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Marker assisted backcross selection for genetic removal of lipoxygenase-2 from popular soybean (*Glycine max*) variety JS 97-52: Parental polymorphism survey and hybridity validation

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Off-flavour associated with soy products is the prime reason for poor utilization of soybean (*Glycine max* L.) seed as the food in the countries outside the traditional bastion of South-East Asia. Barely, 5-7% of the total soybean produced in India is processed for making soy products (Ministry of Food Processing 2010). The undesirable flavour is generated due to the volatile carbonyl compounds released from the hydroperoxidation of the polyunsaturated fatty acids, containing *cis, cis 1,4 penta diene* moiety, by lipoxygenase present in three isozymic forms namely lipoxygenase-1, lipoxygenase-2 and lipoxygenase-3 in soybean seeds. Single dominant gene, i e *Lox1*, *Lox2* and *Lox3* regulates the presence of each of these isozymes, while the absence of these isozymes is due to corresponding null alleles, viz. *lox1, lox2, lox3* (Hildebrand and Hymowitz 1982, Kitamura 1983, Davies and Nielsen 1986). Soy preparations made from null lipoxygenases genotypes are better accepted due to production of very low levels of hexanal compounds. Lipoxygenase-2 is the principal contributor to the off- flavour. Though it is a heat labile but the heat inactivation process at industrial level incurs extra cost, besides affecting the solubility and functionality of proteins. Therefore, development of soybean genotypes free from lipoxygenase-2 is critical for the growth of soy food industries in the country. Recently, soybean genotypes NRC 109 and NRC 110 genotypes have been developed at Directorate of Soybean Research (Kumar *et al.* 2013). However, there is the dire need of the genetic removal of lipoxygenase-2 from the elite soybean varieties, which can be achieved by crossing with donor of null allele of lipoxygenase-2 and backcrossing with the elite variety, which is tedious and time consuming approach. Accelerated introgression of desirable traits through molecular marker assisted backcrossing has been employed in soybean (Concibido *et al.* 2003, Kim *et al.* 2008).

Soybean variety JS 97-52 is an elite soybean cultivar for the Central Zone in India. It has been developed from the cross PK 327 × L129. According to the released proposal of this variety under All India Co-ordinated Research Project on Soybean this genotype has yield potential of 2.5-3.0 tonnes/ha, possesses excellent field emergence as well as longevity and exhibits resistance to yellow mosaic virus disease, root rot, bacterial pustule, charcoal rot, *Cercospora* leaf spot. Moreover, the variety is tolerant to waterlogging conditions. It may be worthwhile to make it lipoxygenase-2 free by transferring *lox2* from PI 596540 (donor of *lox2*) into its genome through marker-assisted backcross (MABC) selection. However, it calls for identification of the target plants with homozygous null allele of lipoxygenase-2 (foreground selection) with maximum recovery of recurrent parent genome (background selection). Foreground selection can be performed using SSR marker tightly linked to *Lox2* locus while for the background selection parental polymorphism survey across the genome is mandatory. In the present study, polymorphism for the elite soybean variety JS 97-52 in combination with PI 596540 using SSR markers from 20 linkage groups (LGps) was surveyed and the F_1 and BC_1F_1 were validated through linked polymorphic SSR markers.

Soybean genotype PI 596540, lacking lipoxygenase-2 activity (*lox2lox2* genotype), was procured from the United States Department of Agriculture (USDA), and was crossed with the cultivar JS 97-52. Genomic DNA was extracted from young leaves of these two genotypes following CTAB (Cetyl trimethyl ammonium bromide) procedure. Purified DNA was subjected to PCR amplification in 10 µl reaction mixture containing 2 µl DNA (20 ng/µl), 1 µl PCR 10x 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

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water. In the thermocycler (MJ Research Thermocycler model PTC100) the 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. and final elongation at 72°C for 10 min. Amplified products so obtained were resolved on 3% metaphore agarose gel, stained with ethidium bromide and analyzed in Gel documentation unit (Syngene). For polymorphism study, 264 SSR markers were selected from the 20 LGps of soybean molecular linkage map (United States Department of Agriculture 2008) and were synthesized from Sigma Aldrich. Kim *et al.* (2004) reported the linkage of Satt522 to *Lox2* locus in soybean while a closer SSR marker Satt656 has been recently reported from our laboratory (Kumar *et al*. 2012). Sequences of SSR marker Satt522 were: forward 5'-GCGAAACTGCCTAGGTTAAAA-3, reverse 5'-TTAGGCGAAATCAACAAT-3' while for Satt656 were : forward 5'-GCGTACTAAAAATGGCAATTATTTGTTG-3', reverse 5'-GCGTGTTTCAGTATTTGGATAATAGAAT-3'.These two SSR markers were employed for hybridity validation and foreground selection.

In both dominant and recessive gene transfer through conventional backcrossing, at each backcross generation, such plants are selected which not only manifest the desired trait from the donor parent but are also visually similar to the recurrent parent. This introduces the error in judgment for selection, which necessitates more number of backcrosses to recover the recurrent parent. In marker aided backcrossing, target plants are selected based on more number of DNA markers from the recurrent parent genome among the population of the plants. This warrants sufficient polymorphism between the recipient and the donor across the genome in any segregating population for background selection. In the present study, parental polymorphism survey which was carried out using 264 SSR markers spanning across 20 LGps for JS 97-52 and PI 596540 exhibited polymorphism to the magnitude of 49.24% with 130 polymorphic SSR markers. Very high level of parental polymorphism observed in our results could be attributed to the diverse genetic background of the two parents. Polymorphism observed with some of the SSR markers is shown in Fig 1.

Furthermore, polymorphic loci were observed on all the 20 LGps across the genome, though the distribution was not uniform. The highest number (11) of polymorphic markers were detected on LGp F followed by M and D1b with 10 and 9 polymorphic markers, respectively (Table 1). Polymorphism is a function of the type of motif (di, tri, tetra, etc.) in the SSRs as well as the number of repeats of the motif in the plants (Xu *et al.* 2013). In the present study, tri-nucleotide repeats containing SSRs were more polymorphic in numbers than others. Of the 130 polymorphic markers, 79 and 48 were with tri-nucleotide and di-nucleotide repeats, respectively while 1 each with tetra-nucleotide and di- as well as tri-nucleotide repeats. In case of tri-nucleotide repeats, motifs with 9-26 repeats, i e $(ATT)_{9\text{-}26}$ exhibited more polymorphism. Among the di-nucleotide repeats, SSR motif with 17-36, i e repeats $(AT)_{17\text{-}36}$ appeared to be more polymorphic than others. Similar results have been recently reported in an earlier study (Kumar *et al*. 2011a).

Of the two SSR markers Satt522 and Sat_074 that have been reported to be linked to *Lox2* locus in earlier study (Kim *et al.* 2004), Satt522 exhibited the distinct polymorphism with amplicons of size 230 and 250 bp for JS 97-52 and PI 596540 respectively. This is in congruence

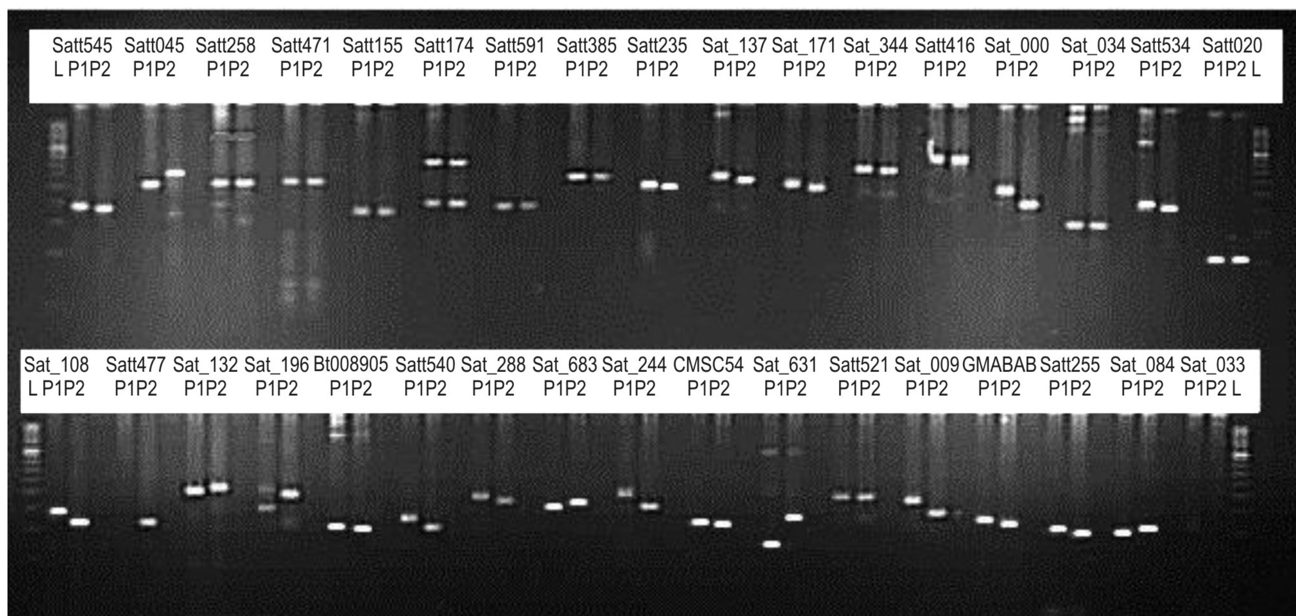

Fig 1 Parental polymorphism between P1: JS97-52 and P2: PI596540 for SSR markers from linkage group A1, B2, O, M and N (PCR products resolved on 3% metaphore)

Table 1 SSR markers from different LGps revealed to be polymorphic for parental combination JS97-52 and PI596540

LGp	No. of markers studied	Polymorphic markers	Polymorphic SSR markers
A1	16	6	Sat_137, Satt684, Satt276, Satt717, Sat_171, Satt042
A2	13	8	Satt421, Sat_294,Satt589, GMENOD28, Satt341, Satt228, Satt089, AW132402
B1	11	6	BE806308, Satt444, Satt509, Satt197, Sat_128, Satt638
B2	11	5	Satt577,Sat_287, Sat_342, Satt687, Sat_009
C1	13	7	Sat_085, Satt646, Satt190, Satt607, Satt399, Sat_416, Sat_337,
C2	14	7	Satt681, Satt281,Sat_213,Satt371,Sat_076, Satt277, Sat_312
D1a	10	5	Satt580, Satt077, Sat_332, Satt267, Satt129
D1b	19	9	Sat_192, Sat_096, Satt266, Satt005, Sat_089, Satt041, Sat_202, Staga002, Satt095
D2	15	4	Sct_192, Sat_292, GMHSP179, Satt311
E	11	5	Satt651, Satt369, Satt598, Sat_172, Satt553
F	19	11	Sat_417, Sat_234, AW756935, Satt656, Satt522, Satt325, Sat_387, Satt193, Satt516, Satt114, Sat_033
G	11	6	Sat_163, Satt288, Satt217,Satt340,Satt303,Satt115
H	11	6	Sat_158, Sctt009, Satt541, Sat_118, Sat_205, Satt442
I	11	2	Sat_170, Satt562
J	10	8	AW310961, Sat674, Satt280, Sct_011, Sat_224, Sat_394,Sat_393, Satt244
K	13	5	Satt539, Satt245, Sat_043, Sat_126, Satt588
L	14	6	Satt232, Satt523, Satt229, Satt513, Sat_245,Satt278
M	14	10	Satt435, Sat_244, Satt626, Satt175, Satt308, Sat_330, Satt551, Satt540, Sat_288, Satt683
N	14	7	Satt080, Satt549, Sat_295, Satt631, Sat_084, Satt255,GMABAB
O	14	7	Sat_132, Satt347, Sat_282, Sat_318, Satt420, Sat_108, Sat_196
	264	130	

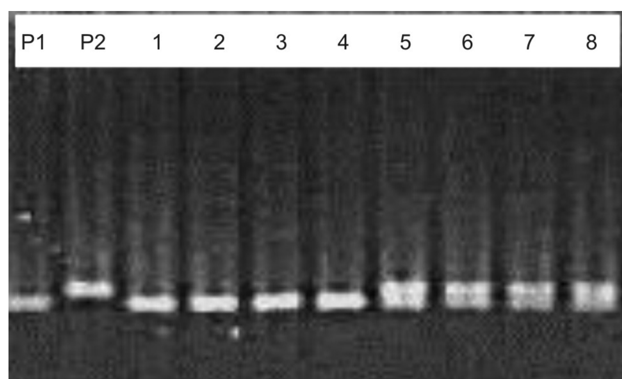

Fig 2 True F_1 plants of JS97-52 x PI596540, confirmed for their truness using polymorphic SSR marker Satt522. P1, P2, lanes 1-4, lanes 5-8 indicate amplicons generated by JS97-52 and PI596540, selfed, and true F_1 plants, respectively

with our earlier report (Kumar *et al*. 2011b). The resolution of PCR products amplified using SSR marker Sat_074 was poor on 3% metaphore gel with amplicon of same size observed for JS 97-52 and PI 596540. Further, hybridity of F_1 plants of the cross JS 97-52 × PI 596540 was tested for the trueness through polymorphic marker Satt522. Twelve true F_1 plants were obtained for the cross JS 97-52 × PI 596540, 4 of which are evident in Fig 2 by the presence of alleles from both the parents. Satt656 which has been recently found to be tightly linked to *Lox2* locus (at 2.4 cM) in the F_2 population derived from JS 97-52 and PI 596540 (Kumar *et al.* 2012) was found to be monomorphic on 3% metaphore gel. However, distinct polymorphism was observed when the PCR products from this SSR marker were resolved on 8% polyacrylamide gel. Therefore, Sattt656 was employed in the foreground selection for identification of target plants with *lox2* allele in the first backcross generation (BC_1F_1) effected by crossing F_1 plants with JS97-52. Further, the selfing of the first backcross generation plants would yield BC_1F_2 plants, and the homozygous recessive (*lox2lox2*) plants found to be morphologically similar to JS 97-52 would be screened for recurrent parent genome content (RPGC) using 130 polymorphic SSR markers identified in the present study. The plants identified with high RPGC and morphologically similar to JS 97-52 would be selected and backcrossed with JS 97-52 for obtaining BC_2F_1 plants and so on till RPGC more than 98% is achieved in plants which are not only similar to JS 97-52 but also carry null allele of lipoxygenase-2 (*lox2*), i e free from lipoxygenase-2 enzyme.

SUMMARY

Lipoxygenase-2 is the prime contributor to the off-flavour associated with the soy products. With the aim to introgress null allele of lipoxygenase-2 into popular soybean variety JS 97-52 through marker assisted backcross selection (MABC), parental polymorphism was surveyed for JS 97-52 (recipient parent) and PI 596540 (the donor of null allele of lipoxygenase-2). A total of 264 SSR markers dispersed across 20 linkage groups were screened. Of these, 130 SSR

markers showed polymorphism. SSR markers Satt522 and Satt656 reported to be in the vicinity of *Lox2* locus were found to be polymorphic for JS 97-52 and PI 596540 and were used for confirming true F_1 and BC_1F_1 plants. The polymorphic SSR markers identified in the study would be utilized for retrieving the genetic background of JS 97-52 in the backcross generations.

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