Theor Appl Genet (2014) 127:2405–2413
DOI 10.1007/s00122-014-2385-9

ORIGINAL PAPER

Selection and molecular characterization of a lipoxygenase-free soybean mutant line induced by gamma irradiation

Kyung Jun Lee · Jung Eun Hwang · Vijayanand Velusamy · Bo-Keun Ha · Jin-Baek Kim · Sang Hoon Kim · Joon-Woo Ahn · Si-Yong Kang · Dong Sub Kim

Received: 28 February 2014 / Accepted: 15 July 2014 / Published online: 5 September 2014

Abstract

Key message **A lipoxygenase-free soybean mutant line (H70) induced by gamma ray was selected and its detailed information about the lipoxygenase was analyzed by comparison of DNA sequence.**

Abstract Soybean seeds contain three lipoxygenase enzymes, which induce a beany or grassy flavor. The elimination of lipoxygenases can reduce the poor stability and off-flavors of soybean oil and protein products. In this study, we selected a soybean mutant (H70) in which the three lipoxygenases had been mutated using gamma rays. To obtain detailed information about the lipoxygenase, we investigated the sequences of the *Lox1*, *Lox2* and *Lox3* genes in H70 compared to the original cultivar, Hwanggum. Comparisons of the sequences of the *Lox1* and *Lox2* genes in H70 with those in a line with normal lipoxygenase (HG) showed that the mutations in these genes affected a highly conserved group of six histidine residues necessary for enzymatic activity. *Lox1* in H70 contained a 74 bp deletion in exon 8, creating a stop codon that prematurely terminates translation. A single point mutation (T-A) in exon 8 of *Lox2* changed histidine (H532, one of the iron-binding ligands essential for *Lox2* activity) to glutamine. The mutation in the *Lox3* gene in H70 was a single-point mutation in exon 6 (A-G), which changed the amino acid from histidine to arginine. This amino acid alteration in *Lox3* was located in the N-terminal barrel, which might play a role in molecular recognition during catalysis and/or proteolysis. These results suggest that gene analysis based on DNA sequencing could be useful for elucidating the lipoxygenase content in soybean mutant lines. Additionally, the soybean mutant line selected in this study could be used to develop soybean cultivars with improved flavor.

Communicated by David A. Lightfoot.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-014-2385-9) contains supplementary material, which is available to authorized users.

K. J. Lee · J. E. Hwang · V. Velusamy · J.-B. Kim · S. H. Kim · J.-W. Ahn · S.-Y. Kang · D. S. Kim (✉)
Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, 1266 Sinjeong, Jeongeup, Jeonbuk 580-185, Republic of Korea
e-mail: bioplant@kaeri.re.kr

K. J. Lee
e-mail: lkj5214@kaeri.re.kr

J. E. Hwang
e-mail: jehwang@kaeri.re.kr

V. Velusamy
e-mail: vijay@kaeri.re.kr

J.-B. Kim
e-mail: jbkim74@kaeri.re.kr

S. H. Kim
e-mail: shkim80@kaeri.re.kr

J.-W. Ahn
e-mail: joon@kaeri.re.kr

S.-Y. Kang
e-mail: sykang@kaeri.re.kr

B.-K. Ha
Division of Plant Biotechnology, College of Agriculture and Life Science, Chonnam National University, Gwangju 500-757, Korea
e-mail: bkha@jnu.ac.kr

Introduction

Gamma ray irradiation is a physical technique used to generate plant mutations that cause potentially useful agronomical traits. Therefore, the use of radiation in plant breeding has mostly been directed at inducing mutations (Ahloowalia and Maluszynski 2001). These mutations have been used for the improvement of major seed-propagated crops, such as wheat, rice, barley, cotton, peanuts, and beans (Ahloowalia and Maluszynski 2001). Soybean mutation breeding was begun in the early 1950s, and four varieties resulting from this method have been released to date (Sigurbjornsson and Micke 1969). Of course, the main objective of soybean breeding varies with the needs of the breeder, but the ultimate objective inescapably centers on yield and quality (Koo 1972).

Lipoxygenases (linoleate:oxygen oxidoreductase; EC 1.13.11.12) are enzymes belonging to a group of nonheme iron-containing proteins that are 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 proteins (Loiseau et al. 2001). Lipoxygenase 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.

Mature soybean seeds contain at least three lipoxygenase isozymes, *Lox1*, *Lox2*, and *Lox3* (Axelrod et al. 1981). Genetic studies have 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). *Lox1* and *Lox2* have 81 % amino acid identity, and *Lox2* and *Lox3* have 74 % identity (Siedow 1991). The *Lox1* and *Lox2* loci were found to be in close genetic linkage on chromosome 13 (LG F), with the *Lox1* and *Lox2* mutant alleles being in the repulsion phase because they were identified in independent germplasms (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).

Spontaneous mutants for single lipoxygenase isozymes have been identified (Hildebrand and Hymowitz 1982; Kitamura 1984), and a number of single, double (Kitamura et al. 1985) and triple null lipoxygenase mutants have been developed by gamma irradiation (Hajika et al. 1992). The protein products from lipoxygenase-free soybeans have more favorable flavor profiles (Kitamura et al. 1983; Davies and Nielsen 1986; Shen et al. 1996; King et al. 1998). The objectives of this study were to select a lipoxygenase-free soybean mutant line induced by gamma irradiation and to determine the molecular basis of the seed lipoxygenase nulls in the selected soybean mutant line.

Materials and methods

Plant material and mutagenesis

The cultivar Hwanggum (HG) and the mutant line (H70) derived from HG were used in this study. cv. Hwanggum's pedigree is Jangdanbaekmok/Yuku3/Clark63/Baekmokjangyup. One thousand seeds of cv. Hwanggum were irradiated with 250 Gy of gamma rays generated using a ^{60}Co gamma-irradiator (150 TBq of capacity; ACEL, Ontario, Canada) at the Korea Atomic Energy Research Institute (KAERI) in 1997. The irradiated seeds and controls were sown at the Radiation Breeding Research Farm. M_1 plants were harvested in bulk. M_2 plants were sown the next year, harvested individually, and carried forward to the M_3 generation as plant-to-row progeny was produced. We selected mutant lines with excellent agricultural characteristics, including yield, disease resistance, and tolerance to environmental stress, during the M_3 to M_{12} generations (Supplementary Fig. 1).

Detection of seed lipoxygenases with a colorimetric assay

A colorimetric assay for lipoxygenases was performed using the method described by Suda et al. (1995), with some modifications. Briefly, the lipoxygenase solution was extracted in 0.5 ml distilled water from 30 mg of dry seeds. The suspension was then centrifuged at 13,000 rpm for 5 min, after which the clear supernatant was collected. In lipoxygenase-1 (Lx1), 100 µl lipoxygenase solution was added to 400 µl substrate solution (200 mM sodium borate buffer pH 9.0, 0.1 mM methylene blue, 10 mM sodium linoleate). In lipoxygenase-2 (Lx-2), 100 µl lipoxygenase solution was added to 400 µl substrate solution (200 mM sodium phosphate buffer pH 6.0, 0.1 mM methylene blue, 12.5 % acetone, 10 mM sodium linoleate, 200 mM DTT). In lipoxygenase-3 (Lx-3), 100 µl lipoxygenase solution was added to 400 µl substrate solution (200 mM sodium phosphate buffer pH 6.6, 10 mM sodium linoleate substrate, 12.5 % β-carotene (50 % saturation)). Three reactions were incubated for 1 min and scored either as clear or dark blue (Lx-1 and Lx-2) or as clear or yellow (Lx-3).

Detection of seed lipoxygenases with SDS-PAGE

Total crude protein was extracted from 100 mg dry seeds in 5 ml 100 mM Tris–HCl buffer (pH 8.0) containing 20 mM

$CaCl_2$. The suspension was centrifuged at 15,000 rpm for 30 min at 4 °C. The clear supernatant was used for SDS-PAGE. For SDS-PAGE, 50 µl of the total crude protein was added to an equivalent amount of 5× SDS-sample buffer (10 % SDS, 50 % glycerol, 1.96 % β-mercaptoethanol, 0.002 % bromophenol blue and 1 M Tris–HCl, pH 6.8). The samples were held in boiling water for 5 min and then centrifuged for 5 min. The supernatants containing total seed protein were used for SDS-PAGE. A 10 µl aliquot of the supernatant was used for 12 % SDS-PAGE in a Mini-PROTEAN 3 Cell (Bio-Rad, CA, USA). After electrophoresis, the gel was stained with 0.25 % Coomassie Brilliant Blue (CBB) prepared in destaining solution (acetic acid:methanol:water, 1:4.5:4.5 v/v), and the gel was destained in the same solution.

Sequencing seed lipoxygenase genes

The primer sets used for sequencing the seed lipoxygenases were designed by Reinprecht et al. (2011), except for the *Lox3*-5′UTR primer set (Table 1). Genomic DNA was extracted from the dry seeds according to the procedure described by Kim et al. (2010). The DNA concentration was determined using the Nanodrop system (Nanodrop, DE, USA). The DNA solution was then diluted to the working concentration with distilled water and stored at −20 °C

Table 1 Soybean seed lipoxygenase gene-specific sequencing primers

Gene	Primer name	Primer sequence (5′-3′)	PCR product size HG/H70 (bp)
Lox1	*Lox1*-P2 F	TAATTCTGGTCCCTCTCTGAATAAG	1,145/1,157
	Lox1-P2 R	AGACACTACGGCCCAAGAAAGCAT	
	Lox1-1 F	CCCAAGAATGAGTTGGAAG	928/948
	Lox1-1 R	ATATTCCTTGCGCTCTCCCG	
	Lox1-2 F	CACCACTTGTGAGTTACAGA	1,253/1,273
	Lox1-2 R	AGATCAAGGGAATCTGCTGT	
	Lox1-3 F	GCAATCTATGGTGATCAAAG	1,032/958
	Lox1-3 R	ATAGTCCTCTATCAGAAGACGAAC	
	Lox1-4 F	AACCCCACATGGAGTTCGT	929/948
	Lox1-4 R	CTAATAGTGCTCACTGCTC	
	Lox1-3′F	GGAACAATGATCCGAGTCTGCAGGG	1,375/1,375
	Lox1-3′F R	GAAGGAACCAGATTCCTTAATCAGTTG	
Lox2	*Lox2*-P1 F	TTTCGATCTTGGCGTTCTTC	1,158/1,333
	Lox2-P1 R	CCTCATCAACACCACTGTCC	
	Lox2-1 F	GTGTCGGGAATCCTGAACAGAG	1,081/1,081
	Lox2-1 R	GTTCTTTGCGCTCTCCTTTTCCATC	
	Lox2-2 F	TCCAAGTGAGACACCAGCAG	1,299/1,303
	Lox2-2 R	CATCTGCTGTTATCTTACTGG	
	Lox2-3 F	CCGATGAAGAATTTGCAAGAG	1,257/1,259
	Lox2-3 R	GCAGATGGATCCTTAATTGCCACT	
	Lox2-4 F	TGCCCCACATGGACTTCGA	712/731
	Lox2-4 R	AGGAATCCCCCTGCAAGTC	
	Lox2-3` F	CCATCCTAACAGTGAGGAAGGGTTGA	1,212/1,182
	Lox2-3` R	CTATGCCTAGCAATGATTACCTTATGATTTC	
Lox3	*Lox3*-5′UTR F	CGTTAAACCAAACATGCACTCA	1,123/1,123
	Lox3-5′UTR R	GAAATGAAACACCGTTAAACGAA	
	Lox3-1 F	GACTTAGTTGGCTCAACACTC	1,115/1,115
	Lox3-1 R	CCACTCTTTGCGTTCTCCAG	
	Lox3-2 F	CAAGTGAGACACCAGCTCCACTA	1,268/1,268
	Lox3-2 R	GGGCTCTAGGTGTTCTTTGGTT	
	Lox3-3′ F	AGTTCCCTCCACGAAGCAAG	1,224/1,224
	Lox3-3′ R	CTTGTCTCCATGACCCACCT	
	Lox3-4 F	GAGAAGATCCTGAACTCCAAGCC	1,037/1,037
	Lox3-4 R	GTTAGTTGCGTCCTGTGATG	

Table 2 Comparison of agricultural traits between HG and H70

Line	Seed coat color	Stem color	Flower color	Days to 50 % flowering (day)[a]	Maturity (day)	Plant height (cm)	No. of node	No. of branch	No. of pod	100-seed weights (g)	Yield (Kg/ha)
HG	Y	P	P	50.5 ± 0.7	128.6 ± 6.5	43.4 ± 4.8	13.7 ± 0.4	4.3 ± 0.6	33.3 ± 3.4	23.2 ± 1.7	206.3 ± 27.5
H70	Y	P	P	50.5 ± 0.7	129.8 ± 1.4	42.9 ± 4.5	13.5 ± 0.4	4.1 ± 0.7	62.9 ± 16.8**	19.2 ± 0.7*	317.1 ± 36.8*

Y Yellow, *P* Purple

[a] Mean ± SD

*, ** Significantly different at $P < 0.05$ and < 0.01, respectively

until use. PCR amplification was then conducted in reaction mixtures that contained 50 ng of the genomic DNA, 2.5 pmol of each forward and reverse primer, 2.5 mM of each dNTP, 1× PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$) and 1 U Taq polymerase in a total volume of 20 µl. The reaction mixture was subjected to the following conditions: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s and extension at 72 °C for 60 s. The PCR products were then resolved by electrophoresis on 1.0 % ethidium bromide-stained agarose gels. The PCR products were cloned using a T&A Cloning kit (RBC, Bioscience). The plasmid DNA was prepared using a commercial kit (Intron, Biotechnology) and sequenced using an ABI 3130 DNA Sequencer (ABI, CA, USA).

Results

Comparison of agricultural traits between HG and H70

The agricultural traits of HG and H70 were evaluated (Table 2). HG and H70 showed the same color of seed coat, stem and flower and the same number of days to 50 % flowering. The growth habits of days to 50 % flowering, maturity, plant height, node number, and branch number were not significantly different between HG and H70. The number of pods and the yield were increased approximately 2 and 3 times, respectively. The 100-seed weight in H70 was smaller than that in HG by approximately 18 %.

Detection of lipoxygenase using SDS-PAGE and a colorimetric assay

To determine the presence or absence of lipoxygenases in the soybean mutant line, we performed SDS-PAGE and colorimetric assays. The HG seed extracts tested positive for all three isozymes, turning the solutions clear. The same tests on distilled water and on the H70 samples indicated that they were negative for *Lox1* (the solution remained blue), negative for *Lox2* (the solution remained blue) and negative for *Lox3* (the solution remained yellow) (Fig. 1a). The seed storage protein profiling using SDS-PAGE showed similar patterns between HG and H70, except at the position just below 100 kDa. HG showed a strong band below 100 kDa, while H70 did not have any protein band in that region (Fig. 1b).

Seed lipoxygenase gene structures of HG and H70

The size of the *Lox1* gene was 6,104 bp in HG and 6,042 bp in H70 (Table 3). The nucleotides from 905 to 910 in HG and from 910 to 915 in H70 comprised the TATA box (TATAA). The start codon (ATG) was located at position 1,025 in HG and at position 1,037 in H70, respectively. The stop codon (TAA) was at 4,852 in HG and at 4,789 in H70. The open-reading frame (ORF) of HG (2,520 bp) encoded 839 amino acids (AA), with an estimated molecular weight of 94,369.3 kDa. The H70 ORF (2,446 bp) encoded 524 AA, with an estimated molecular weight of 58,428.3 kDa.

The size of the *Lox2* gene was 6,140 bp in HG and 6,316 bp in H70 (Table 3). The TATA box was located at nucleotides 956–960 in HG and nucleotides 1,131–1,136 in H70. The start codons of HG and H70 were at 1,076 and 1,251, respectively. The ORFs (2,601 bp) of HG and H70 encoded 867 AA, with estimated molecular weights of 97,274.8 and 97,265.7 kDa, respectively.

The size of the *Lox3* gene was 5,318 in both HG and H70 (Table 3). The TATA box was located at nucleotides 781 to 786 in both HG and H70. The start (ATG) and stop (TGA) codons of HG and H70 were at 863 and 4,952, respectively. The ORF (2,594 bp) encoded 857 AA, with an estimated molecular weight of 96,782.1 kDa in HG and 96,787.1 kDa in H70.

Comparison of *Lox* gene mutations between HG and H70

The *Lox1* gene in H70 showed 99 different nucleotides compared to those in HG (Fig. 2 and Supplemental Table 1). One substitution was detected in exon 2 (C-A), with a histidine (CAT) changed to asparagine (AAT). In the

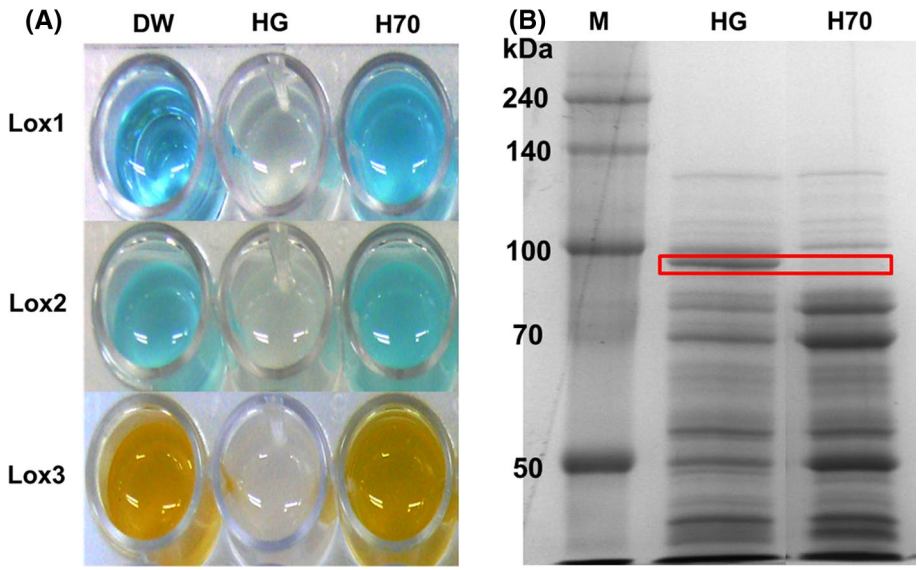

Fig. 1 Chemical assay for lipoxygenase isozymes (**a**) and profiling protein pattern (**b**) between HG and H70. **a** The *color plates* indicate that the lipoxygenase isozymes did not exist in the soybean seeds. **b** The band for lipoxygenase (*Red box*) was not detected in the mutant line. *DW* distilled water, *M* molecular ladder, *HG* hwanggum, *H70* mutant line, *Lox1* lipoxygenase-1, *Lox2* lipoxygenase-2, *Lox3* lipoxygenase-3 (color figure online)

Table 3 Nucleotide sizes of the exons and introns of the three soybean seed lipoxygenase genes for HG and H70

Structure	Gene					
	Lox1		*Lox2*		*Lox3*	
	HG	H70	HG	H70	HG	H70
5′-UTR	1,024	1,036	1,077	1,252	786	786
Exon 1	154	154	244	244	208	208
Exon 2	287	287	287	287	307	307
Exon 3	241	241	241	241	241	241
Exon 4	321	321	318	318	321	321
Exon 5	86	86	86	86	86	86
Exon 6	102	102	102	102	106	106
Exon 7	310	310	307	307	307	307
Exon 8	262	188	264	264	264	264
Exon 9	757	757	754	754	754	754
Total coding region	2,520	2,446	2,601	2,601	2,594	2,594
Intron 1	315	315	344	344	430	430
Intron 2	118	118	127	127	191	191
Intron 3	205	205	226	226	323	323
Intron 4	225	225	151	151	122	122
Intron 5	120	120	215	218	104	104
Intron 6	83	83	106	106	131	131
Intron 7	111	111	88	86	93	93
Intron 8	133	133	85	85	107	107
Total introns	1,310	1,310	1,342	1,343	1,501	1,501
3′-UTR	1,250	1,250	1,120	1,119	437	437
Total DNA	6,104	6,042	6,140	6,315	5,318	5,318

5′-UTR, there were 2 substitutions (T-A and G-A), 3 insertions (AT, TA, and GCATACTAATATA), and 2 deletions (TA and CTT) in H70. A 74 bp deletion occurred in exon 8 of *Lox1* in H70 (Fig. 2). This deletion inserted a stop codon (TAA) at 526 AA, which would result in the premature termination of translation of the *Lox1* protein.

The *Lox2* gene in H70 had 220 different nucleotides compared to HG (Fig. 3 and Supplemental Table 2). The

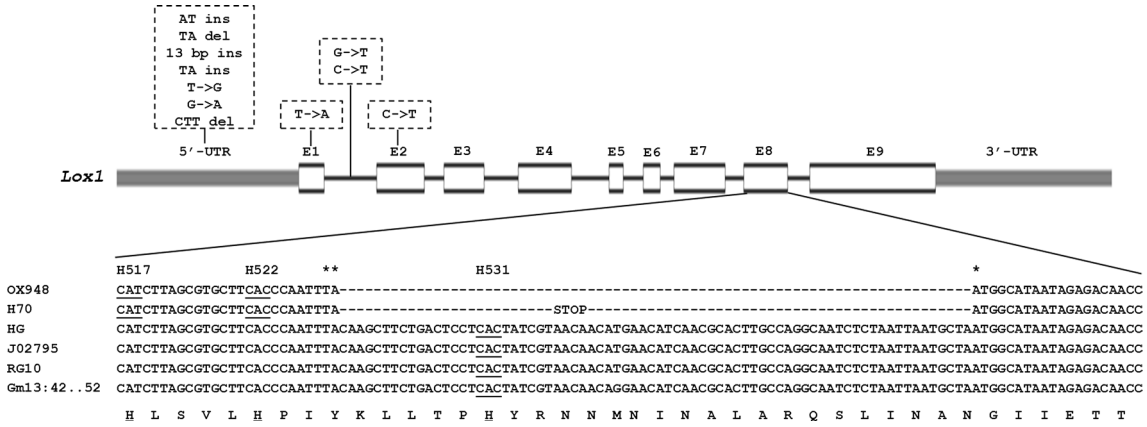

Fig. 2 Structure and sequence of *Lox1* in H70. A 74 bp deletion in exon 8 introduced a stop codon. The amino acids are shown below each codon. ins, insertion; del, deletion. The *dashed boxes* indicate the differences between the HG and H70 sequences. OX948 and RG10, Reinprecht et al. (2011); J02795, Genebank accession, Shibata et al. (1987); Gm13:42.52, Soybase sequence GM13:42327983.42334252, Grant et al. (2010)

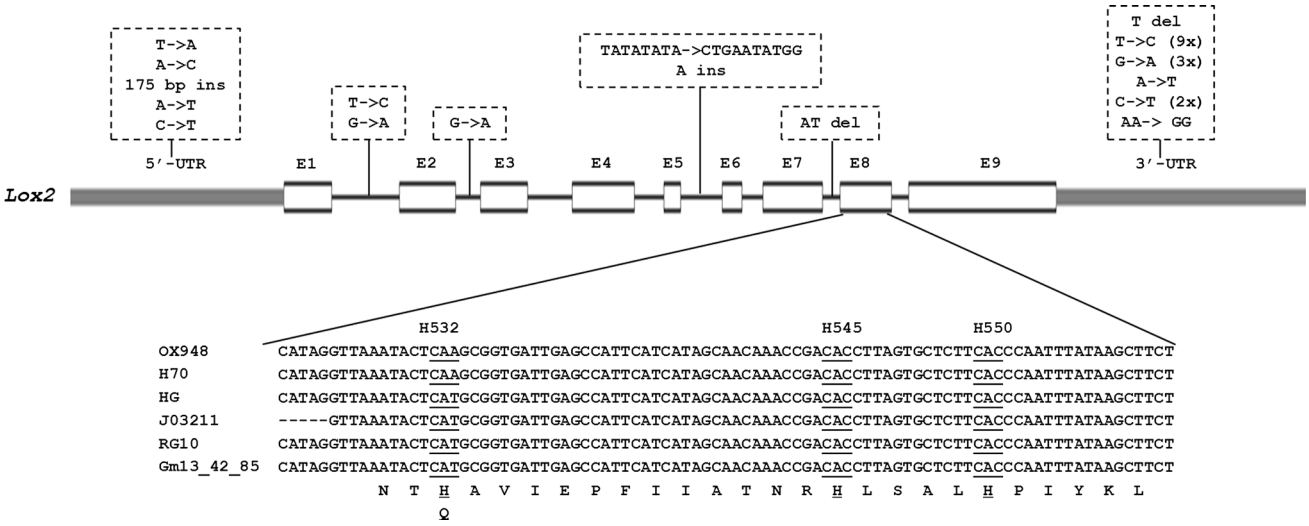

Fig. 3 Structure and sequence of *Lox2* in H70. A point mutation (T-A) in exon 8 changed the amino acid from histidine (H) to glutamine (Q). The amino acids are shown below each codon. *ins* insertion, *del* deletion. The *dashed boxes* indicate the difference between the HG and H70 sequences. OX948 and RG10, Reinprecht et al. (2011); J03211, Genebank accession, Shibata et al. (1988); Gm13_42_85, Soybase sequence GM13:42320689..42326985, Grant et al. (2010)

Lox2 gene showed one SNP in exon 8, which changed the amino acid from histidine (CAT) to glutamine (CAA) (Fig. 3). In the 5′-UTR, 4 substitutions (T-A, A-C, A-T, and C-T) and a 175 bp insertion were detected in H70. In the 3′-UTR, 17 substitutions (9 T-C, 3 G-A, A-T, 2 C-T, and 2 A-G) and a single-nucleotide deletion were different compared to HG. Differences in the intron site between HG and H70 were detected, with 3 substitutions (T-C and 2 G-A), 1 different oligonucleotide (16 nucleotides), 1 insertion (T), and 1 deletion (AT) detected in H70.

Changes to 6 single nucleotides were identified in *Lox3* of H70 (Fig. 4 and Supplemental Table 3). In the intron, 4 substitutions (2 T-C, C-T, and A-G) were detected in H70. One substitution (A-G) each in exon 6 and exon 9 occurred, changing the amino acids from histidine (CAC) to arginine (CGC) and from isoleucine (ATC) to valine (GTC), respectively (Fig. 4).

Different mutant sites of the seed lipoxygenases between H70 and OX948

To determine the uniqueness of the seed lipoxygenase alleles, the H70 sequences were compared with the sequences of other publicly available seed lipoxygenase

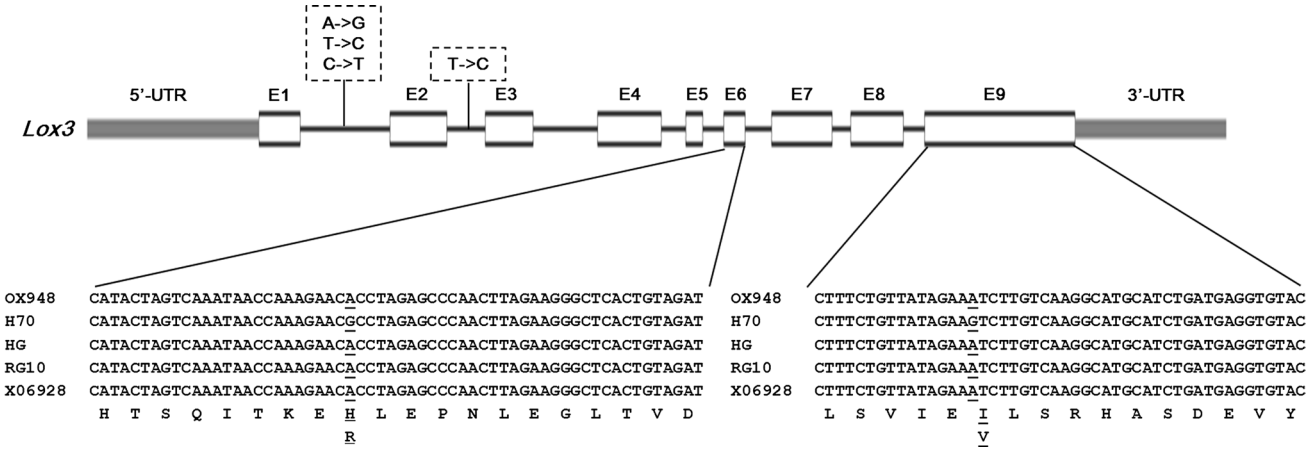

Fig. 4 Structure and sequence of *Lox3* in H70. A point mutation (T-A) in exon 6 changed the amino acid from histidine (H) to arginine (R). The amino acids are shown below each codon. ins, insertion; del, deletion. The *dashed boxes* indicate the difference between the HG and H70 sequences. OX948 and RG10, Reinprecht et al. (2011); X06928. Genebank accession, Yenofsky et al. (1988)

Table 4 Comparison of seed lipoxygenase alleles in H70 with the publicly available lipoxygenase mutant OX948

Gene	Nucleotide position	H70	OX948	Amino acid	bp
Lox1	5′-UTR	T	A		620
			TTT ins		735
	exon 3	AGT	GAG	S-E	1,947
	3-UTR	TTGCAAA	AGGGTTG		4,957–4,963
Lox2	5′-UTR		54 bp del		521–575
			T del		584
			G del		595
			G del		619
	intron 5	CTGAATATGG	TA		3,376–3,385
		AT	C		3,707
Lox3	5′-UTR	T	G		324
		T	C		351
		A ins			416
		A ins			456
		C	T		591
	intron 1	G	A		1,373
		C	T		1,397
		T	C		1,659
	intron 2	C	T		1,873
	exon 6	G	T		3,122
		G	A	H-R	3,190
	exon 9	G	A	I-V	4,621

ins insertion, *del* deletion

genes of OX948 (Table 4). The *Lox1* alleles of H70 and OX948 showed differences including (a) a T-A SNP (620 bp) and a TTT deletion (735 bp) in the 5′-UTR, (b) an AGT-TAG SNP (serine-glutamate, 1,947–1,949 bp) in exon 3 and (c) TTGCAAA-AGGGTTG (4,957–4,963 bp) in the 3′-UTR.

The *Lox2* alleles of H70 and OX948 included differences of (a) a 54 bp deletion (521–575 bp) and single point mutations (T 584 bp, G 595 and 619 bp) in the 5′-UTR and (b) CTGAATATGG-TA (3,376–3,385 bp) and AT-C (3,707–3,708) in intron 5.

Differences in the *Lox3* alleles between H70 and OX948 included (a) 3 single point mutations (T-G 324 bp, T-C 351 bp, and C-T591 bp) and 2 single-nucleotide insertions (A 416 and 456 bp) in the 5′-UTR, (b) 3 (G-A 1,373 bp, C-T 1,397 bp, and T-C 1,659 bp) and 1 (C-T 1,873) single

point mutations in introns 1 and 2, respectively, and (c) 2 [G-T 3,122 bp (silent mutation) and G-A 3,190 bp (histidine-arginine)] and 1 [G-A 4,621 bp (isoleucine-valine)] single point mutations in exons 6 and 9, respectively.

Discussion

Gamma irradiation can be employed as a physical mutagen to induce mutagenesis in plant breeding (Selvi et al. 2007). In the resultant mutant lines, important agronomical traits can be improved, such as flower and seed color, early flowering, lodging, yield and quality, and resistance to diseases, salinity, and cold and heat (Jain et al. 1998). In Japan, two groups of researchers have used gamma irradiation for breeding, breaking the repulsion linkage between loci *lx1* and *lx2* in one case and inducing a change in *LX1* in the other case (Ahloowalia et al. 2004). In our study, the soybean mutant line H70, derived from cv. Hwanggum (HG), was treated with gamma irradiation and developed the traits of high yield and reduced lipoxygenase content. It appeared that H70 developed improved agricultural traits and omitted undesirable factors as a result of gamma irradiation.

We used the *Lox1*, *Lox2*, and *Lox3* sequence information available in Soybase (Grant et al. 2010) to design PCR primers to amplify and clone approximately 1,000 bp from the 5′-UTR to the 3′-UTR regions of these genes according to Reinprecht et al. (2011). In *Lox1*, a 74 bp deletion in exon 8 was detected in H70. This position was the same in OX948 and Jinpoungkong 2 (Lenis et al. 2010). Lenis et al. (2010) and Reinprecht et al. (2011) reported that the 74 bp deletion in exon 8 introduces a stop codon, which would result in early termination of the translation of the *Lox1* protein. The deletion would eliminate the highly conserved H531 and the iron ligands H690, N694 and I839, which are necessary for L-1 enzymatic activity. In *Lox2*, a single point mutation (T-A) at the 1,596 position of the coding sequence led to a change in the amino acid from histidine to glutamine. Wang et al. (1994) and Reinprecht et al. (2011) reported the same single point mutation (T-A) at H532, which resulted in a null *Lox2* phenotype in the mature seeds of Kyushu 111 and OX948, respectively. This replacement of the iron-ligand histidine with glutamine caused structural distortion of the protein and led to its dysfunction (Wang et al. 1994). In *Lox3*, the changes of two amino acids in exon 6 (H-R) and exon 9 (I-V) were detected in H70. Previous studies (Wang et al. 1994; Reinprecht et al. 2011) reported that two single-base substitutions in the promoter region affecting the paired AAATAC boxes are most likely the cause of the *Lox3* null mutation. In our study, however, the original cultivar, HG, had the same a single point mutation and showed *Lox3* activity. We concluded that the null *Lox3* activity in H70 was not affected by the paired AAATAC boxes but rather by a different factor. Skrzypczak-Jankun et al. (1997) suggested that the barrel-like shape and other topological features near region 4 (346–488) corresponded to an N-terminal barrel and might play a role in molecular recognition during catalysis and/or proteolysis. It has been observed that the N-terminal barrel is indispensable for catalysis in soybean lipoxygenase (Steczko et al. 1991). In our results, a single point mutation (A-G) in exon 6 was detected in *Lox3* of H70, causing a change of amino acid 405 from histidine to arginine. We concluded that the changed amino acid in the N-terminal barrel affected Lox-3 activation.

The mutation sites of *Lox1* and *Lox2* in H70 showed similar patterns compared to those of OX948 (Reinprecht et al. 2011). Reinprecht et al. (2011) illustrated that the OX948 parent is a triple null lipoxygenase source obtained by gamma irradiation. Thus, the mutation sites of *Lox1* and *Lox2* in soybeans were thought to be sensitive to gamma irradiation. Chang et al. (2003) reported that nine mutational hotspots at which the same base was mutated simultaneously were found among the 1,941 base pairs of the sequenced region of the *mnp* genes of 4 mutants induced by gamma radiation. Additionally, Wijker et al. (1996) reported that 14 % of the gamma ray-induced mutations were located at the *lacI* gene hot spot at position 620–632. In soybean, 9 base pairs of the Kunitz trypsin inhibitor gene were sensitive to gamma irradiation, and thus were readily substituted DNA sequences (Kim et al. 2010). We observed that the 74 bp deletion in *Lox1* and the T-A point mutation in exon 8 of *Lox2* were changed easily by gamma irradiation.

Soybean seed lipoxygenases affect food quality because they are involved in the production of undesirable grassy and beany aromas and flavors in soybean-containing foods (Gerde and White 2008). The genetic elimination of seed lipoxygenases represents a solution to this problem. In this study, we induced a soybean mutant line using gamma irradiation and selected the H70 line lacking seed lipoxygenase activity. Using the soybean mutant line, we confirmed that a 74 bp deletion which induced nonsense mutation in exon 8 in *Lox1*, a T-A point mutation which it changes amino acid from H to R in exon 8, which affects the binding between lipoxygenase 2 and fatty acid in *Lox2*, and an A-G point mutation which it changes amino acid from H to R in exon 6, which affects the binding between lipoxygenase 3 and fatty acid in *Lox3* were responsible for the null lipoxygenase activity in H70. These results will aid our understanding and characterization of soybean seed lipoxygenases. Additionally, the soybean mutant line, H70, may aid in the development of new varieties without seed lipoxygenase activity.

Author contributions K. J. Lee: Conception and design, Analysis and interpretation, and Writing the article. J. E.

Hwang: Data collection (TA cloning and sequencing). V. Velusamy: Data collection (Plant culture and harvest). B.-K. Ha: Critical revision of the article. J.-B. Kim: Analysis and interpretation (Comparison of DNA sequence). S. H. Kim: Analysis and interpretation (Statistic analysis). J.-W. Ahn: Analysis and interpretation (Comparison of DNA sequence). S.-Y. Kang: Critical revision of the article. D. S. Kim: Conception and design, and Final approval of the article.

Acknowledgments This work was supported by grants from the Nuclear R&D Program by the Ministry of Science, ICT and Future Planning (MSIP) and the R&D program by Korea Atomic Energy Research Institute (KAERI), Republic of Korea.

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

Ethical standards The experiments performed for this publication comply with the current laws of the Republic of Korea.

References

Ahloowalia BS, Maluszynski M (2001) Induced mutations—a new paradigm in plant breeding. Euphytica 188:167–173

Ahloowalia BS, Maluszynski M, Nichterlein K (2004) Global impact of mutation-derived varieties. Euphytica 135:187–2004

Axelrod B, Cheesbrough TM, Laakso S (1981) Lipoxygenase form soybeans: EC 1.13.11.12 Linoleate: oxygen oxidoreductase. Methods Enzymol 71:441–451

Brash AR (1999) Lipoxygenase: occurrence, functions, catalysis, and acquisition of substrate. J Biol Chem 274:23679–23682

Chang HH, Lee YK, Kim JS, Kee KS, Cho KS (2003) Mutation spectrum of manganese (II) peroxidase gene in the *Pleurotus ostreatus* mutants induced by gamma radiation. J Microbiol 41:52–57

Davies CS, Nielsen NC (1986) Genetic analysis of a null-allele for lipoxygenase-2 in soybean. Crop Sci 26:460–463

Gerde JA, White PJ (2008) Lipids. In: Johnson LA, White PJ, Galloway R (eds) Soybeans: chemistry, production, processing, and utilization. AOCS Press, Urbana, pp 193–227

Grant D, Nelson RT, Cannon SB, Shoemaker RC (2010) SoyBase, the USDA-ARS soybean genetics and genomics database. Nucleic Acids Res 38:D483-D486. http://www.soybase.org/

Hajika M, Kitamura K, Igita K, Nakazawa Y (1992) Genetic relationships among the genes for lipoxygenase-1, -2 and -3 isozymes in soybean [*Glycine max* (L.) Merrill] seed. Jpn J Breed 42:787–792

Hildebrand DF, Hymowitz T (1982) Inheritance of lipoxygenase-1 activity in soybean seeds. Crop Sci 22:851–853

Jain SM, Brar DS, Ahloowalia BS (1998) Somaclonal varation and induced mutation in crop iomprovement. Kluwer Academic Publishers, Dordrecht, Great Britain, Dordrecht

Kim DS, Lee KJ, Kim J-B, Kim SH, Song JY, Seo YW, Lee BM, Kang SY (2010) Identification of Kunitz trypsin inhibitor (KTi) mutations using SNAP markers in soybean mutant lines. Theor Appl Genet 121:751–760

King JM, Svendsen LK, Fehr WR, Narvel JM, White PJ (1998) Oxidative and flavor stability of oil from lipoxygenase-free soybean. J Am Oil Chem Soc 75:1121–1126

Kitamura K (1984) Biochemical characterization of lipoxygenase lacking mutants, L-1-less, L-2-less, and L-3-less soybean. Agric Biol Chem 48:2339–2346

Kitamura K, Davies CS, Kitamura N, Nielsen NC (1983) Genetic analysis of a null-allele for lipoxygenase-3 in soybean seeds. Crop Sci 23:924–927

Kitamura K, Kumagai T, Kikuchi A (1985) Inheritance of lipoxygenase-2 and genetic relationships among genes for lipoxygenase-1, -2 and -3 isozymes in soybean seeds. Jpn J Breed 35:413–420

Koo FKS (1972) Induced mutation and plant improvement. Proceedings of a study group meeting Buenos aires (16-20, Nov 1970), IAEA, Vienna

Lenis JM, Gillman JD, Lee JD, Shannon JG, Bilyeu KD (2010) Soybean seed lipoxygenase genes: molecular characterization and development of molecular marker assays. Theor Appl Genet 120:1139–1149

Loiseau J, Vu BL, Macherel MH, Deunff YL (2001) Seed lipoxygenase: occurrence and functions. Seed Sci Res 11:199–211

Reinprecht Y, Luk-Labey S-Y, Yu K, Poysa VW, Rajcan I, Ablett GR, Pauls KP (2011) Molecular basis of seed lipoxygenase null traits in soybean line OX948. Theor Appl Genet 122:1247–1264

Selvi BS, Ponnuswami V, Sumathi T (2007) Identification of DNA polymorphism induced by gamma ray irradiation in Amla (Emblica OYcinalis Gaertn.) grafts of V1M1 and V2M1 generation. J Appl Sci Res 3:1933–1935

Shen N, Fehr W, Johnson L, White P (1996) Oxidative stabilites of soybean oils that lack lipoxygenase. J Am Oil Chem Soc 73:1327–1336

Shibata D, Steczko J, Dixon JE, Hermodson M, Yazdanparast R, Axelrod B (1987) Primary structure of soybean lipoxygenase-1. J Biol Chem 262:10080–10085

Shibata D, Stecsko J, Dixon JE, Andrews PC, Hermodson M, Axelrod B (1988) Primary structure of soybean lipoxygenase L-2. J Biol Chem 263:6816–6821

Siedow JN (1991) Plant lipoxygenase: structure and function. Annu Rev Plant Physiol Plant Mol Biol 42:145–188

Sigurbjornsson B, Micke A (1969) "Progress in mutation breeding", induced mutations in plants. IAEA, Vienna 673

Skrzypczak-Jankun E, Amzel LM, Kroa BA, Funk MO Jr (1997) Structure of soybean lipoxygenase L3 and a comparison with its l1 isoenzyme. Proteins 29:15–31

Start WG, Ma Y, Polacco JC, Hildebrand DF, Freyer GA, Altschuler M (1986) Two soybean seed lipoxygenase nulls accumulate reduced levels of lipoxygenase transcripts. Plant Mol Biol 7:11–23

Steczko J, Donoho GA, Dixon JE, Sugimoto T, Axelrod B (1991) Effect of ethanol and low-temperature culture on expression of soybean lipoxygenase L-1 in Escherichia coil. Protein Expr Purif 2:221–227

Suda I, Hajika M, Nishiba Y, Furuta S, Igita K (1995) Simple and rapid method for the selective detection of individual lipoxygenase isozymes in soybean seeds. J Agric Food Chem 43:742–747

Wang WH, Takano T, Shibata D, Kitamura K, Takeda G (1994) Molecular basis of a null mutation in soybean lipoxygenase 2: substitution of glutamine for an iron-ligand histidine. Proc Natl Acad Sci 91:5828–5832

Wijker CA, Lafleur MVM, Steeg H, Mohn GR, Retèl J (1996) γ-Radiation-induced mutation spectrum in the episomal *lacI* gene of *Escherichia coli* under oxic conditions. Mutat Res 349:229–239

Yenofsky RL, Fine M, Liu C (1988) Isolation and characterization of a soybean (Glycine max) lipoxygenase-3 gene. Mol Gen Genet 211:215–222