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ORIGINAL PAPER

Molecular basis of seed lipoxygenase null traits in soybean line OX948

Yarmilla Reinprecht · Shun-Yan Luk-Labey · Kangfu Yu · Vaino W. Poysa · Istvan Rajcan · Gary R. Ablett · K. Peter Pauls

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Abstract The poor stability and off-flavors of soybean oil and protein products can be reduced by eliminating lipoxygenases from soybean seed. Mature seeds of OX948, a lipoxygenase triple null mutant line, do not contain lipoxygenase proteins. The objective of this study was to determine the molecular basis of the seed lipoxygenase null traits in OX948. Comparisons of the sequences for lipoxygenase 1 (*Lx1*) and lipoxygenase 2 (*Lx2*) genes in the mutant (OX948) with those in a line with normal lipoxygenase levels (RG10) showed that the mutations in these genes affected a highly conserved group of six histidines necessary for enzymatic activity. The OX948 mutation in *Lx1* is a 74 bp deletion in exon 8, which introduces a stop codon that prematurely terminates translation. A single T–A substitution in *Lx2* changes histidine H532 (one of the iron-binding ligands essential for L-2 activity) to glutamine. The mutation in the lipoxygenase 3 (*Lx3*) gene in OX948 is in the promoter region and represents two single base substitutions in a *cis*-acting AAATAC paired box. All three mutations would result in the loss of lipoxygenase activity in mature seed. The seed lipoxygenase gene mutation-based molecular markers could be used to accelerate and simplify breeding efforts for soybean cultivars with improved flavor.

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Y. Reinprecht · S.-Y. Luk-Labey · I. Rajcan · K. Peter Pauls (✉)
Department of Plant Agriculture, University of Guelph, Guelph, ON N1G 2W1, Canada
e-mail: ppauls@uoguelph.ca

G. R. Ablett
University of Guelph, Ridgetown Campus, Ridgetown, ON N0P 2C0, Canada

K. Yu · V. W. Poysa
Agriculture and Agri-Food Canada, Greenhouse and Processing Crops Research Centre, Harrow, ON N0R 1G0, Canada

Introduction

The beany or grassy flavor of soybean seed products is one of the major factors limiting human consumption of soybean (Rackis et al. 1979). Lipoxygenase (linoleate:oxygen oxidoreductase; EC 1.13.11.12) catalyzes the oxidation of unsaturated fatty acids that contain a *cis*, *cis*-1,4-pentadiene structure (Kitamura 1984) to produce conjugated unsaturated fatty acid hydroperoxides, which are converted to volatile compounds associated with undesirable flavors (Start et al. 1986). Eliminating this enzyme from seeds could lead to better quality soybean protein and oil products.

Soybean has three distinct seed lipoxygenase isozymes, lipoxygenase 1 (L-1), lipoxygenase 2 (L-2) and lipoxygenase 3 (L-3). The L-3 protein can be chromatographically separated into L-3a and L-3b isozyme; however, due to virtually indistinguishable properties, they are considered as a single L-3 entity in the literature (Yenofsky et al. 1988). The three lipoxygenases have different pH optima, substrate specificities and reaction products (Axelrod et al. 1981; Feussner and Wasternack 2002).

The seed lipoxygenase isozymes are globular proteins consisting of single polypeptides of similar lengths (Axelrod et al. 1981). L-1 and L-2 have 81% amino acid identity and L-2 and L-3 have 74% identity (Siedow 1991). Substantial differences occur only in their N-termini and in

a stretch of roughly 35 amino acids spanning amino acids 368-401 (L-1). The highest homology occurs in the carboxy-terminal halves of the polypeptides, including a stretch of 27 amino acids from 341 to 367 (L-1), a run of 13 amino acids (ASALHAAVHFGQY; 686–698, L-1), 9 amino acids at the C-terminus (RGIPNSISI) as well as a highly conserved region of 6 histidines within a 38 amino acid span (493-531, L-1).

It has been suggested that the histidine cluster provides the ligands for non-heme iron binding (Shibata et al. 1988). However, in an X-ray analysis of the L-1 isozyme, the iron cofactor was found to be buried and surrounded by three histidines (H499, H504 and H690), a C-terminal isoleucine (I838), asparagine (N694) and water (Minor et al. 1993, 1996). In addition, mutation experiments have shown that a single amino acid replacement at three of the six conserved histidines in soybean L-1 (H499Q, H504Q and H690Q) produced a completely inactive enzyme. Replacement of any one of the other three histidines (H494Q, H522Q and H531Q) produced an enzyme with reduced but detectable enzymatic activity (Steczko et al. 1992). In addition, Kramer et al. (1994) reported that the N713 position was critical for catalytic activity but not for iron binding in L-3. N713A and N713S substitutions completely inactivated the enzyme (Kramer et al. 1994). The only other mutation that produced an inactive enzyme was the deletion of the six COO-terminal amino acids. The terminal COO- provides the fourth iron ligand (I839 in L-1) and the only negative charge in the vicinity of the iron. This suggested that only the mutations that change iron ligands (H499, H504, H690, N694 and I839 in L-1) would produce inactive enzymes.

Spontaneous mutants for single lipoxygenase isozymes have been identified (Hildebrand and Hymowitz 1981; Kitamura 1984) and a number of single, double (Kitamura et al. 1985) and triple null lipoxygenase mutants were developed by gamma (γ)-irradiation (Hajika et al. 1991). Protein products from lipoxygenase-free soybean have more favorable flavor profiles (Kitamuta et al. 1983; Davies and Nielsen 1986; Shen et al. 1996; King et al. 1998).

The molecular basis for seed lipoxygenase nulls have been described in different backgrounds. Wang et al. (1994) identified a single amino acid replacement at H532Q (corresponding to H504 in L-1) in triple null γ-mutant lines (Kyushu 111 and K line) that resulted in inactive L-2 isozymes. Two null alleles have been identified for the *Lx3* gene. Two single nucleotide substitutions in the promoter region of *Lx3* (mutation 2: C–T at −636 and mutation 1: T–A at −585) in Kyushu 111 and the K line, respectively, resulted in a complete nulls for that isozyme (Wang et al. 1995). The lines PI 205085, PI 417458 (*Lx1Lx2lx3*) and Jinpumkong 2 (*lx1lx2lx3*) have a *lx3-a* allele, which contains a frame shift mutation (single G deletion at the position101 in exon 1) that introduces a stop codon and results in premature termination of protein translation (after 41 amino acids, Lenis et al. 2010). Three null alleles have been reported for the *Lx1* gene. The *lx1-a* allele (in PI 408251) has a 74 bp deletion in exon 8 at position 2,752, which introduces a stop codon and terminates protein synthesis after 524 amino acids. In addition to the same 74 bp deletion, the *lx1-c* allele (in Jinpumkong 2) contains seven SNPs and a 3 bp deletion at the 5′ end of the gene. A nonsense mutation (C2880A) in the *lx1-b* allele (in PI 133226) results in an S568STOP change (Lenis et al. 2010).

Lx1 and *Lx2* genes are tightly linked and inherited together, while *Lx3* gene is independently inherited (Davies and Nielsen 1986). We used a recombinant inbred line (RIL) population derived from the cross RG10 x OX948 to confirm that *Lx1* and *Lx2* are on chromosome 13 (linkage group F), and mapped the *Lx3* gene to chromosome 15 (E) (Reinprecht 2002; Reinprecht et al. 2006). Many GenBank records of lipoxygenase genes exist. The complete amino acid sequences for soybean seed lipoxygenase isozymes have been deduced from the nucleotide sequences of their respective cDNAs (Shibata et al. 1987; Shibata et al. 1988; Yenofsky et al. 1988). Yenofsky et al. (1988) also identified and sequenced genomic DNA for soybean *Lx3*. The genomic sequences of the *Lx1* and *Lx2* genes have also been determined and deposited in GenBank (Shin et al. 2007). The lipoxygenase genes contain nine exons and eight introns (Yenofsky et al. 1988). The recent release of complete draft of soybean genome sequence opens new insights into genome organization of this ancient paleopolyploid (Schmutz et al. 2010). Four homologous regions containing 19 lipoxygenase genes are present in soybean genome (Shin et al. 2008). The syntenic region Ks0.1984 containing seed lipoxygenases is found on chromosomes 15 (E) and 13 (F), while the syntenic region Ks0.1827 on chromosome 8 (A2) and chromosome 7 (M) contains vegetative lipoxygenases.

Colorimetric assays (Suda et al. 1995) are convenient for rapidly screening lines for the presence or absence of seed lipoxygenases in soybean breeding programs focussed on selecting cultivars with improved flavor. The colorimetric procedures can be performed on a small piece of the seed, leaving the rest of the seed for planting. However, the colorimetric assays are time consuming, somewhat subjective, prone to errors and cannot identify heterozygotes. In contrast, a screening procedure based on codominant DNA markers developed for lipoxygenase nulls could increase the precision and efficiency of breeding for lipoxygenase-free soybean since the marker patterns can be determined quickly and accurately using DNA extracted from any tissue samples. Kim et al. (2004) developed a single nucleotide polymorphism (SNP) marker for *Lx2*

gene. Markers developed for *Lx1* (Lox1-3) and *Lx3* (Lox3$_{-HaeIII}$) genes by our group (Reinprecht 2002; Reinprecht et al. 2006) have been successfully used to simultaneously select for *lx1*/*lx2* and *lx3* phenotypes accelerating selection for soybean lines for improved soymilk stability and flavor (Luk, 2006). Recently, Lenis et al. (2010) developed molecular marker assays for all three lipoxygenase null alleles in Jinpumkong 2 and several PIs.

Although OX948 and its derivatives are in extensive use by our breeding program at Harrow, the allele status for this line has not been previously investigated. The objective of this work was to determine the molecular basis of the seed lipoxygenase nulls in soybean mutant line OX948. Based on sequence comparisons of the three seed lipoxygenase genes in OX948 and RG10 (wild-type) the lipoxygenase triple nulls in OX948 are likely caused by mutations in the *Lx1*, *Lx2* and *Lx3* genes. This information allowed previously developed gene-based markers for *Lx1* (Lox1-3) and *Lx3* (Lox3$_{-HaeIII}$) to be described more fully and resulted in the development of additional markers (based on new sequence information). Markers developed for these mutations could be used early in a breeding program for marker-assisted selection (MAS) of lines with seed lipoxygenase nulls. Therefore, they have the potential to accelerate and simplify breeding efforts for soybean cultivars with improved flavor.

Materials and methods

Plant material

OX948 is a seed lipoxygenase triple null line (developed at Agriculture and Agri-Food Canada, Greenhouse and Processing Crops Research Centre, Harrow, ON Canada; Buzzel unpublished). This line is a selection from a backcross between Harovinton and a triple null lipoxygenase F_2 plant. OX948's pedigree is Harovinton/3/Century L2L3/Harovinton//triple null lipoxygenase line. The F_1 seed (obtained from Dr. Kitamura—National Agricultural Research Centre, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan) was produced by crossing a selection from the Century_L2L3/Harovinton cross with an unnamed triple null lipoxygenase source (likely in a Suzuyutaka background) obtained by γ-irradiation. RG10 is a seed lipoxygenase wild-type line (developed at the University of Guelph, Ridgetown Campus, Ridgetown, ON, Canada by chemical mutation with ethyl methanesulfonate of the low linolenic acid line C1640; Stojsin et al. 1998). A RIL population (F_5) of 169 individuals was developed from a cross between RG10 and OX948. The population development and field trials were reported by Reinprecht et al. (2005).

Detection of seed lipoxygenases

Seed lipoxygenases were analyzed with colorimetric assays (Suda et al. 1995) and single-dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The presence/absence of seed lipoxygenases was determined using a half-seed technique (Wilcox and Cavins 1985). Individual lipoxygenases were analyzed for two seeds of soybean lines OX948 (seed lipoxygenase triple null), RG10 (seed lipoxygenase wild type) and their F_5 RILs by visually judging the outcome of a slightly modified colorimetric method (Suda et al. 1995) as described in Reinprecht et al. (2005). Total seed proteins of parental genotypes and null RILs (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).

Sequencing seed lipoxygenase genes

Sequences for seed lipoxygenases available in public databases at the initiation of this study [*Lx1* cDNA (GeneBank accession J02795; Shibata et al. 1987), *Lx2* cDNA (GeneBank accession J03211; Shibata et al. 1988), *Lx3* cDNA (GeneBank accession X13302; Yenofsky et al. 1988) and *Lx3* genomic DNA (GeneBank accession X06928; Yenofsky et al. 1988)] were aligned using ClustalW (Chenna et al. 2003) at EBI (http://www.ebi.ac.uk/clustalw). Based on the alignments, primers for each of the three seed lipoxygenase genes were designed [using Primer3 (Rozen and Skaletsky 2000) and synthesized by Sigma–Aldrich Canada (Oakville, ON, Canada)] to amplify all three seed lipoxygenase genes from the seed lipoxygenase triple null mutant line (OX948) and RG10 (a seed lipoxygenase wild-type line) in four overlapping fragments. In addition, the 1-kb flanking sequences of Gm13:42327983..42334252 and Gm13:42320689..42326985 (SoyBase; Grant et al. 2010) were used to sequence 5′ and 3′ untranslated regions (UTR) in *Lx1* and *Lx2* genes, respectively. The primers were used in PCR reactions with genomic DNA isolated from young, freeze-dried leaf tissue from OX948 and RG10 in a 3% (w/v) CTAB (Doyle and Doyle 1990). The PCR reactions were performed in 20 µl volumes containing the 1× PCR buffer and 3 mM $MgCl_2$ (supplied with enzyme), 0.1 mM of each of dNTP (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), 1.6 U *Taq* DNA Polymerase (Invitrogen Canada Inc., Burlington, ON), 5 µM of the forward and reverse primers and 25 ng of soybean genomic DNA, with a PTC-100^{TM} Programmable Thermal Controller (MJ Research Inc., Watertown, MA). The amplification program consisted of an initial 2 min denaturation step at 94°C followed by 35 cycles of denaturation at

94°C for 30 s, annealing at 55–60°C for 45 s and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. After electrophoresis [1% (w/v), agarose gel in 1× TBE buffer], OX948 and RG10 genomic DNA fragments were gel purified [Montage kit (Millipore Corp., Bedford, MA)] and cloned using the TOPO™ (Invitrogen) cloning kit according to the manufacturer's instructions. Plasmid DNA was extracted with QIAprep® Miniprep kit (Qiagen Inc.—Canada, Mississauga, ON) and used as a template for cycle sequencing (CEQ™ 8000 genetic analysis system, Beckman Coulter Inc., Fullerton, CA). Sequences were compared with existing soybean seed lipoxygenase sequences by BLAST searches at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) to ensure that the target genes had been cloned and assembled with the CAP3 program (http://deepc2.psi.iastate.edu/aat/cap/cap.html). The exon/intron structure initially determined by aligning genomic sequences with the available cDNA and *Lx3* genomic sequences was confirmed for *Lx1* (EU028320) and *Lx2* (EU028321) with their genomic sequences that were submitted to the GenBank (Shin et al. 2007) during the current study. The gene structure was also analyzed with the FGenesh 2.6 available at http://www.linux1.softberry.com (Salamov and Solovyev 2000). Translation of nucleotide sequences was generated on ExPASy (http://www.expasy.ch/tools/dna.html). OX948 and RG10 seed lipoxygenase sequences are submitted to the GenBank under accession numbers GU942741 to GU942746. The sequences were compared by BLAST searches against the soybean genome sequence (http://www.phytozome.net/soybean) to determine their positions on soybean sequence map. For each lipoxygenase gene, three level sequence comparisons were performed. First, potential mutations were detected by comparing the mutant OX948 and wild-type RG10 sequences. Once detected, sequence differences in OX948 (relative to RG10) were then compared with the wild-type reference sequences, which were initially used to design sequencing primers [GenBank accession J02795 (cDNA) and SoyBase sequence Gm13:42327983..42334252 (genomic DNA) for *Lx1* gene; GenBank accession J03211 (cDNA) and SoyBase sequence Gm13:42320689..42326985 (genomic DNA) for *Lx2* gene; GenBank accession X06928 (genomic DNA) for *Lx3* gene). Finally, in order to determine unique features of OX948 lipoxygenase mutant null alleles (sequence differences compared with RG10—excluding those if found in reference sequences), OX948 sequences were also compared with the publicly available (GenBank or literature) sequences of other seed lipoxygenase triple null mutants [Jinpumkong 2 (*lx1-c* allele, GQ227543; *lx3-a* allele GQ227544, Lenis et al. 2010; EU028327, Shin et al. 2007 and Kim et al. 2004 for *lx2* allele), GenBank accession D13949 cDNA (*lx2*, Wang et al. 1994) and Kyushu 111/K line (for *lx2*, Wang et al. 1994 and *lx3*, Wang et al. 1995)].

To associate a mutation with the lipoxygenase null phenotypes, several lipoxygenase-free lines (RO115, RO162, OR265, OR297, OR315 and OR322) and wild-type lines with all three seed lipoxygenases present (RO15, RO30, RO61, RO83, RO143 and RO168) from the reciprocal RG10 × OX948 cross were sequenced for the regions affected by the mutations in the lipoxygenase genes (approximately 500 bp).

Marker development and mapping

Sequence information for the seed lipoxygenase gene mutations in OX948 was used to design gene mutation-based markers.

L-1 markers

Based on the *Lx1* mutation, a *Lx1* gene-specific sequence tagged site (STS) marker Lox1-3 was developed using the following PCR primers: forward 5′-GCAATCTATGGT GATCAAAG-3′ and reverse 5′-ATAGTCCTCTATCA GAAGACGAAC-3′. An additional STS marker (Lox1-P2) was developed for the mutation in the promoter region of the *Lx1* gene using the forward 5′-TAATTCTGGTC CCTCTCTGAATAAG-3′ and reverse 5′-AGACACTACG GCCCAAGAAAGCAT-3′ primers.

L-2 markers

The *Lx2* gene mutation-based SNP marker Lox2-M1 was developed for OX948 *Lx2* based on the following PCR primers: forward 5′-gttcatagGTTAAATACTCAA-3′ and reverse 5′-TTTCAACAAGCTCTTCAAT-3′. The forward primer was designed to end at the SNP. An additional STS marker (Lox2-P1) was developed for the mutation in the promoter region of the *Lx2* gene using the forward 5′-TT TCGATCTTGGCGTTCTTC-3′ and reverse 5′-CCTCAT CAACACCACTGTCC-3′ primers.

L-3 markers

The *Lx3* gene mutation-based SNP markers were developed for the OX948 *Lx3* gene using the following PCR primers: common forward 5′-ACGTTAAACCAAACA TGCACTC-3′ and M1 (Lox3-PM1) reverse 5′-GTTTCA TACATAGGTACTTTTGGTT-3′ and M2 (Lox3-PM2) reverse 5′-GAACTTTGTATTGATAATCTGAGTAATA AA-3′. The reverse primers were designed to end at the SNPs. Two additional markers were developed for OX948 *Lx3* gene. A codominant cleaved amplified polymorphic sequence (CAPS) marker Lox3-$_{\text{HeaIII}}$ was developed based on the digestion of the monomorphic Lox3 PCR product (1,224 bp) produced from the following primers: forward

5′-AGTTCCCTCCACGAAGCAAG-3′ and reverse 5′-CTTGTCTCCATGACCCACCT-3′ with *Hae*III restriction endonuclease. The STS marker Lox3-3′ produced with the forward 5′-CGGCTTGATAACCCATTGTT-3′ and reverse 5′-CAAGCATTGTCCCAAACTTC-3′ primers was developed for the deletion at the 3′ UTR in OX948 *Lx3* gene.

The PCR mixtures, PCR programs [except for the annealing steps, which were performed at 60°C (Lox1-3, Lox1-P2, Lox2-P1, Lox3-$_{HaeIII}$ and Lox3-3′), 61°C (Lox2-M1), 62°C (Lox3-PM2) and 65°C (Lox3-PM1) and electrophoresis conditions (except for Lox2-P1, run for 5 h) were the same as those used to amplify the gene fragments for sequencing. The markers were used to screen the 169 F_5 RILs from the RG10 × OX948 cross to map their locations. Linkage analysis was performed using MAPMAKER/EXP version 3.0b (Lander et al. 1987). A minimum LOD score of 3.0 and maximum distance between two markers of 50.0 cM were used to associate loci into linkage groups. Recombination frequencies were converted to centimorgan distances using Kosambi's mapping function (Kosambi 1944). The RG10 × OX948 linkage map was aligned with the soybean Composite_2003 and sequence maps (SoyBase, Grant et al. 2010).

Results

The SDS–PAGE profile for the wild-type line RG10 seed protein extract has three bands, corresponding to the L-3, L-2 and L-1 isozymes, in positions just below the 97 kDa molecular weight marker. In contrast, this molecular weight region of the gel did not have any proteins in the profile obtained from the seed lipoxygenase triple null mutant line OX948 (Fig. 1a). In the colorimetric assays, RG10 seed extracts tested positive for all three isozymes by turning the solutions clear. The same tests for the OX948 samples indicated that they were negative for L-1 (the solution stayed blue), negative for L-2 (the solution stayed blue) and negative for L-3 (the solution remained yellow) (Fig. 1b).

Seed lipoxygenases gene structure

Comparisons of the cloned sequences for all three seed lipoxygenase genes from the triple null mutant line (OX948) and wild-type (RG10) to published sequences and GenBank/SoyBase sequences (Supplemental Figs. S1–S4) confirmed that the amplified DNA fragments corresponded to *Lx1*, *Lx2* and *Lx3*. All three genes were sequenced completely. Gene-specific sequencing primers are listed in Table 1.

The size of the *Lx1* gene is 6,024 bp in RG10 and 5,965 bp in OX948 (Table 2). The 'TATA' box (TATAAA)

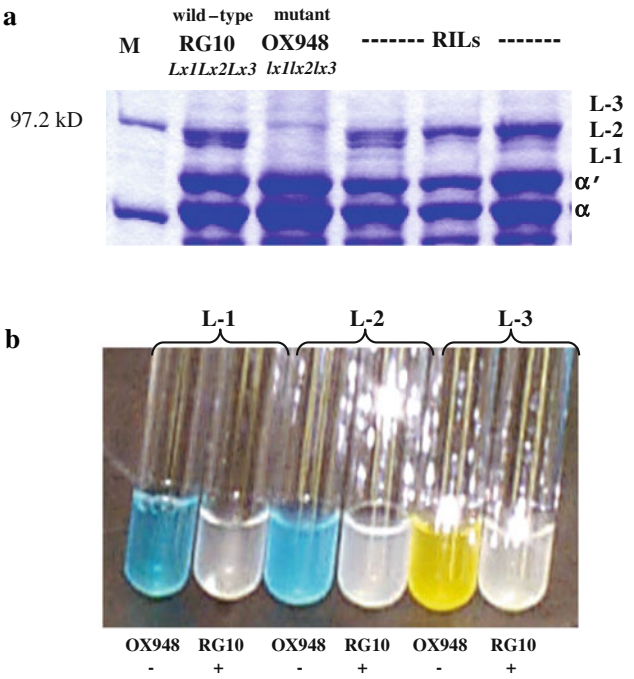

Fig. 1 Seed lipoxygenase assays. **a** SDS–PAGE profiles of lipoxygenases in soybean lines RG10 (wild type), OX948 (lipoxygenase triple null mutant) and several recombinant inbred lines (RILs); L-1 lipoxygenase 1, L-2 lipoxygenase 2, L-3 lipoxygenase 3, α′ and α, subunits of 7-S globulin (β-conglycinin). *M* is the marker lane and shows a band with a molecular weight of 97.2 kDa. **b** Colorimetric assays of seed lipoxygenases for soybean lines RG10 (wild type) and OX948 (lipoxygenase triple null mutant); L-1, L-2 and L-3 seed lipoxygenases 1, -2 and -3, respectively, + presence of seed lipoxygenases and − absence of seed lipoxygenases

is located on nucleotides 847 through 852 in RG10 and from 865 to 870 in OX948. The first methionine (ATG) occurs at position 976 in RG10 and 991 in OX948. The stop codon (TAA) is at 4,805 in RG10 and 4,746 in OX948. The polyadenylation [poly(A)] signal is located at position 4,859 in RG10 and 4,800 in OX948 (Supplemental Fig. S1). The RG10 open reading frame (ORF) (2,520 bp) encodes 839 amino acids with an estimated molecular weight of 94,436 Da. The OX948 ORF (2,446 bp) encodes 524 amino acids with an estimated molecular weight of 58,470 Da.

The size of the *Lx2* gene is 6,056 bp in RG10 and 6,171 bp in OX948 (Table 2). The 'TATA' box occurs between nucleotides 917 through 922 in RG10 and between nucleotides 1,035 to 1,040 in OX948. The first ATG (methionine) occurs at position 1,039 in RG10 and 1,157 in OX948. The stop codon (TAA) is located at 4,975 in RG10 and 5,091 in OX948. The first poly(A) signal is in position 5,021 in RG10 and 5,137 in OX948 (Supplemental Fig. S2). The ORF (2,601 bp) encodes 866 amino acids with an estimated molecular weight of 97,248 Da in RG10 and 97,239 Da in OX948.

Table 1 Soybean seed lipoxygenase gene-specific sequencing primers

Gene	Primer			PCR product size RG10/OX948 (bp)
	ID	Sequence 5′–3′	Position	
Lx1	Lox1-P2	TAATTCTGGTCCCTCTCTGAATAAG	72–1,216	1,145/1,160
		AGACACTACGGCCCAAGAAAGCAT	Gm13:42..52[a]	
	Lox1-1	CCCAAGAATGAGTTGGAAG	132–626	926
		ATATTCCTTGCGCTCTCCCG	J02795[b]	
	Lox1-2	CACCACTTGTGAGTTACAGA	553–1,255	1,254
		AGATCAAGGGAATCTGCTGT	J02795	
	Lox1-3	GCAATCTATGGTGATCAAAG	1,206–1,910	1,032/958
		ATAGTCCTCTATCAGAAGACGAAC	J02795	
	Lox1-4	AACCCCACATGGAGTTCGT	1,874–2,802	930
		CTAATAGTGCTCACTGCTC	J02795	
	Lox1-3′	GGAACAATGATCCGAGTCTGCAGGG	4,800–6,174	1,375
		GAAGGAACCAGATTCCTTAATCAGTTG	Gm13:42..52	
Lx2	Lox2-P1	TTTCGATCTTGGCGTTCTTC	13–1,170	1,158/1,270
		CCTCATCAACACCACTGTCC	Gm13:42..85[a]	
	Lox2-1	GTGTCGGGAATCCTGAACAGAG	99–709	1,081
		GTTCTTTGCGCTCTCCTTTTCCATC	J03211[b]	
	Lox2-2	TCCAAGTGAGACACCAGCAG	621–1,327	1,282
		CATCTGCTGTTATCTTACTGG	J03211	
	Lox2-3	CCGATGAAGAATTTGCAAGAG	1,193–1,955	1,259
		GCAGATGGATCCTTAATTGCCACT	J03211	
	Lox2-4	TGCCCCACATGGACTTCGA	1,953–2,661	712
		AGGAATCCCCCTGCAAGTC	J03211	
	Lox2-3′	CCATCCTAACAGTGAGGAAGGGTTGA	4,967–6,174	1,182/1,181
		CTATGCCTAGCAATGATTACCTTATGATTTC	Gm13:42..85	
Lx3	Lox3-1	GACTTAGTTGGCTCAACACTC	982–2,095	1,114
		CCACTCTTTGCGTTCTCCAG	X06928[b]	
	Lox3-2	CAAGTGAGACACCAGCTCCACTA	2,006–3,273	1,268
		GGGCTCTAGGTGTTCTTTGGTT	X06928	
	Lox3-3′	AGTTCCCTCCACGAAGCAAG	3,194–4,417	1,224
		CTTGTCTCCATGACCCACCT	X06928	
	Lox3-4	GAGAAGATCCTGAACTCCAAGCC	4,356–5,407	1,052
		GTTAGTTGCGTCCTGTGATG	X06928	

[a] SoyBase: *Lx1*, Gm13:42327983..42,334,252 (1 kb flanking); *Lx2*, Gm13:42320689..42,326,985 (1 kb flanking)

[b] NCBI

The size of the *Lx3* gene is 5,608 bp in RG10 and 5,529 bp in OX948 (Table 2). The 'TATA' box is located between nucleotides 706 through 711 in RG10 and from 705 to 710 in OX948. The first ATG occurs at position 787 in RG10 and 786 in OX948. The stop codon (TGA) is at 4,879 in RG10 and 4,880 in OX948. The poly(A) is located at position 4,914 in RG10 and at 4,915 in OX948 (Supplemental Fig. S3). The ORF (2,574 bp) encodes 857 amino acids with an estimated molecular weight of 96,782 Da in RG10 and 96,768 Da in OX948.

All three seed lipoxygenase genes consist of 9 exons and 8 introns (Fig. 2). All introns start with GT and end with AG. The size of exon 1 is different in the three lipoxygenase genes (154 bp in *Lx1*, 244 bp in *Lx2* and 208 bp in *Lx3*). There are also small (3–6 bp) differences in the sizes of exons 4 (−3 bp in *Lx2*), 6 (+6 bp in *Lx3*), 7 (+3 bp in *Lx1*) and 9 (+3 bp in *Lx1*) among the lipoxygenase genes. In contrast, all eight introns have different sizes in the three seed lipoxygenase genes (Table 2; Fig. 2). Introns 1, 2, 3 and 6 are the longest in *Lx3* and the shortest in *Lx1*. The *Lx2* gene is characterized by the longest intron 5 and the shortest introns 7 and 8. The other five introns in the *Lx2* gene are intermediate in size. The *Lx3* gene has the largest total size of introns (1,521 bp in OX948 and 1,519 in

Table 2 Nucleotide sizes of exons and introns of the three soybean seed lipoxygenase genes

Structure	Gene					
	Lx1		*Lx2*		*Lx3*	
	RG10	OX948	RG10	OX948	RG10	OX948
5′-UTR	975	990	1,038	1,156	786	785
Exon 1	154	154	244	244	208	208
Intron 1	315	315	344	344	448	450
Exon 2	287	287	287	287	287	287
Intron 2	118	118	127	127	191	191
Exon 3	241	241	241	241	241	241
Intron 3	205	205	226	226	323	323
Exon 4	321	321	318	318	321	321
Intron 4	225	225	151	151	122	122
Exon 5	86	86	86	86	86	86
Intron 5	120	120	209	209	104	104
Exon 6	102	102	102	102	108	108
Intron 6	83	83	106	106	131	131
Exon 7	308	308	305	305	305	305
Intron 7	111	111	88	86	93	93
Exon 8	264	190	264	264	264	264
Intron 8	133	133	85	85	107	107
Exon 9	757	757	754	754	754	754
3′ UTR	1,219	1,219	1,081	1,080	729	649
Total DNA sequenced	6,024	5,965	6,056	6,171	5,608	5,529
Total coding region	2,520	2,446	2,601	2,601	2,574	2,574
Total introns	1,310	1,310	1,336	1,334	1,519	1,521

RG10) while the *Lx1* and *Lx2* genes have similar total intron sizes (*Lx1* 1,310 bp in both RG10 and OX948; *Lx2* 1,336 bp in RG10 and 1,334 bp in OX948). The *Lx1* ORF encodes the smallest protein (839 amino acids in RG10) while the *Lx2* ORF encodes the largest protein (866 amino acids in RG10) (Supplemental Fig. S4).

Molecular basis of seed lipoxygenase null traits in OX948

To determine the molecular basis of the mutations that resulted in seed lipoxygenase null traits in OX948, all three lipoxygenase genes for the mutant line were isolated, sequenced and compared with the three gene sequences in a wild-type line, RG10. Once detected, sequence differences in OX948 (relative to RG10) were then compared with the wild-type reference sequences initially used to design sequencing primers. Detailed sequence comparisons revealed that the lipoxygenase null traits in OX948 are caused by the mutations in the *Lx1*, *Lx2* and *Lx3* genes.

Lx1 gene mutation in OX948

The *Lx1* gene in the triple lipoxygenase null line (OX948) differs from the sequence in the wild-type line (RG10) in 114 nucleotides (Supplemental Fig. S1). Two substitutions were detected in the coding region. One change in exon 1 (T–A) did not change the amino acid (arginine) coded for and one in exon 2 (C–A) changed a histidine to asparagine. Three nucleotides were changed in exon 3 (AGT codon for serine to GAG codon for glutamic acid) in OX948, RG10, and Gm13:42327983..42334252 (SoyBase) compared with the cDNA sequence J02795 (GenBank accession). Four single nucleotide changes were detected in intron 1 of OX948 (A–T, G–T, C–T and T–A) when compared with the RG10.

A 74 bp deletion was found in exon 8 of the *Lx1* gene in OX948 between positions 1,574–1,649 of the coding sequence in RG10 (where position 1 was assigned to the first nucleotide in the coding region of the RG10 gene and the first methionine in the protein sequence, Fig. 3a). This deletion introduces a stop codon (TAA), which would result in the premature termination of translation of the L-1 protein. The same 74 bp deletion at the beginning of exon 8 was detected in PI 408251(GenBank accession GQ227538) and Jinpumkong 2 (GenBank accession GQ227544) (Lenis et al. 2010). The same deletion was observed in all L-1 null lines with OX948 background (RO115, RO162, OR265, OR297, OR297, OR315 and OR322) but not in wild-type lines (RO15, RO30, RO61, RO83, RO143 and RO168) (Fig. 3b).

Differences between OX948 and RG10 *Lx1* gene were also identified in the 5′ UTR sequences including: seven substitutions (A–G, two times T–A, T–G, two times T–C and G–A), four insertions [two times AT, 13 bp (GCATACTAATATA) and TTT] and TA and CTT deletions in the OX948 *Lx1*. Two additional single nucleotide difference (C–A and A–G) detected in 3′ UTR region of the OX948 *Lx1* gene when compared with RG10 sequence (Supplemental Fig. S1) were also found in Gm13:42327983..42334252 (SoyBase).

Lx2 gene mutation in OX948

A comparison of the *Lx2* genes in mutant (OX948) and wild-type line (RG10) identified a single point mutation (T–A) at position 1,596 in exon 8 of the coding sequence (Fig. 4a). This change resulted in the conversion of the codon for histidine (CAT, H532) to a codon for glutamine (CAA). The mutation of H532, which is one of the iron ligands essential for L-2 activity, would result in dysfunctional L-2 protein. The same mutation was found in the Kyushu 111 and the K line (Wang et al. 1994). The same single base change (T–A) occurred in all L-2 null RILs

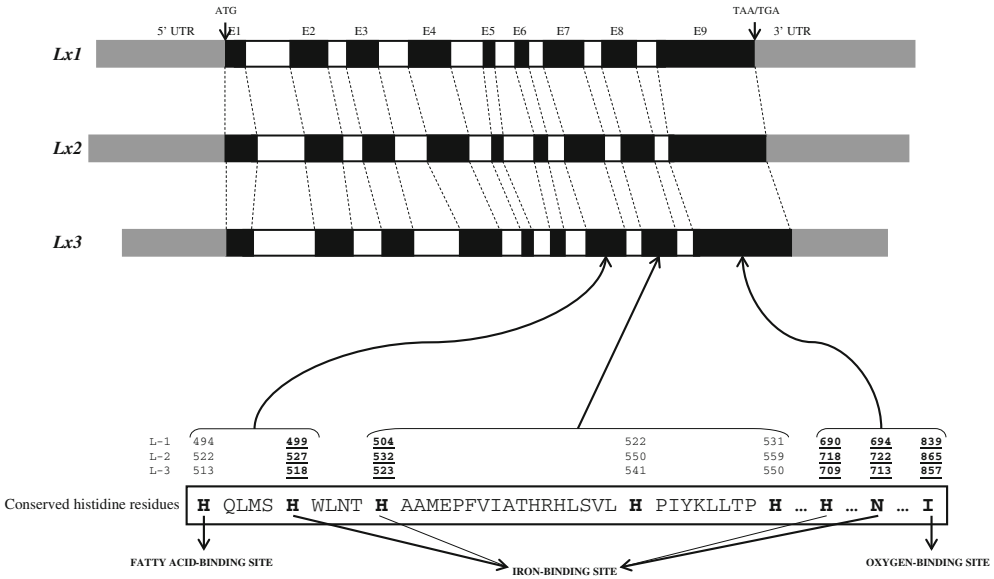

Fig. 2 Structure of seed lipoxygenase genes in soybean (wild type). *Black boxes* represent exons (E1–E9), *clear boxes* represent introns and *gray boxes* represent untranslated regions (UTR). Conserved histidine residues (exons 7, 8 and 9; based on the information from Steczko and Axelrod 1992), the fatty acid-, iron- and oxygen-binding sites are indicated

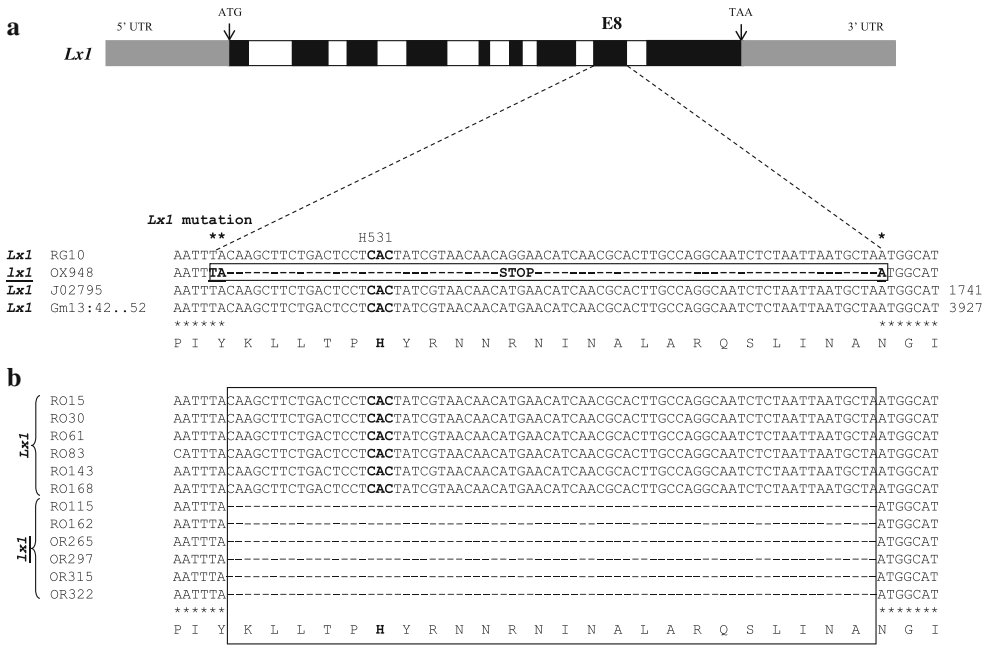

Fig. 3 Sequences surrounding the *lx1* gene mutation in OX948. *Black boxes* represent exons, *clear boxes* represent introns and *gray boxes* represent untranslated regions (UTR). The amino acids are shown below each codon. The location of the mutation is indicated by a *rectangle*. **a** The *lx1* gene mutation is a 74 bp deletion at position 1,574–1,649 of the coding sequence in RG10 that creates a stop codon in exon 8 and would cause premature termination of protein synthesis in OX948; J02795 is from the cultivar Century with normal L-1 activity (Shibata et al. 1987); Gm13:42..52 (Gm13:42327983..42334252) is from the cultivar Williams 82 with normal L-1 activity (http://www.phytozome.net/soybean). **b** Alignments of sequences for segments of the *Lx1* genes in recombinant inbred lines (RILs) from reciprocal crosses between RG10 [normal L-1 (*Lx1*)] and OX948 [no L-1 activity (*lx1*)]

from the RG10 × OX948 cross but not in the RILs with normal L-2 activity (Fig. 4b). When compared with the GenBank accession J03211 (cDNA), both OX948 and RG10 (as well as Gm13:42320689..42326985) have the insertion of GAA at positions 2,557–2,559, resulting in the introduction of a glutamic acid (E852) without changing the reading frame. In addition, both OX948 and RG10 code for threonine (ACA) at the position 678 instead of lysine

(AAA) (Supplemental Fig. S2). An additional single nucleotide differences detected in exon 5 (C–A) of the OX948 *Lx2* gene as compared to RG10 sequence were also found in Gm13:42320689..42326985 (SoyBase).

Differences between OX948 and RG10 were also detected in intron sequences [1 each in introns 4 (C–T) and 5 (G–A) and 2 each in introns 1 (T–C and G–A), 3 (G–A and T–G) and 7 (AT deletion). An additional single nucleotide difference detected in intron 1 (G–A) of the OX948 *Lx2* gene as compared to RG10 sequence (Supplemental Fig. S2) were also found in Gm13:42320689..42326985 (SoyBase). Three nucleotide differences in intron sequences were detected in both OX948 and RG10 (T–C substitution in intron 3 and AT deletion in intron 5) when compared with the SoyBase (Gm13:42320689..42326985) accession.

Differences between OX948 and RG10 detected in the *Lx2* 5′ UTR include: five substitutions (T–A, A–C, T–G, A–T and C–T), 175-bp insertion and five deletions [53 bp and 4 single nucleotides (two times T and two times G)] in OX948 *Lx2* sequence. Differences between OX948 and RG10 sequences in the *Lx2* 3′ UTR were found in 23 places and include: one nucleotide deletion (T) and 21 single nucleotide substitutions in OX948 (T–A, T–G, 9 T–C, 5 G–A, A–T, 2 C–T and 2 A–G) (Supplemental Fig. S2). An additional single nucleotide differences detected in 3′ UTR region (A–G) of the OX948 *Lx2* gene as compared to RG10 sequence were also found in Gm13:42320689..42326985 (SoyBase).

Lx3 gene mutation in OX948

Three single nucleotide differences were detected in the coding region of the OX948 *Lx3* gene as compared to *Lx3* in RG10. A single G–T substitution (exon 6) changed glutamic acid E382 to aspartic acid D382. The second substitution (A–G, exon 7) introduced a *Hae*III restriction site at this position by changing the codon for arginine R463 from AGA to AGG. Another substitution (G–A, exon 9) changed the codon for alanine A797 from GCG to GCA.

In addition, when compared with the GenBank accession X06928, six substitutions were detected in both OX948 and RG10 that resulted in amino acid changes [C–G (exon 1) changes histidine H25 to aspartic acid, C–T (exon 1) changes proline P57 to serine, T–C (exon 2) changes leucine L112 to proline, G–A (exon 3) changes valine V201 to isoleucine, G–A (exon 7) changes glycine G428 to aspartic acid, and C–T (exon 8) substitution changes the codon for

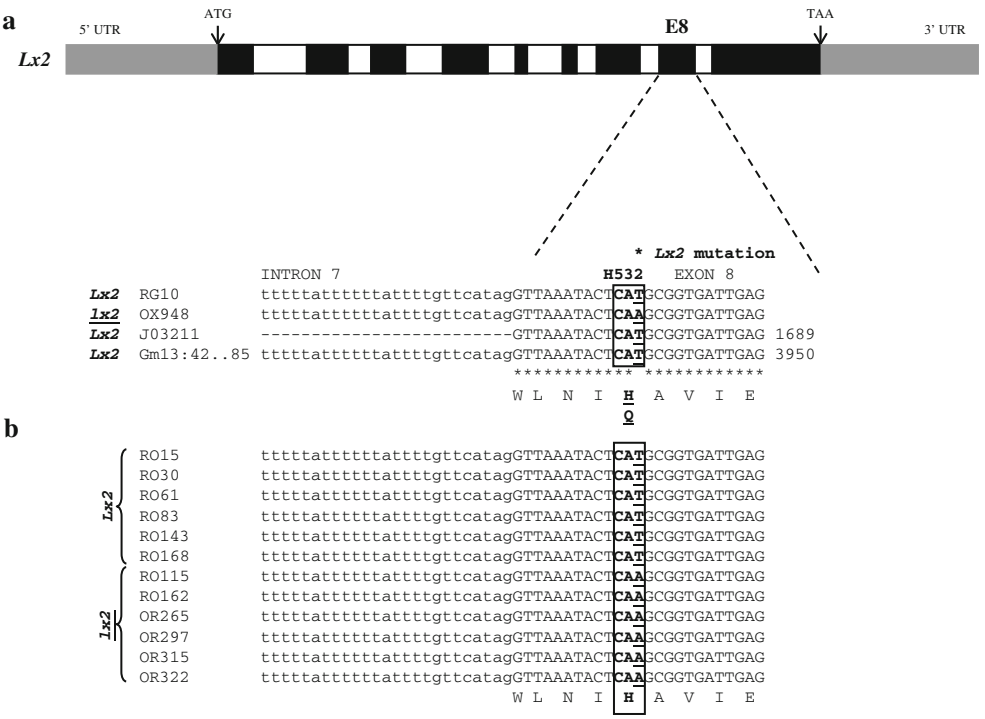

Fig. 4 Soybean seed lipoxygenase sequences surrounding the *lx2* gene mutation in OX948. *Black boxes* represent exons, *clear boxes* represent introns and *gray boxes* represent untranslated regions (UTR). The amino acids are shown below each codon. The location of the mutation is indicated by a *rectangle*. **a** The mutation in the *lx2* gene in OX948 is a single base change (T–A) in exon 8 at position 1,596 of the coding sequence, which changes the iron-ligand histidine H532 to glutamine; J03211 is from an unnamed soybean with normal L-2 activity (Shibata et al. 1988); Gm13:42..85 (Gm13:42320689..42326985) is from the cultivar Williams 82 with normal L-2 activity (http://www.phytozome.net/soybean). **b** Alignments of sequences for segments of the *Lx2* genes in recombinant inbred lines (RILs) from reciprocal crosses between RG10 [normal L-2 (*Lx2*)] and OX948 [no L-2 activity (*lx2*)]

aspartic acid D603 from GAC to GAT]. Three nucleotide differences were detected in introns of OX948 *Lx3* gene as compared to RG10 sequence (AT insertion in intron 1 and G–A substitution in intron 3). An additional difference in intron 4 sequence (G–A substitution) was detected in both OX948 and RG10 when compared with the GenBank accession X06928.

Eleven nucleotide differences were detected between the 5′ UTR sequences of the OX948 and RG10 *Lx3* genes (10 substitutions including: two times C–T, A–G, T–A, 2 times T–G, 2 times T–C, C–A and G–C and an A deletion). Two single base substitutions from T–A at position −584 (M1) and C–T at position −635 (M2) (Fig. 5a) found in the paired AAATAC box in OX948 *Lx3* gene are probably the cause of the lack of L-3 protein in mature OX948 seed. These two point mutations were also present in all L-3 null RILs (Fig. 5b). The same mutations were reported for the Kyushu 111 and the K line (Wang et al. 1995). Two additional single-nucleotide difference (A–T) detected in 5′ UTR region of the OX948 *Lx3* gene when compared with RG10 sequence was also found in GenBank accession X06928. Six substitutions (G–A, 2 times G–C, C–G, A–G and C–T) were detected in both OX948 and RG10 compared to the GenBank accession X06928 (Supplemental Fig. S3).

Differences between the OX948 and RG10 3′ UTR sequences include two substitutions (C–T and A–G) and two deletions (15 and 65 bp). When compared with the GenBank accession X06928 an additional A–G substitution was detected in both OX948 and RG10 3′ UTR sequences (Supplemental Fig. S3).

Seed lipoxygenase null alleles in OX948

The seed lipoxygenase triple null trait in OX948 is caused by the null alleles of all three seed lipoxygenase genes. To determine the uniqueness of these alleles, the OX948 sequences were compared with the sequences of other publicly available seed lipoxygenase null mutants. The characteristics of the OX948 lipoxygenase null alleles including its unique features (sequence differences as compared to RG10—excluding those if found in reference sequences) were presented in Fig. 6.

The lx1 allele

The OX948 *lx1* allele is characterized by (1) a 74 bp deletion at the beginning of exon 8 (the cause of L-1 null phenotype, found also in PI 408251 and Jinpumkong, Lenis et al. 2010), (2) seven SNPs and a 3-bp deletion in the region from 5′ UTR to the end of exon 2 (found also in Jinpumkong 2, Lenis et al. 2010), and (3) a 5′ UTR sequence difference that is unique to the OX948, includes a 2 bp deletion, four insertions and six SNPs (Fig. 6a).

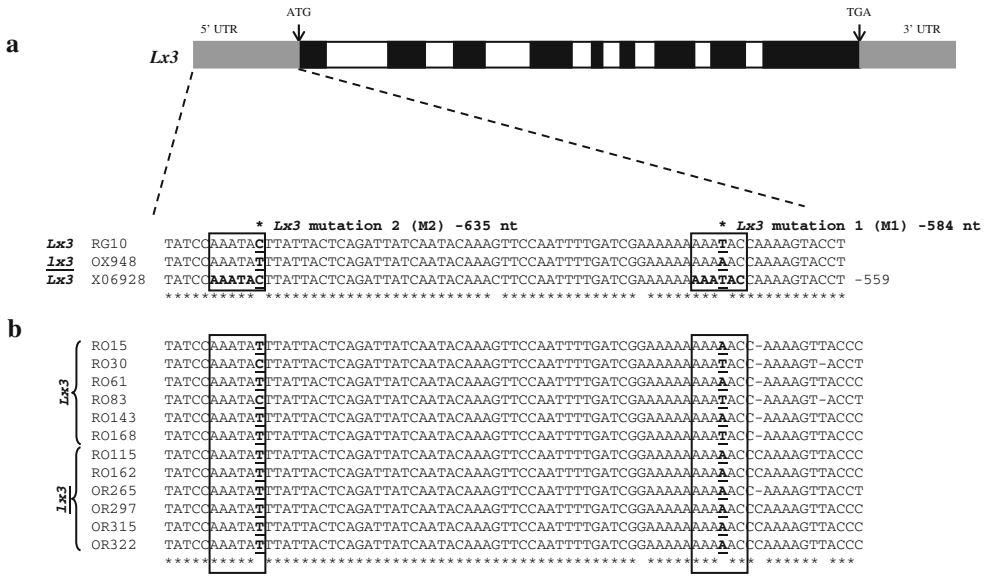

Fig. 5 Soybean seed lipoxygenase sequences surrounding the *lx3* gene mutation in OX948. *Black boxes* represent exons, *clear boxes* represent introns and *gray boxes* represent untranslated regions (UTR). The locations of the mutations are indicated by *rectangles*. **a** The *lx3* gene mutation in OX948 is in the promoter region—two single base substitutions: T–A at the positions −584 (M1) and C–T at the position −635 (M2) in the paired AAATAC box; XO6928 is from the cultivar Dare with normal L-3 activity (Yenofsky et al. 1988). **b** Alignments of sequences for segments of the *Lx3* genes in recombinant inbred lines (RILs) from reciprocal crosses between RG10 [normal L-3 (*Lx3*)] and OX948 [no L-3 activity (*lx3*)]

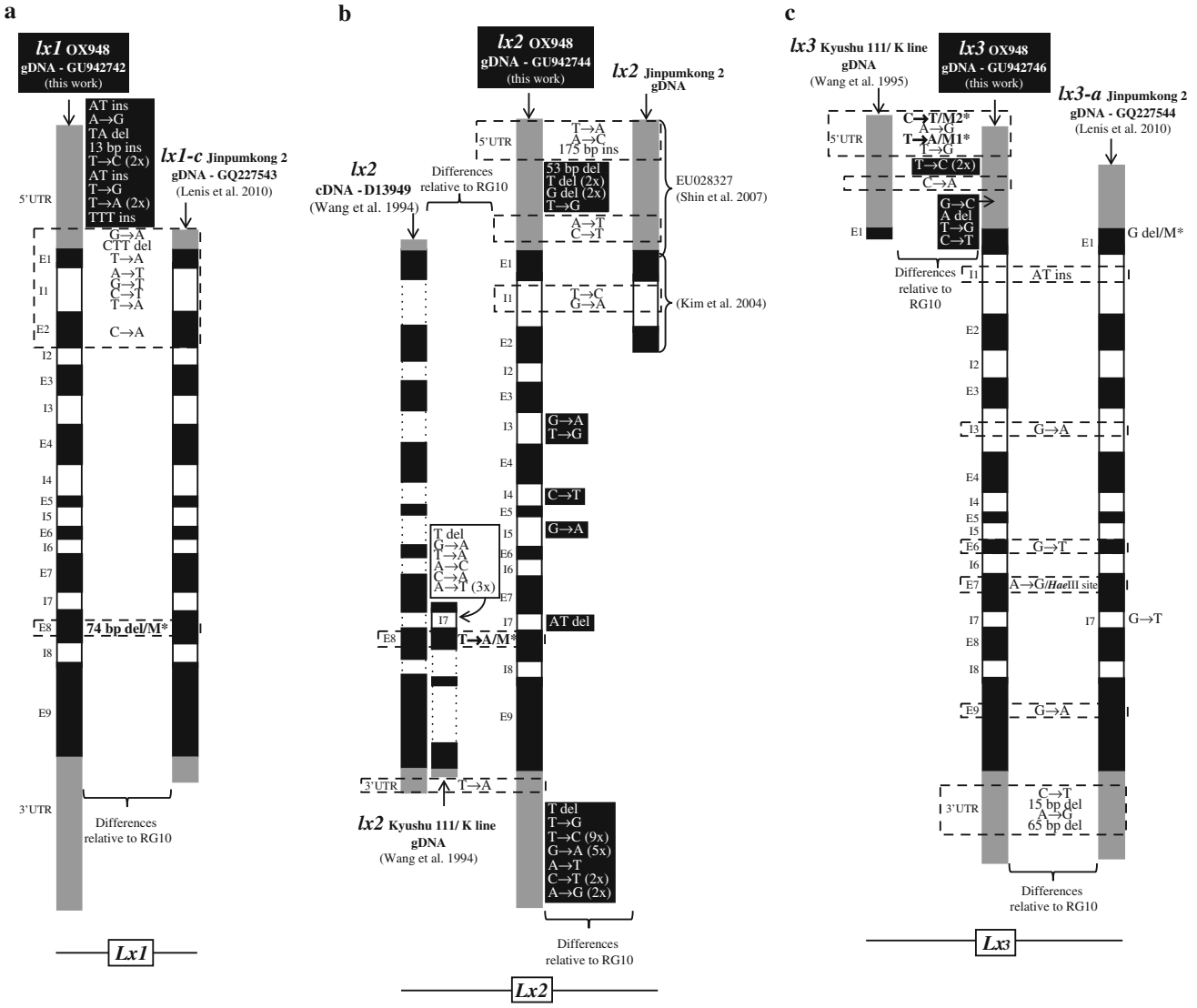

Fig. 6 Comparison of seed lipoxygenase null alleles in OX948 (*lx1lx2lx3*) with the publicly available lipoxygenase mutants. Mutations (in all mutants) are defined as sequence differences from the wild-type RG10 (indicated by *down brace*) and are shown as nucleotides beside the *bars*. Differences (from RG10) shared by alleles are placed in the *middle* and connected by *dashed boxes*. OX948-specific sequence differences are shown in *black boxes*, *bold*. The unique features of OX948 seed lipoxygenase null alleles include: *Lx1*–5′ UTR; *Lx2*–5′ UTR, 3′ UTR and several SNPs in several introns; *Lx3*–5′ UTR. **a** OX948 *lx1* allele (*left*) when compared with the Jinpumkong 2 *Lx1* gene (*lx1-c* allele *right*). **b** OX948 *lx2* allele (*middle*) when compared with the two Jinpumkong 2 *Lx2* partial genomic DNA (gDNA) sequences (*right*) and GenBank accession D13949 (cDNA) and Kyushu 111/K line segmental gDNA sequences (*left*). **c** OX948 *lx3* allele (*middle*) when compared with the Jinpumkong 2 *Lx3* gene sequence (*right*) and Kyushu 111/K line partial gDNA sequence (*left*). Gene, germplasm name, sequence type, GenBank accession (if available) and reference are indicated for each sequence on the *top* (or *bottom* for *lx2* Kyushu 111/K line) of the *bar*. In each *bar*, *black boxes* represents exons (E), *clear boxes* represent introns (I) and *gray boxes* represent untranslated regions (UTR). Not available sequences are indicated by *clear dotted boxes*. Gene mutations are indicated by an *M**

The lx2 allele

The OX948 *lx2* allele includes (1) four SNPs and 175-bp insertion in the 5′ UTR (found also in Jinpumkong 2, Shin et al. 2007), two SNPs in intron 1 (found also in Jinpumkong 2, Kim et al. 2004) and one SNP (T–A, cause of L-2 null phenotype) in exon 8 and one SNP in 3′ UTR (found also in Kyushu 111 and K line, Wang et al. 1994); and (2) sequence differences unique to the OX948 that include five deletions (four 1 and 53 bp) and one SNP at the 5′ UTR, 1 bp deletion and 20 SNPs at the 3′ UTR and 4 SNPs in several introns (2 in intron 3

and 1 in each introns 4 and 5) and 2 bp deletion in intron 7 (Fig. 6b).

The lx3 allele

The OX948 *lx3* allele includes (1) a 2 bp insertion in intron 1, four SNPs in intron 3 and exons 6, 7 and 9 and two SNPs and two deletions (15 and 65 bp) at the 3′ UTR (found also in Jinpumkong 2, Lenis et al. 2010); and (2) sequence differences unique to OX948 that include one deletion and five SNPs at the 5′ UTR (Fig. 6c).

Seed lipoxygenases gene mutation-based markers

L-1 markers

The *Lx1* gene-based sequencing primer pair Lox1-3 revealed a size polymorphism between RG10 and OX948 (Table 3). The size of the PCR fragment in RG10 is 1,032 bp, while the OX948 fragment is 958 bp. The OX948-like fragment (958 bp) was present in all seed lipoxygenase-free RILs with the OX948 background (RO115, RO162, OR265, OR297, OR315 and OR322) while the RG10 fragment (1,032 bp) was present in all wild-type RILs used in this study (data not shown). The development of the second *Lx1* STS marker (Lox1-P2) was based on a 15 bp insertion/deletion difference between the 5′ UTR sequences of RG10 and OX948. It resulted in PCR products of 1,145 bp for RG10 and 1,160 bp for OX948. Both markers mapped to chromosome 13 (F) 3.6 and 3.3 cM, respectively from the *Lx1* gene (Fig. 7), which was mapped previously using the colorimetric score (Reinprecht 2002; Reinprecht et al. 2006). It is likely that there were some phenotyping errors in the colorimetric assay that resulted in the apparent distance between the *Lx1* gene locus and the Lox1-3 or Lox1-P2 markers.

L-2 markers

An SNP marker Lox2-M1 was developed for the mutation in the *Lx2* gene (Table 3). A 629 bp PCR product was amplified from the OX948 mutant, but was absent from wild-type (RG10) reactions. The OX948-like fragment (629 bp) was present in all L-2 null RILs, but absent in wild-type RILs (data not shown). A second marker (Lox2-P1) was developed based on the insertion/deletion in the 5′ UTR of the OX948 *Lx2*. A PCR product of 1,270 bp was present in OX948 and 1,158 bp was amplified from RG10. Both markers mapped to the same location on chromosome 13 (F), 5.0 cM from the *Lx2* gene (Fig. 7), which was mapped previously using the colorimetric score (Reinprecht 2002; Reinprecht et al. 2006). Colorimetric phenotyping errors are likely the reason for the distance between the *Lx2* gene and the mutation-based markers for the gene.

L-3 markers

Initially, a Lox-$_{HaeIII}$ codominant CAPS marker was developed after digestion of the monomorphic Lox3 PCR product (1,224 bp) with restriction endonuclease *Hae*III (Table 3). The RG10 fragment was 754 bp compared to two fragments of 374 and 380 bp for the OX948 parent. A band of 469 bp occurred in both, OX948 and RG10 as well as all F_5 progeny. In addition, two SNPs (Lox3-PM1 and Lox3-PM2) were developed based on the *Lx3* gene mutation. The dominant Lox3-PM1 (226 bp) and Lox3-PM2 (180 bp) fragments were present in OX948 and all L-3 null lines but were missing in RG10 (data not shown). All four markers mapped to chromosome 15 (E). The Lox3-$_{HaeIII}$ marker co-segregated with the *Lx3* gene, the Lox3-3′ marker mapped 0.6 cM from *Lx3* while the Lox3-PM1 was 4.4 cM and the Lox3-PM2 was 10.0 cM apart from *Lx3* gene (Fig. 7), which was mapped previously using the score from the colorimetric assay (Reinprecht 2002; Reinprecht et al. 2006). It is likely that colorimetric phenotyping and some marker ambiguities were responsible for the lack of perfect correspondence between the gene and marker locations.

Discussion

The genomic DNA sequences we obtained for *Lx1*, *Lx2* and *Lx3* in OX948 and RG10 revealed that the seed lipoxygenases in soybean are 75–87% similar (ORF) (Supplemental Figs. S1 to S4). The high degree of similarity led to an expectation that the three genes would have similar intron/exon structures and that they would be similar to the genomic DNA sequence available for *Lx3* (X06928) at the beginning of the study. This was confirmed by aligning our sequences to the Williams 82 *Lx1* (GenBank accession EU028320) and *Lx2* (GenBank accession EU028321) genomic sequences that became available during the course of this study (Shin et al. 2007). All three seed lipoxygenase genes consist of 9 exons and 8 introns as reported previously (Yenofsky et al. 1988). There were 3–6 bp differences in the sizes of exons 4, 6, 7 and 9 among lipoxygenase genes. The most variable was exon 1 which has different sizes in all three lipoxygenase genes. However, as compared to exons, intron sizes are more variable. All eight introns have different sizes in the three seed lipoxygenase genes.

We used the *Lx1* and *Lx2* sequence information available in SoyBase (Grant et al. 2010) to design PCR primers to amplify and clone approximately 1,000 bp from both the

Table 3 Soybean seed lipoxygenase gene-specific markers

Gene	Marker	Primer			PCR product (bp)	
		Sequence 5′–3′	Position	*T* (°C)[a]	RG10	OX948
Lx1	Lox1-P2 (STS)	TAATTCTGGTCCCTCTCTGAATAAG	72–1,216	60	1,145	1,160
		AGACACTACGGCCCAAGAAAGCAT	Gm13:42..52[b]			
	Lox1-3 (STS)	GCAATCTATGGTGATCAAAG	1,206–1,910	60	1,032	958
		ATAGTCCTCTATCAGAAGACGAAC	J02795[c]			
Lx2	Lox2-P1 (STS)	TTTCGATCTTGGCGTTCTTC	13–1,170	60	1,158	1,270
		CCTCATCAACACCACTGTCC	Gm13:42..85[b]			
	Lox2-M1 (SNP)	GTTCATAGGTTAAATACTCA**A**	3,977–4,606	61	Missing	629
		TTTCAACAAGCTCTTCAAT	GU942744[d]			
Lx3	Lox3-PM1 (SNP)	ACGTTAAACCAAACATGCACTC	76–301	65	Missing	226
		GTTTCATACATAGGTACTTTTGGT**T**	X06928[c]			
	Lox3-PM2 (SNP)	ACGTTAAACCAAACATGCACTC	76–255	62	Missing	180
		GAACTTTGTATTGATAATCTGAGTAATAA**A**	X06928			
	Lox3-$_{HaeIII}$ (CAPS)	AGTTCCCTCCACGAAGCAAG	3,194–4,417	60	754 + 469	374/380 + 469
		CTTGTCTCCATGACCCACCT	X06928			
	Lox3-3′ (STS)	CGGCTTGATAACCCATTGTT	5,208–5,683	60	476	396
		CAAGCATTGTCCCAAACTTC	X06928			

[a] Annealing temperature

[b] SoyBase: *Lx1*, Gm13:42327983..42,334,252 (1 kb flanking); *Lx2*, Gm13:42320689..42,326,985 (1 kb flanking)

[c] NCBI

[d] This study

5′ UTR and 3′ UTR regions of these genes. In the *Lx1* gene, the position of the TATA box (TATAAA) is 129 bp in RG10 and 126 bp in OX948 from the first ATG (976 and 991). Since the initiation of transcription in most eukaryotic genes occurs approximately 30 nucleotides downstream from the TATA box, the *Lx1* coding sequence is preceded by approximately 90 and 91 bp 5′ UTR in RG10 and OX948, respectively. The size of the 5′ UTR in the *Lx2* gene is estimated to be similar. The estimated size of 46 bp for the 5′ UTR in *Lx3* corresponds well to that previously reported for this gene (Yenofsky et al. 1988).

The *Lx2* ORF encodes the largest protein (866 amino acids in RG10) while the *Lx1* ORF encodes the smallest protein (839 amino acids in RG10) (Supplemental Fig. S4). This corresponds well with the relative positions of the lipoxygenase proteins in the SDS–PAGE gel (Fig. 1a).

The pedigree of OX948, a seed lipoxygenase triple null mutant, includes an unnamed triple lipoxygenase null source produced by γ-irradiation. The γ-rays (considered low linear energy transfer radiation) normally induce rearrangements and deletions (Bruggemann et al. 1996; Shikazono et al. 1998, 2001). In conventional plant breeding (Chopra 2005), γ-irradiation has been used at low doses to induce mutations in mungbean (Sangsiri et al. 2005), miniature tomato (Matsukura et al. 2007), or to produce four-leaf white clover mutants (Song et al. 2009). However, only few mutants have been characterized at the molecular level (Wilkinson and Crawford 1991; Kieber et al. 1993; Nambara et al. 1994).

Since the molecular basis of the lipoxygenase null alleles for OX948 have not previously been investigated this study characterized the molecular differences in three seed lipoxygenase genes between OX948 and a soybean genotype (RG10) with normal lipoxygenase levels. The 74 bp deletion in exon 8 of the OX948 *Lx1* gene (at the position 1,574–1,649 of the coding sequence) introduces a stop codon, which would result in early termination of translation of the L-1 protein to give a OX948 L-1 protein of 524 amino acids when compared with 839 amino acids in wild-type (RG10) L-1 protein. The deletion would eliminate the highly conserved H531 and iron ligands H690, N694 and I839, which are necessary for L-1 enzymatic activity. Previous studies with soybean L-1 showed that mutations that change iron ligands (H499, H504, H690, N694 and I839) produce inactive enzymes (Steczko et al. 1992; Kramer et al. 1994). The same deletion was observed in all six selected L-1 null lines, but not in the six RILs with L-1 activity. The *lx1* allele in OX948 (GenBank accession GU942742) is likely the same as the *lx1-c* detected in Jinpumkong 2 (GenBank accession GQ227543, Lenis et al. 2010) since it also has the same 3-bp deletion and seven SNPs at the 5′ end of the gene

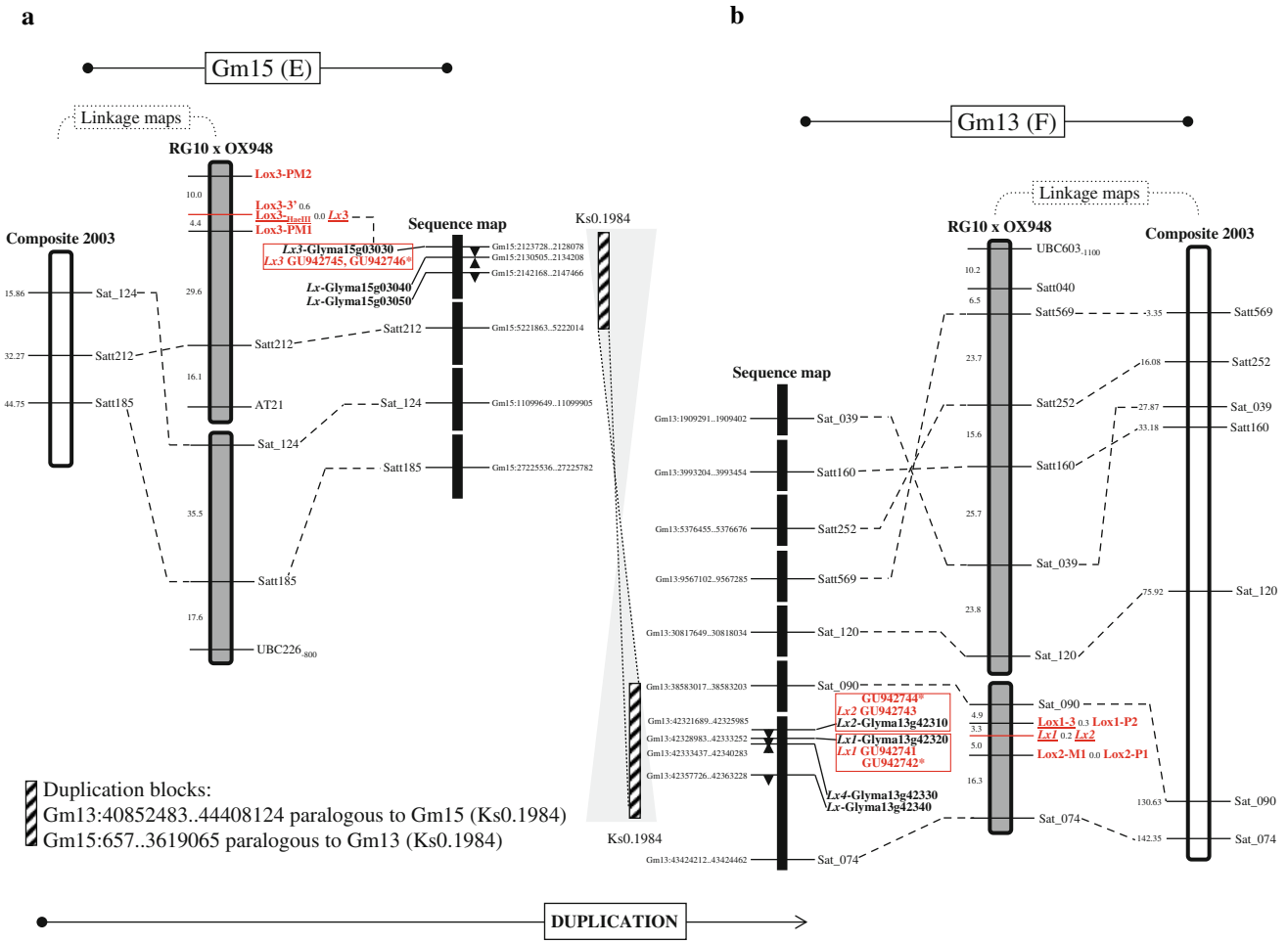

Fig. 7 Positions of the seed lipoxygenase genes and gene mutation-based markers on the RG10 × OX948 linkage map [(shown in *red*, *bold*; previously mapped (Reinprecht 2002; Reinprecht et al. 2006) are *underlined*]. **a** The *Lx3* gene and *Lx3* gene mutation-based markers mapped on chromosome Gm15 (or linkage group E). **b** The *Lx1* and *Lx2* genes as well as *Lx1* and *Lx2* gene mutation-based markers mapped on chromosome Gm13 (or linkage group F). The name of *each map* is indicated on the *top* of the *bar* or *line*. For each chromosome, the RG10 × OX948 linkage map (*gray bar* shown in the *middle*) was aligned with the soybean Composite_2003 map (SoyBase) (*white bar* shown on the *left side* in Gm15 and on the right side in Gm13). *Names* of the *loci* are indicated on the *right side* of each chromosome. Common markers and genes are connected by *dashed lines*. In the RG10 × OX948 map, distances (cM, calculated by the Kosambi function) between markers are indicated on the *left side* of each chromosome. In the Composite_2003 map, the positions of the markers are indicated on the *left side* of each chromosome. In the soybean sequence map (SoyBase), distances [in base pairs (bp)] between markers/genes are indicated on the *left side* of Gm13 and on the *right side* of Gm15. The *arrows* indicate sequence directions of the lipoxygenase genes on the chromosomes. The in silico positions of the OX948 and RG10 lipoxygenase gene sequences (GenBank accessions GU942741 to GU942746) on soybean sequence map (SoyBase) are shown in *red*, *boxed*. Duplicated regions between chromosomes 15 and 13 (Ks0.1984) are indicated by *wide upward diagonal patterned bars*

(Fig. 6a). However, the OX948 sequence is longer when compared with the published Jinpumkong 2 *Lx1* sequence. This allowed us to identify additional differences between the OX948 null allele and a functional *Lx1* gene found in RG10 in the 5′ UTR [6 SNPs, 4 insertions (3, 2 and 13 bp) and 2 bp deletion]. These differences were used to design the new Lox1-P2 marker for the OX948 *lx1* allele. The polymorphism of the previously developed codominant STS marker Lox1-3 (Reinprecht 2002; Reinprecht et al. 2006) is based on the 74 bp deletion in exon 8. In general, this marker amplifies the same region as the recently developed marker based on the *lx1-c* allele in Jinpumkong 2 (Lenis et al. 2010). Therefore, the Lox1-3 marker should also be useful for Jinpumkong 2-based germplasm.

The L-2 null mutation in OX948 is the result of a single point mutation (T–A) at the 1,596 position of the coding sequence, that changes histidine (H532, which is one of the iron ligands essential for L-2 activity) to glutamine. Wang et al. (1994) reported the same single point mutation (T–A) at the H532 that resulted in a null L-2 phenotype in mature seeds of the Kyushu 111 and the K line. This replacement of iron-ligand histidine with glutamine causes structural

distortion of the protein and leads to its dysfunction (Wang et al. 1994). The second difference, found in both OX948 and RG10, compared with GenBank accession J03211 (cDNA) is the insertion of GAA at the position 2,557–2,559, resulting in the introduction of glutamic acid E852 without changing the reading frame. The same insertion was described by Wang et al. (1994) [Suzuyutaka, Kyushu 111, K line and GenBank accession D13949)] and Shin et al. (2007) (GenBank accession EU028321). The OX948 *lx2* gene sequence is the only complete genomic DNA sequence for a L-2 null mutant submitted to GenBank and on the basis of polymorphisms with other *Lx2* sequences in GenBank it appears to be a unique allele. When compared with the available two partial genomic DNA (gDNA) sequences of the 5′ end of the *Lx2* gene in Jinpumkong 2 [1,851 bp (5′ UTR and part of exon 1, GenBank accession EU028327) and 804 bp (intron 1 with parts of exon 1 and 2; Kim et al. 2004)], the OX948 *lx2* allele has five unique deletions (53 bp, two times T and two times G) and one SNP in the 5′ UTR. The deletions at the 5′ end of the gene (compared with the RG10 *Lx2* allele) were used to design the Lox2-P1 marker for the OX948 *lx2* allele. We also identified sequence differences between the intron and 3′ UTR regions of the *Lx2* genes in OX948 and RG10 including: two SNPs in intron 3, one SNP in introns 4 and 5, an AT deletion in intron 7 and a T deletion and 20 SNPs at the 3′ end of the gene (Fig. 6b).

The two single base substitutions in the promoter region affecting the paired AAATAC boxes are probably the cause of the OX948 *Lx3* null mutation. The same T–A base change at position −585 (M1, −584 in OX948 because of one A deletion) and C–T base change at position −636 (M2, −635 in OX948 because of one A deletion) have previously been described for the Kyushu 111 and K line γ-mutants (Wang et al. 1995). It was shown that these substitutions impaired promoter function in tobacco cells, thus explaining the absence of an L-3 protein in plants with the *Lx3* null mutation. The OX948 *lx3* allele differs from the 5′ UTR sequences available for Kyushu 111 and K line in an A deletion and five SNPs. The *lx3* allele in OX948 is also different from the Jinpumkong 2 *lx3-a* allele, by the absence of a frame shift mutation (G deletion) in position 101 in exon 1 (Lenis et al. 2010). However, in comparison to the genotype with normal L-3 activity (RG10) the two triple null mutants share the same differences, including an AT insertion in intron 1, one SNP in intron 3, exon 6, exon 7 and exon 9 and two SNPs and 15 bp and 65 bp deletions at the 5′ UTR of the gene (Fig. 6c). The molecular basis of a previously developed codominant CAPS marker Lox3.$_{HaeIII}$ (Reinprecht 2002; Reinprecht et al. 2006) is the A–G substitution in exon 7, which introduces a *Hae*III restriction site. The deletions at the 5′ UTR were used to design the new Lox3-3′ marker. Both markers could potentially be used with Jinpumkong 2-based germplasm.

In conclusion, the seed lipoxygenase triple null characteristic of OX948 is caused by unique null alleles of three seed lipoxygenase genes. In comparison to published sequences, the unique features of OX948 null alleles include: *Lx1*–5′ UTR (new Lox1-P2 marker); *Lx2*–5′ UTR (new Lox2-P1 marker), 3′ UTR and SNPs in several introns; *Lx3*-one deletion and five SNPs at 5′ UTR.

In the present study, the same *Lx3* mutations were detected in all six selected RG10 × OX948 *Lx3* null RILs. However, these mutations were unexpectedly also detected in some of the six randomly selected RG10 x OX948 L-3 positive RILs. The M2 (−635) mutation was observed in line RO168, while lines RO15, RO61 and RO143 had both mutations in the promoter region (Fig. 5b). There might be several possible reasons for this discrepancy. Cross reaction among lipoxygenase isozymes is possible. All the ambiguous lines cleared the L-1, L-2 and L-3 solutions, and were classified as *Lx1_Lx2_Lx3* positives. Because they contained normal genes for the two other lipoxygenases (*Lx1Lx2*), it is possible that the molecular assay was correct and that the other enzymes reacted with the reagent in the L-3 solution and compensated for the lack of the L-3 isozyme.

Another explanation for the anomalous result may be related to the multiple copies of genes in soybean arising from its tetraploid nature (Shoemaker et al. 2008), which may allow for the existence of an additional copy of the *Lx3* gene. Initially, soybean seed lipoxygenases were designated as four distinct isoforms, including: L-1, L-2, L-3a and L-3b (Siedow 1991). Due to L-3a and L-3b similarity, it was thought that any difference between them arose from posttranslational modification of a protein coded by the same gene (Siedow 1991) because in naturally occurring L-3 mutants, both L-3a and L-3b activities are missing (Kitamura 1984). Based on the tetrad soybean genome structure, four copies of duplicated genes or four homologous regions in soybean are theoretically expected. In fact, two pairs of seed lipoxygenase regions were detected in the soybean genome sequence and, by in silico mapping, nineteen lipoxygenase genes were located on chromosomes 15 (E), 13 (F), 8 (A2) and 7 (M) (Shin et al. 2008). The region Ks0.1984 on chromosomes 15 and 13 are syntenic, as well as region Ks0.1827 on chromosomes 8 and 7 (SoyBase; Grant et al. 2010). Previously, we mapped *Lx1* and *Lx2* to chromosome 13 (F) and *Lx3* to chromosome 15 (E; Reinprecht 2002; Reinprecht et al. 2006). However, there might be additional regions that are amplified by the seed lipoxygenase markers in the Ks0.1827 region on chromosomes 7 and 8.

The colorimetric assay and SDS–PAGE are the most often used conventional techniques for detecting seed

lipoxygenases in soybean. Both techniques gave accurate and reliable assessments of the presence of seed lipoxygenases in wild-type line (RG10) and absence of lipoxygenases in mutant line (OX948). However, procedures required significant amounts of time for sample preparation and running the assays, especially SDS–PAGE. The current study has lead to an understanding of molecular basis of the null alleles for the three seed lipoxygenase genes in OX948. Sequencing the complete seed lipoxygenase genes also allowed us to characterize previously developed *Lx1* and *Lx3* gene-specific marker (Lox1-3 and Lox3-$_{HaeIII}$; Reinprecht 2002; Reinprecht et al. 2006) and to develop *Lx2* and *Lx3* gene mutation-based markers (Lox2-M1, Lox3-PM1 and Lox3-PM2) that are effective in identifying genotypes with any combination of the null or wild-type seed lipoxygenase alleles in material derived from OX948 and RG10. Moreover, based on the additional sequence differences identified between OX948 and RG10 in *Lx1*, *Lx2* and *Lx3*, at least one additional marker was developed for each lipoxygenase gene (Lox1-P2 for the *Lx1* 5′ UTR, Lox2-P1 for the *Lx2* 5′ UTR and Lox3-3′ for the *Lx3* 3′ UTR, respectively). Although the usefulness of these markers might be limited by the allele frequency of the non-causative mutations being present in different soybean germplasm, they will be useful for derivatives of OX948. The usefulness of the CAPS marker Lox3-$_{HaeIII}$ for MAS was confirmed in crosses with the OX948-derived line, OR297 (Luk 2006). This marker was successfully used to verify L-3 status in a breeding program for improved soymilk stability and flavor and to fingerprint populations. The development of the material to the F_4-stage was largely facilitated by this marker. The fidelity of the marker was confirmed by colorimetric assays at each stage. However, the colorimetric testing was not able to distinguish heterozygotes from homozygotes. The usefulness of the marker for screening segregating material would be further increased by eliminating the restriction enzyme digestion step required for a CAPS marker and such a change would substantially reduce the time required to identify the allele status of individuals in a breeding program. Its conversion to SNP marker is underway. In general, having more than one marker for each lipoxygenase gene broadens their applicability to combinations with various soybean germplasms.

Sequencing confirmed that the markers Lox1-i7 (dominant STS) and Lox1-$_{EcoRI}$ (CAPS) were amplifying the same region of *lx1* mutation as Lox1-3 and they were removed from the chromosome 13 (F). Initially mapped as the *Lx2* marker (Reinprecht et al. 2006), sequencing of the Lox2-i78 (dominant STS) revealed that this actually was the *Lx1* marker (similar to the Lox1-3) and was also removed from the chromosome 13 (F). The addition of the *Lx1* and *Lx2* gene-specific markers developed in this study to the chromosome 13 (F) changed some distances as compared to the original RG10 × OX948 map (Reinprecht et al. 2006). The Lox1-3 marker, originally 1.4 cM from the *Lx1* was now, by the addition of Lox1-P2 (0.3 cM apart), placed 3.6 cM from the *Lx1* gene. Newly developed *Lx2*-specific markers, Lox2-M1 and Lox2-P1, co-segregated and mapped 5.0 cM from the *Lx2* gene. Some distances are larger than expected for the gene-specific markers due to possible phenotyping errors with colorimetric assays. This study confirmed tight linkage of the *Lx1* and *Lx2* genes, which are only 0.2 cM apart as compared to 0.6 cM reported in the previous study (Reinprecht et al. 2006). This allows interchangeable application of the *Lx1*- and *Lx2* gene-specific markers. Their positions were also confirmed by sequence in silico mapping. BLAST searches against soybean genome (http://www.phytozome.net/soybean) placed OX948 and RG10 sequences for *Lx1* and *Lx2* genes on chromosome 13 (F) on Glyma13g42320 (*Lx1*, position Gm13:42328983..42333252) and Glyma13g42310 (*Lx2*, position Gm13:42321689..42325985), respectively while the *Lx3* gene sequences were mapped on chromosome 15 (E) on Glyma15g03030 (*Lx3*, position Gm15:2123728..2128078) (Fig. 7). This also allowed us to properly orient the top portion of the chromosome 15 (E), which initially contained a single SSR marker (Satt212).

The markers developed in this study will allow more accurate selection for seed lipoxygenase nulls on the basis of DNA screens rather than relying on phenotypic expression. Therefore, these markers have the potential to simplify and accelerate breeding for soybean lines that are resistant to oxidative degradation.

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Conflict of interest The authors declare that they have no conflict of interest.

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