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Germplasm Detection for *titi* Genotype Using SSR Marker in Soybean

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Abstract

Soybean Kunitz trypsin inhibitor (SKTI) 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. The objective of this research is to confirm SSR marker (Satt228) tightly linked to the *Ti* locus using several germplasm accessions with *TiTi* or *titi* genotypes for MAS in soybean breeding programs. *TiTi* genotypes ('Jinpumkong2', 'Clark', and 'William') had allele1 and *titi* genotypes (PI196168, C242, W60, and PI157440) had allele2 in Satt228 marker analysis. 'Jinpumkong2', 'Clark', and 'William' (*TiTi* genotype) had a 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 the SKTI protein (21.5 kDa size) and allele of Satt228 marker was observed in seven germplasm accessions with different genetic backgrounds. Any recombination between the SKTI protein and allele of the Satt228 marker was not observed. This result indicates that Satt228 marker may effectively utilized to select the plants with the *titi* genotype.

Key words: Kunitz trypsin inhibitor, SSR marker, MAS, soybean

Introduction

Soybean [*Glycine max (L.)* Merr.] is considered a high quality source of oil and protein for food and feed. However, there are several antinutritional factors present in raw, mature soybean seeds. Soybean Kunitz trypsin inhibitor (SKTI) protein has been proposed as one of the major antinutritional factors (Westfall and Hauge 1948). SKTI 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). Proper heat processing is required to destroy protease inhibitors. However, excessive heat treatments may lower amino acid availability. Soybean lines with reduced protease inhibitor content could reduce or eliminate the need for expensive heat treatments and lessen the chance of lowering amino acid availability.

From the USDA germplasm collection, two soybean accessions (PI157440 and PI196168) lacking the SKTI protein have been identified (Orf and Hymowitz 1979). Based on the availability of soybean null lines lacking the SKTI protein, it was suggested that SKTI protein is not essential for soybean growth or development (Jofuku and Goldberg 1989). Five electrophoretic forms of SKTI 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 SKTI have revealed that there are 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 linkage group A2 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 for molecular mapping in soybean (Cregan et al. 1999). Molecular markers tightly linked to desired genes are a valuable tool to detect genotypes of interest,

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saving time and resources (Tanksley et al. 1989). 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. Kim et al. (2004) reported that two flanking RAPD markers (OPO12 and OPC08) were linked to the *Ti* locus at a distance of 16.0 cM. Recently, Moraes et al. (2006) reported that specific DNA primers were linked to the *ti* allele using a single population. One SSR marker tightly linked to *Ti* locus (0 cM) has been identified in a single F2 population (Kim et al. 2006). For markers to be most useful in breeding programs, they should reveal polymorphism or linkage in germplasm accessions with different genetic backgrounds. The objective of this research is to confirm SSR marker tightly linked to the *Ti* locus using several germplasm accessions with *TiTi* or *titi* genotypes for MAS in soybean breeding programs.

Materials and Methods

Plant genotypes

Only two genotypes (PI 157440 and PI 196168) and two near isogenic lines (C242, W60) have been known as soybean genotypes lacking Kunitz trypsin inhibitor protein (*titi* genotype). C242 is a near isogenic line derived from the cultivar 'Clark' and W60 is a near isogenic line derived from the cultivar 'William'. C242 and W60 strains were a generous gift from J. Specht, Professor of Agronomy, University of Nebraska-Lincoln, NE, USA. The origin, seed coat color, SKTI protein, and *Ti* locus of these four strains are shown in Table 1. Cultivars 'Jinpumkong2', 'Clark', and 'William' as wild type genotype with Kunitz trypsin inhibitor protein (*TiTi* genotype) were used. The seeds of all genotypes were planted in the greenhouse in May 2006.

Genomic DNA extraction and SSR marker analysis

Young leaves were collected from the seven genotypes planted in the greenhouse. Genomic DNA was extracted from finely-ground, young leaf tissue by means of a modified CTAB procedure (Saghai Maroof et al. 1984). Approximately 0.75 g of freeze-dried soybean leaf tissue was powdered with a paint shaker. Following the addition of 5 ㎖ extraction buffer [50 m*M* tris, pH 8.0, 0.7 *M* NaCl, 10 m*M* EDTA, 1% (w/v) hexadecyltrimethylammonium bromide, 0.1% (v/v) 2-mercaptoethanol], the slurry was incubated for 60 min at 60 °C with occasional swirling. After incubation, 5 ㎖ of chloroform-octanol (24:1, v/v) was added, and the solution was mixed by inversion and centrifuged at 5200 rpm for 10 min at 4 °C. The aqueous phase was removed and the DNA was precipitated by adding 2/3 volume of isopropanol. Precipitated DNA was spooled on a micropipet, transferred to a tube and washed in 5 ㎖ of a 76% (v/v) ethanol/ 10 m*M* ammonium acetate solution. The DNA was then dissolved in 1.5 ㎖ of 10 m*M* ammonium acetate/0.25 m*M* EDTA. SSR marker Satt228 tightly linked to *Ti* locus with 0 cM (Kim et al. 2006) was used. The sequence of Satt228 marker was 5'-TCATAACGTAAGAGATGGTAAAACT-3 (forward) and was 5'-CATTATAAGAAAACGTGCTAAAGAG-3' (reverse). A 10㎕ PCR reaction contained, 2㎕ genomic template DNA (20 ng/㎕), 2㎕ SSR primer (10 m*M*/㎕), 5 unit *taq* polymerase, dNTP [25 m*M* $MgCl_2$, 25 m*M* dATP, dTTP, dGTP, dCTP], 5X reaction buffer [250 m*M* Tris (pH 8.5), 5 m*M* $MgCl_2$, 100 m*M* KCl, 2.5 ㎎/㎖ BSA, 12.5% ficoll, 1% xylene cyanol], samples were covered with 10㎕ of light mineral oil. The PCR reaction was performed in a MJ research PTC-200 Thermocycler. The thermal profile consisted of 94 °C for 2 min, 94 °C for 45 s, 47 °C for 45 s, 68 °C for 45 s, go to step 2, 38 times, 75 °C for 10 min, 10 °C for 10 min and end. Amplification products were electrophoresed in 2.5% 0.5X TBE agarose gels and were stained with EtBr to reveal DNA segments of varying sizes. Gels were photographed under transmitted UV light.

Determination of Kunitz trypsin inhibitor protein

Ten random, mature seeds from seven genotypes harvested in the greenhouse were selected to extract crude protein for determining the presence or absence of Kunitz trypsin inhibitor proteins. The samples were incubated for 30 min (at room temperature) in 1 ㎖ Tris-HCl, pH 8.0, containing 1.56% v/v β-mercaptoethanol. After centrifugation, 50㎕ of the supernatant was added to an equivalent amount of 5X sample buffer [10% w/v sodium dodecyl sulfate (SDS), 50% v/v glycerol, 1.96% v/v β-mercaptoethanol, 1*M* Tris-HCl, pH 6.8]. The samples were boiled at 97 °C for 5 min and then centrifuged. Two microliters of the supernatant were loaded on 12% acrylamide SDS polyacrylamide gel electrophoresis (SDS-PAGE) medium gels in Owl Separation Systems Inc. (Model : P9DS, Portsmouth, NH, USA). Electrophoresis was performed at 120 V for 7 hrs. Gels were stained overnight in an aqueous solution of 0.25 g Coomassie brilliant blue R250, 10% acetic acid, 45% methanol, and destained with 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 21.5 kDa soybean trypsin inhibitor protein was used to aid recognition of samples lacking the Kunitz trypsin inhibitor protein.

Results and Discussion

Satt228 marker very tightly linked to *Ti* locus at distance of 0 cM was used to screen germplasm accessions 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 ('Jinpumkong2', 'Clark', and 'William') with *TiTi* genotype (Kunitz trypsin inhibitor protein present) are shown in Figure 1A. Also, polyacrylamide gel banding patterns of protein extracted from 10 randomly selected seeds of these seven germplasm accessions used are shown in Figure 1B. *TiTi* genotypes ('Jinpumkong2', 'Clark', and 'William') had allele1, however, *titi* genotypes (PI196168, C242, W60, and PI157440) had allele2 as shown by the analysis of the

PCR profiles obtained with Satt228 marker (Figure 1A). *TiTi* genotypes ('Jinpumkong2', '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 protein gel electrophoresis from the mature seed (Figure 1B). From the comparison of gel electrophoresis for Kunitz trypsin inhibitor protein (Fig. 1B) and banding pattern amplified by Satt228 marker from the genomic DNA (Fig. 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 ('Jinpumkong2', 'Clark', and 'William') which showed 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 showed no 21.5 kDa protein band in electrophoresis of mature seed had allele2 amplified by Satt228 marker from the genomic DNA. Any recombination between the SKTI protein and allele of Satt228 marker was not observed.

At present, protein electrophoresis of mature seed is the method used to select lines lacking the Kunitz trypsin inhibitor protein. Indirect selection based on DNA marker tightly linked to *Ti* locus is an easier and more efficient method than protein electrophoresis for selecting lines lacking Kunitz trypsin inhibitor protein. However, so far few researchers have identified DNA markers linked to *Ti* locus. Kim et al. (2004) reported that two RAPD markers (OPO12 and OPC08) were linked to the *Ti* locus at a distance of 16.0 and 16.6 cM, respectively. Recently, one SSR marker tightly linked to the *Ti* locus with 0 cM was reported (Kim et al. 2006). Moraes et al. (2006) reported a 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 markers designed to detect the absence of the SKTI protein reported by Moraes et al. (2006) was not valid between germplasm accessions of *TiTi* (SKTI protein present) and the *titi* (SKTI protein absent) genotype used in this study. No polymorphism was observed among the tested germplasm accessions. However, cosegregation between the allele of the Satt228 marker and the presence or absence of the SKTI protein in several soybean accessions of *TiTi* and *titi* genotyes was observed (Figs. 1A, 1B). These results indicate that the selection of germplasm accessions or lines lacking the Kunitz trypsin inhibitor protein is possible by using Satt228 marker analysis.

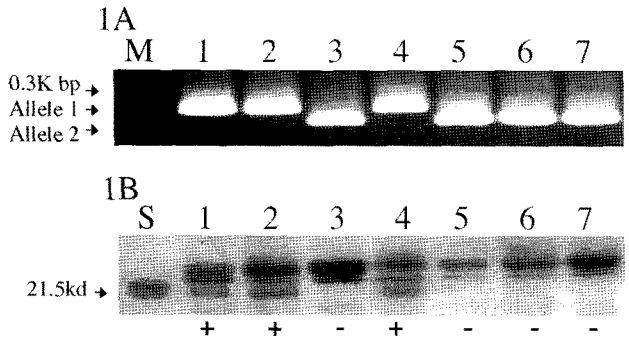

Fig. 1. Pattern of genomic DNA amplification by Satt228 marker using leaf tissue of germplasm accessions (1A) and pattern of polyacrylamide protein gel electrpophoresis extracted from 10 random seeds harvested (1B). M: molecular marker, S: Kunitz trypsin inhibitor protein (Sigma, product number:T6522). 1:'Jinpumkong2'(*TiTi*), 2:'Clark' (*TiTi*), 3:PI196168 (*titi*), 4:'William' (*TiTi*), 5:C242 (*titi*), 6:W60 (*titi*), 7:PI157440 (*titi*). +: presence of SKTI protein and -: absence of SKTI protein.

Table 1. Origin, seed coat color, Kunitz trypsin inhibitor (SKTI) protein, and Ti locus of soybean germplasm accessions used in this study.

Germplasm	Origin	Seed coat color	SKTI protein	*Ti* locus
PI 157440	Korea	Yellow	absent	*titi*
PI 196168	Korea	Yellow	absent	*titi*
C242	USA	Yellow	absent	*titi*
W60	USA	Yellow	absent	*titi*

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