Indian Journal of Experimental Biology
Vol. 51, December 2013, pp. 1125-1129

Deployment of gene specific marker in development of kunitz trypsin inhibitor free soybean genotypes

Vineet Kumar*, Anita Rani & Reena Rawal

Directorate of Soybean Research (ICAR), Indore, 452 001, India

Received 17 December 2012; revised 25 July 2013

Genetic elimination of kunitz trypsin inhibitor in soybean seed would obviate the need for boiling required to inactivate the antinutritional factor and therefore economize the soy processing. PI542044, the source of null variant of kunitz trypsin inhibitor gene is being used in the development of kunitz trypsin inhibitor free soybean genotypes in India. Gene specific marker can expedite the genetic elimination of this undesirable trait from popular soybean genotypes. In the present study, we tested the DNA amplification of soybean genotype PI542044 and kunitz trypsin inhibitor null lines derived from this genotype with a gene specific primer developed from the null variant of PI157740. The amplicons so obtained corresponded to the absence of kunitz trypsin inhibitor protein band on 10% polyacrylamide gel. The gene specific marker also amplified the null allele of template DNA of F_1, BC_1F_1 and BC_2F_1 plants developed during marker assisted introgression of null allele of kunitz trypsin inhibitor into elite soybean cultivar JS97-52. The results presented show the utility of this gene specific marker developed from null allele of kunitz trypsin inhibitor for identification of kunitz trypsin inhibitor free genotypes developed from PI542044, the only source of null variant available in India.

Keywords: Gene specific marker, Kunitz trypsin inhibitor, Soybean

Soybean has been endowed with the sobriquet of 'functional food of the century' as the nutraceuticals-rich bean has been reported to reduce the risk of diabetes, estrogen deficiency-induced cancer, atherosclerosis, osteoporosis etc., besides being packed with basic nutrients which combat the diseases stemming from mal- and under nutrition. Despite these virtues, barely 5-7% of the soybean seeds produced in the country is processed for soyfood and snacks. One of the major deterrents in widespread acceptance of soy foods is the presence of protease inhibitor especially trypsin inhibitor. Kunitz trypsin inhibitor, which accounts for 80% of total trypsin inhibitor activity in soybean seeds, needs to be heat inactivated for improved nutritional quality of the unfermented soy products. For this reason, soybean seeds must be boiled for 20 min followed by drying prior to mixing with wheat to prepare soy fortified flour. Besides, the consignments for export of soybean meal (deoiled cake) for feed purpose also need to be certified for levels of trypsin inhibitor content to meet out the stringent requirement of the buyers in the international market. Though, kunitz trypsin inhibitor is inactivated to best possible level by boiling for 20 min or by toasting, this results in not only 25% increase in energy cost, but also leads to 20% decline in protein solubility. Therefore, availability of soybean seeds genetically free from kunitz trypsin inhibitor is critical to the growth of both soy food and feed industries.

Five electrophoretic forms of kunitz trypsin inhibitor are controlled by multiple alleles Ti^a, Ti^b, Ti^c, Ti^d at single locus. The fifth form does not exhibit a soybean kunitz trypsin inhibitor band A2 and is inherited as recessive allele *ti*. This multiallelic region on chromosome A2 in soybean genome is designated as *Ti* locus. Routine electrophoretic method employed for identification of null kunitz trypsin inhibitor allele is destructive procedure and can't be performed in early generations of plant breeding programme focusing on development of kunitz trypsin inhibitor free soybean varieties. Simple sequence repeats (SSR) markers linked with this trait are being employed for the purpose[1-3]. SSR marker Satt228 has been shown to be at 0.00 and 3.7 cM from the *Ti* locus in the mapping population derived from crossing genotypes Jinpumkong2 and Clark with C242 (the donor of null allele of kunitz tryspin inhibitor)[1]. In the same study, Satt409 was at 4.5 cM away from *Ti* locus in Jinpumkong2 × C242, however no linkage of this marker to *Ti* locus was observed in the second population derived from Clark x C242. Further, SSR marker Satt409 has been reported to be at 4.7 cM from

*Correspondent author
Telephone: 09302106779
Fax: 073-12470520
E-mail: vineetksahni@yahoo.com

Ti locus in the mapping population generated from the crossing of Indian genotype LSb1 with PI542044 (donor for null allele of kunitz tryspin inhibitor)[2]. This variation with regard to the genetic distance of the reported linked SSR marker from the *Ti* locus has been attributed to the structural re-arrangement in the genome in different mapping populations[1,2]. This necessitates the validation of reported markers linked to the locus of interest in every mapping population before its application. However, use of gene specific marker directly selects the allele of interest obviating the need of validation in a specific genetic background prior to embarking upon the marker assisted breeding for the trait using the same donor.

Sequences of the mutant allele for kunitz trypsin inhibitor in genotype PI157740 were identified[4]. Based on these findings, specific marker for null allele of kunitz trypsin inhibitor was designed and employed successfully in identification of heterozygous individuals in backcross population generated from the crossing of cv. Monarca with a breeding line lacking kunitz trypsin inhibitor (with unknown ancestry)[5]. To test the possibility of DNA amplification of the null allele of PI542044, the genotype which has been used as donor in the development of first ever kunitz trypsin inhibitor free soybean genotypes in our country, through the gene specific marker[5], the genomic DNA of PI542044 and the breeding lines derived from this were used as template. At Directorate of Soybean Research, Indore, kunitz trypsin inhibitor free soybean genotypes NRC101 and NRC102 have been developed by crossing kunitz trypsin inhibitor positive cultivar Samrat with PI542044, and these two genotypes are registered with National Bureau of Plant Genetic Resources[6]. In addition, NRC103 and NRC104 were sister breeding lines derived from the same population. Further, five more advanced breeding lines lacking kunitz trypsin inhibitor have been developed by crossing cultivar 'LSb1' with PI542044. In the present study, it was thought worthwhile to test the amplification of the null allele of kunitz trypsin inhibitor through the gene specific marker, in all the afore-mentioned kunitz trypsin inhibitor free genotypes and in null allele carrier plants foreground-selected using linked marker in the ongoing marker assisted backcross (MABC) programme for introgression of the trait into an elite Indian soybean cultivar.

Materials and Methods

Seeds of kunitz trypsin inhibitor free soybean genotypes NRC101, NRC102, NRC103, NRC104, LK-1, LK-2, LK-3, LK-4, LK-5 developed at Directorate of Soybean Research, Indore were sown along with the recipient parent and PI542044, the donor of null allele of kunitz trypsin inhibitor for all the genotypes. Samrat and LSb1 were the recipient genotypes for NRC101, NRC102, NRC103, NRC104 and LK-1, LK-2, LK-3, LK-4, LK-5, respectively. DNA was extracted from the young leaves of 20 days' old plants for genotyping, while the seeds harvested were phenotyped for the presence or absence of kunitz trypsin inhibitor protein by native PAGE.

Phenotyping—The finely ground seed flour (50 mg) of the genotypes lines lacking kunitz trypsin inhibitor and kunitz trypsin inhibitor positive was incubated in 1 mL Tris–HCl buffer (*p*H 8.0) for 30 min and centrifuged. Equal volumes of supernatant and 2x sample buffer containing 20% (v/v) glycerol, 1.96% (v/v) mercaptoethanol, 0.05% bromophenol dye and 1 *M* Tris-HCl (*p*H 6.8) were loaded on 10% acrylamide gel in BioRad electrophoresis system and run at 35 mA for 2 h. Gels were stained overnight in an 0.25% coomassie brilliant blue (R-250) in methanol, water and glacial acetic acid solution in the 45:45:10 ratio, respectively and destained in methanol, water and glacial acetic acid (45:45:10) solution. Standard trypsin inhibitor protein (21.5 kDa) was run in a separate lane for identification of kunitz trypsin inhibitor polypeptide.

Genotyping—Genomic DNA was extracted from the finely ground leaf tissue through cetyl trimethyl ammonium bromide (CTAB) procedure[7]. Crude DNA so obtained was purified following phenol extraction and ethanol precipitation method, quantified spectrophotometrically and dissolved in TE buffer (*p*H 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), 1.0 µL PCR buffer (10X), 1.1 µL $MgCl_2$ (25 mM), 0.1 µL dNTPs (25 mM), 0.4 µL of 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 stained with ethidium bromide using a 96-well horizontal gel electrophoresis unit (Atto Corporation)

and analysed in a gel documentation unit (Syngene). Sequences for gene specific primer of null allele of kunitz trypsin inhibitor[5] and the SSR marker Satt409 and Satt228 reported to be linked to *Ti* locus were synthesised from Sigma-Aldrich and given in Table 1.

Results and Discussion

Gene specific marker for a trait has an advantage over molecular markers linked to the locus of interest due to its precision and specificity. Moreover, the linked SSR marker for a particular trait is not necessarily polymorphic for all the parental combinations and hence its application is limited to the parental combination which exhibits polymorphism for the SSR marker. Further, the linked SSR marker, even if it is polymorphic, drags with it undesirable genes present between the linked SSR marker and the locus of trait. In soybean, linkage of Satt228 and Satt409 to *Ti* locus has been reported with varying genetic distance between the *Ti* locus and the linked SSR marker in the two different mapping populations in the same study[1]. Similarly, genetic distance observed between the *Ti* locus and the Satt409 in the mapping population generated by crossing Indian soybean genotype LSb1 with PI542044[2] was significantly different from the corresponding distance observed in the earlier study[1]. Further, sequence analysis of null allele of kunitz trypsin inhibitor in kunitz trypsin inhibitor free soybean line PI157740 deposited in the gene bank[4] led to the designation of the null allele specific primer for kunitz trypsin inhibitor[5]. This gene specific marker was subsequently employed in the identification of the heterozygous and homozygous recessive plants for kunitz trypsin inhibitor in a population derived from cv. Monarca and a breeding line lacking kunitz trypsin inhibitor[5]. However, the source of null allele of kunitz trypsin inhibitor was not known in this study. Therefore, the amplification of the null allele for kunitz trypsin inhibitor using this gene specific marker needed to be confirmed in PI542044 (the source of null allele of kunitz trypsin inhibitor) and the breeding lines developed for null kunitz trypsin inhibitor trait, before employing it as a standard screening tool to identify kunitz trypsin inhibitor free genotypes. Genomic DNA of the recipient parents (Samrat, LSb1), PI542044 as the donor of null allele for kunitz trypsin inhibitor, kunitz trypsin inhibitor free genotypes NRC101, NRC102, NRC103, NRC104, LK-1, LK-2, LK-3, LK-4, LK-5 recently developed in breeding programme, were amplified using designed gene specific primer[5] and SSR marker Satt409 linked to *Ti* locus. In Fig. 1, lanes 2 and 8 showed the PCR products generated by the genomic DNA of PI542044. The amplicons of 420 bp size were generated by the null allele specific primer for kunitz trypsin inhibitor in the lanes 3, 4, 5, 6 for the genomic DNA of genotypes NRC101, NRC102, NRC103 and NRC104, and in lanes 9, 10, 11, 12, 13 for LSb1 derived kunitz trypsin inhibitor free genotypes LK-1, LK-2, LK-3, LK-4, LK-5 (Fig. 1). No amplicon was observed in the lane 1 and 7 corresponding to the genomic DNA of kunitz trypsin inhibitor positive parents i.e. Samrat and LSb1, respectively. The genomic DNA of the above-mentioned genotypes were amplified using SSR marker Satt409 which has been found to be tightly linked to *Ti* locus in the F_2 mapping population derived from Samrat x PI542044 and LSb1 x PI542044. Satt409 amplified PCR products of 190 bp size for kunitz tryspsin inhibitor positive genotypes (lanes 1 and 7) for Samrat and LSb1, while a fragment size of 170 bp (lane 2 and 8) for the genotype

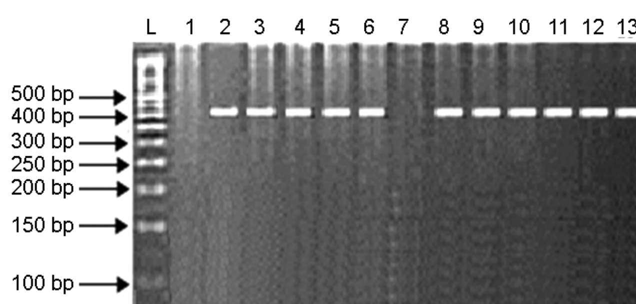

Fig. 1—Amplicons of null allele of kunitz trypsin inhibitor with gene specific marker on 3% metaphor [L is 50 bp DNA ladder, lanes 1 to 13 correspond to Samrat, PI542044, NRC101, NRC102, NRC103, NRC104, LSb1, PI542044, LK-1, LK-2, LK-3, LK-4, LK-5, respectively].

Table 1—Primers utilised for the identification of soybean genotypes with null allele of kunitz trypsin inhibitor.

Primer	Forward Sequence(5′→3′)	Reverse Sequence(5′→3′)
Gene pecific	CTTTTGTGCCTTCACCACCT	GAATTCATCATCAGAAACTCTA
Satt409	CCTTAGACCATGAATGTCTCGAAGAA	CTTAAGGACACGTGGAAGATGACTAC
Satt228	TCATAACGTAAGAGATGGTAAAACT	CATTATAAGAAAACGTGCTAAAGAG

PI542044 (Fig. 2). Lanes 3, 4, 5, 6, 9, 10, 11, 12, 13 which correspond to NRC101, NRC102, NRC103, NRC104, LK-1, LK-2, LK-3, LK-4, LK-5 showed an amplification fragment of 170 bp size similar to the PCR fragment obtained for the donor genotype PI542044. In other words, the kunitz trypsin inhibitor free genotypes which generated PCR amplicons through gene specific markers also amplified PI542044 specific amplicons through linked marker Satt409.

Fig. 3 shows the profile of kunitz trypsin inhibitor polypeptide (21.5 kDa) in the genotypes studied as revealed by PAGE. Presence or absence of null allele specific amplicon in kunitz trypsin inhibitor free and kunitz trypsin positive genotypes shown in Fig. 1 corresponded to the absence or presence of the kunitz trypsin inhibitor protein band in Fig. 3. To confirm the results further, foreground-selected plants through *Ti* locus linked SSR marker Satt 228 in the marker assisted backcross programme for the introgression of null allele of kunitz trypsin inhibitor into elite soybean cultivar JS97-52 from PI542044 were tested for DNA amplification through gene specific marker. Fig. 4 shows the amplicons obtained with the DNA of F_1 (Fig. 4 A), BC_1F_1 (Fig. 4 B), BC_2F_1 (Fig. 4 C) plants derived from JS97-52 x PI542044 and both the parents through gene specific marker in upper panel while the lower panel depicts the same through linked marker Satt228. The amplicons generated by the genomic DNA of F_1, BC_1F_1, BC_2F_1 plants through gene specific marker corresponded to PI542044

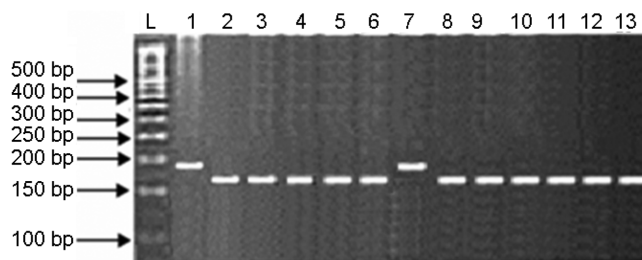

Fig. 2—Amplicons of kunitz trypsin inhibitor positive and negative genotypes along with the lines lacking kunitz tryspin inhibitor amplified using Satt409 (linked marker to *Ti* locus) on 3% metaphor [L is 50 bp DNA ladder, lanes 1 to 13 correspond to Samrat, PI542044, NRC101, NRC102, NRC103, NRC104, LSb1, PI542044, LK-1, LK-2, LK-3, LK-4, LK-5 respectively].

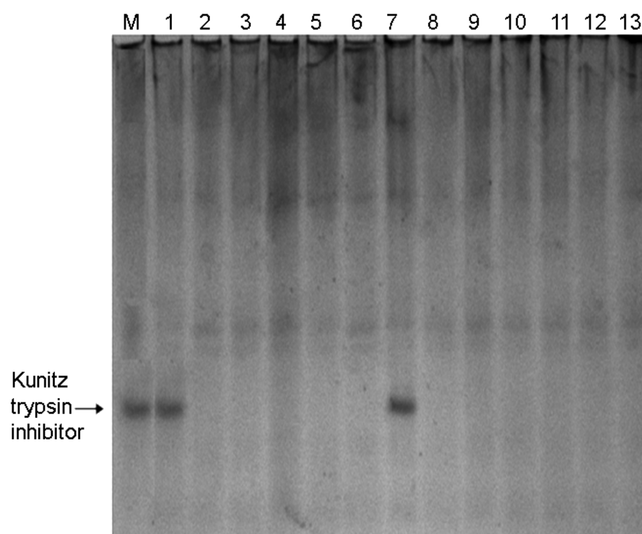

Fig. 3—Kunitz trypsin inhibitor polypeptide on 10% polyacrylamide gel [M is marker for kunitz trypsin inhibitor, lanes 1 to 13 correspond to Samrat, PI542044, NRC101, NRC102, NRC103, NRC104, LSb1, PI542044, LK-1, LK-2, LK-3, LK-4, LK-5 respectively].

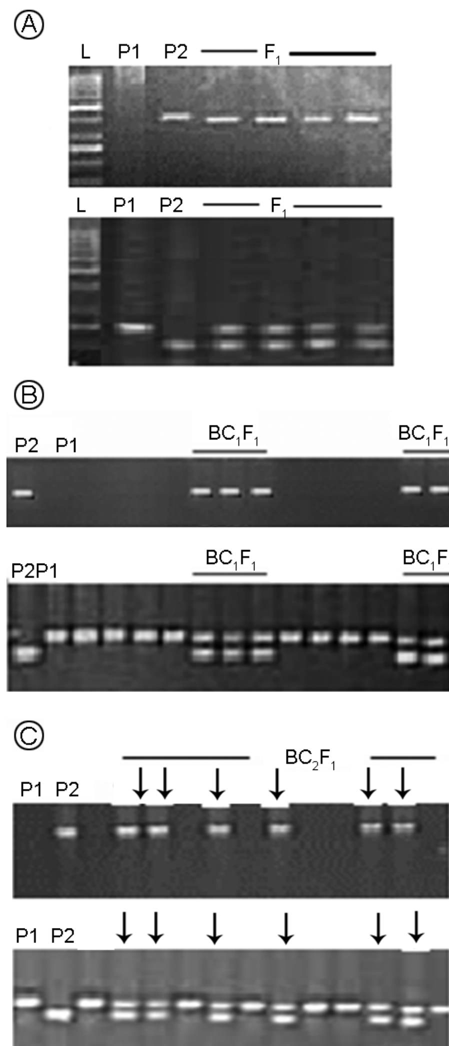

Fig. 4—PCR products on 3% metaphor agarose gel amplified with DNA template of F_1 (4A), BC_1F_1 (4B) and BC_2F_1 (4C) derived from JS97-52 x PI542044 [Upper panel in A, B and C depict amplicons generated with the gene specific primer while the lower panel with the SSR marker Satt228 linked to *Ti* locus].

(donor of null allele) specific amplicons obtained through Satt228.

Therefore, the present results showed that the gene specific marker developed in the original study[5] can be efficiently deployed in the identification of target plants carrying null allele of kunitz trypsin inhibitor in the mapping population derived from the cross between Indian genotypes as recipient parent and PI542044 as the donor for null allele for kunitz trypsin inhibitor in the marker assisted breeding for development of kunitz trypsin inhibitor free soybean in our country.

Acknowledgement

The authors acknowledge Department of Biotechnology, Ministry of Science and Technology, Government of India, for the financial grant provided under the Accelerated Programme in Crop Improvement.

References

1 Kim M S, Park M J, Jeong W H, Nam K C & Chung J, SSR markers tightly linked to the *Ti* locus in soybean [Glycine max (L.) Merr.], *Euphytica*, 152 (2006) 361.

2 Rani A, Kumar V, Mourya V, Singh R & Husain S M, Validation of SSR markers linked to null kunitz trypsin inhibitor allele in Indian soybean, *J Biochem Biotech*, 20(2) (2011) 258.

3 Kumar V, Rani A, Mourya V, Rawal R, Verma K, Shivkumar M, Talukdar A & Lal S K, Marker Assisted Selection for development of kunitz trypsin inhibitor free soybean varieties: I: Parental polymorphism survey using SSR markers, *Indian J Genet Plant Breed*, 71(4) (2011) 372.

4 Jofuku K D, Schipper R D, Goldberg & R B, A frameshift mutation prevents Kunitz trypsin inhibitor mRNA accumulation in soybean embryos, *The Plant Cell*, 1 (1989) 427.

5 Moraes R M A de, Soares T C B, Colombo L R, Salla M F S, Barros J G de A, Piovesan N D, Barros E G C de & Maurilio Alves Moreira M A, Assisted selection by specific DNA markers for genetic elimination of the kunitz trypsin inhibitor and lectin in soybean seeds, *Euphytica,* 149 (2006) 221.

6 Rani A, Kumar V, Husain S M, Pandey S K & Chauhan G S, NRC101 (IC0582901; INGR10054) and NRC102 (IC582902; INGR10055): Soybean (*Glycine max*) Germplasm lines free from kunitz trypsin inhibitor polypeptide, *Indian J Plant Genet Res*, 24(1) (2010) 122.

7 Doyle J J & Doyle J L, Isolation of plant DNA from the fresh tissue, *Focus*, 12 (1990) 14.