BILINGUAL PUBLISHING CO.
Pioneer of Global Academics Since 1984

Journal of Botanical Research
https://ojs.bilpublishing.com/index.php/jbr

ARTICLE

Soybean Improvement for Lipoxygenase-free by Simple Sequence Repeat (SSR) Markers Selection

Valeria Carpentieri-Pipolo[1*] Thales Pereira Barreto[2] Daiana Alves da Silva[3]
Ricardo Vilela Abdelnoor[4] Silvana Rockenbach Marin[4] Giuliano Degrassi[5]

1.Embrapa Trigo, P.O.Box 451, Zip Code 99001-970 Passo Fundo, Rio Grande do Sul, Brazil
2.Syngenta Proteção de Cultivos LTDA. São Paulo, SP, Brazil
3.Agronomic Institute (IAC) - Center for Grain and Fiber - Zip Code 13075-630 - Campinas, SP, Brazil
4.Embrapa Soja, Zip Code 86001970, Londrina, PR, Brazil
5.International Centre for Genetic Engineering and Biotechnology (ICGEB), Industrial Biotechnology Group, Parque Tecnológico Miguelete, San Martín, Buenos Aires, República Argentina

ARTICLE INFO

Article history
Received: 19 January 2021
Accepted: 23 February 2021
Published Online: 30 March 2021

Keywords:
Glycine max
Lipoxygenase isozymes
Molecular markers
MAS

ABSTRACT

Beany flavor of soybean (*Glycine max* (L.) Merr.) is caused by oxidation of polyunsaturated fatty acids by the action of three lipoxygenases (LOX1, LOX2 and LOX3) present in mature seeds. The unpleasant flavor restricts human consumption of soybean products. This problem could be solved through genetic elimination of alleles that code these enzymes. Parental cultivars and two hybrid population were selected and analyzed using genetic markers for alleles locus, encoding Lox_1, Lox_2 and Lox_3 free. The SSR marker Satt 212 confirmed the presence of the homozygous null-allele Lx_3 in the cultivar BRS 213, which were used for hybridization with BR 36. Heterozygote F_1 hybrid plants and homozygous Lx_3 lines in F_2 segregating populations were successfully identified. The SSR markers Sat090 and Sat417 were the most effective diagnostic markers among all SSR markers tested. Satt090 and Satt417 confirmed the presence of the homozygous Lx_2 null-allele in the parental cultivar BRS 213 by flanking Lx_2 loci at 3,00 and 2,77 cM, respectively. The presence of Lx_2 null allele in the F_2 segregating populations between BRS 213 and BRS 155 was successfully identified with a selection efficiency of 98% and have great potential for further application in the Brazilian breeding program aimed at improving soybean seed quality.

1. Introduction

Soybean is the cheapest source of protein with high quality; in addition, it has a low content of saturated fat. Also due to these properties, it is commonly used in the diet of many populations worldwide. Soybean contains several special bioactive ingredients, such as tocopherols, isoflavones, saponins, among others, that can contribute to prevent chronic diseases such as cancer, atherosclerosis, diabetes, Parkinson's, etc. Recently, soy-containing foods have gained approval and are considered as the "functional

**Corresponding Author:*
Valeria Carpentieri-Pipolo,
Embrapa Trigo, P.O.Box 451, Zip Code 99001-970 Passo Fundo, Rio Grande do Sul, Brazil;
Email: valeria.carpentieri-pipolo@embrapa.br

 DOI: https://doi.org/10.30564/jrb.v3i1.2818

food" of this age.

Health-conscious consumers have changed on eating habits and preferences especially in the middle and upper classes. The population aging and the quest for a healthier life have promoted the use of soybean as an alternative to the human diet because of its contribution to reduce the incidence of cardiovascular disease. This has determined an increase of soybean request and consumption for food use worldwide. As a consequence, the market of soybean cultivars with higher agronomic characteristics and seed composition traits has been growing and cultivars of food type are on the demand.

Brazil is the second largest producer with 115.072, 5 million tons produced in 2019, behind only the United States with 123,664 million tons [1]. However, less than 5% of the total soybean produced in Brazil is used for soy foods. The reason is due to the physiology process after post-harvest that affects the seeds with high lipoxygenase (LOX) activity and linoleic acid content with a consequent production of hexanal that even at low concentrations (e.g. 5 ppb), confers unpleasant odor and therefore flavor; this makes difficult the production of soybean-containing food with suitable properties for human consumption. Since lipoxygenase (linoleate: oxygen oxidoreductase EC 1.13.11.12) mediated oxidation of polyunsaturated fatty acids in mature soybean seeds results in unpleasant odor and flavor, lipoxygenase-free seed soybean cultivars specially developed for human consumption can contribute to increase the acceptability of soy foods, for this reason they are becoming important to the food industry and are in demand. The major reason of this off-flavor is the aldehydes and ketones produced by the activity of an iron containing dioxygenase that catalyzes the oxidation of unsaturated fatty acids [2].

Heat treatment has been used commercially to suppress the lipoxygenase activity in order to prevent the beany-flavor generation in soy protein products. However, heat treatment sufficient to inactivate the lipoxygenase action often results in the insolubilization of the soy proteins, generating an unpleasant "cooked" odor. In addition, soybean cultivars lacking the lipoxygenases should become economically valuable due to their enhanced storage stability, since the lipoxygenase-induced oxidative deterioration of protein and oil in soy meals is reduced. A combination of appropriate processing technologies and the new cultivars may enable the production of various soybean-based foods.

There are three major aspects of plant physiology where lipoxygenases have been implicated: growth and development, senescence, and insect wound resistance response and pest resistance. However, it has been demonstrated that lipoxygenase-free seeds regularly develop into normal plants without defects, independently from the role that this enzyme has in the plant physiology [3, 4].

Lipoxygenase is present in mature soybean seeds in the form of three isozymes i.e., lipoxygenase-1 (LOX1), lipoxygenase-2 (LOX2) and lipoxygenase-3 (LOX3). They are encoded by three dominant genes which are inherited in the mendelian way. Functional genes for the isozymes have been assigned and the gene symbols are: Lx_1, Lx_2 and Lx_3. The inheritance and molecular base of lipoxygenase nulls have been investigated in different backgrounds for several authors [5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15]. Spontaneous mutants of the enzymes have been identified and several mutants were developed by gamma radiation [16, 17]. The Lx_1 and Lx_2 loci were found to be in tight genetic link and mapped in the same linkage group, while Lx_3 locus is independent [7].

The development of seed lipoxygenase-free triple null mutant genotype was reported by Hajika et al. [16] and Kitamura [18]. More recently Wang et al. [19] have reported that triple null mutant plants carrying *gmlox1gmlox2gmlox3* triple mutations were generated by clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9). The authors described that the original repulsion-phase linkage in independent sources of mutant alleles at the Lx_1 and Lx_2 loci was broken, resulting in a coupling-phase linkage that eventually led to the development of a triple null lipoxygenase genotype. However, there were no data about the mechanism that generated the genotype without the three seed lipoxygenases.

Accurate evaluation assays are necessary if the appropriate genotype has to be selected for the development of seed lipoxygenase free soybean cultivars. Laborious and time-consuming seed phenotype methods have been used to determine the presence of lipoxygenase in breeding programs. In addition, individuals that are homozygous and heterozygous in the same generation cannot be discriminated using these procedures. In contrast, a screening procedure based on codominant DNA markers developed for lipoxygenase nulls mutation could be used in breeding programs for market-assisted selection (MAS) of lines with seed lipoxygenase nulls. Microsatellite or simple sequence repeat (SSR) marker is a sequence with one to six base pairs repeated in tandem. SSRs consist of small repeat units (1-6 bp) distributed in tandem throughout the genomes and are easily detected by polymerase chain reaction (PCR); they are relatively abundant, have extensive genome coverage, and require a low amount of DNA for amplification, there are 874 SSR markers assembled in ordered positions within the 20 soybean linkage groups. Thus, they have the potential to accelerate and simplify

DOI: https://doi.org/10.30564/jrb.v3i1.2818

breeding efforts for soybean cultivars with improved flavor [13, 14, 15, 20, 21, 22].

This study was undertaken to evaluate the efficiency of microsatellite markers, previously identified as being associated with free-lipoxygenase in Brazilian soybean cultivars lacking seed lipoxygenases to be used as germplasm resource in breeding program focused on selecting cultivars with improved flavor.

2. Material and Methods

2.1 Plant Material and Development of Segregating Population

The plants were grown at greenhouse at Agronomy Department of State University of Londrina, Londrina – PR, at 23º22' latitude south, the phenotyping and genotyping tests were performed at the Laboratory of Biotechnology of Embrapa Soybean, Londrina - PR. $F_{2:3}$ derived populations of 168 RILs were developed by single seed descent from BRS 213 X BR 36 and $F_{2:3}$ derived population from 93 RILs from BRS 213 x BRS 155. Seeds of parental cultivars were obtained from Active Germplasm Bank of Brazilian Agricultural Research Corporation (Embrapa Soybean). The cultivars were chosen for their special characteristics for human consumption. BRS 213 (BR 94-23354 X BR 94-23321) is triple null for lipoxygenase isoenzymes. It was released in 2002 making available excellent raw materials for soy food processing uses, such as soymilk and tofu, due to its lack of beany taste. BR213 has as ancestral the Japanese line triple null seed lipoxygenases (obtained from Dr. Kitamura - National Agricultural Research Centre, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan). The triple null mutant soybean line was produced by gamma-ray irradiation of F_2 plants from a cross between two double mutants: Lx_1- and Lx_3-free and Lx_2- and Lx_3-free [16, 18].

The Brazilian cultivar BR 36 (IAS 4(2) X BR 78-22043) is dominant for the three seeds lipoxygenase genes (Lx_1, Lx_2, Lx_3). It has as clear hilum color, large grains, high protein content and good sensory qualities, which give grains, flour and milk with mild flavor. It is a cultivar preferred by organic growers and it is widely accepted in the soybean market for human [23]. BRS 155 (IAS 4(2) X PI 157440) is a Brazilian cultivar that was released for soy food uses, it has the allele *kti* from the ancestral cultivar Kin-du (PI 157440), a South Korean cultivar that has the *kti* allele. The dominant gene KTI controls an anti-nutritional factor of trypsin inhibitor Kunitz that affects protein digestibility [24]. The presence of the recessive *kti* gene in the BRS 155 allows a reduction in heat treatment, with consequent reduced processing costs and better protein solubility.

Crossing were performed in the summer of 2011 in a greenhouse and F_1 plants were obtained in the winter of 2012. Authentication of hybrid origin of F_1 plants was carried out and they were compared with their female parental. Freshly harvested seeds of these cultivars were analyzed for lipoxygenase content. Statistical analysis of obtained data was performed using the model for a completely randomized design with an unequal replication treatment (each family was considered a treatment). The genetic parameters were estimated using the Genes program [25].

2.2 Detection of Seed Lipoxygenases

To confirm the status of F_1 seeds and to check the segregation of $F_{2:3}$ seeds, non-destructive colorimetric analysis of LOX1 and LOX3 were performed by a procedure based on slightly modified colorimetric method of Suda et al. [26, 9] and single-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS–PAGE). Non-destructive analysis was achieved by cutting with a razor blade a small portion (5 to 10 mg) of cotyledons from the seed, in such a way that the rest of the seed would not lose its germination capacity. LOX1 was extracted by soaking 5 to 10 mg seed cotyledon in 0.25 ml water in a test tube for 3 min. After this period, 0.4 mL of a reaction mixture containing 130 mM sodium borate buffers, pH 9.5, 16 mM Methylene Blue, and 1.5 mM sodium linoleate was added to the tube. After 3 min the solution was checked for bleaching as a result of reduction of Methylene Blue (presence of LOX1) or not (absence of LOX1). LOX3 was extracted by soaking 5 to 10 mg seed cotyledon in 0.25 ml soybean extract containing only LOX1 (the test for LOX3 does not work properly in the absence of LOX1) for 3 min. After this period 0.4 ml reaction mixture containing 130 mM sodium phosphate buffers, pH 6.8, 4% (w/v) β-carotene dissolved in 50% (v/v) acetone, and 1.5 mM sodium linoleate. Bleaching of the β-carotene indicated the presence of LOX3.

Null $F_{2:3}$ families (based on the colorimetric assays) were extracted in lipoxygenase loading buffer [0.0625 mM Tris–HCl (pH 6.8), 2% SDS, 5% (v/v) beta-mercaptoethanol and 10% (v/v) glycerol] and separated by discontinuous (10% - 4%) SDS–PAGE [7]. Genotyping was performed by creating a binary array that was used for statistical analysis and construction of the genetic map.

2.3 Statistical Analysis

The individual segregation of seed lipoxygenases resulted from colorimetric analyses was tested using the

 DOI: https://doi.org/10.30564/jrb.v3i1.2818

chi-square test. Genetic distances between markers were estimated using the Mapmaker EXP V. 3.0 program [27]. A minimum logarithm of odds (LOD) score of 3.0 and maximum distance of 50 cM were chosen to establish the degree of linkage. Recombination fractions were converted to map distances using the Kosambi. LGs were named according to the designations of the consensus USDA map [28]. All analyses were carried out by GQMOL program [29].

2.4 Genotyping Assays

The DNA was extracted from the soybean leaves by the CTAB method [30], and then quantified in a spectrophotometer and stored at 4°C until use.

Out of more than 600 SSR markers developed by Cregan et al. [28], Song et al. [31] and Grant et al. [32] in soybean, 22 pairs of soybean primers flanking the microsatellite regions, previously published, were selected. They were synthesized by Bio Synthesis Inc., Texas, USA. Chromosome 15 (LG E): Satt575, Satt213, Satt651, Satt212, Satt598, Satt573, Sat_136, Satt606. Chromosome Gm13 (LG F): Sat_090, Satt656, Sat_417, Sat_074, Satt395. Chromosome 7 (LG M): Sat_389, Satt404, Sat_391, Satt636, Satt590, Satt201, Satt150, Sat_316, Satt567. The sequences of the Forward and Reverse primers are available at the soybean SoyBase, the USDA-ARS Soybean Genetics and Genomics Database [32]. The primers were chosen because they had presented polymorphism in previous studies [33, 20, 34, 13].

Markers that generated polymorphisms among the parents were amplified in DNA bulks of each family, for instance a solution containing equal amount of DNA from all the plants of the same family. The amplification reactions were carried out in a total volume of 15 µL, containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.4 mM MgCl2; 100 µM of each desoxynucleotide; 0.3 µM of each primer, a unit of *Taq* polymerase and 30 ng genomic DNA. The amplification reactions were carried out in a Perkin Elmer thermocycler, model 9600, programmed for thirty cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; at the end of the 30 cycles, a stage of 7 min at 72°C was performed. The amplification products were separated by electrophoresis in 3% agarose gel, or in 10% native vertical polyacrylamide gels, using a TAE 1X buffer (0.09 M Tris- acetate and 0.002 M EDTA). The amplified SSR fragments of different sizes were considered as different alleles. The fragments were detected by silver staining, following Sanguineti et al. [35].

3. Results and Discussion

The phenotypes for LOX1 and LOX3 of the progenitors used in this work and of $F_{2:3}$ seeds derived from crosses among them were first confirmed by the colorimetric test, as shown in Figure 1. Blue color indicates absence of LOX1(-), yellow indicates presence of LOX1 (+), green indicates the absence of LOX1 (-) and LOX 3 (-); colorless indicates the presence of all lipoxygenases: LOX1 (+), LOX 3 (+).

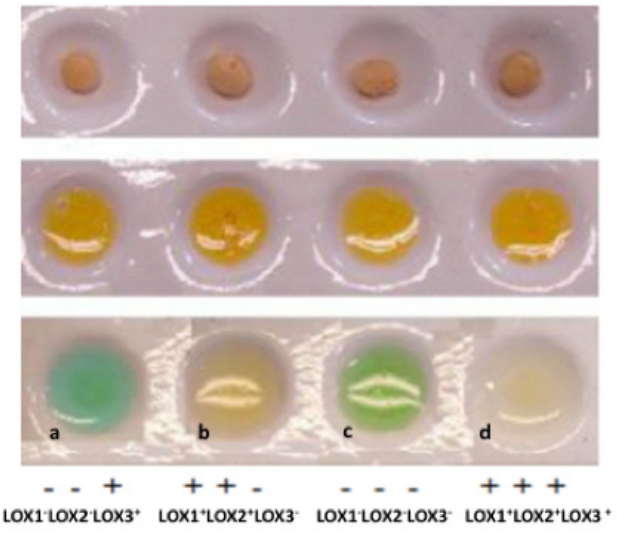

Figure 1. Colorimetric assay of soybean seed lipoxygenase enzymes for soybean in $F_{2:3}$ population from the cross between the soybean cultivars BR 36 and BRS 213, and between BRS155 and BRS213. a-blue indicates absence of LOX 1 (-); b-yellow indicates presence of LOX1 (+); c-green indicates the absence of LOX1 (-) and LOX 3(-); d-colorless indicates the presence of all lipoxygenase (+) LOX 1 (+), LOX 3 (+)

Results indicate a perfect association between the inheritance of homozygous lx_1 or lx_3 and the lack of LOX1 or LOX3 activity (Table 1).

The segregation of the $F_{2:3}$ RILs seeds derived from the cross between the triple null BRS 213 and BR 36 (dominant for the three lipoxygenase) a phenotypic ratio for LOX1 and LOX3 of 15:1 or 153:15 was observed and confirmed by the chi-square test at 1% significance (Table 1). This ratio is expected for two genes, which segregate independently.

The phenotypic segregation between BRS 213 and BRS 155 F2 RILs for the presence of the LOX1 enzymes carried out by the colorimetric test (Figure 1) allowed us to confirm the 3:1 segregation, 69 seeds showed presence of LOX1 and 24 seeds showed the absence of both enzymes; the chi-square test obtained was not significant at 1% probability (Table 1). The colorimetric assay indicated segregation to the presence and absence of lipoxygenase isozymes in the soybean seeds but did not allow detection

DOI: https://doi.org/10.30564/jrb.v3i1.2818

Table 1. Colorimetric determination of presence or absence of LOX1 and LOX3 activity in $F_{2:3}$ soybean seeds population from the cross between the cultivars BR 36 and BRS 213 and between BRS155 and BRS 213.

Population $F_{2:3}$	Phenotype	Genotype	Expected [a]	Observed [b]	X^2	P
BR 36 X BRS213	Presence $LOX1^+LOX3^+$ And heterozygous $LOX1^+LOX3^-$	$Lx_1Lx_1Lx_3Lx_3$ and $Lx_1lx_1Lx_3lx_3$	157,5	153	$5,76^{ns}$	0,12
	Absence LOX1-LOX3-	$lx_1lx_1lx_3lx_3$	10,5	15		
BR 155 X BRS213	Presence LOX1+	Lx_1Lx_1 and Lx_1lx_1	70,0	69	$0,032^{ns}$	0,86
	Absence LOX1-	lx_1lx_1	23,5	24		

[a]Expected segregation ratio, [b]Observed segregation ratio; X^2 Chi-square value, [ns]Non significant at 1% probability; P: probability.

Table 2. Segregation analysis of three SSR markers (Satt212, Satt417and Sat_090) linked to the *Lx* genes in the $F_{2:3}$ population from the cross between the soybean cultivars BR 36 and BRS 213, and between BRS155 and BRS 213.

$F_{2:3}$ Population	LG	Marker	Genotype	Expected[a]	Observed [b]	X^2	P
BR36XBRS213	F	Satt212	lx_1lx_1,lx_3lx_3	38,75:77,50:38,75	27:79:43	$3,98^{ns}$	0,14
BRS155XBRS213	E	Satt417	lx_1lx_1	22,75:45,50:22,75	22:44:25	$0,29^{ns}$	0,86
		Sat_090	lx_1lx_1	23:46:23	23:46:23	$0,00^{ns}$	1,00

LG: Linkage group of linkage map of the soybean genome (Cregan et al., 1999); [a]Expected segregation ratio for co-dominant markers, X^2 Chi-square value, [ns]Non significant at 1% probability; P: probability.

of heterozygous individuals (Figure 1).

Although colorimetric analysis is useful during the breeding process to eliminate lipoxygenase from soybean seeds, it is very limited because no heterozygous seeds can be identified. Identification of heterozygous seeds can be extremely important in a backcross breeding program specially when the trait of interest is recessive. This information can speed up the creation of new cultivars because there is no need of self-fertilization during the odd numbered generations.

In the present study, we investigated two different seed-expressed lipoxygenases (Lx_1 and Lx_3), expressed in the Brazilian parental soybean cultivars BRS 213, BR 36 and in $F_{2:3}$ derived populations. Several authors reported the inheritance of seed lipoxygenases in soybean, showing that they are present on the syntenic region Ks0.1984 containing chromosomes 15 (E) and 13 (F). Lx_1 and Lx_2 genes are tightly linked and inherited together and the Lx_1 and Lx_2 genes are mapped as single major genes to the same location on chromosome Gm13 (LG F) while Lx_3 gene is independently inherited, mapped on chromosome Gm15 (LG E) [13]. Kim et al. [20, 34] found the Lx_2 locus positioned on one end of chromosome 13 (LG F), flanked by the SSR markers Satt522 and Sat074 and the Lx_3 locus at the linkage group M next to the marker Satt150. However, Reinprecht et al. [13] found Lx_2 locus flanked by the SSR markers Sat_090 and Sat_074 on chromosome 13 (LG F) and the loci Lx_3 was mapped on the chromosome 15 (linkage group E) next to the Satt212 [13, 14, 15]. Thus, in order to validate the SSR markers reported previously by Kim et al. [33], Kim et al. [20], Reinprecht et al. [13], Lenis et al., [14] and Reinprecht et al. [15], we surveyed the DNA

 DOI: https://doi.org/10.30564/jrb.v3i1.2818

polymorphism in the parents cultivars and RIL $F_{2:3}$ derived population generated from the cross between BRS 213 ($lx_1lx_2lx_3$) and BR36 ($Lx_1Lx_2Lx_3$) and BRS 213 ($lx_1lx_2lx_3$) and BRS 155 ($Lx_1Lx_2Lx_3$). Additional SSR markers in the neighboring genomic region of Lx_1 and Lx_3 were tested.

Among the 22 SSR primer pairs used in PCR amplification of DNA for the parents lines BRS 213, BR 36 and BRS155, only eight produced polymorphic DNA fragments showing 31% of parental polymorphism. Nevertheless, seven SSR markers which have shown polymorphisms in the parents cultivars had shown poor segregation in the $F_{2:3}$ population and the data were excluded. The SSR primers pair previously used by Kim et al. [33] linked with Lx_1 (Lx_2) have shown polymorphism in the parental lines BRS 213 and BR 36 but did not in the RIL $F_{2:3}$ derived population. However, the RIL $F_{2:3}$ population derived from cross BRS155 and BRS 213 presented polymorphism to the primers linked to Lx_1,(Lx_2) locus but did not reveal polymorphism to the marker linked to lx_3 locus.

Therefore, the RIL population $F_{2:3}$ derived from the cross between BR36 and BRS213 were used to map locus Lx_3 and the RIL population $F_{2:3}$ derived from cross between BRS155 and BRS213 were used to map the locus Lx_1(Lx_2).

The poor polymorphism observed in the current study appeared to be quite similar to the one found by other authors [36, 13, 14, 15, 21, 37]. This approach was explained previously based on the fact that the commercial soybean cultivars fell into 17 allelic classes from five genotypes and the pedigree of these genotypes are closely related. Moreover, the use of markers in breeding depends on their being polymorphic, because there is no warranty that the markers identified in one population are polymorphic in different populations.

The level of polymorphism in soybean depends on the genetic background of the parents used to develop the populations, as well as on the type of marker used in a study [36]. Low polymorphism observed in this study could be explained by the fact that all three populations share the similar ancestral, all of them are descended either from Century L1L2 as source of null lipoxygenase [38, 18] or from a selection of this cultivar.

Lenis et al. [14] developed gene specific molecular markers assay for genotyping of three triple null soybean lipoxygenase free breeding lines IA2040LF, 8AR-56061 and 935F203. They concluded that there were no new mutations in the US lines, but the lipoxygenase genes took part in the triple null quality of the ancestral triple null lipoxygenase Jinpumkong 2 [20,33] that the new soybean lines are derived and the genetic recombination was the mechanism that broke the tight repulsion-phase linkage between Lx_1 and Lx_2 loci, allowing the combination of three independent *lx* mutant alleles in the lipoxygenase-free.

The PCR product of parental cultivars BRS 213 and BRS 36 showed polymorphism to the SSR marker Satt212. The SSR marker Satt212 appeared linked and codominant segregation for the 149 RILs (Figure 2). The populations showed normal segregation of 1:2:1 ratio with a Chi-squared value of 3,98 (P = 13,67) (Table 1). It was also observed that the Lx_3 locus segregated independently of the Lx_1 loci. A genetic map was constructed using genotyping data of Satt212 and phenotyping data for Lx_3 locus (Figure 3). The Lx_3 locus showed complete association between the inheritance of homozygous *lx* mutant alleles and the lack of lipoxygenase activity. Lx_3 free has been located on chromosome Gm15 linkage group E in the classical linkage map of soybean [28]. The distance of Satt212 from Lx_3 gene was 24,1 cM, corresponding with the distance assigned by Reinprecht et al. [13]. Though it is the first report of a map position for LOX3 free in Brazilian commercial cultivar. However, the discovery of still closer SSR marker will make the assisted selection for this gene more accurate.

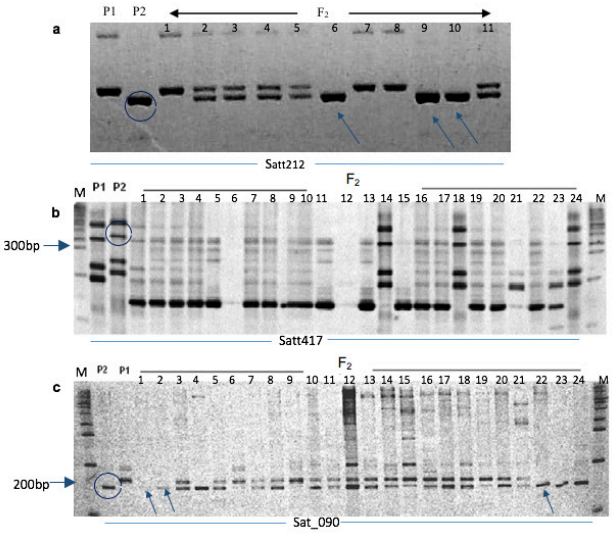

Figure 2. Amplification products of three SSR markers linked to the soybean lipoxygenase gene. M is a 100 bp DNA ladder. a. Segregation pattern of DNA fragments in parents P_1 BR 36 and P_2 BRS 213 $F_{2:3}$ RIL population using SSR markers Satt212, lanes 6, 9 and 10 absence of LOX3; b. Segregation pattern of DNA fragments in parents P_1 BRS 155 and P_2 BRS 213 and F_2 RIL using SSR markers Satt 417, all lines shows presence of LOX1; c. Segregation pattern of DNA fragments in parents P_1 BRS 155 and P_2 BRS 213 and $F_{2:3}$ RIL using SSR markers Sat_090, lines 1, 2, 4 and 22 shows absence of LOX1. The arrow depicts band fragment size of lipoxygenase triple null mutant from BRS 213

DOI: https://doi.org/10.30564/jrb.v3i1.2818

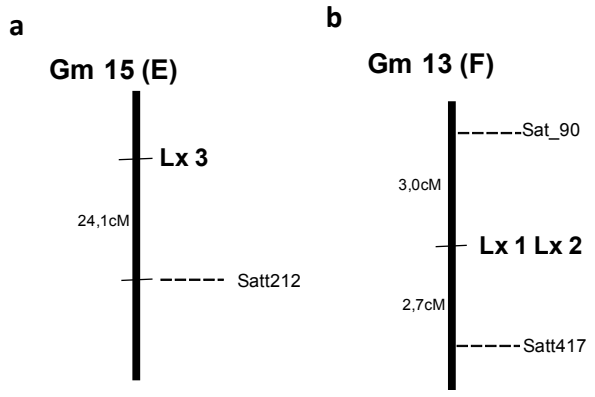

Figure 3. a. Position of the seed lipoxygenase Lx_3 gene mutation-based markers in the $F_{2:3}$ population BRS 213(lx_1, lx_2; lx_3) X BR 36 ($Lx_1Lx_2Lx_3$). Lx_3 is mapped on chromosome Gm15 or linkage group E. b. Position of the seed lipoxygenase Lx_1 and Lx_2 gene mutation-based markers in the $F_{2:3}$ population BRS 213(lx_1, lx_2; lx_3) X BRS 155 ($Lx_1Lx_2Lx_3$). Lx_1 and Lx_2 are mapped on chromosome Gm13 or linkage group F. The linkage map was aligned with the soybean composite_2003 map (www.soybase.org) and linkage map of the soybean genome (Cregan et al., 1999). Name of the locus is indicated on the right side of each chromosome. Marker is connected by dashed line

The markers for the mutation in the Lx_1 gene and those previously reported in the vicinity, Sat_074, Sat_090, Satt395, Sat417, Satt656, were tested for parental polymorphism in the lines BRS 213 and BRS 155 and were found to be polymorphic to the parental lines. The others markers tested did not reveal polymorphism on the parental cultivars. Genotyping of Lx_1 against the 93 RILs was successfully conducted using the SSR markers Sat_090 and Satt417. Sat_090 Showed normal segregation of 1:2:1 ratio with Chi-squared of 0,00 (P = 100%) and Satt417 showed a normal segregation of 1:2:1 ratio with a Chi-squared of 0,297 (P = 86,21%) (Table 1). Representative gel of amplification pattern obtained for Sat_090 and Satt417 using the genomic DNA of the parents BRS 213 and BRS 155 and the $F_{2:3}$ population is shown in Figure 2.

The Lx_1 gene for LOX1 has been mapped to chromosome 13 LG F in the molecular soybean genetic map [28]. A genetic map was constructed using genotyping data of Sat090 and Satt 417. Lx_1 loci has been located on linkage group F in the classical linkage map of soybean [28]. This indicates that Lx_1 is located nearly at the same position as Lx_2 in our study (Figure 3). Sat090 was found to be linked with Lx_1 and Lx_2 loci at a distance of 3,00 cM and 2,77 cM respectively, the two SSR markers are flaking the Lx_1, Lx_2 in interval distance of 5,77 cM (Figure 3). This is in accordance with the observation of Kim et al. [20] and Reinprecht et al. [13, 15].

Satt090 and Satt417, on the same linkage group, presented high efficiency in MAS because they are very close to Lx_1, Lx_2 genes. In addition, the fact that the two markers are flanking the genes increases selection efficiency. Phenotypic selection is influenced by the environment and the number of plants that can be selected is limited, while assisted selection by molecular markers does not have such limitations. Therefore, the SSR markers validated in this study will allow more accurate selection for seed lipoxygenase nulls on base of DNA screens rather than relying on phenotypic expression with the potential to simplify and accelerate selection for further application in the Brazilian breeding program aimed at improving soybean seed quality.

4. Conclusions

The absence of lipoxygenase is due to the action of three recessive genes (Lx_1, Lx_2 and Lx_3) and in the RILs population between BR 36 and BRS 213 and between BRS 155 and BRS 213 the nulls genes come from BRS 213.

The results confirm the linkage of SSR marker Satt212, linkage group E, with the locus Lx_3 in the RILs population derived from BR 36 and BRS 213, at distance of 24.1 cM.

The RILs population derived from BR 36 and BRS 213 did not present polymorphism to the SSR markers of LGF, thus it was not possible mapping Lx_1 and Lx_2 loci.

For the RILs population derived from BRS 213 and BRS 215, the SSR markers Satt090 and Satt417, linkage group F, were found to be linked with the Lx_1, and Lx_2 at a distance of 3,00 cM and 2,77 cM, respectively.

Funding

The authors would like to acknowledge CAPES (Coordination for the Improvement of Higher Education Personnel) and CNPq (Brazilian National Council for Scientific and Technological Development) for the master students grants.

References

[1] CONAB – Companhia Nacional De Abastecimento (2020), "Acompanhamento da safra brasileira de grãos – Safra 2020/2021", CONAB, Brasília, 8(2),1-62.

[2] Hildebrand, D.F. (1989), "Lipoxygenases", Physiol. Plant. 76(2),249-253. https://doi.org/10.1111/j.1399-3054.1989.tb05641.x

[3] Hajika, M., Kitamura, K., Igita, K., Nakazawa, and Y. (1992), "Genetic Relationships among the Genes for Lipoxygenase-1, -2 and -3 Isozymes in Soybean

 DOI: https://doi.org/10.30564/jrb.v3i1.2818

[Glycine max (L.) MERRILL] Seed", Jpn. J. Breed., 42(4), 787-792.

[4] Wang, C., Croft, K.P.C., Jarlfors, U., and Hildebrand, D.F. (1999), "Subcellular localization studies indicate that lipoxygenases 1 to 6 are not involved in lipid mobilization during soybean germination", Plant Physiol. 120(1),227-236.

[5] Hildebrand, D.F., and Hymowitz, T. (1981), "Two soybean genotypes lacking lipoxygenase-1", J. Am. Oil Chem. Soc. 58(5),583-586.

[6] Hildebrand, D.F., and Hymowitz, T. (1982), Inheritance of Lipoxygenase-I activity in soybean seeds, Crop Sci. 22(4),851-853.

[7] Kitamura, K., Davies, C.S., Kaizuma, N., and Nielsen, N.C. (1983), "Genetic analysis of a null-allele for lipoxygenase-3 in soybean seeds", Crop Sci. 23(5),924-927.

[8] Kitamura, K. (1984), "Biochemical of lipoxygenase lacking mutants, L1-Less, L2-Less and L3-Less soybeans", Agricul. Biol. Chem.,48(9),2339-2346.

[9] Kitamura, K., Kumagai, T., and Kikuchi, A. (1985), "Inheritance of lipoxygenase- 2 and genetic relationships among genes for lipoxygenase-1, -2 and -3 isozymes in soybean seeds", Jpn. J. Breed. 35(4),413-42.

[10] Davies, C.S., and Nielsen, N.C. (1986), "Genetic analysis of a null - allele for Lipoxygenase - 2 in Soybean[1]", Crop. Sci. 26(3),460-463.

[11] Davies, C.S., Nielsen, S.S., and Nielsen, N.C. (1987), "Flavor improvement of soybean preparation by genetic removal of lipoxygenase-2", J. Am. Oil Chem. Soc. 64(10),1428-1433.

[12] Wang, W.H., Takano, T., Shibata, D., Kitamura, K., and Takeda, G. (1994), "Molecular basis of a null mutation in soybean lipoxygenase 2: substitution of glutamine for an iron-ligand histidine", Proc. Natl. Acad. Sci. USA 91(13),5828-5832.

[13] Reinprecht, Y., Poysa, V.W., Yu, K., Rajcan, I., Ablett, G.R., and Peter Pauls, K. (2006), "Seed and agronomic QTL in low linolenic acid, lipoxygenase-free soybean (Glycine max (L.) Merrill) germplasm", Genome 49(12),1510-1527.

[14] Lenis, J.M., Gillman, J.D., Lee, J.D., Shannon, J.G., and Bilyeu, K.D. (2010), "Soybean seed lipoxygenase genes: molecular characterization and development of molecular marker assays", Theor. App. Genet. 120,1139-1149.

[15] Reinprecht, Y., Luk-Labey, S.Y., Yu, K., Poysa, V.W., Rajcan, I, Ablett, G.R., and Peter Pauls, K. (2011), "Molecular basis of seed lipoxygenase null traits in soybean line OX948", Theor. Appl. Genet., 122,1247-1264.

[16] Hajika, M., Igita, K., and Kitamura, K. (1991), "A line lacking all the seed lipoxygenase isozymes induced by gamma-ray irradiation", Jpn. J. Breed. 41(3),507-509.

[17] Lee, K.J., Hwang, J.E., Velusamy, V., Ha, B.K., Kim, J.B., Kim, S.H., Ahn, J.W., Kang, S.Y., and Kim, D.S. (2014), "Selection and molecular characterization of a lipoxygenase-free soybean mutant line induced by gamma irradiation", Theor. Appl. Genet. 127(11),2405-13.

[18] Kitamura, K. (1991), "Spontaneous and induced mutations of seed proteins in soybean (Glycine max L. Merrill)", Gamma Field Symposia 30,61-69.

[19] Wang, J., Kuang, H., Zhang, Z., Yan, L., Zhang, M., and Guan, Y. (2020), "Generation of seed lipoxygenase-free soybean using CRISPR-Cas9", Crop J. 8(3),432-439.

[20] Kim, M.Y., Ha, B.K., Jun, T.H., Hwang, E.Y., Van, K., Kuk, Y.I., and Lee, S.K. (2004), "Single nucleotide polymorphism discovery and linkage mapping of lipoxygenase-2 gene Lx2 in soybean", Euphytica 135,169-177.

[21] Shin, J.H., Van, K., Do Kim, K., Lee, Y., Jun, T., and Lee, S. (2012), "Molecular sequence variations of the lipoxygenase-2 gene in soybean", Theor. Appl. Genet. 124,613-22.

[22] Carpentieri-Pipolo, V. (2015), "UEL 175: a novel lipoxygenase-free soybean cultivar with kunitz trypsin inhibitor absence", Crop. Breed. Appl. Biotechnol. 15(3),191-192.

[23] Pípolo, A.E., Almeida, L.A., Kiihl, R.A.S., Carrão-Panizzi, M.C., Gomide, F.B., Miranda, L.C., Arias, C.A.A., Toledo, J.F.F., Kaster, M., Carneiro, G.E.S., Yorinori, J.T., Domit, L.A., Dias, W.P., and Almeida, A.M.R. (2005), "BRS 257 - nova cultivar para alimentação humana",Reuni*ã*o de Pesquisa de Soja da Regi*ã*o Central do Brasil. Cornélio Procópio. Resumos. Londrina: Embrapa Sojap. 371. (Embrapa Soja. Documentos, 257).

[24] Carrão Panizzi, M.C., Pípolo, A.E., Mandarino, J.M.G., Arantes, N.E., Garcia, A., De Toledo Benassi, V., Arias, C.A., Kaster, M., De Oliveira, M.F., Oliveira, M.A., De Toledo, J.F., Moreira, J.U.V. and Carneiro, G.E. De S. (2009), "Breeding specialty soybean cultivars for processing and value-added utilization at Embrapa in Brazil", World Soybean Research Conference, Beijing. Developing A Global Soy Blue Print For A Safe Secure And Sustainable Supply: Proceedings, Chinese Academy Of Agricultural Sciences: Institute Of Crop Science. Beijing, China.

[25] Cruz, C.D. (1997), "Programa Genes - Aplicativo

DOI: https://doi.org/10.30564/jrb.v3i1.2818

Computacional em Genética e Estatística", Viçosa, MG: Editora UFV, v1. 442 p.

[26] Suda, I., Hajika, M., Nishiba, Y., Furuta, E., and Igita, K. (1995), "Simple and rapid method for the selective detection of individual lipoxygenase isozymes in soybean seeds", J. Agric. Food Chem. 43(3),742-747.

[27] Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E., and Newberg, L. (1987), "Mapmaker: an interactive computer package for constructing genetic linkage maps of experimental and natural populations", Genomics 1,174-181.

[28] Cregan, P.B., Jarvik, T., Bush, A.L., Shoemaker, R.C., Lark, K.G., Kahler, A.L., Kaya, N., Van Toai, T.T., Lohnes, D.G., Chung, J., and Specht, J.E. (1999), "An integrated genetic linkage map of the soybean genome", Crop. Sci. 39(5),1464-1490.

[29] Cruz, C.D. (2011), "Programa GQMOL: programa para análise de genética quantitativa e molecular", Viçosa: UFV, http://arquivo.ufv.br/dbg/gqmol/gqmol.htm

[30] Klein et al., 1988 (reference CTAB method DNA extraction from plants)

[31] Song, Q.J., Marek, L.F., Shoemaker, R.C., Lark, K.G., Concibido, V.C., Delannay, X., Specht, J.E., and Cregan, P.B. (2004), "A new integrated genetic linkage map of the soybean", Theor. Appl. Genet. 109,122-8. **DOI: 10.1007/s00122-004-1602-3.**

[32] Grant, D., Nelson, R.T., Cannon, S.B., and Shoemaker, R.C. (2010), "SoyBase, the USDA-ARS soybean genetics and genomics database", Nucleic Acids Res. 38(1), D843-D846. https://doi.org/10.1093/nar/gkp798.

[33] Kim, S.D., Kim, Y.H., Park, K.Y., Kim, H.T., Lee, Y.H., Lee, S.H., Seung, Y.K., Kim, H.S., Hong, E.H., and Kim, Y.S. (1997), "A new beany tasteless soybean variety "Jinpumkong 2" with good quality", RDA J. Crop Sci. 39(2),112-115.

[34] Kim, Y.S., Moon, J.K., Cho, J.H., Kim, H.T., Park, K.Y., and Kim, S.J. (2006), "Improvement of special characters on three elite soybean cultivars by markers-assisted selection", Treat. Crop Sci. 7,197-210.

[35] Sanguineti, C., Dias-Neto, E., and Simpson, A.J.G. (1994), "RAPD silver staining and recovery of PCR products separated on polyacrylamide gels", Biotechniques 17(5),914-921.

[36] Akkaya, M.S., Shoemaker, R.C., Specht, J.E., Bhagway, A.A. and Cregan, P.B. (1995), "Integration of simple sequence repeat DNA markers into a soybean linkage map", Crop. Sci. 35(5), 1439-1445.

[37] Mandal, S., Nandita, S., Rajarani, A.P., and Santha, I.M. (2013), "Molecular cloning, characterization and expression of lipoxygenase 2 (lox-2) isozyme from India soybean [(Glycine max(L.) Merrill] cv. Pusa 16", Indian J. Biochem. Biophys. 50(1),54-63.

[38] Hajika, M., Igita, K., and Kitamura, K. (1995), "Induction of a Soybean (Glycine max (L.) Merrill) line lacking all seed lipoxygenase isozymes" JARQ 29:73-76.

 DOI: https://doi.org/10.30564/jrb.v3i1.2818