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Genetic elimination of Kunitz trypsin inhibitors (KTI) from DS9712, an Indian soybean variety

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Abstract

In this study, the null allele of Kunitz trypsin inhibitor i.e. *kti* was transferred from PI542044, a germplasm line free from KTI in to a popular Indian soybean variety DS9712 through marker assisted backcross breeding (MABB) approach. Following foreground selection with 3 SSR markers viz., Satt228, Satt429 and Satt409 that are linked to *kti*, and background selection with 93 polymorphic SSR markers in BC_1F_1 and BC_2F_1 generations, target plants were selected that had 96-98% recovery of the recurrent parent genome (RPG). In BC_2F_2 generation, plants homozygous the for target allele (*kti kti*) were identified and harvested individually. In BC_2F_3 generation, seed proteins of the selected lines were extracted and analyzed through native polyacrylamide gel electrophoresis (PAGE) and confirmed absence of the KTI peptides. Four lines were identified that were free from Kunitz trypsin inhibitor but retained nearly all of the phenotypic features of DS9712. This study exemplified successful elimination of KTI from soybean seeds through MABB approach.

Key words: KTI, MABB, anti-nutritional factor, soybean, recombinant selection

Introduction

Soybean [*Glycine max* (L) *Merr*.] contains 38-44% protein and 18-23% oil, which makes it unique among the cultivated crops. Besides mineral and tocopherol, it contains flavonoides and other essential elements that help keeping a good health of the regular consumers. However the soybean seeds contain certain anti-nutritional factors such as Kunitz trypsin inhibitor (KTI), hemaglutinins, phytase, etc. that deserve elimination for safe use of food or feed. Biochemically, KTI is a monomeric and non-glycosylated protein weighing 21.5 kDa, and containing 181 amino acid residues [1]. Its presence in the food is reported to have adverse effect on the growth of the tested animals primarily through inhibition of trypsin in the digestive track. Therefore, it is important to eliminate the KTI from the seeds. The usual process of eliminating KTI is through heat treatment as it is heat labile. However, it is not fully effective in elimination of KTI as about 20% residues left depending upon intensity and duration of heat treatment [2]. Besides, it is not economical as it adds extra cost to the processors. Therefore, genetic elimination has been found to be the best option for elimination of KTI from the soybean seeds. In this study, we could eliminate the KTI from soybean seeds through marker-assisted backcross breeding approach.

Materials and methods

Plant materials

Soybean germplasm line PI542044 and a North Indian variety DS9712 was used in this program. The DS9712 contains higher amount of trypsin inhibitor (TI) (83.37 mg g^{-1} of seed meal) while PI542044 contains nearly no KTI [2]. Therefore, the PI542044 was used as donor of null allele of TI and the DS9712 was used as recipient parent.

Foreground markers

Three SSR markers viz., Satt228, Satt409 and Satt429, linked to the null allele of KTI (*kti*) has been reported by Kim *et al.* [3]. All the markers flank the target allele within 0-10cM on chromosome 8, and hence were used for foreground selection.

Background markers

A set of 290 SSR markers were used in order to estimate

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the recovery of the recurrent parent genome (RPG). The markers were randomly picked up from across the soybean genome; however to avoid linkage drag, more markers were used from the carrier chromosome. Sequence of the markers were obtained from soybase (www.soybase.org) and synthesized through local vendors.

Molecular genotyping

DNA was extracted from the younger leaves of the parents and progeny plants in each generation following CTAB method [4]. Quality and quantity of the DNA samples was tested following standard procedures. DNA amplification was done through PCR using protocol described by Kumar *et al*. [5]. Amplicons were size separated using metaphore-agarose gel and documented through Gel-Doc system. The data were analyzed using appropriate software and the graphical genotype was prepared through GGT2.0 software [6].

Breeding scheme

The germplasm line PI543044 was crossed with DS9712 and the F_1 seeds were harvested. The DNA sample collected from the F_1 plants were used to test hybridity of the plants using polymorphic SSR markers. The F_1 plants were crossed to DS9712 to develop BC_1F_1 seeds. The plants in BC_1F_1 generation were subjected to foreground selection with the linked markers. The selected plants were analyzed with background markers to estimate recovery of the RPG. Plants with higher recovery of the RPG were crossed back with the recurrent parent. The plants in BC_2F_1 generation were further subjected to foreground and background selection. Plants with higher recovery of RPG were allowed for selfing. The BC_2F_2 plants were harvested separately and sown in the next generation (BC_2F_3) as individual family row. Plants homozygous for the kti locus were harvested individually. The seeds of BC_2F_4 plants were subjected to biochemical analysis to confirm absence of KTI peptide in the seeds.

Results and discussion

Presence of KTI in the seeds of soybean has restricted its applicability to a larger extent in the food and feed industries of the world. Its negative impact on the tested animal's health and growth has raised safety concern of the consumers. It has been reported that ingestion of KTI often results in increased pancreatic secretion and hypertrophy of the pancreas. Further, it leads to internal loss of enzyme due to increased secretion in to the digestive tubes. Therefore, it is advocated for elimination of KTI from the seeds through genetic means, which is safe, stable and economic for its better and safer consumption of soy-based foods and feeds. In this study, the allele of *KTI* in DS9712, which contributed towards accumulation of trypsin in the seed, was substituted with *kti*, the null allele of KTI through marker-assisted backcross breeding approach. The null *kti* allele was transferred from PI542044, a germplasm line collected from the Directorate of Soybean Research (DSR), Indore, through repeated backcrossing with DS9712 coupled with selection with the linked molecular markers. The background markers depicted the status of genome recovery of the recurrent parent, i.e. DS9712.

Hybridity testing and parental polymorphism

The three markers viz., Satt228, Satt409 and Satt429, which were reported to be linked with *kti* were initially tested for its applicability in DS972 and PI542022. All the three markers produced polymorphic bands, and were fit to use as foreground markers in the subsequent generations (Fig. 1). These markers were also used for testing hybridity of the plants in F_1 generation. The hybrid plants produced heterozygous bands representing paternal and maternal alleles while the self-fertilized plants produced only one band which represented the maternal allele.

Out of the 290 SSR markers used for background study, only 93 appeared to be polymorphic (32.06% polymorphism) (Table 1). Poor level of polymorphism indicated existence of lesser genetic distances between the two genotypes. Indian soybean genotypes usually show lower level of polymorphism owing to their genetic relatedness in ancestry [7, 8]. The donor and the recipient genotypes used in this study varied in their

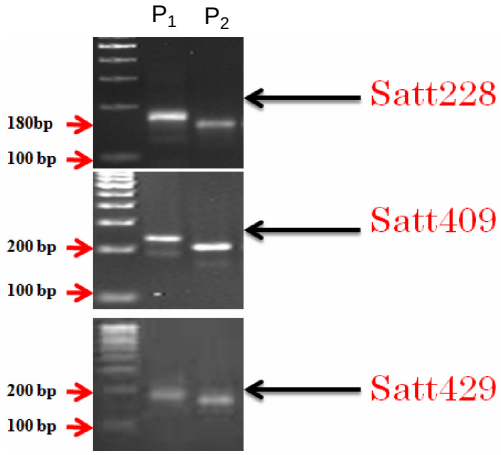

Fig. 1. Polymorphic bands produced in recurrent parent DS9712 (P1) and the donor parent PI542044 (P2)

Table 1. Distribution of background selection markers across soybean genome

Chr. No.	No. of markers selected	No. of polymorphic markers	Polymorphism (%)
1	17	5	29.41
2	18	4	22.22
3	15	4	26.66
4	16	4	25.00
5	20	9	45.00
6	13	4	30.76
7	12	4	33.33
8	17	4	23.52
9	13	5	38.46
10	13	4	30.76
11	19	6	31.57
12	15	4	26.66
13	11	4	36.36
14	16	4	25.00
15	12	4	33.33
16	12	7	58.33
17	11	5	45.55
18	14	4	28.57
19	12	4	33.33
20	14	4	28.57
Total	290	93	32.06

morpho-phenotypic traits as well. On average, 4 polymorphic markers per chromosome were left for background study. However, more polymorphic markers were there on the carrier chromosome. It facilitated recombinant selection so as to avoid the linkage drag to the greatest extent. Plants with recombination on either side of the target allele were also recovered. Such plants will have least linkage drag, if at all, any.

Genome recovery in BC_1 and BC_2 generation

The plants in the BC_1 and BC_2 generations were first tested with foreground markers for presence or absence of the target locus i.e. *kti* (Fig. 2). The plants so selected were only subjected to background selection. In BC_1F_1, average RPG recovery ranged from 70.43-87.63%. Similarly, the average RPG recovery in BC_2F_1 ranged from 93.01-98.82% (Table 2). In this approach, recovery of the recurrent parent genome was much higher than what is expected through conventional approach. For

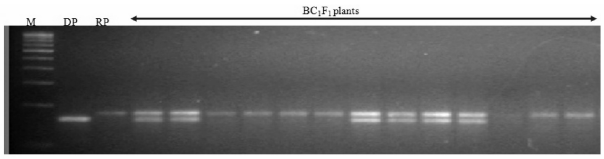

Fig. 2. Banding pattern observed in the BC_1F_1 plants. As expected, heterozygous and recurrent parental bands were observed. DP: Donor parent; RP: Recurrent parent

example, in conventional backcrossing program, average recovery of the recurrent parent genome in BC_2F_1 is 87.5%; however, it was 98.82% in this study. Higher recovery of the RPG is the result of selective backcrossing of the plants with the recurrent parent. The genomic status of the selected plants has been depicted graphically (Fig. 3). It is clear from the figure that baring a few, all the recurrent parent alleles have been recovered in the selected plants. It exhibits the power of MABB approach in reconstituting the genome of the recurrent parent.

Phenotypic and agronomic performance

The plants selected in this study not only had the genomic similarity, but also had the phenotypic and agronomic similarities with the recurrent parent i.e. DS9712. Their yield and other attributes were also at par with DS9712. However, the selected plants were 5-7 days earlier to DS9712, which is desirable for the North Indian plain zone. Usually, DS9712 matures in 120 to 125 days, however, the lines developed here matured in 110-115 days. It will be useful to the farmers as their land will become free early for growing the next crop.

Biochemical testing

Absence of KTI peptides in the seeds of the selected plants were tested thorough polyacrylamide gel electrophoresis (PAGE). For this matter, the seed proteins were extracted from the seeds and were tested in native PAGE. At least 4 plants were found to have no KTI as it did not show the corresponding band for KTI in the gel. It thus confirmed the result of marker-assisted selection.

It is always difficult to transfer a recessive allele through conventional backcross breeding approach as it needs selfing after every backcross generation followed by screening of the plants for presence of the recessive alleles. Thus it needs more time and resources to develop a line with the target recessive allele. However, molecular marker linked to the target recessive allele help eliminate the trouble of selfing after

Table 2. Genomic status with recovery of recurrent parental genome in BC_2F_1 plants

Plant No.	A (%)	H (%)	RPG* (%)	Plant No.	A (%)	H (%)	RPG* (%)
1	91.39	8.60	95.69	19	89.24	10.75	94.26
2	95.69	4.30	97.84	20	88.17	11.82	94.08
3	92.47	7.50	96.23	21	89.24	10.75	94.26
4	86.02	13.97	93.01	22	92.47	7.52	96.23
5	86.02	13.97	93.01	23	91.39	8.60	95.69
6	96.77	3.22	98.38	24	88.17	11.82	94.08
7	91.39	8.60	95.69	25	87.01	12.90	93.54
8	90.32	9.67	95.16	26	89.24	10.75	94.26
9	88.17	11.82	94.08	27	90.32	9.67	95.16
10	87.09	12.90	93.54	28	93.54	6.45	96.77
11	93.54	6.45	96.77	29	93.54	6.45	96.77
12	94.62	5.37	97.31	30	95.69	4.30	97.84
13	87.09	12.90	93.54	31	93.54	6.45	96.77
14	84.90	15.05	92.47	32	86.02	13.97	93.01
15	86.02	13.97	93.01	33	88.17	11.82	94.08
16	86.02	13.97	93.01	34	95.69	4.30	97.84
17	90.32	9.67	95.16	35	97.84	2.15	98.92
18	90.32	9.67	95.16	36	92.42	7.52	96.23

*A: homozygous recipient allele; H: heterozygous allele; RPG: recurrent parent genome recovered

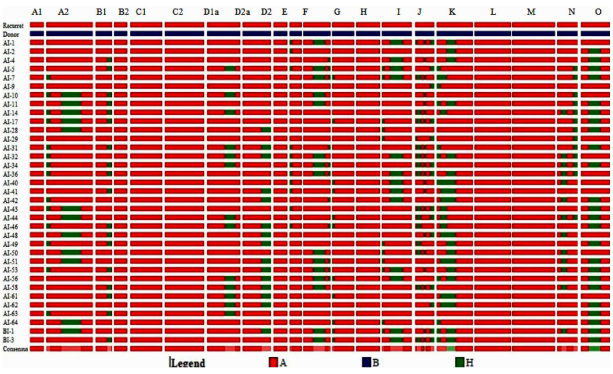

Fig. 3. Graphical genotype of the BC_2F_1 plants. Barring a few loci, recurrent parental genome (red color) got recovered

every backcross generation. Further, it takes lesser time to transfer the target allele and reconstitute the genome of the recurrent parent through MABB. This study marks successful transfer of null allele of KTI through marker-assisted backcross breeding approach for the development of Kunitz trypsin inhibitor free soybean genotype.

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