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ORIGINAL ARTICLE

Validation of SSR markers linked to null kunitz tryspin inhibitor allele in Indian soybean [*Glycine max* (L.) Merr.] population

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Abstract Kunitz trypsin inhibitor, a proteinaceous antinutritional factor present in soybean seeds, is responsible for inferior nutritional quality of raw soybean and incompletely processed soy products. The objective of the present investigation was to validate the SSR markers (Satt228 and Satt409) reported to be linked to *Ti* locus in an Indian soybean population generated from the cross between soybean cultivar LSb1 (*TiTi*) and PI542044 (*titi*). Parental polymorphism was surveyed using Satt409, Satt228 and 5 SSR markers in the neighbouring genomic region of *Ti* locus. A portion of the cotyledon of F_2 seeds was used for analyzing the presence or absence of kunitz trypsin inhibitor polypeptide electrophoretically while the remaining portion containing the embryo was used for raising the F_2 plants (104) for the development of mapping population. The SSR marker Satt228 reported to be tightly linked with *Ti* locus was not found to be polymorphic for the parents used in our study. Satt409 was found to be linked with *Ti* locus at 4.7 cM. Besides, a new marker Satt538 was found to be linked with *Ti* locus at a distance of 17.8 cM. Thus, the SSR marker Satt409 can be useful for Marker Assisted Selection for transferring titi allele in the background of Indian soybean genotypes.

Keywords Kunitz trypsin inhibitor · Soybean · SSR markers · *Ti* locus

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Abbreviations

SSR	Simple sequence repeat
KTI	Kunitz trypsin inhibitor
LOD	Log of odds
PAGE	Polyacrylamide gel electrophoresis
MAS	Marker assisted selection

Introduction

Soy food is being acclaimed as 'functional food of the century' as it contains nutraceutical components viz. isoflavones, tocopherols, lecithin and saponins that have been implicated in reducing the risk of major killer diseases like atherosclerosis, breast cancer, diabetes and osteoporosis. However, barely 5% of the total soybean produced in the country is processed into soyfoods. One of the major constraints in acceptance of soy foods is the presence of several anti-nutritional factors in soybean seed with trypsin inhibitors such as kunitz trypsin inhibitor (21 kDa) and bowman-birk factor (8 kDa) as the major proteinaceous antinutrients (Kunitz 1945; Birk 1961). Kunitz trypsin inhibitor (KTI), a non glycosylated monomeric protein, constitutes about 80% of the trypsin inhibitor activity and is also known as SBTI-A_2. Though kunitz trypsin inhibitor is heat labile but its active form has been found to be responsible for the growth inhibition, pancreatic hypertrophy and hyperplasia in experimental animals (Liener and Kakade 1980; Furukawa et al. 1987; Ge and Morgan 1993). In industries, thermal treatment is employed to bring down the trypsin inhibitor content of the soy-based products; however, this process has its own limitations. There is always a level of residual activity of this anti-nutrient depending upon the level of temperature and duration of

heating. More importantly, thermal treatment affects the protein solubility (Anderson 1992) and is cost-ineffective for the soy processing units. Furthermore, in India, soybean is often recommended to mix with wheat (1:9) for making *chapati* flour at household level. This necessitates the extra time-consuming step of boiling of beans for inactivation of kunitz trypsin inhibitor prior to grinding with wheat grain. As none of the 94 released varieties of soybean in India was found to be free from kunitz trypsin inhibitor in our laboratory; therefore, development of soybean genotypes free from this antinutritional factor is one of the major plant breeding objectives in augmenting utilization of soybean in food and feed in the country.

Five electrophoretic forms of soybean kunitz inhibitor have been reported to be controlled by multiple alleles Ti^a, Ti^b, Ti^c and Ti^d at single locus (Hymowitz and Hadley 1972; Orf and Hymowitz 1979). The fifth form does not exhibit a soybean trypsin inhibitor-A2 band and is inherited as a recessive allele designated as *ti*. In the plant-breeding programme focusing on the development of kunitz inhibitor free soybean variety, SSR (simple sequence repeat) markers linked with this trait can be used for Marker Assisted Selection (MAS) as routine gel electrophoresis method employed for identification of kunitz trypsin inhibitor polypeptide may damage the embryo while removing a part of seed tissue for analysis. Kim et al. (2006) reported the linkage of two SSR markers Satt228 and Satt409 with *Ti* locus in two mapping population viz. Jinpumkomg2 x C242 and Clarke x C242 and another SSR marker Satt429 was found to be linked with *Ti* locus in the former population only. This study also showed that though two SSR markers Satt228 and Satt409 were found to be linked with *Ti* locus in both the populations but the distances of the markers from the *Ti* locus were different in two populations. It was pertinent to validate the linkage of these two markers with *Ti* locus in Indian soybean genotypes before using them in the MAS (Marker Assisted Selection) for development of kunitz trypsin inhibitor free soybean varieties. Thus the present investigation was undertaken to validate the linkage of Satt228, Satt409 and the neighbouring SSR markers with *Ti* locus in a mapping population developed from LSb1 (an early maturing Indian soybean variety with *TiTi* genotype) and PI542044 (soybean germplasm accession with *titi* genotype).

Materials and methods

Soybean genotype PI542044, which has null allele of kunitz trypsin inhibitor, was procured from United States Department of Agriculture. Soybean cultivar LSb1, which has kunitz trypsin inhibitor protein, was crossed with PI542044 in the fields of Directorate of Soybean Research (Indian Council of Agricultural Research), Indore. F_1 plants were raised in the glasshouse to produce F_2 seeds.

Phenotyping

A portion of the cotyledon from each of the F_2 seeds obtained from 2 F_1 plants was removed, without damaging the embryo, with a sharp cutter to phenotype electrophoretically for the presence or absence of kunitz trypsin inhibitor polypeptide. The finely ground cotyledonary tissue was incubated in 1 ml Tris–HCl buffer (pH 8.0) for 30 min. and centrifuged. Equal volumes of supernatant and 5x sample buffer containing 50% v/v glycerol, 1.96% v/v mercaptoethanol, 0.05% bromophenol dye and 1 M Tris–HCl (pH6.8) were loaded on 10% acrylamide gel in BioRad electrophoresis system and run at 35 mA for 2 hrs. Gels were stained in an aqueous solution of coomassie blue, acetic acid and methanol overnight and destained in acetic acid and methanol mixture. Standard trypsin inhibitor protein (21.5 kDa) was run in a separate lane for identification of kunitz trypsin inhibitor polypeptide.

Genotyping

F_2 seeds containing the embryo were planted in the glass house and after 10–15 days of planting, the tender young leaves were collected from 104 individual plants of the segregating population. Genomic DNA was extracted from the finely ground leaf tissue by means of CTAB (Cetyl trimethyl ammonium bromide) procedure (Doyle and Doyle 1990). Crude DNA obtained above was purified following phenol extraction and ethanol precipitation method, quantified spectrophotometrically and suitably dissolved in TE buffer (pH 8.0). The PCR was performed in a MJ Research Thermocycler model PTC100 and the reaction mixture (10 μl) contained 2 μl DNA (20 ng/μl), PCR 10X buffer (1 μl), 1.1 μl $MgCl_2$ (25 mM), 0.1 μl dNTPs (25 mM), 0.4 μl each forward and reverse SSR primers (30 ng/μl), 0.068 μl Taq DNA polymerase (3 units/μl), 4.932 μl distilled water. Initially, DNA was denatured at 94°C for 2 min. followed by 30 cycles each consisting of denaturation at 94°C for 1 min, primer annealing at 50°C for 2 min., primer elongation at 72°C for 3 min. Finally, elongation was carried out at 72°C for 10 min. Amplified products so obtained were resolved on 3% metaphore agarose gel using a 96-well horizontal gel electrophoresis unit (Atto Corporation) and analysed in a gel documentation unit (Syngene). Two SSR markers Satt228 (154.11 cM) and Satt409 (145.57 cM) from LGA2 reported to be linked to *ti* and the 5 neighbouring SSR markers viz. Satt_294 (139.97 cM), Satt _347 (158.39 cM), Satt538 (159.63 cM), Satt429 (162.03 cM), Satt378 (165.73 cM) were amplified for parental polymorphism survey. Subsequently, only

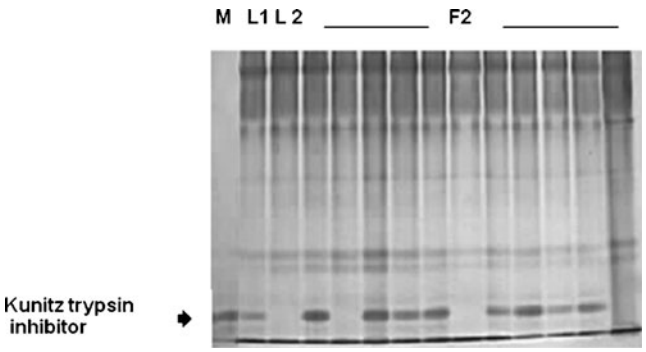

Fig. 1 Native polyacrylamide gel electrophoresis (PAGE) profile of proteins extracted from parents and F_2 seeds. Lanes L1 and L2 depict LSb1 and PI542044, respectively while M is the marker protein for kunitz trypsin inhibitor

polymorphic markers (Satt294, Satt409, and Satt538) were amplified with the genomic DNAs of mapping population.

Linkage of the polymorphic SSR markers (Satt294, Satt409 and Satt538) with target gene was determined using MAPMAKER version 3.0 software as given by Lander et al. (1987). Linkage was considered significant if the logarithm of odds (LOD) score was >3.0. The Kosambi mapping function was used to convert recombination fraction to map distance among the markers and the target gene (Kosambi 1944).

Results and discussion

The electrophoretic banding pattern of kunitz trypsin inhibitor protein (KTI) of the parents and the F_2 seeds was shown in Fig. 1. The polypeptide band at 21.5 kDa position corresponding to the kunitz trypsin inhibitor protein is present in the parent cultivar LSb1 but absent in PI542044. The segregation of F_2 seeds for presence or absence of kunitz trypsin inhibitor protein in the population LSb1 (*TiTi*) x PI542044 (*titi*) is given in Table 1. Out of the 104 F_2 seeds, 76 exhibited presence of kunitz trypsin inhibitor polypeptide while remaining 28 lacked kunitz trypsin inhibitor polypeptide ($\chi 2$=0.205, P=0.50–0.70). These observations fit 3:1 ratio proving that the character is controlled by single gene.

Ti locus has been located on group 9 in the classical linkage map of soybean (Hildebrand et al. 1980; Kiang 1987), which has been assigned to the linkage group A2 of the USDA/Iowa State University soybean molecular linkage map (Cregan et al. 1999). Kim et al. (2006) have reported the linkage of SSR

Table 1 Observed and expected segregation in F_2 population LSb1 x PI542044 for the presence or absence of the kunitz trypsin inhibitor protein

	Titi/*TiTi*	*titi*	$\chi 2$	*P*-value
Observed	76	28	0.205	070—0.50
Expected	78	26		

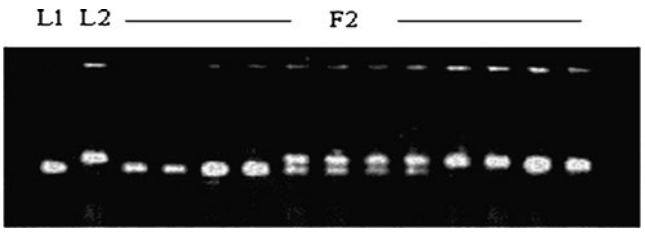

Fig. 2 Segregating pattern of DNA fragments in parents and F_2 population using SSR marker Satt409. Lane L1 depicts PI542044 while L2 exhibits LSb1

markers Satt228 and Satt409 with *Ti* locus. The present investigation was undertaken to validate the SSR markers linked to *Ti* locus viz. Satt228 and Satt409. In addition, 5 SSR markers viz. Satt_294, Satt _347, Satt538, Satt429, Satt378 in the vicinity of Satt228 and Satt409 were also tested for parental polymorphism for LSb1 and PI542044. Satt_294, Satt_409, and Satt538 were found to be polymorphic for the parental lines. Satt228 reported to be tightly linked in two mapping populations viz. Jinpumkomg2 x C242 (source of null allele of kunitz trypsin inhibitor protein) and Clark x C242 (source of null allele of kunitz trypsin inhibitor protein) studied by Kim et al. (2006) did not reveal polymorphism for cultivar LSb1 and PI542044 in our results. All the three polymorphic markers viz. Satt_294, Satt_409, and Satt538, used for genotyping F_2 individuals, showed a normal segregation of 1:2:1.

Representative gel of the amplification pattern obtained for Satt409, using genomic DNA of parents (PI542044; LSb1) and F_2 plants is shown in Fig. 2. A genetic map was constructed using genotyping data of Sat_294, Satt409, Satt538 and phenotyping data for *Ti* locus using MAPMAKER version 3.0 (Fig. 3). Satt409 was found to be

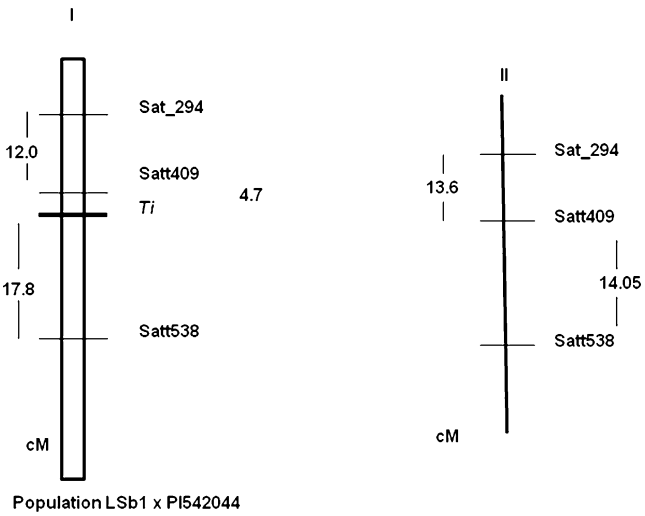

Fig. 3 Molecular linkage map A2 of *Ti* locus using population derived from the cross of LSb1 (*TiTi*) x PI542044 (*titi*)-I. Map was constructed using MAPMAKER. II shows linkage map given by USDA 2008 (bldg6.arsusda.gov/cregan/soymap.html for comparison)

linked with the *Ti* locus at a distance of 4.7 cM. This is in accordance with the observation of Kim et al. (2006) who mapped Satt409 at a distance of 4.5 cM from *Ti* locus in mapping population derived from Jinpumkomg2 x C242. However, in the other population derived from Clark x C242, a mapping distance of 18.2 cM for Satt409 and *Ti* locus was observed. In addition to Satt409, two other SSR markers Satt538 and Sat_294 mapped close to *Ti* locus in the order Satt_294-Satt409-*Ti*-Satt538 which is similar to the linkage map given by USDA (2008) (Fig. 3). However, compared to the USDA linkage map of A2, the mapping distance was more between Satt538 and Satt409 and lesser for Sat_294 and Satt409 in the present study. It is noteworthy to mention that the SSR marker Satt538 showing linkage with *Ti* locus at a distance of 17.8 cM in this study was not reported earlier.

Thus, the results confirm the linkage of SSR marker Satt409 with *Ti* locus in the present population of LSb1 x PI542044. Though it is a useful marker for the introgression of kunitz trypsin inhibitor null allele into popular soybean varieties; however the discovery of still closer SSR markers will make Marker Assisted Selection more accurate.

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