**Length polymorphisms of simple sequence repeat (SSR) markers in assisted selection for lipoxygenase-free in soybean**

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**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 Lox1, Lox2 and Lox3 free. The SSR marker Satt212 confirmed the presence of the homozygous null-allele *Lx3* in the cultivar BRS 213, which were used for hybridization with BR 36. Heterozygote F1 hybrid plants and homozygous Lx3 lines in F2 segregating populations were successfully identified. The SSR markers Sat090 and Sat417 was the most effective diagnostic marker among the all SSR markers tested. Satt090 and Satt417 confirmed the presence of the homozygous *Lx2* null-allele in the parental cultivar BRS 213 by flanking *Lx2* loci at 3,00 and 2,77 cM, respectively. The presence of Lx2 null allele in The F2 segregating populations between BRS 213 and BRS 155 were 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.

Index terms: *Glycine max*, lipoxygenase isozymes, molecular markers, markers assisted selection

**Introduction**

Soybean is the most economical source of high-quality protein and low saturated fat and is present in the regular diet of the people in South-East Asia. Soybean seeds has several special bioactive ingredients, like tocopherols, isoﬂavones, saponins, that can stave oﬀ the onset of chronic diseases like cancer, atherosclerosis, diabetes, Parkinson etc. In the recent past, soy food is gaining acceptance and is being recognized as the ‘functional food’ of the century. Health-conscious consumers have changed on eating habits and preferences specially in the middle and upper classes. The population aging, the quest for a healthier life, have started using soybean as alternative in their diet because it reduces the risk of cardiovascular disease, and the worldwide demand for food-quality soybeans has increased. As a result, soybean cultivars with both desirable seed composition traits and superior agronomic characteristics are growing market and cultivars of food type are on the demand.

Brazil is the second largest producer with 115.072,5 million of tons produced in 2019, behind only the United States with 123,664 million tons (CONAB, 2020). However, less than 5% of the total soybean produced in the country is processed into soy foods. The reason is due the physiology process after post-harvest for many seeds with high levels of lipoxygenase (LOX) enzymes and linoleic acid such as the production of hexanal that even when present in foods in very low concentrations (e.g. 5 ppb), has a highly undesirable odor and therefore flavor, creating great difficulties in the production of acceptable food products containing soybean. Lipoxygenase (linoleate: oxygen oxidoredutase EC 1.13.11.12) mediated oxidation of polyunsaturated fatty acids in mature soya seeds results in unpleasant therefore lipoxygenase-free seed soybean cultivars specially developed for human consumption can contribute to increases the acceptability of soy foods, are of significant importance to the food industry and are in demand. The major reason of this off-flavor is an iron containing dioxygenase that catalyzes the oxidation of unsaturated fatty acids resulting in the formation of aldehyde and ketone compounds (Hildebrand, 1989).

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, and can generate 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 fields of plant physiology where lipoxygenases have been implicated: growth and development, senescence, and insect wound resistance response and pest resistance. Regardless of the actual role lipoxygenases may play in the physiology of soybean seeds, lipoxygenase-free seeds have been demonstrated to develop into normal plants without defect (Hajika et al. 1992; Wang et al. 1999).

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: *Lx1*, *Lx2* and *Lx3*. The inheritance and molecular base of lipoxygenase nulls have been investigated in different backgrounds for several authors (Hildebrand and Hymowitz, 1981; Hildebrand and Hymowitz 1982; Kitamura *et al*. 1983; Kitamura, 1984; Kitamura et al., 1985; Davies and Nielsen, 1986; Davies et al., 1987; Wang et al. 1994; Reinprecht et al. 2006; Lenis et al. 2010; Reinprecht et al. 2011). Spontaneous mutants of theenzymes have been identified and several mutants were developed by gamma radiation (Hajika et al., 1991; Lee et al 2014). The *Lx1* and *Lx2* loci were found to be in tight genetic link and mapped in the same linkage group, while *Lx3* locus is independent (Kitamura et al. 1983).

The development of seed lipoxygenase-free triple null mutant genotype was reported by Hajika et al. (1991); Kitamura (1991) and more recently Wang et al (2020) have reported that was generated by clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9) triple nulls mutants plants carrying gmlox1gmlox2gmlox3 triple mutations. The authors described that the original repulsion-phase linkage in independent sources of mutant alleles at the *Lx1* and *Lx2* loci was broken, resulting in a coupling-phase linkage that eventually led to the development of a triple null lipoxygenase genotype. However, they mentioned that the mechanism involved in the induction of the genotype lacking the three seed lipoxygenases was unknown.

The development of seed lipoxygenase free soybean cultivars requires accurate evaluation assays to select for the appropriate genotypes. The presence of lipoxygenases in breeding programs has been determined by laborious and time-consuming seed phenotype methods. In addition, these procedures are not able to discriminate between individuals that are homozygous and heterozygous in the same generation. 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 breeding efforts for soybean cultivars with improved flavor (Reinprecht et al., 2006, Lenis et al., 2010, Reinprecht et al., 2011; Kim et al., 2004; Shin et al., 2012; Carpentieri-Pipolo, 2015).

This study was undertaken to evaluated 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.

**Material and methods**

*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. F2:3 derived populations of168 RILs were developed by single seed descent from BRS 213 X BR 36 and F2:3 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 soyfood 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 F2 plants from a cross between two double mutants: *Lx1-* and *Lx3*-free and *Lx2*- and *Lx3*-free (Hajika et al., 1991; Kitamura et al., 1991).

The Brazilian cultivar BR 36 (IAS 4(2) X BR 78-22043) is dominant for the three seeds lipoxygenase genes (*Lx1, Lx2*, *Lx3*). 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 (Pípolo et al. 2005). BRS 155 (IAS 4(2) X PI 157440) is a Brazilian cultivar that was released for soyfood uses, it has the allele ti 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 tripsin inhibitor Kunitz that affects protein digestibility (Carrão-Panizzi et al. 2009). 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 F1 plants were obtained in the winter of 2012. Authentication of hybrid origin of F1 plants was carried out and they were compared with their female parental. Freshly harvested seeds of these cultivars were analyzed for lipoxygenase content. The data obtained were analyzed statistically 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 (Cruz, 1997).

*Detection of seed lipoxygenases*

To confirm the status of F1 seeds and to check the segregation of F2:3 seeds, non-destructive colorimetric analysis of LOX1 and LOX3 were performed by a procedure based on slightly modified colorimetric method of Suda et al. (1995) (Kitamura et al. 1985) 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 buffer, 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 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 buffer, pH 6.8, 4% (w/v) -carotene dissolved in 50% (v/v) acetone, and 1.5 mM sodium linoleate. Bleaching of the -carotene indicated the presence of LOX3.

Null F2:3 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) -mercaptoethanol and 10% (v/v) glycerol] and separated by discontinuous (10% - 4%) SDS–PAGE (Kitamura et al. 1983). Genotyping was performed by creating a binary array that was used for statistical analysis and construction of the genetic map.

*Statistical Analysis*

The individual segregation of seed lipoxygenases resulted from colorimetric analyses was tested using the chi-square test. Genetic distances between markers were estimated using the Mapmaker EXP V. 3.0 program (Lander et al., 1987**)**. A minimum logarithm of odds (LOD) score of 3.0and 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 (Cregan et al., 1999)**.** All analyses were carried out by GQMOL program (Cruz, 2011).

*Genotyping assays*

The DNA was extracted from the soybean leaves by the CTAB method (Klein et al., 1988), 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. (1999), Song et al. (2004) and Grant et al. (2010) 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 (Grant et al., 2010). The primers were chosen because they had presented polymorphism in previous studies (Kim et al., 1997; 2004; 2006; Reinprecht et al., 2006).

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 the Sanguineti et al. (1994).

**Results and Discussion**

The phenotypes for LOX1 and LOX3 of the progenitors used in this work and of F2:3 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 (+).

Results indicate a perfect association between the inheritance of homozygous *lx1*  or *lx3* and the lack of LOX1 or LOX3 activity ( Table1).

The segregation of the F2: RILs 3 seeds derived from the cross between the triple null BRS 213 and BR 36 (dominant for the three lipoxygenase) a phenotypic ratio for LOX1and 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 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 (*Lx1* and *Lx3*), expressed in the Brazilian parental soybean cultivars BRS 213, BR 36 and in F2:3 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). *Lx1* and *Lx2* genes are tightly linked and inherited together and the *Lx1* and *Lx2* genes are mapped as single major genes to the same location on chromosome Gm13 (LG F) while *Lx3* gene is independently inherited, mapped on chromosome Gm15 (LG E) (Reinprecht 2002; Reinprecht et al., 2006). Kim et al. (2004; 2006) found the *Lx2* locus positioned on one end of chromosome 13 (LG F), flanked by the SSR markers Satt522 and Sat074 and the *Lx3* locus at the linkage group M next to the marker Satt150. However, Reinprecht et al. (2006) found *Lx2* locus flanked by the SSR markers Sat\_090 and Sat\_074 on chromossome 13 (LG F) and the loci *Lx3*was mapped on the chromossome 15 (linkage group E) next to the Satt212 (Reinprecht et al., 2006; Lenis et al., 2010 and Reinprecht et al 2011). Thus, in order to validate the SSR markers reported previously by Kim et al. (1997), Kim et al. (2004), Reinprecht et al. (2006), Lenis et al., (2010) and Reinprecht et al (2011), we surveyed the DNA polymorphism in the parents cultivars and RIL F2:3 derived population generated from the cross between BRS 213 (*lx1lx2lx3*) and BR36 (*Lx1Lx2Lx3*) and BRS 213 (*lx1lx2lx3*) and BRS 155 (*Lx1Lx2Lx3*). Additional SSR markers in the neighboring genomic region of *Lx1* and *Lx3*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 F2:3 population and the data were excluded. The SSR primers pair previously used by Kim et al. (1997 and 1984) linked with *Lx1* (*Lx2)* have shown polymorphism in the parental lines BRS 213 and BR 36 but did not in the RIL F2:3 derived population. However, the RIL F2:3  population derived from cross BRS155 and BRS 213 presented polymorphism to the primers linked to *Lx1,*( *Lx2* *)* locus but did not reveal polymorphism to the marker linked to *lx3* locus.

 Therefore, the RIL population F2:3  derived from the cross between BR36 and BRS BRS213 were used to map locus *Lx3* and the RIL population F2:3  derived from cross between BRS155 and BRS BRS213 were used to map the locus *Lx*1*( Lx2)*.

The poor polymorphism observed in the current study appeared to be quite similar to the one found by other authors (Akkaya et al., 1995; Reinprecht et al., 2006; Lenis et al., 2010; Reinprecht et al., 2011; Shin et al., 2012; Mandal et al., 2013). This approach was explained previously based on the fact of 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 (Akkaya et al., 1995). 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 (Hajika et al., 1995; Kitamura et al., 1991) or from a selection of this cultivar.

Lenis et al. (2010) 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 (Kim el al., 1997; 2004) that the new soybean lines are derived and the genetic recombination was the mechanism that broke the tight repulsion-phase linkage between *Lx1* and *Lx2*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 *Lx1*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. *Lx3* free has been located on chromosome Gm15 linkage group E in the classical linkage map of soybean (Cregan et al., 1999). The distance of Satt212 from *Lx*3 gene was 24,1 cM, corresponding with the distance assigned by Reinprecht et al. (2006). Though it is the first report of a map position for LOX3 free in Brazilian commercial cultivar. However, the discover of still closer SSR marker will make the assisted selection for this gene more accurate.

The markers for the mutation in the *Lx1* 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 *Lx1* 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 F2:3 population is shown in Figure 2.

The *Lx1* gene for LOX1 has been mapped to chromosome 13 LG F in the molecular soybean genetic map (Cregan et al., 1999). A genetic map was constructed using genotyping data of Sat090 and Satt 417. *Lx1* loci has been located on linkage group F in the classical linkage map of soybean (Cregan et al., 1999). This indicates that *Lx1* is located nearly at the same position as *Lx2* in our study (Figure 3). Sat090 was found to be linked with *Lx1* and *Lx2* loci at a distance of 3,00 cM and 2,77 cM respectively, the two SSR markers are flaking the *Lx1, Lx2* in interval distance of 5,77 cM (Figure 3). This is in accordance with the observation of Kim et al. (2004) and Reinprecht et al. (2006; 2011).

Satt090 and Satt417, on the same linkage group, presented high efficiency in MAS because they are very close to *Lx1*, *Lx2* 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.

**Conclusions**

The absence of lipoxygenase is due to the action of three recessive genes (*Lx1, Lx2 and Lx3*) and in the RILs population between BR 36 and BRS 213 and between BRS 155 and BRS 2013 the nulls genes come from BRS213.

The results confirm the linkage of SSR marker Satt212, linkage group E, with the locus *Lx3 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 LG F, thus it was not possible mapping *Lx1* and *Lx2* loci.

For the RILs population derived from BRS 213 and BRS215, the SSR markers Satt090 and Satt417, linkage group F, were found to be linked with the *Lx1,*and *Lx2* at a distance of 3,00 cM and 2,77 cM, respectively.



**Figure 1.** Colorimetric assay of soybean seed lipoxygenase enzymes for soybean in F2:3 population from the cross between the soybean cultivars BR 36 and BRS 213, and between BRS155 and 213. **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 (+).

**Table 1**. Colorimetric determination of presence or absence of LOX1 and LOX3 activity in F2:3 soybean seeds population from the cross between the cultivars BR 36 and BRS 213 and between BRS BRS155 and 213.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Population F2:3 | Phenotype | Genotype | Expected a | Observed b | *X*2 | P |
| BR 36 X BRS213 | Presence LOX1+LOX3+And heterozygous LOX1+LOX3- | *Lx1Lx1Lx3Lx3* and *Lx1lx1Lx3lx3* | 157,5 | 153 | 5,76ns | 0,12 |
|  | Absence  LOX1-LOX3- | *lx1lx1lx3lx3* | 10,5 | 15 |  |  |
| BR 155 X BRS213 | Presence LOX1+  | *Lx1Lx1* and *Lx1lx1* | 70,0 | 69 | 0,032ns | 0,86 |
|  | Absence  LOX1- | *lx1lx1* | 23,5 | 24 |  |  |

aExpected segregation ratio, bObserved segregation ratio; *X*2 Chi-square value, nsNon 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 F2:3 population from the cross between the soybean cultivars BR 36 and BRS 213, and between BRS155 and 213.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| F2:3 Population | LG | Marker | Genotype | Expecteda | Observed b | *X*2 | P |
| BR36XBRS213 | F | Satt212 | *lx1lx1,lx3lx3* | 38,75:77,50:38,75 | 27:79:43 | 3,98ns | 0,14 |
|  |  |  |  |  |  |  |  |
| BRS155XBRS213 | E | Satt417 | *lx1lx1* | 22,75:45,50:22,75 | 22:44:25 | 0,29ns | 0,86 |
|  |  | Sat\_090 | *lx1lx1* | 23:46:23 | 23:46:23 | 0,00ns | 1,00 |

LG: Linkage group of linkage map of the soybean genome (Cregan et al., 1999); aExpected segregation ratio for co-dominant markers, *X*2 Chi-square value, nsNon significant at 1% probability; P: probability.

**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 P1 BR 36 and P2 BRS 213 F2:3 RIL population using SSR markers Satt212, lanes 6, 9 and 10 absence of LOX3; **b**. Segregation pattern of DNA fragments in parents P1 BRS 155 and P2 BRS 213 and F2 RIL using SSR markers Satt 417, all lanes shows presence of LOX1; **c**. Segregation pattern of DNA fragments in parents P1 BRS 155 and P2 BRS 213 and F2:3 RIL using SSR markers Sat\_090, lanes 1, 2, 4 and 22 shows absence of LOX1. The arrow depicts band fragment size of lipoxygenase triple null mutant from BRS 213.



**Figure 3.** **a.** Position of the seed lipoxygenase *Lx3* gene mutation-based markers in the F2:3 population BRS 213(*lx1*, *lx*2; *lx3*) X BR 36 (*Lx1Lx2Lx3*). *Lx3* is mapped on chromosome Gm15 or linkage group E. **b**. Position of the seed lipoxygenase *Lx1* and*Lx2* gene mutation-based markers in the F2:3 population BRS 213(*lx1*, *lx*2; *lx3*) X BRS 155 (*Lx1Lx2Lx3*). *Lx1* and*Lx2* are mapped on chromosome Gm13 or linkage group F. The linkage map was aligned with the soybean composite\_2003 map ([www.soybase.org](http://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.

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