Development and commercialization of kunitz trypsin inhibitor-free Indian soybean (*Glycine max* L.) genotypes

Soybean is one of the most economical sources of basic nutrients like protein, vitamins, minerals, polyunsaturated fatty acids, etc. for ensuring nutritional security. More importantly, soybean seeds are rich in nutraceutical components like isoflavones, tocopherols and lunasin that keep the major killer diseases like breast cancer, diabetes, atherosclerosis and osteoporosis at bay. Despite these virtues, barely 5–7% of the total soybean produced in the country is processed for preparing soy products[1]. One of the major deterrents in the utilization of the bean as food is the presence of antinutritional factor, trypsin inhibitor, 80% activity of which is ascribed to kunitz trypsin inhibitor polypeptide, which is controlled by a single dominant gene. This anti-nutrient is heat-labile, but the heat-inactivation process – which is carried out by boiling for 20 min – not only incurs extra cost to the processors but also causes insolubilization of valuable soy proteins. Further, to prepare soy-fortified wheat flour, only the boiled soybean, which has been subsequently air-dried, is recommended to blend with wheat grains in the ratio 1 : 9 (soybean : wheat) prior to grinding. This step of boiling followed by air-drying being time-consuming, is not followed at the household level. However, fermented soy products like tempeh, miso and natto, which are in vogue only in Southeast Asian countries, contain very low levels of this antinutritional factor due to the release of protease by fungal cultures. Therefore, delivery of kunitz trypsin inhibitor-free soybean seeds to processors in India is critical for boosting soy-based products, whose processing does not involve fermentation. For this purpose, genotype PI542044, which is a donor of null allele of kunitz trypsin inhibitor was procured at the Directorate of Soybean Research, Indore, from the United States Department of Agriculture. Plant was in poor genetic background and was agronomically not adaptable to Indian condition. But the genotype being free from kunitz tryspin inhibitor was crossed with farmers' variety 'Samrat', and was advanced to F_7 generation. The plants showing null allele of kunitz trypsin inhibitor in each generation were selected using SSR marker Satt228 linked to *Ti* locus[2] and null allele (*ti*)-specific marker[3]. Table 1 provides the sequences of Satt228 and null allele for kunitz trypsin inhibitor. Both linked SSR marker and gene-specific marker have been validated in Indian soybean mapping population in our laboratory[4,5]. Phenotyping in the advanced lines NRC101 and NRC102 was carried out through native polyacrylamide gel (10%). For genotyping, genomic DNA extracted from the young leaves of NRC101, NRC02, Samrat (the recipient) and PI542044 (the donor for null allele of kunitz trypsin inhibitor) through CTAB method[6] was used as template for amplification using the primer specific for the null allele of kunitz trypsin inhibitor. The oligonucleotides sequence of this null allele-specific marker was synthesized from Sigma Aldrich (Bengaluru). The PCR reactions were performed in a thermocycler (model PTC100); the reaction mixture (10 µl) contained 2 µl DNA (20 ng/µl), 1 µl PCR (10×) buffer, 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 (3U/µl) and 4.932 µl distilled water. Initially, DNA was denatured at 94°C for 1 min followed by 30 cycles, each comprising denaturation at 94°C for 2 min, primer annealing at 50°C for 2 min and primer elongation at 72°C for 3 min. Finally, elongation was carried out at 72°C for 10 min. The PCR products were resolved on 3% metaphore gel. The amplicons of 420 bp size observed in NRC101 and NRC102 were similar to the donor parent PI542044 (left panel, Figure 1). Samrat, the kunitz trypsin inhibitor-positive parent, did not generate any amplicon. This confirmed the transfer of null allele of kunitz trypsin inhibitor in NRC101 and NRC02. Figure 1 (right panel) shows the profile of kunitz trypsin inhibitor polypeptide (21.5 kDa) in genotypes Samrat, PI542044, NRC101 and NRC102. Presence or absence of null allele-specific amplicon (420 bp) in kunitz trypsin inhibitor-free and kunitz trypsin-positive genotypes (left panel, Figure 1) corresponds to the absence or presence of the kunitz trypsin inhibitor protein band (right panel, Figure 1). Both the kunitz trypsin inhibitor-free genotypes NRC101 and NRC102 attain harvest maturity between 85 and 90 days and bear a yield potential of more than 2.0 tonne/ha and registered with the National Bureau of Plant Genetic Resources, New Delhi under accession numbers INGR 10054 and INGR 10055 respectively. Figure 2 shows the crop of both the kunitz trypsin inhibitor-free soybean genotypes grown in the field and the freshly harvested seeds obtained.

The genotypes NRC101 and NRC102 were launched formally for commercialization in the Directorate of Soybean Research–industry interface held at the Directorate of Soybean Research. Indian Tobacco Company (ITC) Limited, which has forayed aggressively into soybean in the recent past, sought the license of kunitz trypsin inhibitor-free genotypes NRC102 from the Directorate, after confirming the claim for absence of the antinutrient at its Research and Development Centre. The seeds of kunitz trypsin inhibitor-free soybean genotype NRC102 were transferred to ITC after signing a memorandum of understanding (MoU) between the Directorate and ITC at Hyderabad. In the cropping season 2014, ITC raised this genotype at three centres, viz. Sehore, Ujjain and Vidisha in

Table 1. Forward and reverse sequences of primer specific to *lox2*

Primer	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)
Gene-specific	CTTTTGTGCCTTCACCACCT	GAATTCATCATCAGAAACTCTA
Satt228	TCATAACGTAAGAGATGGTAAAACT	CATTATAAGAAAACGTGCTAAAGAG

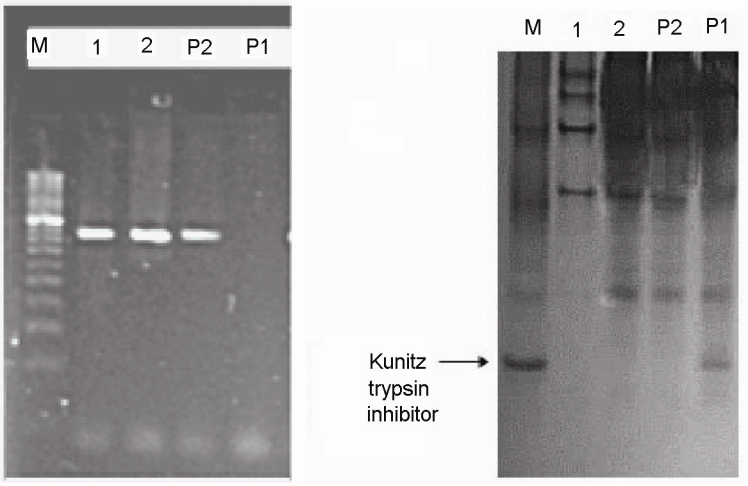

Figure 1. (Left panel) PCR products generated by the gene-specific marker. (Right panel) Kunitz trypsin inhibitor polypeptide as resolved on 10% native PAGE. Lane M (left panel) DNA ladder; (right panel), Standard kunitz trypsin inhibitor polypeptide. Lanes 1, 2, P1, P2 correspond to NRC101, NRC102, PI542044 and Samrat respectively in both panels.

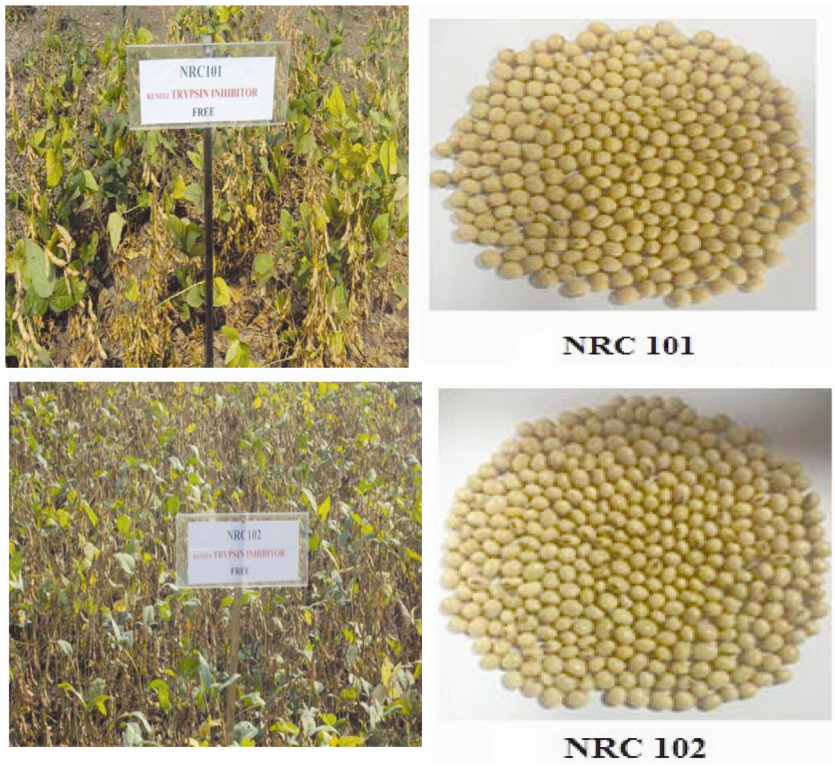

Figure 2. (Left) Crop of NRC101 (top) and NRC102 (bottom) grown in the field. (Right) Freshly harvested seeds of NRC101 (top) and NRC102 (bottom).

Madhya Pradesh (MP) – the hub of soybean cultivation in the country – under contract farming. Likewise, M/S Ruchi Soy Industries Limited, Mumbai, one of the major players in soy processing industry, after confirmation of the absence of the anti-nutrient in NRC101 at their end, sought the license of this kunitz trypsin inhibitor-free genotype from the Directorate. The seeds of NRC101 were transferred to M/S Ruchi Soy Industries Limited after signing a MoU with the Directorate. The company has planted the material in a research farm at Maheshwar, MP.

The Directorate of Soybean Research has also spearheaded a programme for marker-assisted introgression of null allele of kunitz trypsin inhibitor into five popular varieties – JS97-52, NRC7, JS93-05, MACS450, DS97-52 – in collaboration with participating centres, viz. Agarkar Institute, Pune and Indian Agricultural Research Institute, New Delhi, under the aegis of accelerated crop improvement programme of the Department of Biotechnology, New Delhi thereby ridding off these varieties of kunitz tryspin inhibitor. The Directorate plans to undertake the elimination of kunitz trypsin inhibitor from other major varieties of soybean from different agro-climatic zones through marker-assisted backcrossing.

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