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Genetic removal of trypsin inhibitor and lipoxygenase isozymes form soybean seeds (*Glycine max*) by simple sequence repeat marker assisted selection

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Genet. Mol. Res. 22 (3): gmr19145
Received March 08, 2023
Accepted June 29, 2023
Published August 24, 2023
DOI http://dx.doi.org/10.4238/gmr19145

ABSTRACT. Kunitz trypsin inhibitor (KTI), which affects protein digestibility and lipoxygenase isozymes, responsible for the off-flavor associated with soy-based foods, are two undesirable factors present in soybean seeds. These unpleasant factors are usually inactivated by heat treatment. However, heat treatment does not completely eliminate these factors; in addition it may decrease protein solubility and may incur extra energy costs. Genetic elimination of these factors could be an alternative to heat treatment. This study aimed to select soybean lines free of KTI and lipoxygenase isozymes in the seeds. The population under study was obtained by crossing the BRS 213 cultivar, which shows low lipoxygenase activity, with BRS 155, a KTI lacking cultivar. $F_{2:3}$ hybrid populations were selected and analyzed using DNA markers for the identification of recessive alleles that encode the absence of KTI and the three lipoxygenase enzymes (LOX1, LOX2 and LOX3). $F_{2:3}$ segregating populations were successfully identified with the KTI specific marker with 100% efficiency. However,

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the *KTi/kti*-gene-specific marker does not allow for the identification of heterozygous and homozygous genotypes with dominant *KTi* alleles; the the simple sequence repeat (SSR) markers Satt228 and Satt409, which bind tightly to the *KTi* locus, were much more suitable diagnostic marker for screening plants in segregating populations. 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 SSR markers used in this study could be efficiently used in marker assisted selection in a breeding program aimed at improving soybean seed quality.

Key words: *Glycine max*; Kunitz trypsin inhibitor; Lipoxygenase isozymes; Molecular markers; Soybean

INTRODUCTION

Soybean (*Glycine max*) is a source of high quality oil and protein for food and feed and is one of the most important and widely consumed legume crops in the world. Brazil is the second largest producer with 115.072.5 million tons produced in 2020, behind only the United States with 123.664 million tons (CONAB, 2021).

The majority of soybean produced is used or exported to be used in animal feed; less than 5% of the soybeans produced in Brazil are processed into soy food. However, raw soybean cannot be used for monogastric animal feeding because of the presence of anti-nutritional factors that decrease its nutritional value. The major anti-nutritional factor in soybean seeds is the Kunitz trypsin protease inhibitor (KTI), a member of the serine protease inhibitor family that can inhibit the activity of trypsin (Gupta, 1987; Liener, 1989).

Trypsin inhibitor activity in soybean seeds feeding poultry and livestock inhibits their growth and weight gain and causes pancreatic hypertrophy. For soybean seeds with high KTI, a preliminary heat, before use of the seeds is recommended, heat treatment effectively destroys is required to inactivate the trypsin inhibitor enzyme. This procedure has the disadvantage of decreased levels of available amino acids with additional increasing final product cost (Liencer, 1995; Palacios et al, 2004; Chung, 2011; Kumar et al, 2013; 2015).

The organization and regulation of the genes encoding KTI have been elucidated (Orf & Hymowitz, 1979; Walling et al., 1986; Jofuku et al., 1989; Krishnan, 2001; Moraes et al., 2006; Kumar et al., 2015; Bulatova et al., 2019; Choi et al., 2020). Although soybean, during the plant life cycle, expresses at least 10 distinct genes for KTI, Kti_3 is the mutant gene that encodes the predominant trypsin inhibitor in soybean seeds (Jofuku et al., 1989).

The reason soy-based foods are not appreciated in many western nations, e.g. Brazil and North America is due to undesirable grassy and beany flavors in foods containing soybean due to oxidation of polyunsaturated fatty acids resulting from seed lipoxygenase activity that even at low concentrations confers unpleasant odor and flavor (Hajika et al. 1991, Hajika et al. 1992, Carrão-Panizzi and Kitamura 1995; Carpentieri-Pipolo, 2015; Carpentieri-Pipolo et al 2021). Thus, soy products made of soybeans with lipoxygenases-free seeds specially developed for human consumption can contribute to increase the

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acceptability of soy foods, for this reason they are becoming important to the food industry and are in demand.

The consumption of legumes is also in evidence in vegetarian and vegan diets and in those looking for health. According to the GFI (2021) there is a global trend that shows the plant-based market reducing the consumption of animal protein in substitution by alternative Brazilian proteins. This new habit represents an influential consumer group, which increased from 29% in 2018 to 50% in 2018.

Mature soybean seeds contain three forms of lipoxygenase isozymes, i.e., lipoxygenase-1 (LOX1), lipoxygenase-2 (LOX2) and lipoxygenase-3 (LOX3). They follow the principle of Mendelian inheritance and are encoded by three dominant genes Lx_1, Lx_2 and Lx_3. The inheritance and molecular base of lipoxygenase nulls have been investigated in different backgrounds by 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; Hajika et al., 199, Wang et al. 1994; Reinprecht et al. 2006; Lenis et al. 2010; Reinprecht et al. 2011; Lee et al 2014. Moreover, recently, the successful application of the CRISPRCas9 system for development of targeted soybean lipoxygenase free mutagenesis of three Lox genes (*Lox1*, *Lox2*, and *Lox3*) has been reported by Wang et al (2020). Loci were knocked out in an elite cultivar Huachun 6 and transgene-free mutants were obtained by screening the T2 generation of lipoxygenase-free mutant lines (GmLox-28 and GmLox-60).

Soybean lines with null lipoxygenase enzymes and trypsin inhibitor absence have also been developed using DNA markers as a screening procedure on in breeding programs for assisted selection (MAS), achieving success in accelerate and simplify breeding efforts (Reinprecht et al., 2006, Lenis et al., 2010, Reinprecht et al., 2011; Kim et al., 2004; Shin et al., 2012; Carpentieri-Pipolo, 2015; Carpentieri-Pipolo et al 2020).

This study was undertaken to evaluate the efficiency of microsatellite markers, previously identified as being associated with a lack of lipoxygenase and KTI antinutritional factor to be used as a germplasm resource in Brazilian soybean breeding programs focused on generation of new cultivars with improved flavor and nutritional quality.

MATERIAL AND METHODS

Plant material and development of segregating population

Ninety three recombinant $F_{2:3}$ progenies derived from the soybean cultivars BRS 213 and BRS 155 were developed by single seed descent method and were grown at greenhouse at Agronomy Department of State University of Londrina, Londrina – PR, at 23º22' latitude south. The parentals 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. BR 213 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 (Hajika et al., 1991; Kitamura et al., 1991). 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 Kunitz trypsin inhibitor 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.

Authentication of hybrid origin of F_1 plants was carried out and they were compared with their female parents. 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 (Cruz, 1997). $F_{2:3}$ family phenotyping and genotyping tests were performed at the Laboratory of Biotechnology of Brazilian Agricultural Research Corporation, Embrapa Soybean, Londrina - PR.

Detection of seed lipoxygenases

To confirm the status 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 Kitamura et al. (1985) and Suda et al. (1995) and one 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 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 buffer, pH 6.8, 4% (w/v) β-carotene were 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) β-mercaptoethanol and 10% (v/v) glycerol] and separated by discontinuous (10% - 4%) SDS–PAGE (Kitamura et al., 1983).

Extraction of seed proteins and SDS-PAGE analysis

Ten milligrams of each seed were used for protein extraction with 500μL of 50 mM Tris-HCl buffer, pH 8.2, containing 10 mM CaCl2. After centrifugation at 13.600 g for 15 min, the supernatant was used for the SDS-PAGE analysis. SDS-PAGE analysis was performed according to the discontinuous system described by Laemmli (1970) with slight modifications proposed by Moraes et al (2006). The separation gel consisted of 14% polyacrylamide in Tris-HCl 0.9 M, pH 8.8, containing 0.1% SDS. The stacking gel consisted of 6% polyacrylamide in Tris- HCl 0.15 M, pH 6.8, containing 0.1% SDS. The

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electrophoresis was conducted for 5 hours at 120 V in Tris-HCl 0.05 M buffer, pH 8.3, containing 0.192 M glycine and 0.1% SDS. The proteins were visualized after staining with 0.15% Coomassie Blue R-250 in 45% methanol and 9% acetic acid and destaining in a 7.5% acetic acid and 25% methanol solution.

Genotyping assays

DNA was extracted from soybean leaves of each 93 $F_{2:3}$ family by the CTAB method (Keim et al., 1988), quantified in a spectrophotometer, and stored at 4°C until use. Identification of genetic constitution at *KTi* locus (*KTiKTi, KTikti* and *ktikti*) of samples was carried out, based on the published sequence of soybean trypsin inhibitor *KTi3* gene (GenBank accession), using both *KTi* null allele-specific marker (Forward: 5'–CTTTTGTGCCTTCACCACCT–3' and reverse: 5'–GAATTCATCATCAGAAACTCTA–3' reverse) and linked SSR marker Satt228, Satt 333, Satt 409 and Satt 429 were used (Krishnan, 2001; Moraes et al., 2006; Kim et al., 2006).

Twenty-two pairs of soybean primers flanking the *Lx1*, *Lx2* and *Lx3* microsatellite regions, previously published (Kim et al., 1997; Kim et al., 2004; Kim et al., 2006; Reinprecht et al., 2006) were selected. 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 SSR markers linked to the *kti* and *Lx1* (*Lx2*) locus are positioned in A2 and F, respectively, linkage group (LGs) corresponding consensus map for soybean (Cregan et al., 1999; Grant et al., 2010). The sequences of the Forward and Reverse SSR markers, used in this study, were obtained at the SoyBase, USDA-ARS Soybean Genetics and Genomics Database (Grant at al., 2010) and primer markers were synthesized by Bio Synthesis Inc., Texas, USA.

Markers that generated polymorphisms among the parents were amplified in $F_{2:3}$ plants of each 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 deoxynucleotide; 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 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 ethidium bromide, following Sanguineti et al. (1994).

Statistical Analysis

The individual segregation of seed lipoxygenases resulting from colorimetric analyses was tested using the chi-square test. Phenotype data obtained were analyzed with the Genes program (Cruz, 2006). 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 3.0 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 (Creegan et al., 1999). All analyses were carried out by the GQMOL program (Cruz, 2011).

RESULTS AND DISCUSSION

Electrophoresis of seed storage proteins of the parents and the $F_{2:3}$ seeds are shown in Figure 1. The electrophoretic banding pattern from the studied soybean seeds revealed the presence of the polypeptide band at 21.5 kDa position, corresponding to the kunitz trypsin inhibitor protein (KTI) present in the parent cultivar in BRS 213 but absent in BRS155.The segregation of $F_{2:3}$ seeds for presence or absence of kunitz trypsin inhibitor protein obtained by SDS-PAGE analyze in the population BRS 213 (*KTiKTi*) x BRS 155 (*ktikti*) is given in Table 1. Out of the 93 $F_{2:3}$ seeds, 70 exhibited presence of kunitz trypsin inhibitor polypeptide while remaining 23 lacked kunitz trypsin inhibitor polypeptide (X^2=0.04, P=95%) (Table1). These observations fit a 3:1 ratio proving that the character is controlled by a single gene.

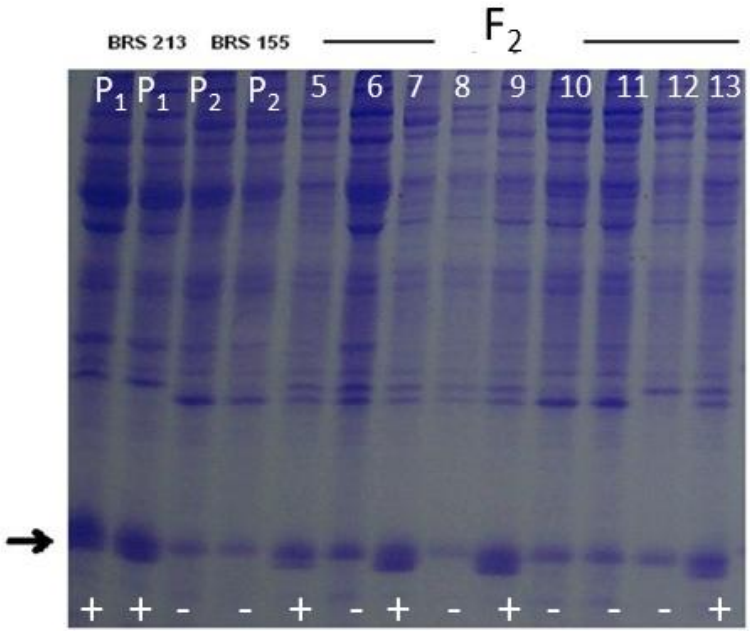

Figure 1. SDS-PAGE analyses of kunitz trypsin inhibitor KTI in soybean seeds. Samples P_1 and P_2 are the progenitors BRS 213 and BRS 155 respectively, and 5 to 13 the individuals from the $F_{2:3}$ RIL segregating population. The seeds 6, 8, 10, 11 and 12, do not express the *Kti* gene, therefore they are recessive homozygotes (-). The samples 5, 7, 9 and 13 are dominant homozygotes (+). The band for the 21 kDa molecular component KTI is indicated by an arrow.

Molecular analysis of *Kti* locus encoding KTI was carried out with four SSR markers located on linkage group A2 of the USDA/Iowa State University soybean molecular linkage map (Cregan et al. 1999), the markers were selected on base published data (Kim et al 2006, Moraes et al 2006 and Bulatova et al 2019). Kim et al. (2006) reported Satt228 and Satt409 to be tightly linked in the mapping populations Jinpumkomg2 x C242

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(source of null allele of kunitz trypsin inhibitor protein) and Clark x C242. Bulatova et al. (2019) assessed the populations derived from two soybean cultivars Lastochka x Ascasubi and Lastochka x Hilario for kunitz trypsin inhibitor protein with biochemical and molecular markers (Satt 228, Satt409 and *KTi/kti* gene specific). They found that the two soybean cultivars from Italy Hilario and Ascasubi, showed the lowest levels of trypsin units inhibited, and $F_{2:3}$ segregating populations were successfully identified using Satt228, and it confirmed the presence of the homozygous null-allele *kti/kti* in cultivars Ascasubi and Hilario. We studied the *Kti* locus and the gene *KTi/kti* with *Kti* locus. In addition, two SSR Markers, Satt333 and Satt429, in the vicinity of Satt228 and Satt409 were also tested.

Amplification products of these markers during PCR analysis with DNA from soybean cultivar with low KTI (BRS155) and cultivar BRS 213 with high KTI are shown in Figure 2. All four polymorphic markers Satt228, Satt333, Satt409 and Satt429, used for genotyping $F_{2:3}$ individuals, clearly distinguished between soybean genotypes on the basis of the *Kti* alleles and showed a normal segregation of 1:2:1 (Table 1).

Table 1. Observed and expected segregation analysis of SSR markers in the $F_{2:3}$ RIL population from soybean cultivars BRS 213 and BRS155, for the presence or absence of kunitz trypsin inhibitor *KTi/kti* and lipoxygenases Lx_1, Lx_2 and Lx_3 genes.

Population $F_{2:3}$	Genotype/ SSR Marker	N[a]	Observed[b]	Expected[c]	X^2	P-value (%)
BRS213xBRS155	*KTi/kti*	93	70:23	3:1	0.04	95.l2
	Satt228	93	28:38:22	1:2:1	2.455	29.3
	Satt333	93	28:46:12	1:2:1	6.372*	4.13
	Satt429	93	30:36:23	1:2:1	4.348	11.37
	Satt409	93	24:46:17	1:2:1	1.414	49.31
	KTi/k*ti*, gene specific marker	93	32:38:22	1:2:1	6.32*	
BRS213xBRS155	Lx_1, Lx_2	93	69:24	3:1	0.032 [ns]	85.74
	Sat_090	92	23:46:23	1:2:1	0.000	100.00
	Satt417	91	22:44:25	1:2:1	0.297	86.21

[a] Total number of individuals observed; [b] observed segregation ratio for co-dominant marker; [c] expected ratio; X^2 Chi –square value; P: probability; * significant at 1% probability; [ns]non significant at 1% probability.

PCR analysis of $F_{2:3}$ of hybrid plants in the presence of both parents confirmed that plants of cultivar BRS 213 have genotypes with the dominant alleles *KTi/KTi*, while with plants of BRS 155 are homozygotes with recessive null-allele *kti/kti*. Among progenies of the $F_{2:3}$ hybrid population, all genotypes with the dominant and recessive alleles of the *KTi* locus were identified (Figure 2). However, *KTi/kti*-gene-specific marker does not allow for the identification of homo- and heterozygous genotypes with dominant alleles *KTi*, which makes it difficult to apply this marker in segregating populations (Figure 2). In contrast, the SSR marker Satt228 and Satt409, tightly to *KTi* locus, was a much more suitable diagnostic marker for the further screening of plants in segregating populations, for the identification of homozygote progenies with null-allele *KTi* and production of the best non-segregating breeding lines with low KTI in seeds. In the hybrid combination, BRS 213 x BRS 155, PCR analysis of DNA from $F_{2:3}$ plants and SSR marker Satt228 and Satt409 confirmed the presence of the null-allele *kti* from parental BRS 155, as well as segregants with null-alleles, *KTi/kti*, they showed very clear presence/absence of bands in a simple discrimination pattern.

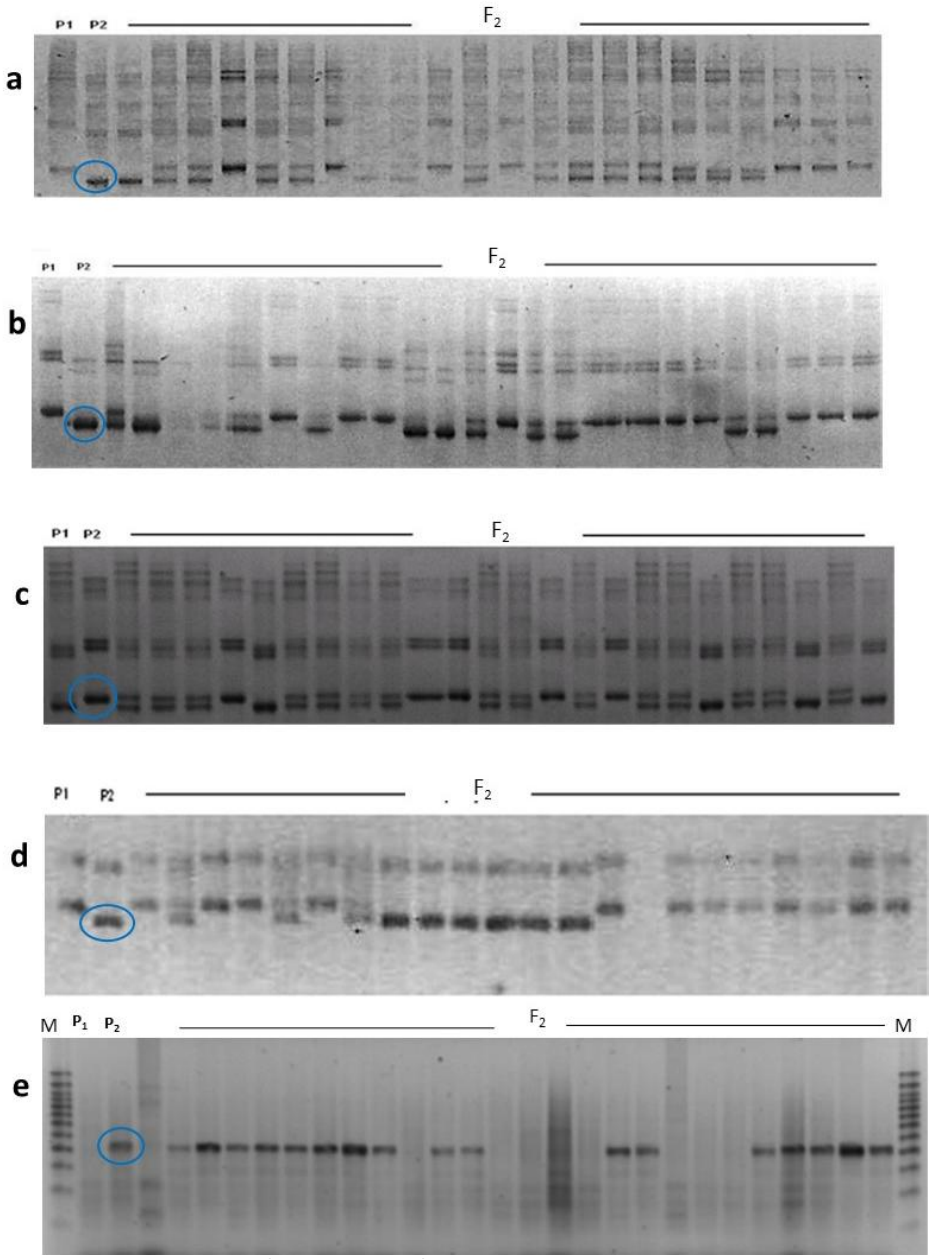

Figure 2. Agarose gel electrophoresis of PCR products for kunitz trypsin inhibitor *Kti* gene in $F_{2:3}$ population derived from the cross of BRS 213 x BRS 155. The samples P_1 (dominant homozygote) and P_2 (recessive homozygote) are the progenitors BRS 213 (*KTiKTi*) and BRS 155 (*ktikti*) respectively, whereas $F_{2:3}$ are individuals of the segregating population BRS 213 x BRS 155. M, Marker 50 bp (DNA Ladder, 50-1000 bp). Segregation pattern of DNA fragments in parents P_1 BRS 213 and P_2 BRS 155 and $F_{2:3}$ using SSR markers (a) Satt228, (b) Satt333, (c) Satt409, (d) Satt429 and (e) *KTi/kti*-gene specific marker. Recessive homozygote DNA fragments from parental BRS155 are indicated by blue circle.

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The comparison of segregation analyses using seed storage proteins and the molecular SSR marker Satt228, S488 and the gene specific *KTi/kti* locus revealed full consensus and confirmed the Mendelian monogenic-type inheritance in the studied $F_{2:3}$ populations of BRS 213 and BRS 155 (Table 1). Thus, they can be used for the selection of genotypes with the null allele of the *KTi* locus in seeds. SSR markers are well known as being polymorphic, with codominant inheritance, and therefore can be widely used for genotype identification and selection of desired traits (Bulatova et al., 2019).

A genetic map was constructed using genotyping data of Satt228, Satt333, Satt409 and Satt429, linking the *KTi* locus at a distance of 4.7 cM (Figure 3). The distance between the SSR marker and the gene of interest can vary depending on the type of population (Kim et al., 2004; Kim et al., 2006; Kumar et al., 2015; Bulatova et al., 2019). For example the genetic distance between Satt228 and *Ti3* varied between 0 and 3.7 cM in two different populations. Another codominant SSR marker Satt409 could also be successfully used instead but it was mapped to a region more genetically distant from the *Ti3* locus at 4.5–21.9 cM (M), meaning that there would be a greater chance of unwanted recombinants (Bulatova et al. 2019). Kim et al. (2006) mapped Satt409 at a distance of 4.5 cM from *KTi* locus in mapping population derived from Jinpumkomg2 x C242. However, in the other population derived from Clark x C242, a mapping distance of 18.2 cM for Satt409 and *KTi* locus was observed.

Therefore, either electrophoresis of storage proteins or SSR markers can support the reliable selection of new promising breeding lines, and the choice will depend on the cost or convenience as preferred by researchers.

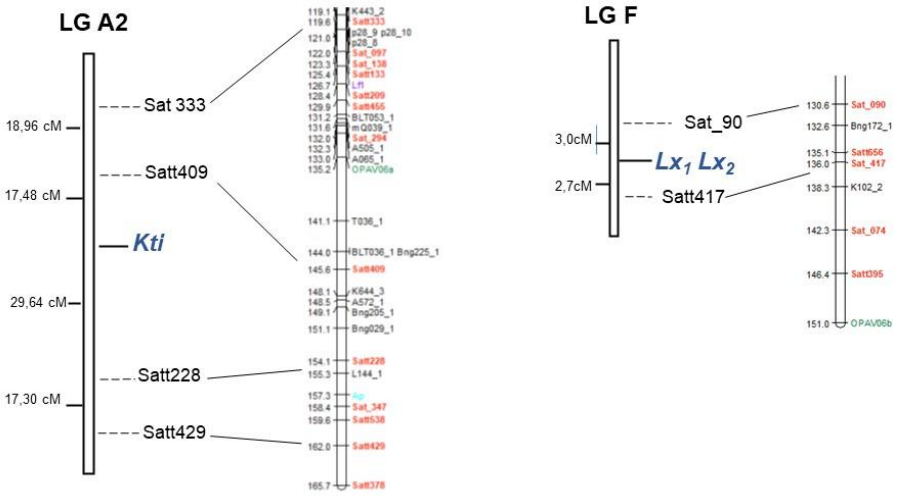

Figure 3. Molecular linkage map for kunitz trypsin inhibitor *Kti* and lipoxygenase Lx_1 and Lx_2 locus using $F_{2:3}$ population derived from the cross of BRS 213 x BRS 155. Position of the *Kti* gene based markers in the $F_{2:3}$ population (BRS 213 (*KTiKTi*) x BRS 155 *ktikti*). *Kti* is mapped on linkage group (LG) A2. 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 the linkage group (LG) 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.

The markers Satt228, Satt409 tightly linked to the *KTi* locus, and the gene-specific marker *KTi/kti* can be perfectly used as diagnostic markers for MAS of genotypes with the null-allele *KTi* and they are useful marker for the introgression of kunitz trypsin inhibitor null allele into brazilian soybean varieties. Biochemical analysis of seed storage proteins using polyacrylamide gel electrophoresis is very accurate and can be used at the seed stage whilst also saving the viable part of the line for multiplication. Therefore, it is important to enrich the initial pool of recombinant lines with the null allele of the *kti* locus because a large part of the lines can be removed at the early stage of the breeding process.

The phenotypic segregation between BRS 213 and BRS 155 $F_{2:3}$ families for the presence of the LOX1 enzymes carried out by the colorimetric test 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.

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, especially when the trait of interest is recessive. This information can speed up the creation of new cultivars because there is no need for self-fertilization during the odd numbered generations.

In the present study, we investigated two different seed-expressed lipoxygenases expressed in the Brazilian parental soybean cultivars BRS 213, BRS 155 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) (Kim et al., 2004; Reinprecht et al., 2006; Kim et al., 2006; Reinprecht et al, 2011) 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. (2006) 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 (Reinprecht et al., 2006; Lenis et al., 2010; 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 $F_{2:3}$ derived population generated from the cross between BRS 213 ($lx_1lx_2lx_3$) and BRS 155 ($Lx_1Lx_2Lx_3$). Additional SSR markers in the neighboring genomic region of Lx_1, Lx_2 and Lx_3 were also tested.

Among the 22 SSR primer pairs used in PCR amplification of DNA for the parent lines BRS 213 and BRS 155, 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. , 1997 linked with Lx_1 (Lx_2) have shown polymorphism in the parental lines BRS 213 but

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not in the RIL $F_{2:3}$ derived population. However, the $F_{2:3}$ 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 $F_{2:3}$ population derived from the cross between BRS 155 and BRS 213 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 (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 that the commercial soybean cultivars fell into 17 allelic classes from five genotypes and the pedigree of these genotypes are closely related (reference) 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 935F2:303. 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 Lx_1 and Lx_2 loci, allowing the combination of three independent *lx* mutant alleles in the lipoxygenase-free.

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 other markers tested did not reveal polymorphism on the parental cultivars. Genotyping of Lx_1 against the $F_{2:3}$ population 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 1.

The Lx_1 gene for LOX1 has been mapped to chromosome 13 LG F in the molecular soybean genetic map (Creegan et al., 1999). 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 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 flaking the Lx_1, Lx_2 in interval distance of 5.77 cM (Figure 2). 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 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. The cultivars BRS 213 with absence of LOX1, LOX2 and BRS 155, with significantly less lipoxygenase KTI, than other studied soybean germplasms, are most valuable as donors of genetic resources to the modern soybean breeding program.

The use of the diagnostic SSR marker is based on regular PCR and is very simple and quick. However, this process must be improved and significantly sped up via the use of MAS in the initial steps of selections, using a suitable diagnostic marker strongly associated with the null-allele *kti*, and lx_1/lx_2 to produce new soybean cultivars with improved seed quality.

CONCLUSIONS

For the $F_{2:3}$ population derived from BRS 213 and BRS 215, the SSR marker Satt228 and Satt 409, and *KTi/kti* gene specific linkage group A2, were identified as the most effective diagnostic markers, helping to select homozygous lines with the null allele *Kti*, for kunitz trypsin inhibitor, in $F_{2:3}$ segregated populations to enable the reduction of kunitz trypsin inhibitor KTI and improvement of seed quality. The SSR markers Satt090 and Satt417, linkage group F, were found to be tightly linked with the nulls lipoxygenase Lx_1 and Lx_2 alleles, at a distance of 3.00 cM and 2.77 cM, respectively, and were identified as the most effective diagnostic markers to select homozygous soybean lines with null levels of lipoxygenases. These markers could be efficiently used in MAS.

ACKNOWLEDGMENTS

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 funding fellowships for D. Alves da Silva and T. Barreto,.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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