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Identification of Molecular Markers Linked to *Ti* Locus in Soybean

Myung Sik Kim, Min Jung Park, Jung Gyu Hwang, Soo Ho Jo, Mi Suk Ko, and Jong Il Chung†

Department of Agronomy, Research Institute of Life Sci., Gyeongsang National University, Chinju 660-701, Korea

ABSTRACT : Soybean is a major source of protein meal in the world. Kunitz trypsin inhibitor (KTI) protein is responsible for the inferior nutritional quality of unheated or incompletely heated soybean meal. The objective of this research was to identify RAPD markers linked to KTI protein allele using bulked segregant analysis. Cultivar Jinpumkong2 (*TiTi*) was crossed with C242 (*titi*, absence of KTI protein) and F_1 seeds were planted. The F_1 plants were grown in the greenhouse to produce F_2 seeds. Each F_2 seed from F_1 plants was analysed electrophoretically to determine the presence of the KTI protein band. The present and absent bulks contained twenty individuals each, which were selected on the basis of the KTI protein electrophoresis, respectively. Total 94 F_2 individuals were constructed and 1,000 Operon random primers were used to identify RAPD primers linked to the *Ti* locus. The presence of KTI protein is dominant to the lack of a KTI protein and Kunitz trypsin inhibit protein band is controlled by a single locus. Four RAPD primers (OPAC12, OPAR15, OPO12, and OPC08) were linked to the *Ti* locus. RAPD primer OPO12 was linked to *Ti* locus, controlling kunitz trypsin inhibitor protein at a distance of 16.0 cM. This results may assist in study of developing fine map including *Ti* locus in soybean.

Keywords: Kunitz trypsin inhibitor, RAPD marker, BSA, soybean

Soybean protein is a major component of the diet of food producing animals and is increasingly important in the human diet. The trypsin inhibitor proteins have been proposed as one of the major antinutritional factors present in raw mature soybean (Westfall & Hauge, 1948). Proper heat processing is required to destroy protease inhibitors. Excessive heat treatment may lower amino acid availability and reduce animal weight gain. Soybean lines with reduced protease inhibitor content could reduce or eliminate the need for expensive heat treatment and lessen the chance of lowering amino acid availability. Several different trypsin inhibitors have been reported to be present in soybeans (Birk, 1961; Frattali & Steiner, 1968; Kunitz, 1945), but much of the soybean trypsin inhibitor (SBTI) activity is thought to be due to the protein SBTI-A_2 (Rackis *et al.*, 1962), which generally is known as the Kunitz trypsin inhibitor. Soybean Kunitz trypsin inhibitor (SKTI) is a small, monomeric and non-glycosylated protein containing 181 residues. This 21.5 kDa non-glycosylated protein was first isolated and crystallized from soybean seeds by Kunitz (1945).

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 & Hadley, 1972; Orf & Hymowitz, 1979). Orf & Hymowitz (1979) found that the fifth form does not exhibit a soybean trypsin inhibitor-A_2 band and is inherited as a recessive allele designated *ti*. They also found that crude seed protein from seeds that lacked a soybean trypsin inhibitor-A_2 band had a 30 to 50% reduction in trypsin inhibitor activity compared with 'Amsoy 71' that has the soybean trypsin inhibitor-A_2 band. The SKTI gene has been located on 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).

The development of molecular tools has facilitated the task of identifying chromosomal regions related to particular traits. Plant breeders can use molecular markers to select indirectly individuals in segregating populations that carry a gene for a favorable trait if a tight linkage exists between a marker locus and the genetic locus controlling that trait. Marker assisted selection allows the breeder to bypass laborious and/or costly phenotypic screens. Bulked segregant analysis is an effective strategy for identifying linkage between genetic markers and qualitative traits (Michelmore *et al.*, 1991). A population of F_2 individuals segregating for two alleles of a single gene (or marker) can be sorted into two classes comprised of the contrasting homozygous genotypes. If the DNA bulks corresponding to those two classes display a polymorphism for a genetic marker, linkage between the marker and the segregating locus can be inferred. Molecular markers linked to SKTI allele were not published. The objective of this research was to use bulked segregant analysis to identify RAPD markers linked to SKTI allele.

†Corresponding author (Phone) +82-55-751-5421 (E-mail) jongil@gshp.gsnu.ac.kr <Received November 17, 2004>

MATERIALS AND METHODS

Plant materials

Cultivar used in the cross was Jinpumkong2 that has Kunitz trypsin inhibitor protein band (*TiTi*). C242 used as male was a genotype free of the Kunitz trypsin inhibitor protein (*titi*). Cultivar Jinpumkong2 was crossed with C242 and F_1 seeds were planted. The F_1 plants were grown in the greenhouse to produce F_2 seeds.

Determination of Kunitz trypsin inhibitor genotype

Each F_2 seed from F_1 plants was analysed electrophoretically to determine the presence (KTI - '+') or absence (KTI - 'null') of the band. A sample of about 10 mg separated from the F_2 soybean seeds except for embryo 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 in 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^{TM}, Product Code : M4038) containing the 21.5 kDa soybean trypsin inhibitor protein, was used to aid recognition of samples lacking the Kunitz protein band.

DNA extraction, PCR, and construction of bulks

F_2 seeds tested for Kunitz trypsin inhibitor protein band were planted in the field on May, 2004. Young leaves were collected from the 94 individual F_2 plants and parent 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). The PCR protocol followed that described by Williams *et al.* (1990), except for minor modifications in the thermocycler temperatures and times. The PCR reaction was performed in a MJ research PTC-200 Thermocycler. The thermal profile consisted of 2 cycles of 1 minute at 92 °C, 22 second at 42 °C, and 70 second at 72 °C, followed by 39 cycles of 16 second at 92 °C, 22 second at 42 °C, and 70 second at 72 °C, before ending with 1 cycle of 5 minutes at 72 °C. Amplification products were electrophoresed in 1.2 % TBE agarose gels and were stained with EtBr to reveal DNA segments of varying sizes Gels were photographed under transmitted UV light. For the construction of present and absent Kunitz trypsin inhibitor protein band bulks, the methodology of bulked segregant analysis (BSA), described by Michelmore *et al.* (1991) was used. The present and absent bulks contained twenty individuals each, which were selected on the basis of the Kunitz trypsin inhibitor protein electrophoresis, respectively.

Genetic linkage analysis

Primers that distinguished the bulks and the parents were tested on the entire F_2 population. A linkage map of RAPD markers was constructed using the computer program MAPMAKER v. 3.0 (Lander *et al.*, 1987) from the marker data obtained from 94 F_2 progenies. 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 .

RESULTS AND DISCUSSION

The banding patterns of Kunitz trypsin inhibit protein that appeared in the parents and F_2 seeds from the cross are shown in Fig. 1. Jinpumkong2 parent had band in 21.5 KDa position and the band was segregated in F_2 seeds.

The summarized data for the observed and expected distri-

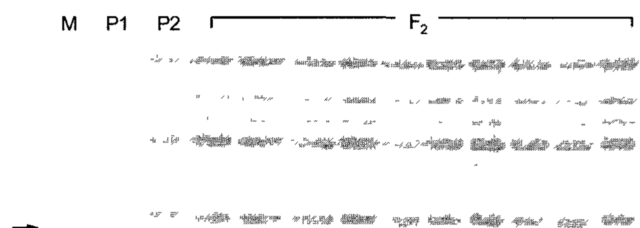

Fig. 1. Polyacrylamide gels of protein extracted from parents and F_2 seeds. P1 (Jinpumkong2) and P2 (C242) are parents and arrow points to the Kunitz trypsin inhibitor band (21.5 KDa).

Table 1. Observed and expected segregation of F_2 seeds from selfed F_1 soybean plants from the cross Jinpumkong2 (*TiTi*) × C242 (*titi*) for the presence or absence of the Kunitz inhibit protein band.

F_2 seeds		F_2 electrophoretic bands		χ^2	Probability
		*Ti_*	*titi*		
No.	Observed	72	26	0 12	0 7 - 0.8
98	Expected	73 5	24.5		

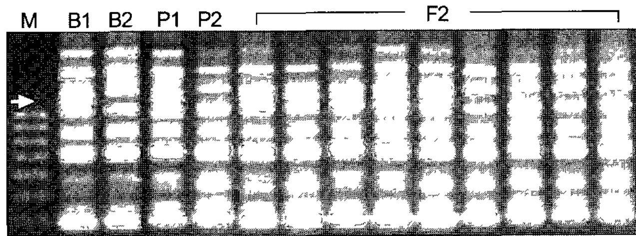

Fig. 2. Patterns of segregating DNA fragment for RAPD primer OPAC12 in both parents, bulked parents, and F_2 population. P1 is Jinpumkong2 (*TiTi*) and P2 is C242 (*titi*) B1 is bulked of present Kunitz trypsin inhibitor protein individuals and B2 is bulked of absent individuals. Arrow is polymorphic band

bution of F_2 seeds are shown in Table 1. The data support that the presence of Kunitz trypsin inhibit protein is dominant to the lack of a Kunitz trypsin inhibit protein and Kunitz trypsin inhibit protein band is controlled by a single locus (Orf & Hymowitz, 1979).

Of the 1,000 RAPD primers tested on the two parents of cultivar Jinpumkong2 (*TiTi*) and C242 (*titi*), 124 polymorphic primers were identified. Approximately 12 % primers produced polymorphic DNA fragment differences between the parents. Only 35 polymorphic primers were identified between the parental DNA bands in the bulked DNA samples. Among 35 primers, only 16 primers were shown polymorphism between parents and bulked parents simultaneously. These primers was segregated in 94 F_2 population. Four seeds were not germinated. Fig. 2 represents some examples of segregating DNA fragment for RAPD markers (OPAC12) in parents, bulked parents and F_2 population.

A genetic map was constructed from the 16 segregating RAPD markers. Of the 16 primers, 10 RAPD primers were found to be genetically linked. These markers coalesced into 3 linkage groups. Linkage group 3 showed that RAPD primer OPO12 was linked to *Ti* locus, controlling Kunitz trypsin inhibitor protein at a distance of 16.0 cM (Fig. 3).

Also, RAPD primers OPAC12, OPAR15, and OPC08 were linked to *Ti* locus. Hildebrand *et al.* (1980) reported that *Ap* locus, controlling seed acid phosphatase was linked to *Ti* locus with a crossover frequency of 16.2 %. Kiang

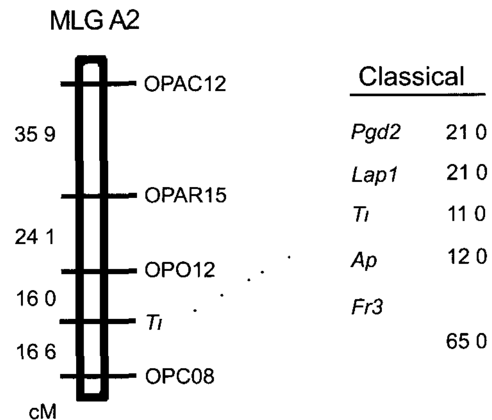

Fig. 3. Molecular linkage map A2 (Cregan *et al*, 1999) of *Ti* locus defined using 94 F2 population derived from the cross of Jinpumkong2 (*TiTi*) and C242 (*titi*). Map was constructed using MAPMAKER/EXP (LOD 4.0 maximum distance 50 cM). Marker loci names are on the right and Kosambi map distances are on the left

(1987) reported that the recombination frequency between *Ap* and *Ti* was estimated at 6.6 ± 0.5 %. Also, he found that the map distance between *Lap1* locus, controlling leucine aminopeptidase and *Ti* locus was calculated to be 16.3 ± 1.0 map units. Linkage group 3 including *Ti* locus constructed in this study should to be A2 of the USDA/Iowa State University soybean molecular linkage map (Cregan *et al.*, 1999).

High trypsin inhibitor levels in soybeans have negative nutritional impact in both in food and feed applications. The most common way of inactivating Kunitz trypsin inhibitor protein is by heat treatment. However, this method lowers the solubility of other soybean seed proteins and results in the loss of essential amino acids. The availability of soybean lines lacking the Kunitz trypsin inhibitor and RAPD markers identified linked to the *Ti* locus may assist others in their investigations of the function and role of Kunitz trypsin inhibitor protein in soybean breeding program.

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