the parents and the 273 individual F₅ plants lacking the Kunitz trypsin inhibitor protein derived from the cross of 'Jinpumkong2' (*TiTi*) and C242 (*titi*). After harvesting at maturity, SDS-PAGE electrophoresis using crude protein extracted from ten random F₆ seeds of each F₅ plants and parents was performed to detect Kunitz trypsin inhibitor protein of size 21.5 kDa. DNA banding pattern of the Satt228 marker and polyacrylamide gel banding patterns of the protein is shown in Figure 6 (A and B). The P₁ parent (Jinpumkong2) had the AA genotype (allele 1) and the P₂ parent (C242) had BB genotype (allele 2) for Satt228 marker. All 273 individual F₅ plants were shown only to have the BB genotype (A of Figure 6). This indicated all 273 F₅ progenies had the *titi* genotype and contained no Kunitz trypsin inhibitor protein. Also, the P₁ parent had Kunitz trypsin inhibitor protein of 21.5 kDa size and the P₂ parent did not have the KTI protein (B of Figure 6). All 273 individual F₅ plants did not have the Kunitz trypsin inhibitor protein of 21.5 kDa size (B of Figure 6). Amplification patterns obtained from the Satt228 marker using genomic DNA of 17 individual F₅ plants derived from the cross of Hannamkong (*TiTi*) and C242 (*titi*) and polyacrylamide gel banding patterns of protein extracted from the mixture of 10 random F₆ seeds harvested from each F₅ plant are shown in Figure 6 (C and D). The P₁ parent (Hannamkong) had a AA genotype while the P₂ parent (C242) had the BB genotype (C of Figure 6). All 17 individual F₅ plants derived from cross of Hannamkong and C242 showed only the BB genotype pattern for Satt228 marker analysis (C of Figure 6). This indicated all 17 F₅ progenies had the *titi* genotype and no Kunitz trypsin inhibitor protein. For the protein analysis, the P₁ parent had the 21.5 kDa Kunitz trypsin inhibitor protein while the P₂ parent did not have the KTI protein in polyacrylamide protein (D of Figure 6). All 17 individual F₅ plants did not have the 21.5 kDa Kunitz trypsin inhibitor protein within their F₆ seed samples (D of Figure 6). Amplification patterns by the Satt228 marker using genomic DNA of 45 individual F₅ plants derived from the cross of GS06 (*TiTi*) and C242 (*titi*) and polyacrylamide gel banding patterns of protein extracted from the mixture of 10 random F₆ seeds harvested from each F₅ plant are shown in Figure 6 (E and F). The P₁ parent (GS06) had the AA genotype and the P₂ parent (C242) had the BB genotype (E of Figure 6). All 45 individual F₅ plants derived from the cross of GS06 and C242 displayed only the BB genotype pattern for marker Satt228 (E of Figure 6). Also, the P₁ parent had the 21.5 kDa Kunitz trypsin inhibitor protein and the P₂ parent did not have the KTI protein in polyacrylamide protein gel from mature seeds (F of Figure 6). All 45 individual F₆ seeds harvested from same individual F₅ plants did not have Kunitz trypsin inhibitor protein of 21.5 kDa size (F of Figure 6). This indicated all 45 F₅ progenies had the *titi* genotype and contained no Kunitz trypsin inhibitor protein. Using marker Satt228, amplification patterns from 56 individual F₅ plants derived from the cross of 20M183 (*TiTi*) and C242 (*titi*) and

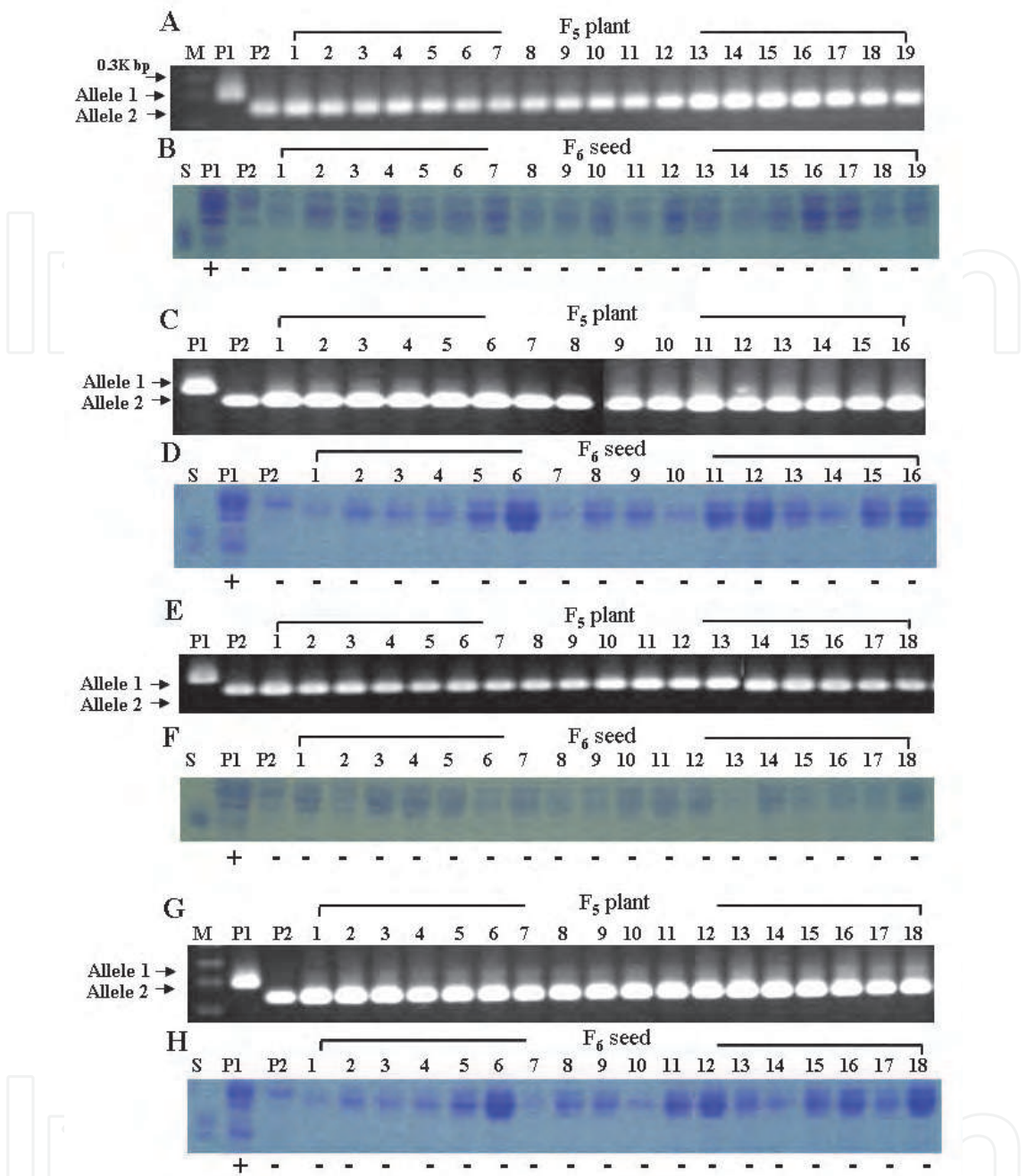


Fig. 6. Pattern of genomic DNA amplification by Satt228 marker using leaf tissue of parent and individual F₅ plants (A, C, E, G) and pattern of polyacrylamide protein gel using protein extracted from parents and 10 random seeds of individual F₆ seed harvested (B, D, F, H). A and B, P1: 'Jinpumkong2' and P2: C242; C and D, P1: 'Hannamkong' and P2: C242; E and F, P1:GS06 and P2: C242; G and H, P1:20M183 and P2: C242. M; molecular marker, S; Kunitz trypsin inhibitor protein (Sigma, product number: T6522), +; present of KTI protein, -; absent of KTI protein.

polyacrylamide gel banding patterns of protein extracted from the mixture of 10 random F₆ seeds harvested from each F₅ plant are shown in Figure 6 (G and H). The P₁ parent (20M183) had the AA genotype while the P₂ parent (C242) had the BB genotype (G of Figure 6). All 56 individual F₅ plants were shown only to have the BB genotype pattern for the Satt228

marker (G of Figure 6). This indicated all 56 F₅ progenies had the *titi* genotype and contained no Kunitz trypsin inhibitor protein. For the protein analysis the P₁ parent had the Kunitz trypsin inhibitor protein of 21.5 kDa, while the P₂ parent did not have the KTI protein (H of Figure 2). All 56 individual F₅ plants did not have the 21.5 kDa Kunitz trypsin inhibitor protein based upon their 10 random F₆ seed samples (H of Figure 6).

Segregation patterns of genomic DNA amplification by the Satt228 marker using young leaf tissue of four parents and several individual F₅ plants (A, C, E, G) and patterns of polyacrylamide protein gel using protein extracted from 10 random seeds of four parents and individual F₆ harvested (B, D, F, H) are shown in Figure 6. Satt228 marker analysis showed the four female parents had the allele 1 (AA genotype) while the C242 male parent has the allele 2 (BB genotype). In seed, four parents had KTI protein and C242 had not KTI protein of 21.5 kDa. A total of 391 F₅ plants derived from the four crosses (273 plants from Jinpungkong2 × C242, 17 plants from Hannamkong × C242, 45 plants from GS06 × C242, and 56 plants from 20M183 × C242) all have the allele 2 (BB genotype) for Satt228 marker. The 391 individual F₆ seeds harvested from same individual F₅ plants are also absent of the KTI protein. Complete cosegregation between the Satt228 marker allele and the *Ti* locus was observed in these four different populations (Kim et al., 2008).

4. Conclusion

Soybean Kunitz trypsin inhibitor (KTI) protein is a small, monomeric and non-glycosylated protein containing 181 amino acid residues and is responsible for the inferior nutritional quality of unheated or incompletely heated soybean meal. *Ti* gene controls the presence or absence of KTI protein. SSR marker tightly linked to the *Ti* locus was identified and was confirmed in two ways. Two mapping populations were developed. Population 1 was derived from a cross between cultivar Jinpungkong2 (*TiTi*) and C242 (*titi*). Population 2 was made from a mating between cultivar Clark (*TiTi*) and C242. Each F₂ seed from F₁ plants was analysed electrophoretically to determine the presence of the KTI protein band. Twelve DNA markers (4 RAPD, 4 AFLP, and 3 SSR) and *Ti* locus were found to be genetically linked in population 1 consisted with 94 F₂ individual plants. Three SSR markers (Satt409, Satt228, and Satt429) were linked with *Ti* locus within 10 cM. Satt228 marker was tightly linked with *Ti* locus. Satt228 marker was tightly linked within 0 - 3.7 cM of the *Ti* locus. Using several germplasms with *TiTi* or *titi* genotypes, Satt228 marker was confirmed. *TiTi* genotypes ('Jinpungkong2', 'Clark', and 'William') had allele1 and *titi* genotypes (PI196168, C242, W60 and PI157440) had allele2 in Satt228 marker analysis. 'Jinpungkong2', 'Clark', and 'William' (*TiTi* genotype) had Kunitz trypsin inhibitor protein of 21.5 kDa size and PI196168, C242, W60, and PI157440 (*titi* genotype) did not have the band in protein gel electrophoresis from the mature seed. Cosegregation between KTI protein (21.5 kDa size) and allele of Satt228 marker was observed in seven germplasms with different genetic background. This result indicates that Satt228 marker may effectively utilized to select the plant with *titi* genotype. Also, Satt228 marker tightly linked to the *Ti* locus was confirmed in four different F₅ populations. Four female parents (*Glycine max* L. cv. Jinpungkong2, Hannamkong, GS06, 20M183) of *TiTi* (KTI protein present) genotype and one male parent C242 of *titi* (KTI protein absent) genotype were used. Four different populations of F₂ plants free of KTI protein were advanced to the F₅ generation. Satt228 marker analysis showed the four female parents had the allele 1 (AA genotype) while the C242 male parent has the allele 2 (BB genotype). In seed, four parents had KTI protein and C242 had not KTI protein of 21.5 kDa. A total of 391 F₅ plants derived from the four crosses (273 plants from Jinpungkong2 × C242,

17 plants from Hannamkong × C242, 45 plants from GS06 × C242, and 56 plants from 20M183 × C242) all have the allele 2 (BB genotype) for Satt228 marker. The 391 individual F₆ seeds harvested from same individual F₅ plants are also absent of the KTI protein. Complete cosegregation between the Satt228 marker allele and the *Ti* locus was observed in these four different populations. The objective of this research was to identify and to confirm a SSR marker tightly linked to the *Ti* locus for MAS breeding in different genetic populations and germplasms. So far, KTI free new soybean cultivars (Gaechuck#1, Gaechuck#2 and Jinnong#1) have been developed using Satt228 marker.

5. Acknowledgment

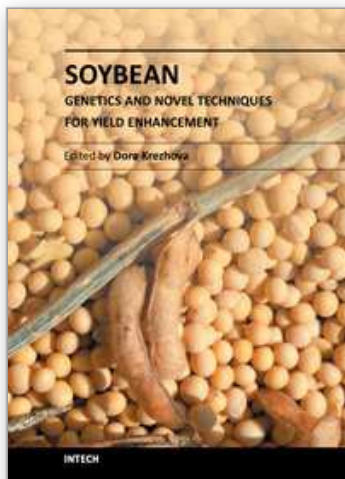
Soybean genotype C242 and W60 was a generous gift from James E. Specht, professor of Agronomy, University of Nebraska-Lincoln, U.S.A.

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