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ORIGINAL PAPER

Soybean seed lipoxygenase genes: molecular characterization and development of molecular marker assays

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Abstract Soybean seeds contain three lipoxygenase (Lox) enzymes that are controlled by separate genes, *Lox1*, *Lox2* and *Lox3*. Lipoxygenases play a role in the development of unpleasant flavors in foods containing soybean by oxidation of polyunsaturated fatty acids. Null alleles for all three enzymes have been identified, *lox1*, *lox2* and *lox3*, and are known to be inherited as simple recessive alleles. Previous studies determined that a missense mutation rendered Lox2 inactive; however, the genetic cause of either *lox1* or *lox3* mutation was not known. The objectives of this study were the molecular characterization of both *lox1* and *lox3* mutant alleles and the development of molecular markers to accelerate breeding for Lox-free soybean varieties. We identified two independent mutant alleles as the genetic causes of the lack of Lox1 in seeds of two *lox1* mutant soybean lines. Similarly, a mutant allele that truncates Lox3 in a *lox3* mutant soybean line was identified. Molecular markers were designed and confirmed to distinguish mutant, wild type, and heterozygous individuals for *Lox1*, *Lox2* and *Lox3* genes. Genotype and Lox phenotype analysis showed a perfect association between the inheritance of homozygous *lox* mutant alleles and the lack of Lox activity. Molecular characterization of a seed-lipoxygenase-free soybean line led to the discovery that an induced recombination event within the *Lox1* gene was responsible for breaking the tight linkage in repulsion phase between mutant alleles at the *Lox1* and *Lox2* loci. The molecular resources developed in this work should accelerate the inclusion of the lipoxygenase-free trait in soybean varieties.

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Introduction

Lipoxygenases are enzymes belonging to a group of non-heme iron containing proteins widely distributed in plants, fungi and animals (Brash 1999). In plants, lipoxygenases can be found in all organs, representing up to 2% of soybean seed protein (Loiseau et al. 2001). Lipoxygenases catalyze the oxidation of polyunsaturated fatty acids such as linoleic (18:2) and α-linolenic (18:3) to produce unsaturated fatty acid hydroperoxides (Brash 1999; Liavonchanka and Feussner 2006; Song et al. 1990). Products of the lipoxygenase pathway have been shown to have a role in a variety of plant processes such as vegetative growth; wounding, herbivore and pathogen attack responses and also in mobilization of storage lipids during germination (Porta and Rocha-Sosa 2002).

In germinating soybean seeds, lipoxygenases were found not to be involved in lipid mobilization (Wang et al. 1999).

The lack of substantial oxygenation of polyunsaturated fatty acids during the germination process supports the idea that soybean seed lipoxygenases might have been recruited to function as storage proteins despite their intact but obsolete enzymatic capacity (Siedow 1991; Wang et al. 1999). 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).

Seed lipoxygenases are of significant importance to the food industry, and lipoxygenase-free soybean varieties are in demand. The oxidation products resulting from seed lipoxygenase activity have been associated with the development of undesirable grassy and beany flavors in products containing protein from soybean and other legumes' seeds (Gerde and White 2008; Robinson et al. 1995; Sessa 1979; Wilson 1996).

Mature soybean seeds contain primarily three lipoxygenases, Lox1, Lox2 and Lox3 (Axelrod et al. 1981). Genetic studies demonstrated that the absence of each enzyme is under the control of three null alleles, *lox1*, *lox2* and *lox3*, which are inherited as simple recessive alleles (Davies and Nielsen 1986; Hildebrand and Hymowitz 1982; Kitamura et al. 1983, 1985). In addition to a single characterized source of the *lox2* mutant allele, two single mutant soybean lines for both *Lox1* and *Lox3* genes have been reported and are available in the USDA's National Plant Germplasm System (Davies and Nielsen 1986; Hildebrand and Hymowitz 1982; Kitamura et al. 1983, 1985). The *Lox1* and *Lox2* loci were found to be in tight genetic linkage on chromosome 13 (LG F), with *lox1* and *lox2* mutant alleles being in the repulsion phase since they were identified in independent germplasm (Davies and Nielsen 1986; Hildebrand and Hymowitz 1982; Kitamura et al. 1985). The *Lox3* locus, on chromosome 15 (LG E), segregates independently of *Lox1* and *Lox2* (Davies and Nielsen 1986; Hajika et al. 1992; Kitamura et al. 1985). The original repulsion-phase linkage in independent sources of mutant alleles at the *Lox1* and *Lox2* loci was broken, resulting in a coupling-phase linkage that eventually led to the development of a triple null lipoxygenase genotype (Hajika et al. 1991; Kitamura 1991). The mechanism involved in the induction of the genotype lacking the three seed lipoxygenases, however, was unknown (Hajika et al. 1991).

The soybean lipoxygenase gene family is thought to have expanded by ancient polyploidy followed by a recent soybean-specific duplication. Consequently, the level of similarity and sequence conservation between regions containing *Lox* genes is high (Shin et al. 2008). Nineteen soybean *Lox* genes were analyzed and found to be distributed across only four chromosomes, with each one containing between three to seven *Lox* genes (Shin et al. 2008).

The molecular basis of the null *lox2* genotype was reported to be a T2849A missense mutation, which resulted in the substitution of glutamine for histidine in a highly conserved histidine-rich motif. Although the mutation did not prevent gene transcription, the structure and function of the protein was severely affected, resulting eventually in its degradation (Wang et al. 1994). The genetic basis of both Lox1- and Lox3-free genotypes has not been previously reported.

The development of seed-lipoxygenase-free soybean cultivars requires accurate evaluation assays to select for the appropriate genotypes. The presence or absence of lipoxygenases in breeding programs has been determined by laborious and time consuming seed phenotyping methods. In addition, these procedures are not able to discriminate between individuals that are homozygous wild type and heterozygotes in the same generation. Consequently, tests to study the genetic makeup of the parents must be performed on the progeny, adding both time and expense to the selection process. Methods to phenotype for seed lipoxygenase status include SDS–polyacrylamide gel electrophoresis, immunological assays, and the relatively faster and simpler colorimetric assays (Kitamura et al. 1983; Narvel et al. 2000; Suda et al. 1995). A single nucleotide polymorphism (SNP) marker that co-segregated perfectly with the *Lox2* locus was previously developed, but it was not specific for the *lox2* causative mutation (Kim et al. 2004).

The primary objective of this work was to determine the molecular basis of the null mutations in soybean *Lox1* and *Lox3* genes. In addition, we wanted to design molecular marker assays perfectly associated with the causative mutations for the three soybean seed *Lox* genes as well as to investigate the genetic process involved in the generation of a genotype lacking the three soybean seed lipoxygenases, 'Jinpumkong 2' (Kim et al. 1997).

In this study, two independent mutations were identified as the genetic causes of the lack of Lox1 activity in seeds of *lox1* mutants, while a common mutation was observed to be responsible for the Lox3-free phenotype in all *lox3* mutants. Perfect molecular marker assays were designed to distinguish mutant from wild type alleles for *Lox1*, *Lox2* and *Lox3* genes and showed a complete association between the inheritance of homozygous *lox* mutant alleles and the lack of lipoxygenase activity. Finally, it was observed that Jinpumkong 2 has the same mutations found in the single mutant sources and that recombination within *Lox1* locus led to the creation of this triple mutant cultivar.

Materials and methods

DNA isolation and PCR for sequencing *Lox1* and *Lox3*

Genomic DNA was isolated from ~30 mg dried seed tissue using the DNeasy Plant Mini Kit (Qiagen Sciences Inc., Germantown, MD, USA) and used at 5–50 ng per

PCR amplification. PCR was carried out using Ex *Taq* according to manufacturer's recommendations (TaKaRa, Otsu, Shiga, Japan) in a PTC-200 thermocycler (MJ Research/Bio-Rad, Hercules, CA, USA) using the following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1.5 min. PCR products were analyzed by gel electrophoresis (1% agarose gel) to verify for size and ensure specific amplification. PCR products were isolated with the QIAprep Spin Miniprep kit (Qiagen Sciences Inc.) and sequenced at the University of Missouri DNA Core facility. Primers used for amplification and sequencing are listed in Supplementary Table 1.

Sequence analysis

Sequences were imported into ContigExpress program of Vector NTI Advance 10 (Invitrogen, Carlsbad, CA, USA), trimmed, assembled and manually evaluated for disagreements between 'Williams 82' (Bernard and Cremeens 1988) reference sequence and imported sequence contigs. Putative SNPs and deletions were verified by at least two independent PCR amplifications. To evaluate the effect of changes in coding sequence at the protein level, the program ExPASy translate tool was used (http://ca.expasy.org/tools/dna.html). Sequence alignments were generated using the AlignX software (Invitrogen). *Lox2* and *Lox3* gene models were obtained from the whole soybean genome assembly (http://www.phytozome.net/soybean) and verified for consistency with accessions GenBank:D13949.1 and GenBank:EU028322.1, respectively. The *Lox1* gene model was determined through the alignment of genomic and coding sequence of accession GenBank:EU028320.1 using the AlignX software (Invitrogen) since we found discrepancies in both sequence and predicted gene model between NCBI entries and the whole soybean genome assembly.

Plant material and development of segregating populations

Study of the genetic basis of the mutations at the soybean seed *Lox* genes and development of molecular marker assays to identify wild type and mutant alleles were carried out with soybean lines PI 408251, PI 547877 and PI 133226 (single mutants for *Lox1* gene); PI 86023 (single mutant for the *Lox2* gene); PI 205085 and PI 417458 (single mutants for *Lox3* gene) and Jinpumkong 2 as well as breeding lines IA2040LF, 8AR-56061 and 935F203 (null for the three soybean seed lipoxygenase genes *Lox1*, *Lox2* and *Lox3*). Seeds of single mutant accessions were obtained from the USDA's National Plant Germplasm System (http://www.ars-grin.gov/npgs/index.html), while seeds of Jinpumkong 2 were donated by Dr. Young-Hyun Hwang (Division of Plant Biosciences, Kyungpook National University, Republic of Korea). Jinpumkong 2 is a Korean cultivar that was released for soyfood uses, such as soymilk, due to its lack of beany taste (KRDA 2009). Seeds of breeding lines IA2040LF, 8AR-56061 and 935F203 were provided by Dr. John Schillinger (Schillinger Genetics).

In the summer of 2006, Jinpumkong 2 was crossed with 'M23' (Takagi and Rahman 1996), a line with normal lipoxygenase activity. F_1 seeds were sent to Costa Rica, and F_2 seed was produced during the winter of 2006–2007. F_2 recombinant inbred lines (RILs) were advanced to F_5 by single seed descent method in Costa Rica from the summer of 2007 to early 2008. In 2008, F_5 seed from single F_4 plants was planted at the Lee farm, University of Missouri-Delta Center, MO, USA.

An additional $F_{3:4}$ NIL population derived from the cross M23 × Jinpumkong 2 was developed. Seven F_2 plants were randomly selected, and F_3 seed from each F_2 plant was used for further analysis.

Sampling DNA and seed harvest

FTA PlantSaver cards (Whatman Inc., Florham Park, NJ, USA) were used to take DNA samples from 129 plants of the F_5 RIL population and from six plants from each of the seven F_3 NILs, according to manufacturer's recommendations. Progeny of each single plant from which a DNA sample was collected were harvested in the fall of 2008 for lipoxygenase activity assays.

Lox1 genotyping assay

PCR was carried out in a PTC-200 thermocycler (MJ Research/Bio-Rad) using the following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s, followed by a melting curve from 70°C to 85°C, with readings taken every 0.1°C. Reactions were carried out in 20 µl containing: 5–50 ng DNA template, primers 5′-ACCGACATCTTAGCGTGCTT-3′ and 5′-AAAAAGGTTGTCTCTATTATGCCAT-3′ (0.375 µM final concentration), buffer (40 mM Tricine-KOH (pH 8.0), 16 mM KCl, 3.5 mM $MgCl_2$, 3.75 µg ml^{-1} BSA, 200 µM dNTPs), 1.25 µM EvaGreen (Biotium Inc., Hayward, CA, USA), 5% DMSO and 0.2X Titanium *Taq* polymerase (BD Biosciences, Palo Alto, CA, USA). Samples homozygote for the *lox1* mutant allele produced a melting peak at 76°C compared to a peak at 81.5°C for wild type plants. Heterozygotes produced both the 76 and 81.5°C melting peaks. To verify PCR product size differences, products were separated on 2% agarose gel and approximate size was evaluated by comparison to a Flashgel 100 bp DNA marker

(Lonza, Rockland, ME, USA). Expected product size was 130 and 56 bp for wild type and mutant alleles, respectively.

Lox2 and *Lox3* genotyping assays

SimpleProbe assays are based on the disassociation kinetics of SimpleProbe oligonucleotides (Roche Applied Sciences, Indianapolis, IN, USA) transitioning from a fluorescent bound to target state to a nonfluorescent unbound state. SimpleProbes were designed using the Lightcycler Probe Design Software, version 1 (Roche Applied Sciences) and purchased from Roche Applied Sciences. Asymmetric PCR was carried out to generate more single stranded DNA for probe binding. *Lox2* genotyping assays were performed with an asymmetric mixture of primers [forward (5′-CTG GCCAAAGCTTATGTGGT-3′) and reverse (5′-GCGTTG ATGTTCATGGTGTC-3′) primers at 0.1 and 1 μM final concentration, respectively]; the *Lox2* SimpleProbe was 5′-SPC-GTTAAATACTCAAGCGGTGATTGAGCCATT CA-phosphate-3′. *Lox3* genotyping reactions were carried out similarly: forward (5′-CATAGTAGTGTTGGTGGG TTGC-3′) and reverse (5′-TGTTGAGCCAACTAAGTC GAGA-3′) primers at 1 and 0.1 μM final concentration, respectively; the *Lox3* SimpleProbe was 5′-SPC-TAA TTCCCCCAACGCTGGTTACGCTA-phosphate-3′. Reactions were carried out in a total volume of 20 μl containing template, primers, SimpleProbe (0.4 and 0.2 μM final concentration for *Lox2* and *Lox3*, respectively), buffer (40 mM Tricine-KOH (pH 8.0), 16 mM KCl, 3.5 mM $MgCl_2$, 3.75 μg ml^{-1} BSA, 200 μM dNTPs), 5% DMSO and 0.2X Titanium *Taq* polymerase (BD Biosciences). Genotyping reactions were performed using a Lightcycler 480 II real time PCR instrument (Roche Applied Sciences), using the following PCR parameters: 95°C for 5 min followed by 40 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s and then a melting curve from 55 to 72°C in the *Lox2* assay and from 58 to 73°C in the *Lox3* assay. Fluorescence was read after each cycle and every 0.1°C during the melting curve analysis (MCA). A mismatch between the probe and the target sequence results in altered disassociation kinetics of the probe. Consequently, each genotype produces a characteristic melting profile, as measured by the melting temperature (T_m) of the first negative derivative of the fluorescence value change with increasing temperature. In the SimpleProbe assays, *Lox2* alleles produced a T_m of 64°C and *lox2-a* alleles produced a T_m of 68°C; *Lox3* alleles produced a T_m of 66°C and *lox3-a* produced a T_m of 62°C.

Phenotyping assays

Colorimetric assays were used to detect the activity of Lox1 and Lox3 in two segregating populations. Phenotypic assays were performed with $F_{4:6}$ and $F_{3:4}$ seeds of the RIL and NIL populations, respectively. Dyes, substrate and test solutions were prepared as outlined by Narvel et al. (2000), with slight modifications: (1) Reactions were performed in 96 deep well 2 ml plates (Fisherbrand, Denmark), 2) The volume of soaking water as well as of test solutions were halved and (3) Lox3 assays were scored after 15 min. Four individual progeny seeds from each plant were tested. Preliminary experiments showed that for the Lox3 assay, scoring samples as long as 30 min after addition of test solution was very difficult as differences in coloration of samples were not always clear. However, discrimination of samples was best between 5 and 15 min after adding test solution. Also, for the Lox3 test, in addition to triple-null seeds, single mutants for Lox3 were included as controls. The inclusion of the additional control was needed to correct *lox3* single mutant samples for non-specific bleaching of the test solution.

Results

Genomic arrangement of soybean seed lipoxygenase genes

With the availability of the whole soybean genome assembly (http://www.phytozome.net/soybean), we were able to determine the disposition of soybean seed lipoxygenase genes in the genome (Fig. 1). *Lox1* and *Lox2* (Glyma13g42320 and Glyma13g42310 in the phytozome soybean browser, respectively) are located on chromosome 13/LG F along with two other genes with lipoxygenase domains (Glyma13g42330 and Glyma13g42340) in a cluster that spans 41.54 kb. The distance between *Lox1* and *Lox2* is only 2,998 bp (Fig. 1a). *Lox3* (Glyma15g03030) is located on chromosome 15/LG E in another lipoxygenase-rich gene cluster that covers 23.74 kb and includes two other genes that also have lipoxygenase domains (Glyma15g03040 and Glyma15g03050) (Fig. 1b). The gene models for *Lox1, Lox2* and *Lox3* represent 3,830, 3,939 and 4,095 bp of sequence, respectively, from start to stop codon, and each gene contains nine exons.

Genetic mutations in *Lox1* and *Lox3* genes

We used gene-specific PCR to amplify and sequence *lox1* from PI 408251 and PI 133226 (*lox1, Lox2, Lox3*) and *lox3* from PI 205085 and PI 417458 (*Lox1, Lox2*, *lox3*). Genomic sequence from cultivar Williams 82 (*Lox1, Lox2*, *Lox3*) was used as reference sequence (http://www.phytozome.net/soybean).

For the *lox1* allele in PI 408251, we identified a 74 bp deletion that starts in exon 8 at genomic position

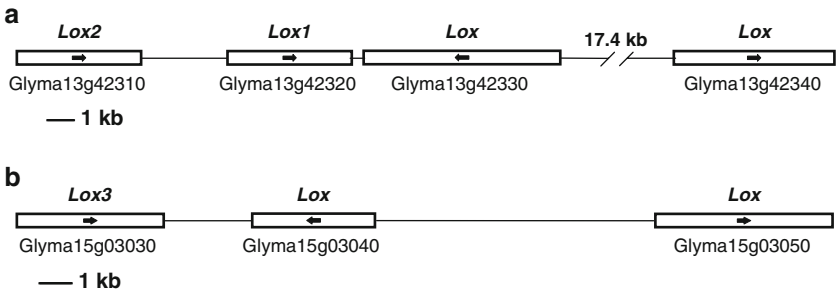

Fig. 1 Genomic arrangement of soybean seed lipoxygenase genes. Cluster of *Lox* genes in **a** chromosome 13 and **b** chromosome 15. Genes are represented by *white boxes*. *Arrows* in *white boxes* indicate orientation of genes. *Lox* genes covered in this work are denoted with their name, i.e., *Lox1*, *Lox2* and *Lox3*; *Lox* genes not covered in this study are denoted as *Lox*. Code below *white boxes* representing genes corresponds to gene name in the whole soybean genome assembly (http://www.phytozome.net/soybean). Gene clusters are drawn to scale

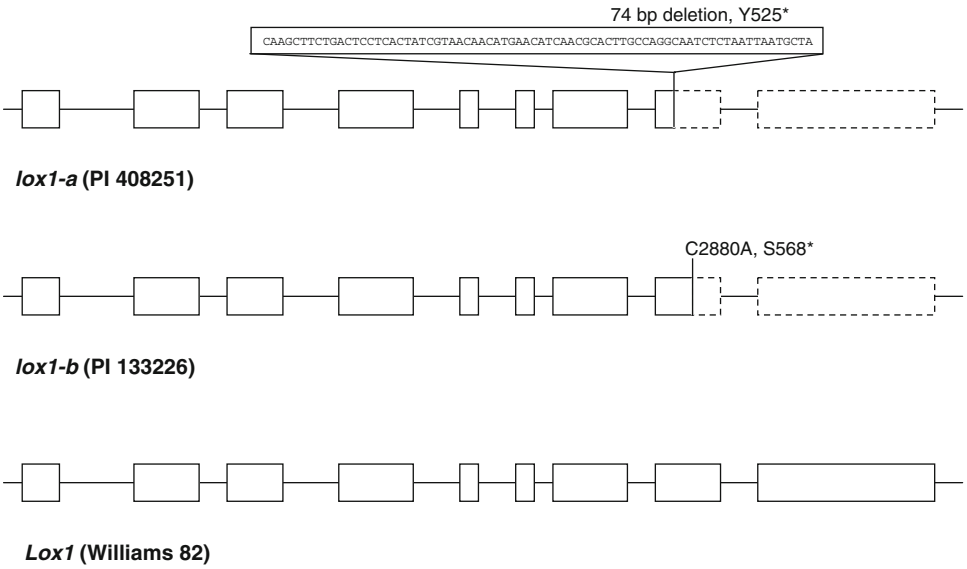

Fig. 2 *Lox1* gene structure and polymorphisms observed in *lox1* mutant alleles. Williams 82 (wild type), PI 408251 (*lox1* single mutant) and PI 133226 (*lox1* single mutant) alleles at the *Lox1* locus. PI 408251 (*lox1-a*) presents a 74 bp deletion starting at genomic position 2752 relative to start codon. The deletion creates an immediate premature stop codon and a truncated protein of 524 residues compared to the reference Lox1 protein of 839 residues. PI 133226 (*lox1-b*) has a C2880A nonsense mutation relative to start codon that results in a S568STOP change and premature truncation of Lox1 protein to 567 amino acids. *Boxes* represent exons; *lines between boxes* represent introns; *boxes with dashed lines* represent exons with untranslated regions in predicted Lox1 proteins from *lox1* mutant alleles

2752, relative to start codon (GenBank accession GQ227538). The deletion creates an immediate premature stop codon and a truncated protein of 524 residues, compared to the reference Lox1 protein containing 839 amino acid residues (Fig. 2). For the *lox1* allele in PI 133226, a nonsense mutation, C2880A relative to start codon, results in an S568STOP change (Fig. 2; GenBank accession GQ227539). The *lox1* 74 bp deletion found in PI 408251 was absent in PI 133226, and PI 408251 did not contain the C2880A mutation found in the PI 133226 *lox1* allele. No other changes were identified in the *lox1* alleles compared to the reference Williams 82 *Lox1* sequence.

Characterization of the *lox3* allele in PI 205085 and PI 417458 revealed an identical mutation in both sources: a single base deletion of a guanine in a run of five guanine nucleotides, within exon 1, from position 97 to 101 relative to start codon, compared to the wild type *Lox3* reference sequence (GenBank accessions GQ227541 and GQ227542). This guanine deletion results in a frame shift at position 101 that prematurely truncates the protein after only 41 amino acids (Fig. 3). No other changes were identified in the *lox3* allele compared to the reference Williams 82 *Lox3* sequence.

We will refer to the *lox1* mutant alleles from PI 408251 and PI 133226 as *lox1-a* and *lox1-b*, respectively.

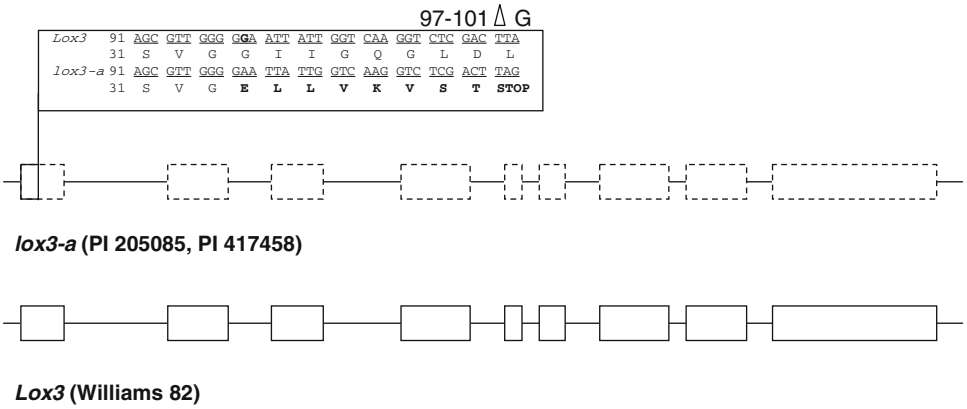

Fig. 3 *Lox3* gene structure and polymorphisms observed in *lox3* mutant allele. Williams 82 (wild type) and PI 205085 and PI 417458 (*lox3* single mutants) alleles at the *Lox3* locus. PI 205085 and PI 417458 (*lox3-a*) present a single guanine deletion in a run of five guanines starting at genomic position 97 relative to start codon. As a result, a frame shift is introduced at position 101 (*bold* nucleotide) that prematurely truncates the protein at position 42. Amino acid residues resulting from the frame shift are in *bold*. Numbers in the *left margin* of genomic and protein sequences indicate nucleotide and amino acid residue number, respectively. *Boxes* represent exons; *lines between boxes* represent introns, *boxes with dashed lines* represent exons with untranslated regions in predicted Lox3 protein from *lox3-a* mutant allele

Similarly, we will refer to the *lox3* mutant allele found in PI 205085 and PI 417458 as *lox3-a*. Finally, the *lox2* mutant allele present in PI 86023, previously reported by Wang et al. (1994), we will term *lox2-a*.

Development of molecular marker assays

Molecular marker assays were designed to distinguish the mutant *lox1-a, lox2-a,* and *lox3-a* alleles from the wild type alleles for *Lox1*, *Lox2* and *Lox3* genes (Fig. 4). Due to the high degree of sequence similarity among the genes, we performed gene-specific PCR and then discriminated between wild-type and mutant alleles of each gene using the characteristic melting profile produced from SimpleProbe disassociation for *Lox2* and *Lox3* assays (see "Materials and methods"). For the *Lox1* assay, gene-specific primers were designed to flank the region containing the 74 bp deletion in *lox1-a* mutants and discriminate genotypes using MCA of the amplification products in the presence of the fluorescent dye EvaGreen or based on resolution of product size differences. MCA using DNA from plants homozygous for the *lox1-a* allele gave a distinctive melting peak of 76°C compared to a peak at 81.5°C for DNA from Williams 82. Heterozygotes produced both the 76 and 81.5°C melting peaks (Fig. 4a). For the *lox2-a* and *Lox2* alleles, MCA using the SimpleProbe assay yielded melting peaks at 68 and 64°C for individuals that were homozygous, respectively. Heterozygous *lox2-a*/*Lox2* plants showed both melting peaks at 68 and 64°C (Fig. 4b). Finally, a SimpleProbe assay designed to distinguish *Lox3* from *lox3-a* showed, in a MCA, melting peaks at 66 and 62°C for homozygous wild type and mutant plants, respectively, while both melting peaks were observed in heterozygous individuals (Fig. 4c). The molecular marker assays reliably distinguished homozygous mutant, homozygous wild-type, and heterozygous genotypes for the *Lox1*, *Lox2* and *Lox3* genes.

Genetic recombination within *Lox1* gene locus in Jinpumkong 2

The hurdle to the development of a *lox1, lox2, lox3* triple mutant genotype was the tight linkage between the *Lox1* and *Lox2* loci. A single lipoxygenase-free soybean line was recovered after a complex experiment that included gamma-ray mutagenesis of mixed F_2 seeds of crosses between *lox1, lox3* and *lox2, lox3* double mutants (Hajika et al. 1991). However, the mechanism involved in the induction of the genotype lacking the three seed lipoxygenases was unknown (Hajika et al. 1991). Subsequently, one additional triple mutant lipoxygenase genotype was developed from mutagenesis of a *lox2*, *lox3* double mutant, but that line appears to be unrelated to the cultivar Jinpumkong 2 and the lipoxygenase-free breeding lines available for investigation (Kitamura 1991).

Sequence characterization and genotyping of the Jinpumkong 2 seed lipoxygenase genes enabled the reconstruction of the events leading to the combination of *lox1*, *lox2*, and *lox3* mutant alleles. The Jinpumkong 2 *lox2* allele was genotyped to contain the H532Q missense mutation from PI 86023. The Jinpumkong 2 *lox3* allele was identical to the *lox3-a* allele found in PI 205085 and PI 417458 (GenBank accession GQ227544).

Characterization of *lox1* from Jinpumkong 2 revealed a unique *lox1* mutant allele. Although Jinpumkong 2 contained the *lox1* 74 bp deletion identical to that in

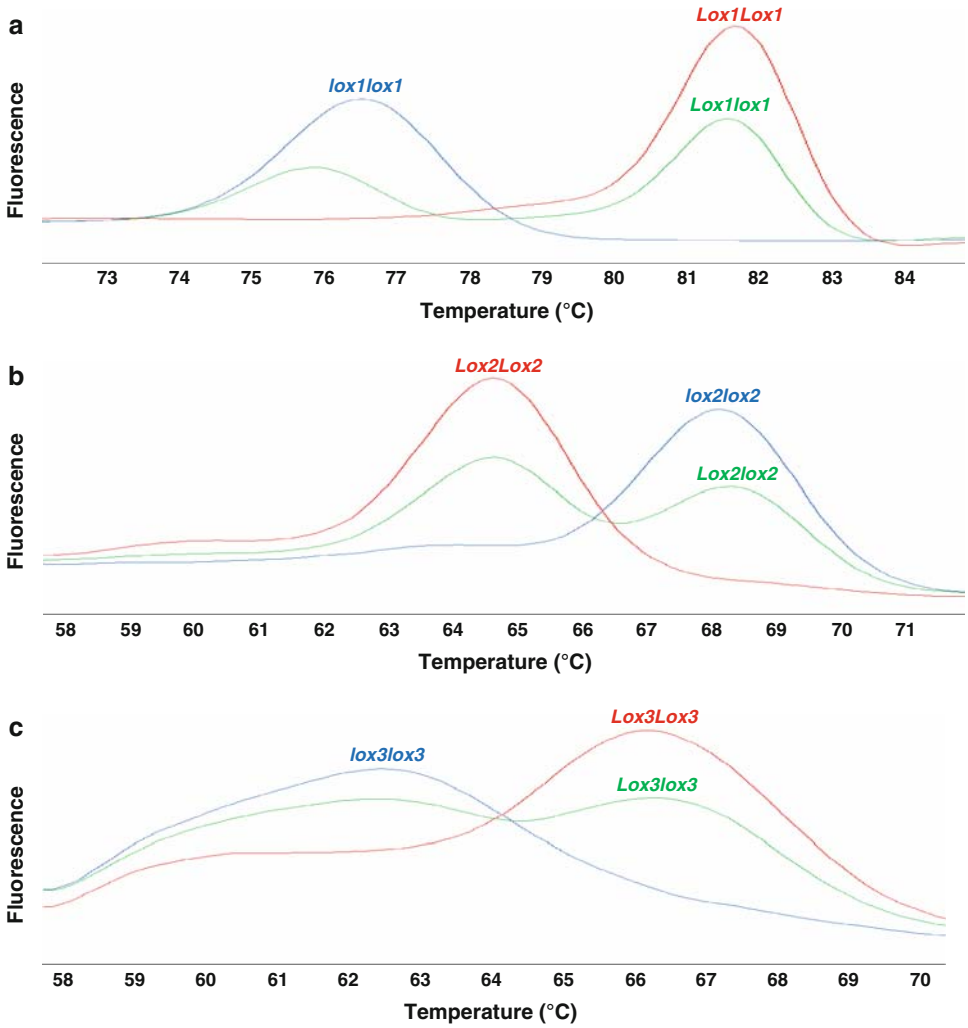

Fig. 4 Genotyping results using molecular marker assays for soybean seed lipoxygenase genes. **a** Typical results for the *Lox1* genotyping assay. Homozygous *Lox1* and PI 408251-derived *lox1* individuals give peaks at 81.5 and 76°C, respectively. Heterozygotes show both peaks. **b** Typical results for the *Lox2* genotyping assay. Homozygous *Lox2* and *lox2* individuals give peaks at 64 and 68°C, respectively. Heterozygotes show both peaks. **c** Typical results for the *Lox3* genotyping assay. Homozygous *Lox3* and *lox3* individuals give peaks at 66 and 62°C, respectively. Heterozygotes show both peaks

PI 408251, an additional seven SNPs and a 3 bp deletion were also present in the 5′ end of the Jinpumkong 2 *lox1* gene (Fig. 5; GenBank accession GQ227543).

The *Lox2* gene resides ~3 kb upstream of the *Lox1* gene (Fig. 1). The source of the Jinpumkong 2 *lox2* mutation was PI 86023 (*Lox1*, *lox2*, *Lox3*), which produces active Lox1 enzyme. We verified that the PI 86023 *Lox1* allele does not contain either the PI 408251 74 bp deletion or the PI 133226 C2880A mutation. However, sequence characterization of the PI 86023 *Lox1* gene revealed a number of missense, silent and intron SNPs compared to the reference Williams 82 *Lox1* sequence and the PI 408251 and PI 133226 *lox1* mutant alleles (Fig. 5; GenBank accession GQ227540). A contiguous subset of these *Lox1* polymorphisms from PI 86023 (seven SNPs and a 3 bp deletion) was present in the Jinpumkong 2 *lox1* allele upstream from the 74 bp deletion (Fig. 5).

The existence of a novel mutant allele of *lox1* in Jinpumkong 2 containing elements of both the PI 86023 *Lox1* gene and the PI 408251 *lox1* gene is consistent with a recombination event which occurred within the *Lox1* locus that broke the repulsion linkage to couple the two mutant *lox1* and *lox2* alleles from independent sources in a single line (Fig. 5). The last shared *Lox1* SNP present in both Jinpumkong 2 and PI 86023 is at position 472 relative to start codon, and sixteen SNPs and two single base deletions beginning at position 1185 relative to start codon are present only in the PI 86023 *Lox1* allele (Fig. 5). The novel mutant allele at the *Lox1* locus from Jinpumkong 2 will be referred to as *lox1-c*.

Finally, genotyping of three different Lox-null soybean breeding lines (i.e., IA2040LF, 8AR-56061 and 935F203) that are currently used to develop marketable lipoxygenase-free soybean varieties revealed they contain identical combination of *lox* mutations to that found in Jinpumkong 2.

Together with the previously identified mutation in *Lox2* present in the Lox2-free accession PI 86023 (Wang et al. 1994), there are now a total of five seed *lox* mutant alleles, and three of them have perfect molecular markers (i.e., specific to the causative mutation), one for each of the three seed *Lox* loci (Table 1).

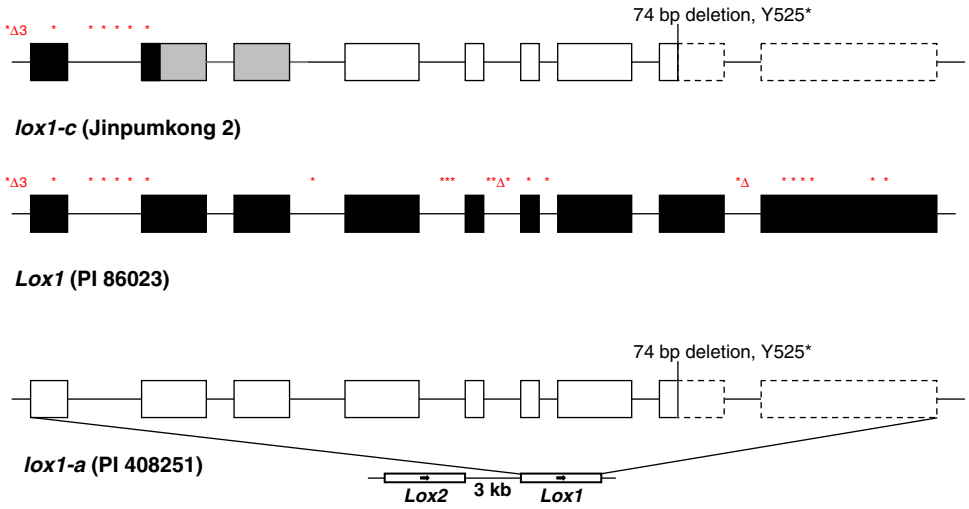

Fig. 5 Comparison of Jinpumkong 2, PI 86023 and PI 408251 alleles at the *Lox1* locus. Jinpumkong 2 contains *lox1-c*, a unique *lox1* mutant allele with elements of both the PI 86023 *Lox1* gene and the PI 408251 *lox1-a* gene. Jinpumkong 2 has the *lox1* 74 bp deletion identical to that in PI 408251 as well as seven SNPs and a 3 bp deletion present in the 5′ end of the PI 86023 *Lox1* gene. *Asterisks* represent SNPs found in PI 86023. The symbol Δ^n represents a deletion of 'n' nucleotides when 'n' is higher than one. *Boxes* represent exons; *lines between boxes* represent introns. *Black boxes* represent genomic regions from PI 86023; *white boxes* represent genomic regions from PI 408251; *light gray boxes* and *lines* represent recombination region within *Lox1* locus; *boxes with dashed lines* represent exons with untranslated regions in predicted Lox1 proteins from Jinpumkong 2 and PI 408251

Table 1 Listing of mutant alleles at the three soybean seed *Lox* genes

	Lipoxygenase genes			
Genotype	***Lox1***[a]	***Lox2***	***Lox3***	**Reference**
PI 133226	*lox1-b*^{C2880A}	*Lox2*	*Lox3*	This work
PI 408251	*lox1-a*$^{\Delta 74bp}$	*Lox2*	*Lox3*	This work
PI 547877	*lox1-a*$^{\Delta 74bp}$	*Lox2*	*Lox3*	This work
PI 86023	*Lox1*	*lox2-a*^{T2849A}	*Lox3*	Wang et al. (1994)
PI 417458	*Lox1*	*Lox2*	*lox3-a*^{G101}	This work
PI 205085	*Lox1*	*Lox2*	*lox3-a*^{G101}	This work
Jinpumkong 2	*lox1-c*$^{\Delta 74bp}$	*lox2-a*^{T2849A}	*lox3-a*^{G101}	This work

[a] Superscript designates type of mutation; C2880A, C to A change at position 2880; Δ74 bp, 74 bp deletion starting at position 2752; T2849A, T to A change at position 2849; G101Δ, deletion of a guanine resulting in a frame shift at position 101. All mutations are relative to start codon, where A = 1. Uppercase designates wild-type alleles. Mutant *lox* alleles molecularly characterized in this work are indicated by black background and white fonts. Novel PI 86023 *Lox1* allele, characterized in this work, is indicated by light gray background and black fonts

Association analysis for seed lipoxygenase phenotype and *Lox1* and *Lox3* genotypes

To determine the consistency of the molecular marker assays developed to distinguish mutant from wild type alleles for *Lox1*, *Lox2* and *Lox3* genes, we carried out an association analysis between the genotype of individuals of two segregating populations and the seed lipoxygenase phenotype of their progeny. The segregating populations were derived from a cross between M23 (*Lox1, Lox2, Lox3*) and Jinpumkong 2 (*lox1, lox2, lox3*). Population 1 was an $F_{4:5}$ RIL population containing 129 individuals, and population 2 was a set of seven $F_{2:3}$ NILs with 42 individuals; two individuals of the NIL population were not analyzed. We genotyped samples from these populations for the *lox1*, *lox2*, and *lox3* alleles and phenotyped for seed Lox1 and Lox3 activity in the progeny (see "Materials and methods"). Results indicated a perfect association between the inheritance of homozygous *lox1* or *lox3* alleles and the lack of either Lox1 or Lox3 activity, respectively (Table 2). It was also observed that the *Lox3* locus segregated independently of the *Lox1* and *Lox2* loci. On the contrary, *Lox1* and *Lox2* loci were inherited as if they were on the same locus, i.e., no independent segregation occurred, but a tight coupling-phase linkage existed (data not shown).

Table 2 Colorimetric determination of presence (+) or absence (−) of Lox1 and Lox3 activity in progeny seeds derived from genotyped parental plants

Parent genotype		Observed no. of progeny seeds with each possible Lox phenotype[a]			
No. of plants	Genotype	Lox1(+) Lox3(+)	Lox1(+) Lox3(−)	Lox1(−) Lox3(+)	Lox1(−) Lox3(−)
27	*Lox1 Lox1 Lox3 Lox3*	108	0	0	0
8	*Lox1 Lox1 Lox3 lox3*	28[b]	4[b]	0	0
32	*Lox1 Lox1 lox3 lox3*	0	128	0	0
2	*Lox1 lox1 Lox3 Lox3*	5[c]	0	3[c]	0
3	*Lox1 lox1 Lox3 lox3*	12[d]	0	0	0
10	*Lox1 lox1 lox3 lox3*	0	30[e]	0	10[e]
28	*lox1 lox1 Lox3 Lox3*	0	0	112	0
14	*lox1 lox1 Lox3 lox3*	0	0	40[f]	16[f]
45	*lox1 lox1 lox3 lox3*	0	0	0	180

[a] For phenotype determination, four seeds of each parental plant were evaluated. Homozygous wild type genotypes are indicated by white background and black fonts. Homozygous mutant genotypes are indicated by black background and white fonts. Heterozygous genotypes are indicated by light gray background and black fonts

[b] χ^2 for 3 Lox3(+): 1 Lox3(−) = 2.67; not significant at $p < 0.05$

[c] χ^2 for 3 Lox1(+): 1 Lox1(−) = 0.17; not significant at $p < 0.05$

[d] χ^2 for 9 Lox1(+) Lox3(+): 3 Lox1(+) Lox3(−): 3 Lox1(−) Lox3(+): 1 Lox1(−) Lox3(−) = 6.14; not significant at $p < 0.05$

[e] χ^2 for 3 Lox1(+): 1 Lox1(−) = 0.00; not significant at $p < 0.05$

[f] χ^2 for 3 Lox3(+): 1 Lox3(−) = 0.38; not significant at $p < 0.05$

Discussion

Recent phylogenetic analysis has revealed that the lipoxygenase gene family expanded by two rounds of whole genome duplication, resulting in gene members with a high degree of sequence and structure similarity (Shin et al. 2008). In our study, we characterized three different seed-expressed lipoxygenase genes (*Lox1, Lox2* and *Lox3*), which possess a high degree of identity at the amino acid level (81.1% between Lox1 and Lox2, 70.7% between Lox1 and Lox3 and 74.5% between Lox2 and Lox3). Based on the soybean genome assembly (http://www.phytozome.net/soybean), we determined that the distance between *Lox1* and *Lox2* loci is ~3 kb. The minute separation between the two loci explains the tight linkage reported in previous genetic studies (Davies and Nielsen 1986; Hajika et al. 1992; Kitamura et al. 1985). Hajika et al. (1991) were able to obtain a soybean line lacking all the seed lipoxygenases by gamma-ray irradiation of F_2 plants from a cross between two double mutants: Lox1- and Lox3-free × Lox2- and Lox3-free. However, the mechanism involved in the induction of the triple mutant was unknown to the authors. Point or gene mutation, chromosomal deletion and crossing-over are among the possible events that could have allowed them to obtain the triple mutant line.

Jinpumkong 2 is a null for the three seed lipoxygenases (Kim et al. 1997). By means of gene-specific PCR and sequence comparison, we determined that no new mutation or chromosomal deletion containing any of the three seed *Lox* genes took part in the triple null quality of Jinpumkong 2, leaving genetic recombination between *Lox1* and *Lox2* loci as a viable alternative. Comparison of *Lox1* sequence between Jinpumkong 2 and PI 86023 (donor of *lox2-a* allele) indicates that these two accessions share unique sequence polymorphisms in the 5′ end of the *Lox1* gene that are not present either in Williams 82 (reference sequence) or in *lox1* single mutants (PI 408251 and PI 133226). Moreover, after the last common polymorphism between Jinpumkong 2 and PI 86023 at position 472, relative to start codon, the sequences between the triple mutant and PI 408251 become identical, while the PI 86023 *Lox1* sequence contains additional unique polymorphisms. This strongly implicates that genetic recombination within the *Lox1* locus, creating a novel *lox1* mutant allele, is the mechanism that allowed the combination of *lox1*, *lox2* and *lox3* alleles in Jinpumkong 2 despite the donor repulsion-phase linkage between the alleles at the *Lox1* and *Lox2* loci (*Lox1, lox2* and *lox1, Lox2*).

Mutant soybean lines lacking Lox1 (Hildebrand and Hymowitz 1981, 1982), Lox2 (Davies and Nielsen 1986; Kitamura et al. 1985) and Lox3 (Kitamura et al. 1983) have been identified, and the inheritance of the activity of each of the enzymes was studied. Also, the molecular basis for Lox2 deficiency was previously investigated (Wang et al. 1994) and reported to be a missense T2849A mutation,

which results in a H532Q substitution in a highly conserved histidine-rich motif. Such a substitution does not prevent the expression of the gene, but affects protein structure, function and stability (Wang et al. 1994). This is the first report describing the molecular basis for Lox1- and Lox3-null phenotypes.

Seed lipoxygenases affect food quality since they are involved in the production of undesirable grassy and beany aroma and flavor in soybean-containing foods (Gerde and White 2008). The genetic elimination of seed lipoxygenases represents a solution to this problem. To develop Lox-free soybean cultivars, accurate evaluation assays to select for appropriate genotypes are required. The methods that have been used to characterize soybean lines for presence/absence of seed lipoxygenases include detection of the Lox proteins in single dimension SDS–polyacrylamide gels using electrophoresis and crude seed extracts with either total protein staining or by Westerns with specific lipoxygenase antibodies, and colorimetric enzymatic assays, based on the pH of the test solution for which the corresponding lipoxygenase has optimum activity (Kitamura et al. 1983; Narvel et al. 2000; Suda et al. 1995). While these methods test phenotypes, none of them analyze the genetic makeup of evaluated soybean lines. In each case of the phenotypic assays, samples which have a heterozygous genotype are not distinguishable from homozygous wild-type individuals. We report herein the development of perfect molecular marker assays for each of the three soybean seed *Lox* genes. Association analysis showed a complete agreement between genotype and phenotype of evaluated individuals, validating the robustness of our assays.

In our hands, colorimetric assays were consistent except when evaluating Lox2 activity. We were unable to assign the correct phenotype to several known wild type samples for Lox2, which failed to react in the test solution. One factor affecting the accuracy of the colorimetric assay for Lox2 was seed age: the older the seed, the more inconsistent the test was. However, even with recently harvested seeds, the assay remained inconsistent. Seed age had no impact on the call accuracy of our molecular marker assays. An additional advantage of our molecular markers is that homozygous mutant, heterozygous, and homozygous wild-type genotypes could be clearly differentiated in the same generation. On the contrary, for the phenotypic assays, progeny tests must be performed to definitely discern the genetic makeup of individuals, representing a considerable increase in both the number of samples to analyze and time.

As a result of this work, three *lox* mutant alleles, one for each of the soybean seed *Lox* genes, can be easily and quickly detected by means of perfect molecular markers. Furthermore, the potential of these assays becomes more evident by the fact that these *lox* mutant alleles were found to be present in lipoxygenase-free soybean breeding lines currently in use. The utilization of molecular markers herein reported will allow soybean breeders to directly select for mutant alleles in early generations of segregating populations allowing more efficient introgression of the lipoxygenase-free trait in their soybean varieties.

Conclusions

In this work, the genetic basis of mutations in *Lox1* and *Lox3* soybean genes were investigated. Two independent mutations, a 74 bp deletion and a C2880A nonsense mutation, were responsible for the premature truncation of the Lox1 protein in mutants. In contrast, all *lox3* mutants showed a single base deletion introducing a frame shift at position 101 which resulted in a premature stop codon. Co-dominant molecular marker assays perfectly associated with the causative mutations were developed for *Lox1*, *Lox2* and *Lox3* genes, providing a quick, inexpensive and accurate means to assess the genetic makeup of individuals. Finally, it was elucidated that genetic recombination was the mechanism that broke the tight repulsion-phase linkage between *Lox1* and *Lox2* loci, allowing the combination of three independent *lox* mutant alleles in the lipoxygenase-free variety Jinpumkong 2.

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