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RESEARCH ARTICLE

Genetic Elimination of Off-Flavour Generating Lipoxygenase-2 Gene of Soybean through Marker Assisted Backcrossing and Its Effect on Seed Longevity

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ABSTRACT Lipoxygenase-2 present in soybean seeds is the prime contributor to off-flavour generated during the processing of soy products. Genetic elimination of this undesirable component is important as the heat inactivation not only incurs extra cost but also affects the protein solubility. The present study was aimed at eliminating lipoxygenase-2 from cultivar 'JS97-52' through marker assisted introgression of null allele of *Lox2* from PI596540 (*lox2lox2*). Foreground selection in BC_1F_1, BC_2F_1 and BC_3F_1 of the cross 'JS97-52' × PI596540 was carried out using *lox2* specific marker, while *lox2* specific marker in combination with SSR marker Satt656 tightly linked with *Lox2* locus was employed for identification of homozygous recessive plants (*lox2lox2*) in BC_2F_2 and BC_3F_2. Background selection performed using 150 polymorphic markers resulted in development of 12 Lox2-free soybean lines ($BC_3F_{2:3}$ seeds) exhibiting recurrent parent genome content in the range of 97.66-98.66%. Qualitative and quantitative assays confirmed the absence of Lox2 in introgressed lines (ILs). The ILs were at par in days-to-flowering, days-to-maturity,100-seeds weight, yield and protein content with the recurrent parent but showed significant improvement in seed longevity over the latter.

Keywords Lipoxygenase-2, Marker assisted backcrossing, Soybean, Seed longevity

INTRODUCTION

Soybean being one of the most economical source of protein can mitigate mal- and under-nutrition in developing countries. It ideally fits in the dietary regimes of people who seek nutritious but follow vegetarian/vegan diet. Moreover, plethora of studies revealed the protective effects of soybean several bioactive compounds like isoflavones, Bowman-Birk factor, tocopherols, lecithin, lunasin, saponins etc., against cancer, atherosclerosis, diabetes, osteoporosis etc. (Kumar *et al*. 2010; Messina 2016). These health-promoting attributes of soybean have earned this crop the 'function food' status. However, soy products have not been able to permeate into daily diet of masses across the world as indicated by the fact that barely 10% of the total global production of soybean is utilized in food uses, most of it confined to East and South East Asia where soy products are in the staple diet since centuries ago. Besides the presence of antinutritional factor Kunitz trypsin inhibitor and allergens, such as α subunit of β-conglycinin (Gly m Bd 68K) and P34 glycoprotein (Gly m Bd 30K) in soybean which deters the utilization of soybean for human consumption, beany flavour associated with soy products is also responsible for the aversion of people to the inclusion of this bean in the daily diet in several countries including India. The biochemical basis of the generation of this off-flavour is the catalytic oxidation of 1,4-Z,Z-pentadiene-containing polyunsaturated fatty acids *viz*. linoleic

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and α linolenic acid by seed lipoxygenases. The hydroperoxides so formed are further metabolized by hydroperoxide lyases, thereby generating volatile aldehyde and ketone compounds, which impart characteristic off/beany flavour to the soy products (Baysal and Demirdoven 2007).

Soybean seed lipoxygenase exists in three forms *viz*. lipoxygenase-1, lipoxygenase-2 and lipoxygenase-3 which differ in their substrate specificity, pH optima and electrophoretic mobility. Null forms of these three seed lipoxygenases are controlled by single recessive *loci*, designated as *lox*1, *lox*2 and *lox*3 (Kitamura *et al*. 1983; Davies and Nielsen 1986). Heat inactivation of lipoxygenases at industrial level is not only cost-ineffective but also affects functionality of proteins (Macleod and Ames 1988). Therefore, genetic elimination of lipoxygenase-2 is the key to spur utilization of soybean in food uses. Of the three lipoxygenases, lipoxygenase-2 is mainly responsible for the generation of n-hexanal (Nishiba *et al*. 1995; Mellor *et al*. 2010). Genetic removal of lipoxygenase-2 has been reported to improve the flavor of soy products considerably (Davies *et al*. 1987; Wilson 1996). Soymilk and *tofu* prepared from IA2011, a commercially available Lox2-free soybean variety developed by Iowa State, have been demonstrated to be devoid of beany flavour (Shurtleff and Aoyagi 2014). Soybean genotypes, namely, PI086023, PI596540 for null allele for lipoxygenase-2 have been reported (Davies and Nielsen 1986; Pfeiffer *et al*. 1997). These accessions were procured by ICAR-National Bureau of Plant Genetic Resources, New Delhi, India and delivered to ICAR-Indian Institute of Soybean Research, India for developing lipoxygenase-2 free soybean which would give reduced off-flavour under the plant breeding programme focusing on development of food-grade soybean. Using PI086023 as the donor parent for lipoxygenase-2, first lipoxygenase-2 free soybean NRC109 was developed in India by crossing it with early maturing Indian soybean genotype 'Samrat' (Kumar *et al*. 2013). Subsequently, it was thought worthwhile to introgress null allele of lipoxygenase-2 (*Lox2*) into high yielding soybean varieties through Marker assisted backcrossing (MABC). This plant breeding approach comprising of foreground and background selection is an expeditious process over conventional backcross breeding to transfer a trait in the desirable genetic background, and has been employed for introgression of useful traits in soybean (Zhu *et al*. 2007; Kim *et al*. 2008; Kumar *et al*. 2015). *Lox*2 locus has been mapped on LG 'F', and SSR markers tightly linked to *Lox*2 locus were identified (Kim *et al*. 2004; Kumar *et al*. 2014) and SSR marker specific to null allele of *Lox*2 were designed (Reinprecht *et al*. 2011; Shin *et al*. 2012). This facilitated and expedited the foreground selection of the individual plants carrying null allele (*Lox*2*lox*2/*lox*2*lox*2) in plant population segregating for lipoxygenase-2. In the presented work, we employed MABC approach for introgression of null allele of lipoxygenase-2 (*lox*2) from germplasm accession PI596540 into a high yielding Indian soybean variety 'JS97-52.' For this purpose, sufficient number of polymorphic markers (*loci*) between the genetically diverse donor (PI596540) and recipient ('JS97-52') parents reported previously (Rawal *et al*. 2014) were employed to recover the gene pool from the recurrent parent.

MATERIALS AND METHODS

Parent materials

Recipient genotype

Soybean variety 'JS97-52' is a high yielding variety released for cultivation in Central India. Though, this variety is lipoxygenase-2 positive like other varieties released for cultivation under All India Co-ordinated Research Project, but it gives excellent field emergence and shows resistance against multiple diseases, such as yellow mosaic virus disease, root rot, bacterial pustule, charcoal rot, cercospora leaf spot and target leaf spot. Further, the variety is tolerant to water-logging conditions, which the crop has to face in the event of heavy precipitation in a very short period during the monsoon season. The genotype flowers in 47-50 days and attains maturity in 105 days in the agroclimatic conditions of Central India.

Donor genotype

PI596540, lacking lipoxygenase-2 activity (*lox*2*lox*2 genotype), was procured from United State Department of Agriculture was used as a donor. This genotype is derived from Camp × ('Vance'^{2} × L_2-3) and was developed at Kentucky Agricultural Experimental Station (Pfeiffer *et al*. 1997). In agroclimatic conditions of Central India, this

genotype flowers in 27-29 days and reaches harvest maturity in 85 days.

Molecular markers for foreground and background selection

To identify the individual plants harbouring the target allele in first filial generations in the backcross progenies BC_1F_1, BC_2F_1 and BC_3F_1, null allele of *Lox*2 (*lox*2) specific primer given by Reinprecht *et al*. (2011) was employed. SSR marker Satt656 which was identified to be tightly linked with *Lox*2 locus (Kumar *et al*. 2014) was used in conjunction with null allele specific marker for the identification of homozygous recessive plants (*lox*2*lox*2) in BC_2F_2 and BC_3F_2 generations. Oligonucleotide sequences of null allele (*lox*2) specific marker and linked marker Satt656 are given in Table 1. For the background selection, 150 polymorphic SSR markers between 'JS97-52' and PI596540 were used, some of them have been reported earlier (Rawal *et al*. 2014). SSR markers were selected from 20 LGs of soybean molecular linkage map as given by United State Department of Agriculture (bldg6.arsusda.gov/cregan/soymap.html). The synthesis of the molecular markers was outsourced to Sigma Aldrich, Bangalore.

DNA extraction and PCR conditions

Genomic DNA was extracted from young leaves of the two parental genotypes F_1s and backcross progenies following CTAB (Cetyl trimethyl ammonium bromide) procedure. DNA was purified and its concentration was quantified using spectrophotometer (Model UV 1601, Shimadzu) and final concentration was adjusted to ~25 ng/μL. Polymerase Chain Reaction (PCR) was carried out for amplification of the genomic DNA using linked (Satt656) and polymorphic SSR markers, in 10 μL reaction mixture containing 2 μL DNA (25 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 (3 U/μL) and 4.932 μL distilled water. DNA was denatured in the thermocycler (*Lifepro* Bioer) at 94℃ for 2 minutes, followed by 30 cycles each consisting of denaturation at 94℃ for 1 minute, primer annealing at 50℃ for 2 minutes, primer elongation at 72℃ for 3 minutes and final elongation at 72℃ for 10 minutes, using linked and polymorphic SSR markers. PCR conditions for amplification through null allele specific marker were: initial denaturation at 94℃ for 2 minutes followed by 35 cycles each consisting of denaturation at 94℃ for 30 seconds, primer annealing at 58.4℃ (*lox*2 specific) for 45 seconds, primer elongation at 72℃ for 1 minute and final elongation at 72℃ for 10 minutes. Amplified products from gene specific marker were resolved on 1% agarose, while PCR amplified products through SSR marker Satt656 and all other SSR markers were resolved using 8% polyacrylamide gel (PAGE) and 3% metaphor agarose, respectively. The images were analyzed in Gel documentation unit (Syngene).

Backcrossing

Due to the difference of about 20 days in days-to-50% flowering of the donor and the recipient parent, staggered sowing of 'JS97-52' and PI596540 was done to synchronise the buds of 'JS97-52' with the flowering stage of PI596540. Crosses were effected between the parent plants by transferring the pollens from PI596540 to the stigma of JS97-52 to generate F_1 seeds. True F_1 plants confirmed using polymorphic SSR marker Satt522 (LG 'F') were subjected to first backcrossing using 'JS97-52' as a female parent. Foreground-selected BC_1F_1 individuals were subjected to background selection and the individuals carrying maximum recurrent parent genome (RPG) were used as

Table 1. Oligonucleotide sequences of *Lox*2 linked and null allele specific molecular marker along with their corresponding annealing temperature.

Marker	Primer sequences	Annealing temperature (℃)
Satt656	F-5′GCGTACTAAAAATGGCAATTATTTGTTG-3′ R-5′GCGTGTTTCAGTATTTGGATAATAGAAT-3′	50.0
*lox*2 specific (Reinprecht *et al*. 2011)	F 5′-GTTCATAGGTTAAATACTCAA-3′ R 5′-TTTCAACAAGCTCTTCAAT-3′	58.4

male parent for effecting BC_2F_1 generation. BC_2F_1 individuals with the target allele and with highest % RPG content were selfed. Foreground-selected BC_2F_2 homozygous recessive individuals (*lox*2*lox*2) were used to pollinate 'JS97-52' to advance to BC_3F_1 generation. True BC_3F_1 individuals carrying *lox*2 allele were selfed to obtain BC_3F_2 generation.

Assessment of recurrent parent genome content

For assessing the RPGC for an individual plant surveyed through a set of SSR markers (*loci*), score '1' was given to heterozygous locus (H), while the locus homozygous for recurrent parent (A) was given a score of '2'. SSR markers exhibiting homozygosity for the donor parent (B) were given '0' score. Percent RPGC was computed by summing up the score of an individual plant given for all the *loci* divided by total number of alleles (2 × number of SSR markers surveyed).

Phenotyping

Qualitative determination of Lox2 isozyme

Soybean flour of 'JS97-52', PI596540 and ILs was subjected to dye bleaching assay as given by Suda *et al*. (1995). To ascertain the phenotype, minimum 5 individual seeds from each plant were analysed. For this purpose soybean flour (5 mg) from each seed was weighed into a test tube and 0.5 mL of distilled water was added. The mixture was stirred lightly and allowed to stand for 1-10 minutes. Dye-substrate solution 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 dye, 5 mL of 10 mM sodium linoleate substrate and 5 mL of acetone in a 100 mL glass stoppered bottle. In the test tube containing sample, 2 mL of the dye-substrate solution was added and the change in blue colour of the solution was observed.

Quantitative estimation of Lox2 isozyme activity

Quantitative estimation of MABC-derived Lox2-null ILs was carried out through enzymatic assay for the absence of lipoxygenase-2 isozyme. Crude extract was prepared by homogenisation of 0.5 g of the defatted soy flour with 50 mL of sodium phosphate buffer (0.2 M, pH 6.8) in a microtissue polytron homogenizer at 20,000 rpm for 20 minutes at 0-4℃. The homogenised solution was centrifuged at 10,000 rpm for 10 minutes at 4℃. The supernatant so obtained was used as crude extract for the enzymatic assay following the method given by Axelrod *et al*. (1981). Reaction mixture consisted of 2.8 mL sodium phosphate buffer (0.2 M, pH 6.1), suitable volumes of crude extract and 10 mM sodium arachidonate as a substrate. The change in absorbance was recorded in spectrophotometer (Model UV1601, Shimadzu) at 238 nm after every 15 seconds. One unit of enzyme was taken as equivalent to the amount of enzyme that generated an increase in absorbance of 1.0 per minute at 238 nm.

Agronomic parameters

For this purpose, seeds were sown in single row plot of 3 m length maintaining plant-to-plant and row-to-row distance of 5 and 45 cm, respectively, in random block design. The dates of 50% percent flowering and the harvest maturity of each genotype in each replicate were recorded. Yield of each genotype in each replicate was recorded and expressed in Kg/hac. Randomly drawn 100 seeds from the harvest of from each replicate were weighed to determine 100 seeds weight. For assessing the seed longevity, the seeds were stored at ambient temperature in polythene bags for a period of two years. Two hundred seeds of each genotype were drawn in 3 replicates immediately after the harvest, and test for germination percentage immediately, and after a period of 12 and 24 months. For this purpose, soybean seeds were placed on sterile damp germination paper and incubated at 28°C for 4 days in a seed germinator. Germination percentage was calculated as follow: Total number of germinated seeds divided by total number of seeds tested multiplied by 100.

Statistical analysis

All statistical analyses were carried out using *SAS 9.3*.

RESULTS

One hundred fifty polymorphic SSR markers (their respective LGp/chr and the position (cM) on the LGp/chr is given in Table 2) were found for the parental combination

Table 2. Details of 150 SSR markers, spanning soybean genome, deployed for backcross selection.

LGp (*Chr*)	NO of SSR markers	SSR markers with position (cM) on the LGp (*chr*)
LGpA1 (*chr*5)	9	Sat_137 (0.00 cM), Satt684 (3.54 cM), Satt368 (14.37 cM), Satt276 (17.16 cM), Satt042 (27.66 cM), Satt717 (51.95 cM), Sat_171 (57.79 cM), Satt545 (71.39 cM), Satt211 (95.68 cM)
LGpA2 (*chr*8)	10	Satt589 (33.96 cM), GMENOD2B (58.44 cM), AW132402 (67.86 cM), Satt341 (77.70 cM), Satt089 (87.57 cM), Satt233 (100.09 cM), Satt329 (110.94 cM), Satt421 (115.93 cM), Sat_294 (131.97 cM), Satt228 (154.11 cM)
LGPB1 (*chr*11)	10	BE806308 (0.00 cM), Satt509 (32.51 cM), Satt638 (37.80 cM), Satt197 (46.39 cM), Sat_128 (53.41 cM), Satt597 (73.77 cM), Sct_026 (78.13 cM), Satt444 (85.92 cM), Satt665 (96.36 cM), Sat_331 (125.74 cM)
LGpB2 (*chr*14)	8	Satt577 (6.05 cM), Sat_342 (20.31 cM), Sat_287 (31.88 cM), Sat_355 (66.24 cM), Satt556 (73.21 cM), Sat_009 (78.66 cM), Satt560 (97.92 cM), Satt687 (113.61 cM)
LGpC1 (*chr*4)	5	Sat_337 (32.10 cM), Satt607 (67.03 cM), Satt646 (70.52 cM), Sat_085 (76.91 cM), Sat_042 (82.51 cM)
LGpC2 (*chr*6)	6	Satt681 (3.15 cM), Satt281(40.3 cM), Sat_213 (90.93 cM), Satt277 (107.59 cM), Sat_312 (112.85 cM), Satt371 (145.48 cM)
LGpD1a (*chr*1)	7	Sat_332 (5.25 cM), Satt320 (46.8 cM), Satt267 (57.34 cM), Satt580 (62.37 cM), Satt468 (69.91 cM), Satt077 (77.49 cM), Satt129 (109.67 cM)
LGpD1b (*chr*2)	10	Sat_096 (0 cM), Sat_279 (3.79 cM), Satt095 (25.6 cM), Satt266 (59.61 cM), Satt005 (75.29 cM), Satt041 (84.04 cM), Sat_183 (112.63 cM), Sat_202 (118.86 cM), Staga002 (126.45 cM), Sat_192 (135.26 cM)
LGpD2 (*chr*17)	4	Sct_192 (11.77 cM), Sat_292 (75.29 cM), Satt311(84.62 cM), GMHSP179 (99.04 cM)
LGpE (*chr*15)	5	Sat_124 (15.86 cM), Satt651 (32.1 cM), Sat_172 (42.74 cM), Satt369 (56.27 cM), Satt553 (67.92 cM)
LGp F (*chr*13)	10	Satt193 (3.42 cM), Sat_240 (25.58 cM), Satt516 (44.42 cM), Satt595 (50.24 cM), Sat_234 (66.55 cM), Sct_033 (74.13 cM), Sct_188 (85.30 cM), Satt144 (102.08 cM), AW756935 (124.88 cM), Satt395 (146.42 cM)
LGp G (*chr*18)	10	Satt163 (0.00 cM), Sat_163 (10.06 cM), Satt217 (18.25 cM), Sat_315 (27.48 cM), Satt324 (33.26 cM), Satt115 (43.78 cM), Satt303 (53.42 cM), Satt400 (63.28 cM), Satt472 (94.84 cM), Sat_372 (107.75 cM)
LGp H (*chr*12)	9	(27.64 cM), Satt009 (38.89 cM), Satt442 (46.95 cM), Satt541 (53.35 cM), Sat_205 (68.18 cM), Sat_158 (73.46 cM), Satt637 (85.79 cM), Satt181 (91.12 cM), Sat_180 (104.37 cM)
LGpI (*chr*20)	4	Satt562 (22.84 cM), Satt354 (46.22 cM), Satt650 (63.33 cM), Sat_170 (75.00 cM)
LGpJ (*chr*16)	8	AW310961 (5.19 cM), Satt674 (15.95 cM), Sat_339 (27.97 cM), Satt280 (38.7 cM), Satt380 (43.01 cM) Sctt011 (62.89 cM), Sat_224 (75.13 cM), Sat_393 (90.33 cM)
LGpK (*chr* 9)	7	Satt539 (1.8 cM), Sat_119 (17.11 cM), Satt349 (42.39 cM), Satt710 (51.00 cM), Sat_043 (61.67 cM) Sat_126 (108.2 cM), Satt588 (117.02 cM)
LGpL (*chr*19)	7	Sat_301(11.12 cM), Satt523 (27.92 cM), Satt278 (31.22 cM), Sat_150 (53.67 cM), Satt229 (93.89 cM), Satt513 (106.37 cM),Sat_245 (115.07 cM)
LGpM (*chr*7)	8	Satt435 (38.94 cM), Sat_244 (48.86 cM), Satt626 (58.60 cM), Satt175 (66.99 cM), Sat_288 (76.41 cM), Satt551 (95.45 cM), Satt308 (130.76 cM), Sat_330 (140.69 cM)
LGpN (*chr*3)	6	Satt641 (29.28 cM), Sat_084 (36.86 cM), Satt080 (45.14 cM), GMABAB (73.1 cM), Sat_295 (95.00 cM), Satt022 (102.06 cM)
LGpO (*chr*10)	7	Sat_196 (0.00 cM), Sat_132 (8.75 cM), Sat_318 (24.61 cM), Satt420 (49.71 cM), Satt345 (59.43 cM), Sat_282 (63.81 cM), Sat_108 (129.3 cM)

JS97-52 × PI596540. For the introgression of null allele of *Lox*2 into 'JS97-52' from PI596540 (donor of *lox*2 allele), crosses were effected between 'JS97-52' (female) and PI596540 (male). Table 3 details about the foreground and background selection at various stages of backcross generation. Screening of F_1 generation using polymorphic SSR marker Satt522 led to identification of 12 true F_1 plants.

Foreground and background selection

First Backcross generation

True F_1 plants were used as male parents to pollinate recurrent parent 'JS97-52' to effect BC_1F_1 generation. BC_1F_1 individuals (165 plants) grown in the field were subjected to foreground selection using null allele specific marker given by Reinprecht *et al*. (2011) (Fig. 1a). This led to the identification of 59 true BC_1F_1 individuals (*Lox*2*lox*2). Of these, 24 plants exhibiting morphological similarity with the recipient parent *i.e*. 'JS97-52', were selected for first background selection using 150 polymorphic SSR markers (Table 2) for 'JS97-52' and PI596540, across the genome. This revealed recurrent parent genome content to the range of 72-83%.

Second Backcross generation

To further minimise the genomic segments from the donor parent, 5 BC_1F_1 individuals exhibiting RPGC in the range of 80-83% were used as male parent to pollinate recurrent parent 'JS97-52'. Foreground selection of BC_2F_1 individuals (127) with null allele specific marker resulted in the confirmation of 54 true BC_2F_1 plants (*Lox*2*lox*2) carrying null allele of *Lox*2 (Table 3). Among these, 16 plants were morphologically similar to recurrent parent. Nine out of these 16 BC_2F_1 individuals which showed recombination with flanking marker Satt522 (located on the other side of *Lox*2 locus with respect to the position of Satt656) were subjected to background selection using 51-60 SSR markers, which were heterozygous in the previous backcross generation (BC_1F_1). This revealed retrieval of 84-94.33% of recurrent parent genome in the selected BC_2F_1 individuals. However, 5 plants exhibiting RPGC between 91.33-94.33% were selfed. BC_2F_2 generation (1109) was raised and screened with null allele specific marker for identification of plants carrying null allele of lipoxygenase-

Table 3. Details of foreground and background selection in different generations.

Generation	No. of plants screened	No. of plants confirmed	Background selection		RPGC (%)	No. of Plants selected based on high % RPGC	Plants selected based on high % RPGC
			No. of plants selected	No. of primers			
F_1	98	12 (*Lox2lox2*)	-	-	-	-	-
BC_1F_1	165	59 (*Lox2lox2*)	24	150	72.00-83.00	5	80.00-83.00
BC_2F_1	127	54 (*Lox2lox2*)	9	51-60	84.00-94.33	5	91.33-94.33
BC_2F_2	1109	250 (*lox2lox2*)	35	17-26	89.33-96.66	8	94.33-96.66
BC_3F_1	96	31 (*Lox2lox2*)	31	8-13	96.66-98.00	5	98.00
BC_3F_2	1200	260 (*lox2lox2*)	260	6	96.33-98.66	12	97.66-98.66

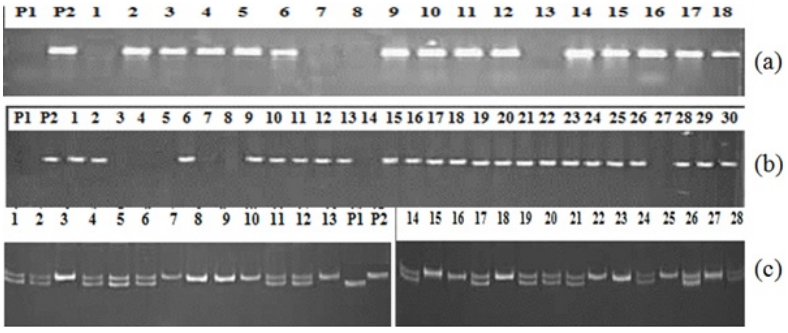

Fig. 1. Foreground selection in BC_1F_1 (a) and BC_3F_2 (b) generations using null allele specific marker on 1% agarose gel and in BC_3F_2 (c) generation using linked marker Satt656 on 8% polyacrylamide gel.

2. However, application of null allele specific marker did not distinguish *Lox*2*lox*2 from *lox*2*lox*2 plant individuals. Therefore, the plants found to carry null allele of *Lox*2 were, subsequently, surveyed with tightly linked marker Satt656 (flanking *Lox*2 locus), which led to the identification of 250 homozygous recessive (*lox*2*lox*2) plants. Of these, 35 plants similar to recurrent parent in morphology when subjected to background selection (III^{rd}) using 17-26 polymorphic SSR markers, which were heterozygous in the BC_2F_1 generation, revealed a range of 89.33-96.66% RPGC.

Third Backcross generation

To enhance the recovery of recurrent parent ('JS97-52') genome, pollens from 8 BC_2F_2 plants exhibiting more than 94.33% RPGC were used to effect BC_3F_1 generation. Foreground selection of BC_3F_1 plants using gene specific marker resulted in identification of 31 true BC_3F_1 plants carrying *lox*2 allele (*Lox*2*lox*2), which were subjected to background selection at 8-13 *loci*, which were either heterozygous or both the alleles were from the donor parent genome in the previous background selection (BC_2F_2 generation). Five plants which exhibited 98% retrieval of recurrent parent genome were selfed. BC_3F_2 generation was raised and subjected to foreground selection first using null allele specific marker (Fig. 1b). The plants identified to harbour null allele of *Lox*2 (*Lox*2*lox*2/*lox*2*lox*2) were subsequently surveyed using tightly linked marker Satt656 (Fig. 1c). This resulted in the identification of 260 homozygous recessive plants (*lox*2*lox*2), all of which were surveyed using 6 SSR markers, heterozygous in the previous background selection (BC_3F_1). This showed a recovery of 96.33-98.66% of recurrent parent genome. However, 12 lines ($BC_3F_{2:3}$) exhibiting recurrent parent genome recovery of 97.66-98.66% were selected as the ILs for confirmation of absence of lipoxygenase-2 through dye bleaching and quantitative test.

Carrier Chromosome Analysis *vis-a-vis* linkage drag

For analysing the recovery of recurrent parent genome in ILs on the chromosome carrying *Lox*2 locus, 10 polymorphic SSR markers (Satt193, Sat_240, Satt516, Satt595, Sat_234, Sct_033, Sct_188, Satt144, AW756935 and Satt395) other than Satt656 and Satt522 (used for foreground selection) on the LGp F (*chr* 13) were surveyed (Fig. 2). SSR markers Sat_240, Satt516, Satt595, Sat_234 and Satt395 were fixed for the recurrent parent genome in all the 12 ILs. However, Satt193, Sct_033, Sct_188 and Satt144 were not fixed for the recurrent parent in all the ILs, as some of the ILs exhibited either heterozygosity or were found to carry both the alleles from the donor parent (PI596540) at one or

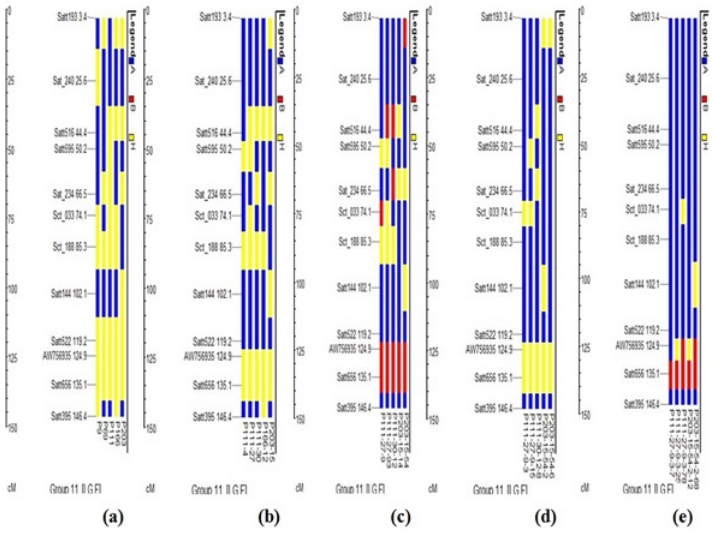

Fig. 2. Graphical genotyping of selected plants in BC_1F_1 (a), BC_2F_1 (b), BC_2F_2 (c), BC_3F_1 (d) and BC_3F_2 (e) generations on carrier chromosome (LG 'F' i.e. *chr* 13) through GGT 2.0 (Berloo 2008).

Table 4. Comparison of the biochemical and agronomic parameters of JS97-52 and JS97-52 derived Lox2- free ILs.

Genotype	Dye bleach assay*	Lox2 units/g defatted flour	Protein %	Days-to-flowering	Days-to-maturity	100 SW g	Yield Kg/ha	Germination %		
								months		
								0	12	24
JS97-52	+ve	450.2^{b}	40.2^{b}	50^{b}	105^{b}	9.5^{b}	2635^{b}	84.6^{a}	71.0^{a}	30.3^{a}
PI596540	−ve	40.00^{a}	38.0^{a}	28^{a}	85^{a}	8.4^{a}	1256^{a}	86.0^{a}	85.5^{b}	55.0^{b}
JPILX2F1	−ve	35.24^{a}	39.7^{b}	47^{b}	103^{b}	9.4^{b}	2686^{b}	87.2^{a}	80.4^{b}	50.7^{b}
JPILX2F2	−ve	39.87^{a}	40.0^{b}	48^{b}	104^{b}	9.6^{b}	2572^{b}	84.4^{a}	85.4^{b}	50.0^{b}
JPILX2F3	−ve	41.39^{a}	39.9^{b}	47^{b}	103^{b}	9.2^{b}	2589^{b}	86.0^{a}	85.4^{b}	54.4^{b}
JPILX2F4	−ve	37.21^{a}	40.2^{b}	50^{b}	104^{b}	9.3^{b}	2627^{b}	87.0^{a}	87.3^{b}	55.2^{b}
JPILX2F5	−ve	38.56^{a}	39.0^{b}	51^{b}	106^{b}	9.2^{b}	2610^{b}	90.1^{b}	82.1^{b}	54.3^{b}
JPILX2F6	−ve	43.78^{a}	40.1^{b}	48^{b}	107^{b}	9.5^{b}	2589^{b}	87.3^{a}	90.0^{c}	52.3^{b}
JPILX2F7	−ve	45.65^{a}	39.5^{b}	51^{b}	103^{b}	9.4^{b}	2592^{b}	90.0^{b}	87.3^{b}	51.6^{b}
JPILX2F8	−ve	38.21^{a}	40.0^{b}	50^{b}	104^{b}	9.7^{c}	2612^{b}	90.2^{b}	86.2^{b}	50.5^{b}
JPILX2F9	−ve	46.21^{a}	39.6^{b}	48^{b}	105^{b}	9.4^{b}	2691^{b}	90.4^{b}	84.4^{b}	53.4^{b}
JPILX2F10	−ve	41.45^{a}	40.5^{b}	49^{b}	102^{b}	9.5^{b}	2618^{b}	90.5^{b}	87.6^{b}	55.2^{b}
JPILX2F11	−ve	38.61^{a}	39.7^{b}	47^{b}	105^{b}	9.4^{b}	2605^{b}	85.1^{a}	83.9^{b}	49.0^{b}
JPILX2F12	−ve	44.32^{a}	40.1^{b}	50^{b}	104^{b}	9.4^{b}	2598^{b}	86.2^{a}	82.0^{b}	51.3^{b}

*'+ve' and '−ve' indicate the bleaching and persistence of methylene blue dye, respectively. Values are mean of triplicate observation. Values given superscripted with different alphabets are significantly ($P < 0.05$) different.

more of these *loci*. Further, in BC_3F_2 generation an SSR marker AW756935 (124.9 cM) which has been mapped between flanking SSR markers *viz*. Satt522 (119.2 cM) and Satt656 (135.1 cM) (USDA 2008), was also found to be fixed for the recurrent parent allele in one IL, namely, P111-27-9-3-7. However, 4 ILs exhibited heterozygosity at locus AW756935, and remaining 7 ILs exhibited donor parent alleles in homozygous state. Therefore, line P111-27-9-3-7 exhibited minimum introgression of donor parent genome on the carrier chromosome.

Qualitative and quantitative estimation of Lox2 in ILs

Results of dye-bleaching test performed using the soy flour from parent genotypes *viz*. 'JS97-52', PI596540 and 12 ILs are presented in Table 4. Dye-substrate (methylene blue-sodium linoleate) solution containing soy flour from the recurrent parent ('JS97-52') exhibited bleaching of dye within 1 minute. On the contrary, soy flour of PI596540 and ILs did not cause fading of the dye even after 7 minutes. Stability of dye-color in MABC-derived Lox2-free ILs confirmed the absence of lipoxygenase-2 isozyme. Lipoxygenase-2 activity per gram of defatted soy flour in the recipient parent 'JS97-52' (*Lox2Lox2*) and donor parent PI596540 (*lox2lox2*) was 450.21 and, 40 units per gram of defatted flour, respectively, while 12 ILs exhibited negligible activity of 35.24-46.21 units/g of defatted flour, which was at par with the donor parent PI596540 (Table 4). Qualitative data of parents and ILs corresponded well with the results of quantitative assay, which confirmed the absence of lipoxygenase-2 in ILs. Further, the protein content in most of the ILs was at par with the recurrent parent 'JS97-52'. Results presented in Table 4 also showed that the germination percentage of the recurrent parent was at par with the ILs immediately after the harvest. After storage at ambient temperature for 12 months, recurrent parent registered 71% germination, which plummeted to 30.3% after 24 months. On the contrary, % germination among 12 ILs was 82.0-90.0% and 49.0-55.2% after 12 and 24 months of storage.

DISCUSSION

Despite being increasingly recognized as the economical source of protein to address under-nutrition in developing countries and as 'functional food' to stave off broad spec-

trum of diseases across the world, utilization of soybean in food uses stands minuscule. Off-flavour associated with the soy products deter masses to incorporate of soybean in daily diet in several countries including India. The off-flavour is ascribed to the aldehyde and ketone compounds released during the oxidation of polyunsaturated fatty acids by the lipoxygenases present in the seed. Of the three lipoxygenases, lipoxygenase-2 is the prime contributor to off-flavor as the isozyme generates maximum n-hexanal-producing 13-hydroperoxides (Mellor *et al*. 2010). Genetic elimination of lipoxygenase-2 is critical for the flavour improvement of soy products (Davies *et al*. 1987). Like the other two isozymes, expression for *Lox*2 is also governed by single dominant gene and the sources of recessive form have also been identified (Kitamura *et al*. 1983; Davies and Nielsen 1986). Two flanking SSR markers, namely, Satt522 and Satt656 around *Lox*2 locus, have been identified to be linked to *Lox*2 at a distance of 9.3 and 2.7 cM on LG 'F' (*chr* 13) in the mapping population derived from 'JS97-52' × PI596540 in the previous study (Kumar *et al*. 2014). Further, null allele specific marker for *lox2* has been developed by Reinprecht *et al*. (2011). Since the parentage of donor genotype PI596540 used in the present work is Camp x ('Vance'^{2} × L_2-3), and the L_2-3 is the progeny of PI086023, we could successfully amplify the genomic DNA of the null plants (*lox*2*lox*2) using null allele specific marker given by Reinprecht *et al*. (2011) and deployed it in carrying out foreground selection in BC_1F_1, BC_2F_1 and BC_3F_1 generation. In BC_2F_2 and BC_3F_2 generation, null allele specific marker in conjunction with linked SSR marker Satt656 enabled the identification of homozygous recessive plants (*lox*2*lox*2). The high level of SSR polymorphism (49.24%) detected between the genome of 'JS97-52' and PI596540 in our previous report (Rawal *et al*. 2014) was exploited for faster recovery of the recurrent parent genome using marker assisted background selection.

Background retrieval

First background selection in 24 individuals morphologically similar to 'JS97-52' in first backcross generation using 150 polymorphic SSR markers identified across 20 LGs exhibited RPGC in the range of 72-83% against the expected average recovery (75%). Kim *et al*. (2008) employed 26 and 21 SSR markers in the first background selection, with the objective of introgressing *rxp* locus responsible for imparting bacterial leaf pustule resistance into 2 soybean varieties 'Hwangkeumkong' and 'Taekwangkong' and reported 76.9-96.1 and 61.9-95.2% recurrent parent recovery, respectively. Higher RPGC recovery in the first background selection in both the varieties compared to our results may be ascribed to relatively lesser number of SSR markers employed by Kim *et al*. (2008). We conducted second background selection in the BC_2F_1 generation using 51-60 SSR markers, as the remaining SSR markers were fixed for the recurrent parent. This led to the recovery of 84-94.33% RPGC with some of the plants showing RPGC, exceeding the expected recovery of 87.5% at this stage. In a marker assisted backcross breeding program in maize focusing on development of low phytic acid genotype, retrieval of RPGC to the magnitude of 92.15% in BC_2F_1 generation has been reported (Naidoo *et al*. 2012). Kim *et al*. (2008) reported background recovery of 82.2-91.5 and 85.5-93.3% during the introgression of *rxp* locus responsible for imparting bacterial leaf pustule resistance in varieties 'Hwangkeumkong' and 'Taekwangkong'. The range of recovery of RPGC in this study is in proximity with our observation in BC_2F_1 generation. Third background selection carried out in BC_2F_2 (*lox*2*lox*2) progenies using 17-26 loci, which remained in heterozygous condition in BC_2F_1 generation, revealed RPGC higher/lower compared to their corresponding BC_2F_1 parent. Lower recurrent parent genome recovery in some of BC_2F_2 (homozygous recessive for *lox*2 allele) progenies is due to fixation of heterozygous alleles towards donor parent genome while higher % RPGC compared to their corresponding BC_2F_1 parent is attributed to the realization of homozygosity for recurrent parent at several heterozygous loci. As we did not observe recurrent parent genome content recovery more than 96.66% in BC_2F_2 generation; therefore, third backcross generation was effected to enhance the RPGC recovery. Background selection in BC_3F_1 generation using 8-13 SSR markers, which were heterozygous or fixed towards donor parent genome in BC_2F_2 generation, resulted in identification of plants with 96.66-98% RPGC. The last background selection performed in BC_3F_2 homozygous recessive (*lox*2*lox*2) individuals using

remaining heterozygous loci (6) led to identification of 12 BC_3F_2 lines with 97.66-98.66% RPGC.

Minimizing the linkage drag

In the presented work, PI596540 as the donor parent of null allele of *Lox*2 exhibited short-stature and poor yield. Therefore, development of Lox2- null ILs with minimum linkage drag was sought. Satt656 and Satt522 are two flanking markers around *Lox*2 locus at 2.7 and 9.3 cM, respectively (Kumar *et al*. 2014). The former SSR marker was employed for foreground selection, while plants exhibiting recombination with Satt522 in BC_2F_1 generation were carried to BC_3 generation to minimize the linkage drag in the ILs. One IL (P111-27-9-3-7), which showed recovery of SSR marker AW756935 (124.9 cM) mapped between flanking SSR markers *viz*. Satt522 (119.2 cM) and Satt656 (135.1 cM), carried minimum donor parent genomic content, except the region governing the expression of trait.

Biochemical and agronomic evaluation

The data pertaining to the biochemical and agronomic characters of ILs and both the parents are presented in Table 4. Soy flour from ILs ($BC_3F_{2:3}$) did not cause bleaching of the dye colour in qualitative assay. Quantitative estimation of lipoxygenase-2 showed negligible activity of lipoxygenase-2 at par with the donor parent PI596540 (Table 4). Negligible activity of lipoxygenase-2 observed in donor parent PI596540 and ILs may be ascribed to the presence of other lipoxygenase-like enzymes as discussed by Iassonova *et al*. (2009) or any other metabolic pathway involved in the generation of n-hexanal (Mellor *et al*. 2010). All the JS97-52-derived lipoxygenase-2 free ILs were at par with the recurrent parent in days-to-50% flowering (47-50 days), days-to-maturity (102-107 days), and yield (2572-2686 kg/ha). Both the donor and the recurrent parent were small seeded, and the null Lox-2 ILs exhibited the same 100 seed weight as that of the recurrent parent. However, seed longevity of ILs was better than recurrent parent as indicated by the higher germination percentage of the former than the latter after 12 and 24 months of storage at ambient temperature. Suppressed lipoxygenase activity has also been reported to enhance seed longevity during storage in other crops such as tobacco (Li *et al*. 2018), rice (Ma *et al*. 2015). This may be attributed to the reduction in lipid peroxidation of cell membrane due to lower levels of lipoxygenase-produced reactive oxygen species.

In the presented work, introgression of null allele of *Lox*2 into a high yielding soybean cultivar 'JS97-52' by deploying marker assisted foreground and background selection was expeditiously accomplished. Null Lox2 introgressed lines showed same biochemical and morpho-physical traits as that of the recurrent parent except the improved seed longevity of the former over the latter. Use of these Lox2-free lines as initial raw material in soy processing industries would deliver soy-based products with reduced off-flavour and the seeds of these breeding lines can be stored for longer period under ambient conditions. More importantly, these ILs can also be used for pyramiding Lox2-null with null forms of other two lipoxygenase isozymes *viz*. lipoxygenase-1 and lipoxygenase-3 for development of triple null-lipoxygenase soybean.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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