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Identification of simple sequence repeat markers tightly linked to lipoxygenase-2 gene in soybean

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Lipoxygenase-2, controlled by single dominant gene, is prime cause of off-flavour generated in soy products. The objective of the present investigation was to validate the SSR markers (Sat_074 & Satt522) reported to be linked to *Lox2* locus and to identify tightly linked SSR markers in F_2 mapping population 1 and population 2, generated from crosses between Indian soybean varieties JS97-52 and JS93-05 as the conventional parents (*Lox2Lox2*) and PI596540 as the source of null allele of lipoxygenase-2 (*lox2lox2*), respectively. Parental polymorphism was surveyed using SSR markers Sat_074, Satt522 and 7 SSR markers in the neighbouring genomic region of *Lox2* locus. $F_{2:3}$ seeds from each F_2 individual in both the mapping populations were phenotyped for the presence/absence of lipoxygenase-2 isozyme. The SSR marker Sat_074 was not found to be polymorphic for the parental combination JS97-52 × PI596540. Satt522 was found to be at 9.3 and 13.5 cM from *Lox2* locus in mapping population 1 and 2, respectively. We found two additional new SSR markers Sat_417 and Satt656 closely linked to *Lox2* locus. SSR marker Sat_417 was found to be at a close distance of 6.9 and 6.6 cM from *Lox2* locus; while Satt656 was identified to be tightly linked to *Lox2* locus at a distance of 2.7 and 2.1 cM in the mapping population 1 and 2, respectively. Thus, the SSR marker Satt656 can be employed in marker assisted selection for transferring null allele (*lox2*) of the lipoxygenase-2 in the background of popular Indian soybean cultivars.

Keywords: Lipoxygenase-2, *Lox2* locus, off-flavour, soybean, SSR markers

Introduction

Despite being rich source of basic nutrients and numerous health-promoting nutraceuticals, barely 5-7% of the total soybean produced in the country finds its usage in preparation of soy food products. One of the major constraints in utilization of soybean in food usage is the off-flavour generated while processing soy products from the beans. It is ascribed to the hexanal compounds resulting from the catalytic oxidation of the polyunsaturated fatty acids by the lipoxygenase present in the seed. Lipoxygenase in soybean seed exists in three isozymic forms; lipoxygenase-1, lipoxygenase-2 and lipoxygenase-3, which differ in their pH optima, substrate specificity and mol wt. Lipoxygenase-1, also known as class I isozyme, is active at alkaline pH (9.0), while lipoxygenase-2 as well as lipoxygenase-3 are active at neutral pH and are collectively designated as class II isozymes. Presence of each of the three isozymes is controlled by single dominant genes, *viz.*, *Lox1*, *Lox2* and *Lox3*, and their absence is ascribed to corresponding null alleles (*lox1, lox2, lox3*). Soy preparations made from null lipoxygenases genotypes are better accepted due to production of very low levels of hexanal compounds[1]. Heat inactivation of the lipoxygenases employed at industrial level not only incurs extra cost but also affects the solubility and functionality of proteins[2]. Therefore, development of lipoxygenases-free genotypes through genetic elimination is the key to get rid of the beany-flavour and boost utilization of soybean in food usage.

Though all the three lipoxygenase isozymes catalyse the release of hexanal compounds, lipoxygenase-2 is mainly responsible for the generation of off-flavour imparting *n*-hexanal in soybean[3]. Genetic removal of lipoxygenase-2 from the seed has been reported to be more effective in improving the flavour of soy products[4]. In the plant breeding programme aiming for the development of lipoxygenase-2 free soybean genotypes, conventional colorimetric method used for identification of lipoxygenase-2 free segregants necessitates fine grinding of the seed, which cannot be performed in early generations. Marker-assisted selection (MAS) using SSR (simple sequence repeat) markers tightly linked to lipoxygenase-2 (*Lox*2) locus can be used for development

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of lipoxygenase-2 free soybean varieties. Foreground selection using SSR markers tightly linked to the *Lox2* locus, followed by background selection using polymorphic SSR markers across the genome can expedite the recovery of gene pool of the recurrent variety in a backcross breeding programme aimed at introgression of null allele of lipoxygenase-2 in existing high yielding soybean varieties. Trisomic analysis performed in an earlier investigation[5] showed the presence of *Lox2* gene on chromosome 13, which corresponded to linkage group F in the molecular genetic map[6]. Position of *Lox2* has been shown on one end of linkage group F flanked by Sat_074 and Satt522 in a previous report[7]. It was thought worthwhile to validate the linkage of the SSR markers Sat_074 and Satt522 with *Lox2* locus in populations generated by crossing two lipoxygenase-2 positive Indian varieties, *viz.*, JS97-52 and JS93-05, with PI596540 as source of recessive allele (*lox2*) of lipoxygenase-2, and more importantly to investigate the neighbouring SSR markers for identifying an SSR marker tightly linked to *Lox2* locus.

Material and Methods

Soybean genotype PI596540, lacking lioxygenase-2 activity (*lox2lox2*), was procured from United State Department of Agriculture (USDA). Two lipoxygenase-2 positive (*Lox2Lox2*) Indian soybean varieties JS97-52 and JS93-05 were crossed with PI596540 in the fields of Directorate of Soybean Research, Indore. F_1 plants obtained from the crosses JS97-52 × PI596540 and JS93-05 × PI596540 were raised in the glasshouse to harvest F_2 seeds. One hundred eight F_2 individuals for the cross JS97-52 × PI596540 and 104 F_2 individuals for JS93-05 × PI596540 constituted mapping population 1 and mapping population 2, respectively. Tender leaves from the 15- to 20-d-old F_2 plants of both the mapping populations were collected for genotyping. $F_{2:3}$ seeds harvested from each of the F_2 plants of two mapping populations were used for phenotyping.

Phenotyping

Lipoxygenase-2 activity in the $F_{2:3}$ seeds obtained from F_2 plants of population 1 and population 2 was tested following the standard procedure[8]. For this purpose, minimum 20 individual seeds from each plant were analysed to ascertain the phenotype. Test was conducted as follows: (i) Soybean flour (5 mg) was weighed into a test tube, (ii) 0.5 mL of distilled water was added and the mixture was stirred lightly and allowed to stand for 3-10 min, (iii) dye substrate was prepared for 20 samples by mixing 154.25 mg of dithiothreitol, 25 mL of 200 mM sodium phosphate buffer (pH 6.0), 5 mL of 100 µM methylene blue, 5 mL of 10 mM sodium linoleate substrate and 5 mL of acetone in a 100 mL glass stoppered bottle, (iv) 2 mL of the above prepared dye substrate was added to the test tube, and (v) after 3 min, the colour of the solution was checked visually. In the test tubes containing lipoxygenase-2 positive seed flour, the blue colour of the dye bleached due to the quenching of the free radicals generated by the action of lipoxygenase-2 on polyunsaturated fatty acids; while in the lipoxygenase-2 free soy flour, the blue colour of the dye persisted.

Genotyping

Genomic DNA was extracted from the finely ground leaf tissue of tender young leaves collected from 10- to 15-d-old seedlings by means of CTAB procedure[9]. 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 reactions were performed in a Thermocycler model PTC100 and the reaction mixture (10 µL) contained: 2 µL DNA (20 ng/µL), PCR 10× buffer (1 µL), 25 mM $MgCl_2$ (1.1 µL), 25 mM dNTPs (0.1 µL), 0.4 µL each forward and reverse SSR primers (30 ng/µL), 0.068 µL Taq DNA polymerase (3 U/µL), and distilled water (4.932 µL). Initially, DNA was denatured at 94°C for 3 min, followed by 30 cycles, each cycle comprising of denaturation at 94°C for 1 min, primer annealing at 50°C for 2 min., primer elongation at 72°C for 3 min. Final elongation was carried out at 72°C for 10 min. Two SSR markers Sat_074 and Satt522 from LG F reported to be linked with *Lox2* locus and 7 neighbouring SSR markers from linkage group F were amplified by the above procedure for parental polymorphism survey. Subsequently, only polymorphic markers were amplified with the genomic DNA of F_2 individuals of mapping population 1 and 2. Barring Satt656, which showed best resolution on 8% polyacrylamide gel for JS97-52 and PI596540, PCR products obtained from all other SSR markers 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).

Statistical Analysis

Linkage of the polymorphic SSR markers with the *Lox2* locus was determined using MAPMAKER version 3.0[10]. Linkage was considered significant if the logarithm of odds (LOD) score was >3.0. The

Kosambi mapping function[11] was used to convert recombination fraction to map distance among the markers and the target gene.

Results and Discussion

Segregation observed for presence or absence of lipoxygenase-2 activity in $F_{2:3}$ seeds for mapping population 1 (JS97-52 × PI596540) and mapping population 2 (JS93-05 × PI596540) is summarized in Table 1. In case of mapping population 1, out of 108 F_2 individuals, 30 tested positive (*Lox2Lox2*), 25 tested negative (*lox2lox2*) and 53 showed segregation (*Lox2lox2*) for lipoxygenase-2 activity. Similarly, in mapping population 2, out of 104 F_2 individuals, 27 tested positive (*Lox2Lox2*), 27 tested negative (*lox2lox2*) and 50 were segregants (*Lox2lox2*) for lipoxygenase-2 activity. These observations conform to a segregating ratio of 1:2:1, indicating lipoxgenase-2 activity is controlled by single gene. In an earlier investigation[5], trisomic studies showed the location of *Lox2* on chromosome 13, which corresponded to linkage group F in the molecular-genetic map[6]. Another study[7] showed SSR markers Satt522 and Sat_074 at genetic distance of 14.5 and 8.6 cM, respectively from *Lox2* locus. Therefore, in the present investigation, we tested the parental combinations JS97-52 × PI596540 (source of *lox2*) and JS93-05 × PI596540 (source of *lox2*) for polymorphism using Sat_074 and Satt522 and 7 neighbouring SSR markers, *viz.*, Satt657, Satt218, AW756935, Sat_090, Satt656, Sat_417 and Satt395. However, Sat_074 was not found to be polymorphic for the parental combination JS97-52 × PI596540. Only Sat_417, Satt656, Satt657, Satt 522 were found to be polymorphic for both the parental combinations (JS97-52 × PI596540 & JS9305 × PI596540). All these polymorphic markers conformed to a normal genetic segregation ratio of 1:2:1 in both the mapping populations.

Table 1—Observed and expected segregation of $F_{2:3}$ seeds from selfed F_1 soybean plants for the lipoxygenase-2 activity

	JS97-52 x PI596540		JS93-05 x PI596540	
Genotype	Observed	Expected	Observed	Expected
Lox2Lox2	30	27	27	26
Lox2lox2	53	54	50	52
lox2lox2	25	27	27	26
$\chi 2$	0.496		0.152	
Probability	0.70-0.80		0.90-0.95	

Genetic maps were constructed using phenotyping data for *Lox2* locus and genotyping data for the mapping population 1 (JS97-52 × PI596540) using Sat_417, Satt656, Satt522 and Satt657, and for mapping population 2 (JS93-05 × PI596540) using Sat_074, Sat_417, Satt656, Satt522 and Satt657, through MAPMAKER version 3.0. SSR marker Sat_074 reported at a distance of 14.5 cM from *Lox2* locus in the earlier study[7] was found to be at genetic distance of 11.7 cM from this locus in mapping population derived from JS93-05 × PI596540. Satt656 was found to be linked to *Lox2* locus at a distance of 2.7 cM in mapping population JS97-52 × PI596540 and at 2.1 cM in mapping population JS93-05 × PI596540. Moreover, the orientation of Satt656 with respect to *Lox2* locus was same in both the mapping populations. Representative gels of the amplification pattern obtained for Satt656 using genomic DNA of parents and F_2 plants (*Lox2Lox2*, *Lox2lox2* & *lox2lox2*) for the mapping population 1 and 2 are shown in Figs 1 and 2, respectively. It is evident from

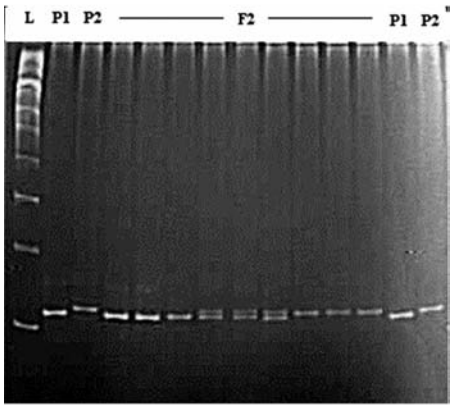

Fig. 1—Patterns of segregating DNA fragments using SSR marker Satt656 in parents and F_2 population of JS97-52 × PI596540 on 8% polyacrylamide gel. P1 is JS97-52 (*Lox2Lox2*) and P2 is PI596540 (*lox2lox2*). The lanes showing double fragments correspond to the heterozygotes.

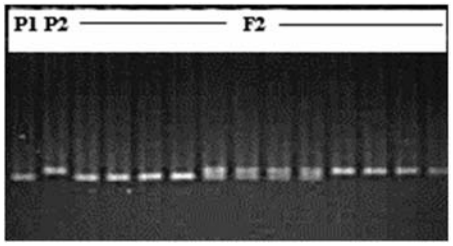

Fig. 2—Patterns of segregating DNA fragments using SSR marker Satt656 in parents and F_2 population of JS93-05 × PI596540 on 3% metaphor. P1 is JS93-05 (*Lox21Lox2*) and P2 is PI596540 (*lox2lox2*). The lanes showing double fragments correspond to the heterozygotes.

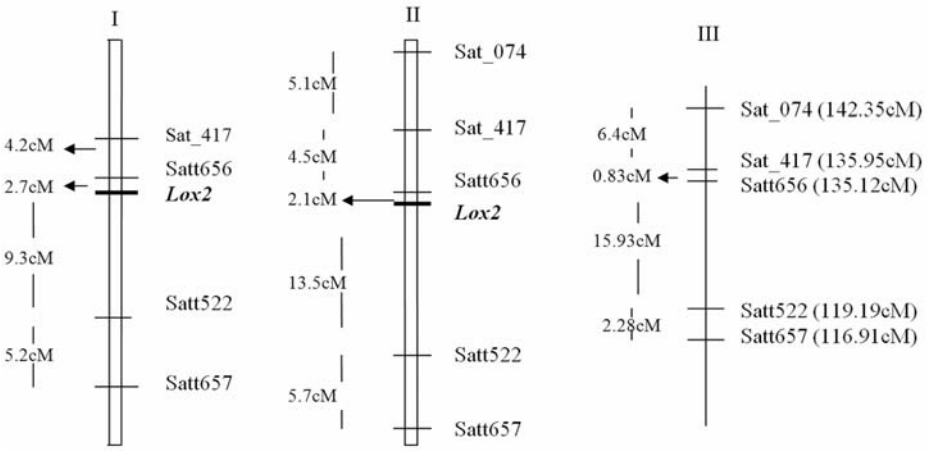

Fig. 3—Molecular linkage map F of *Lox2* locus defined using population 1 (I) derived from the cross between JS97-52 (*Lox2Lox2*) × PI596540 (*lox2lox2*) and population 2 (II) derived from the cross between JS93-05 (*Lox2Lox2*) × PI596540 (*lox2lox2*) [Map was constructed using MAPAKER]. III shows linkage map given by USDA 2008. [bldg6.arsusda.gov/ cregan/soymap.html]

the Fig. 3 that the order of the SSR markers mapped to the *Lox2* locus in our study was the same as reported in the molecular linkage map given by USDA[12]. The genetic distance observed between SSR markers Sat_417 and Satt656 was 3.37 and 3.67 cM more in population 1 and population 2, respectively, compared to the corresponding distance between these SSR markers depicted in the molecular linkage map given by USDA[12]. For mapping population 1 and population 2, Satt522 and Satt657 were found to be 5.2 and 5.7 cM apart, respectively; while the corresponding distance between these SSR markers has been shown to be 2.28 cM in the original molecular linkage map (Fig. 3) The distance computed between Satt656 and Satt522 was 12.0 and 15.6 cM for population 1 and population 2, respectively. These values are comparable to the distance (15.93 cM) depicted between these two SSR markers in the molecular linkage map given by USDA[12]. Satt522 reported to be linked at a distance of 14.5 cM with *Lox2* locus in the earlier study[7] was found to be at a closer distance of 9.3 and 13.5 cM from *Lox2* locus in the mapping population derived from JS97-52 × PI596540 and JS93-05 × PI596540, respectively.

In conclusion, compared to the SSR markers Sat_074 and Satt522 earlier reported to be linked to *Lox2*, our study revealed two new SSR markers Sat_417 and Satt656, which were found to be more tightly linked to *Lox2* locus in both the soybean mapping populations generated in the study. Therefore, Satt656 present at a distance of 2.1 cM from *Lox2* locus may be exploited for introgression of recessive allele *(lox2)* of lipoxygenase-2 into two high yielding Indian soybean varieties JS97-52 and JS93-05 through marker assisted backcross breeding.

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