Inheritance of Lipoxygenase-2 and Genetic Relationships among Genes for Lipoxygenase-1, -2 and -3 Isozymes in Soybean Seeds

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The mode of inheritance of lipoxygenase-2 (L-2) in soybean seeds was determined from progeny of a cross between PI 86023 (L-2-less) and Suzuyutaka (normal) soybeans. Immunological and electrophoretic analyses of F₂ and F₃ seeds from the cross indicated that the absence of L-2 isozyme was due to a single allele recessive to an allele controlling the presence of the isozyme. The gene symbols, Lx_2 and lx_2 are assigned to the alleles for the presence and absence of L-2 isozyme, respectively. Electrophoretic analysis of a total of 474 F₂ seeds from the reciprocal crosses between PI 86023 (L-2-less) and Tohoku No. 74 (L-3-less) indicated that the Lx_2 locus is independent of the Lx_3 locus. Electrophoretic analysis of a total of 157 F₂ seeds from the cross between PI 86023 and a L-1·L-3-less double mutant line indicated that the Lx_3 locus is independent of the Lx_1 and Lx_2 loci, respectively, being consistent with the no linkage between the Lx_2 and Lx_3 loci indicated by the analysis of F₂ seeds from the former reciprocal crosses. In this study, two types of double mutant seeds were identified that lacked both L-1 and L-3, and both L-2 and L-3 isozymes at the same time. However, no double mutant seeds were identified that lacked both L-1 and L-2 isozymes, so far. The L-1·L-3-less and L-2·L-3-less double mutant seeds germinated, grew and matured normally to produce F₃ seeds that lacked both L-1 and L-3, and both L-2 and L-3 isozymes, respectively.

KEY WORDS : *G. max* L. MERILL soybean, lipoxygenase isozymes, inheritance, beany-flavor, quality improvement.

Introduction

Lipoxygenase is known to catalyze the oxidation of unsaturated fatty acids and polyunsaturated lipids containing cis-cis-1, 4-pentadiene moieties. Normal soybean seeds contain at least three lipoxygenase isozymes, called lipoxygenase-1 (L-1), lipoxygenase-2 (L-2), and lipoxygenase-3 (L-3) (AXELROD 1981). These enzymes seem to be responsible for the generation of objectionable grassy-beany flavors in soybean protein products, since the oxidation products, hydroperoxides, caused by the enzymes further decompose to middle chain aldehydes and alcohols that are major constituents of the grassy-beany flavors (FUJIMAKI *et al.* 1965, ARAI *et al.* 1970, WOLF 1975, SESSA 1979, SASAKI and CHIBA 1983).

Heat treatment has been used mainly to inactivate the lipoxygenase actions causing the lipid oxidation and the beany-flavor generation during food processing of soybeans. However, the heat treatment often causes poor protein solubility resulting in poor food functionality of soybean protein (WOLF 1975, SASAKI and CHIBA 1983). Thus, the lipoxygenase problems are not entirely solved as yet, and seem to be the biggest obstacle to greater use of soy products in food (RACKIS *et al.* 1970, RACKIS *et al.* 1979, WOLF 1975).

Recently, HILDEBRAND and HYMOWITZ (1981) found the L-1-less soybean cultivars, PI 133226 and PI 408251, and HILDEBRAND and HYMOWITZ (1982) demonstrated that the L-1-less characteristic is due to the single recessive allele, lx_1 . KITAMURA *et al.* (1983) found

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the L-3-less soybean cultivars, Wasenatsu and Ichigowase, and demonstrated that the L-3-less characteristic is due to the single recessive allele, lx_3 . Moreover, KITAMURA *et al.* (1983) found the L-2-less soybean cultivar, PI 86023. The preliminary genetic analysis showed that the absence of L-2 isozyme was due to a single recessive allele (KUMAGAI and KITAMURA 1984). By the use of the three types of the lipoxygenase lacking mutants, we have been attempting to breed soybeans having low levels of lipoxygenase activity aiming at improving the quality of soybean.

In the present investigation, we report the results of genetic analysis of a null-allele for lipoxygenase-2 and the genetic relationships among the genes for the three lipoxygenase isozymes. Further, we describe two types of double mutant seeds obtained in this study that lack both L-1 and L-3, and both L-2 and L-3 isozymes, respectively.

Materials and Methods

Crosses. A cross was made between Suzuyutake (normal) and PI 86023 (L-2-less) soybeans to study the mode of inheritance of L-2 isozyme. To study genetic relationships among the genes for lipoxygenase isozymes, the following crosses were made: 1) Tohoku No. 74 (L-3-less) $\times$ PI 86023 and its reciprocal, the former parent was recently identified as a L-3-less cultivar in this laboratory, 2) a L-1·L-3-less double mutant line $\times$ PI 86023, the former parent was a F_3 plant from one of the F_3 seeds that lacked both L-1 and L-3 isozymes obtained from a cross between Wasenatsu (L-3-less) and PI 408251 (L-1-less). All crosses were made in the greenhouse. The F_2 seeds were obtained from selfed F_1 plants grown at the Iwate University Experimental Farm during the summers of 1982 and 1983. The F_3 seeds from the crosses Suzuyutaka $\times$ PI 86023, and Tohoku No. 74 $\times$ PI 86023 were obtained from selfed F_2 plants grown in the greenhouse during the winter of 1982.

Lipoxygenase analysis. Individual F_2 and F_3 seeds were crushed in paper sacks with a hammer. For half-seed analysis, the distal portions (about 40 mg) of the seeds were cut off with a razor blade, and were ground in buffer with a small mortar and pestle. The seeds containing the embryonic axes were planted in the greenhouse. The presence and absence of L-2 isozyme of the F_2 seeds from the cross between Suzuyutaka and PI 86023 were determined by both double immunodiffusion using an anti-L-2 serum and the improved sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) techniques as previously described (KITAMURA 1984). Since the presence and absence of the three lipoxygenase isozymes can be readily and accurately judged by the improved SDS-PAGE using only 5–10 mg of seed sample, the F_3 seeds from the above cross and the F_2 seeds from the other crosses were analyzed using only the electrophoretic technique.

Lipoxygenase fractionation and assay. The isozyme fractionation and assay of crude extracts from the seeds of $lx_1lx_1 \cdot lx_3lx_3$ and $lx_2lx_2 \cdot lx_3lx_3$ double mutants were carried out as previously described (KITAMURA 1984).

Results

Inheritance of L-2. Forty nine F_2 seeds from the cross between Suzuyutaka and PI 86023 were examined for L-2 isozyme by the immunological and the electrophoretic analyses. The segregation of the F_2 seeds for the presence or absence of the isozyme well

Table 1. Segregation of L-2 in F_2 seeds from the cross between Suzuyutaka and PI 86023

Genotype	Number of seeds	Expected
Lx_2-	35	36.75
lx_2lx_2	14	12.25

χ^2 for 3 Lx_2- : 1 lx_2lx_2 = 0.33 ; $P > 0.50$

Table 2. Segregation of the L-2 null-allele in F_3 seeds from the cross between Suzuyutaka and PI 86023

F_2 parents		F_3 seeds	
Number of plants examined	Genotype	Lx_2-	lx_2lx_2
8 ¹⁾	Lx_2Lx_2	146	0
20 ¹⁾	Lx_2lx_2	418 ²⁾	144 ²⁾
7	lx_2lx_2	2 ³⁾	208

1) χ^2 for 1 Lx_2Lx_2 : 2 Lx_2lx_2 = 0.285
 $P > 0.50$

2) χ^2 for 3 Lx_2- : 1 lx_2lx_2 from Lx_2lx_2
= 0.116 $P > 0.50$

3) Contamination or outcrossing

crosses between Tohoku No. 74 and PI 86023 were examined for L-2 and L-3 isozymes by the improved SDS-PAGE. The joint segregation of the presence or absence of the both isozymes well fitted to the expected 9 : 3 : 3 : 1 ratio for independent inheritance (Table 3). These data indicate that the Lx_2 locus is independent of the Lx_3 locus.

Thirty three double mutant seeds that lacked both L-2 and L-3 isozymes were obtained. Thirty two of the 33 F_2 seeds germinated normally. All the F_2 plants grew and matured to produce at least 10 F_3 seeds per plant in the greenhouse during the winter of 1983.

Relationships among Lx_1 , Lx_2 and Lx_3 loci. F_2 seeds from the cross between the L-1·L-3-less double mutant line and PI 86023 were examined for the presence or absence of the three lipoyxygenase isozymes by the improved SDS-PAGE. The joint segregations of the presence or absence of L-2 and L-3, and of L-1 and L-3 isozymes well fitted to the expected 9 : 3 : 3 : 1 ratio for independent inheritance (Table 4). These data indicate that the Lx_3 locus is independent of Lx_1 and Lx_2 loci, respectively, being consistent with the F_2 data listed in Table 3. Two types of double mutant F_2 seeds were identified to lack both L-1 and L-3, and both L-2 and L-3 isozymes, respectively. The six L-1·L-3-less and the nine L-2·L-3-less double mutant seeds obtained from the above cross germinated, grew and matured normally to produce F_3 seeds in the greenhouse.

On the other hand, the joint segregation of the presence or absence of L-1 and L-2 isozymes did not fit to the expected ratio for independent inheritance. No seed lacking L-1 and L-2 isozymes was identified. This indicates that the Lx_1 locus tightly links with the Lx_2 locus, otherwise that embryos of $lx_1lx_1 \cdot lx_2lx_2$ genotype are possibly lethal in the process of seed development.

Characterization of L-1·L-3-less and L-2·L-3-less soybeans.

fitted to the expected ratio of 3 : 1, suggesting that the absence of L-2 is recessive to the presence of L-2 (Table 1). The inheritance of L-2 isozyme was further investigated by the electrophoretic analysis of F_3 seeds produced by the F_2 seed embryos with cotyledons of known phenotypes (Table 2). Segregation of the F_3 seeds followed the expected 1 : 0, 3 : 1, and 0 : 1 ratios for homozygous dominant, heterozygous, and homozygous recessive genotypes of the F_2 seeds, respectively, as anticipated with a single recessive gene.

From the above results, we conclude that the absence of L-2 isozyme was due to a homozygous allele, lx_2 , that was recessive to a allele, Lx_2 , required for the presence of L-2 isozyme in the seed.

Relationship between Lx_2 and Lx_3 loci.

A total of 474 F_2 seeds from the reciprocal

Two types of double mutant F_2 plants whose genotypes were $lx_1lx_1 \cdot lx_3lx_3$ and $lx_2lx_2 \cdot lx_3lx_3$, respectively, produced normal F_3 seeds. Individual F_3 seeds from the F_2 plants of the $lx_1lx_1 \cdot lx_3lx_3$ and $lx_2lx_2 \cdot lx_3lx_3$ genotypes, respectively, were shown to lack both L-1 and L-3, and both L-2 and L-3 isozymes, respectively (Fig. 1).

Further, crude extracts from the L-1·L-3-less and the L-2·L-3-less soybean seeds were examined for the lipoxxygenase isozyme activities using DEAE-Sephacel columns. The elution profiles shown in Fig. 2-A and 2-B demonstrated that both the L-1 and L-3, and both the L-2 and L-3 isozyme activities completely disappeared in the L-1·L-3-less and the

Table 3. Observed and expected segregation of F_2 seeds for the presence and absence of L-2 and L-3

Genotype of progeny	Tohoku No. 74 ($Lx_2Lx_2lx_3lx_3$)	PI 86023 ($lx_2lx_2Lx_3Lx_3$)
	×	×
	PI 86023 ($lx_2lx_2Lx_3Lx_3$)	Tohoku No. 74 ($Lx_2Lx_2lx_3lx_3$)
Lx_2-Lx_3-	91 (96.75) ¹⁾	182 (169.875)
$Lx_2-lx_3lx_3$	34 (32.25)	53 (56.625)
$lx_2lx_2Lx_3-$	35 (32.25)	46 (56.625)
$lx_2lx_2lx_3lx_3$	12 (10.75)	21 (18.875)
Number of F_2 seeds	172	302
Chi-square 9 : 3 : 3 : 1		
Value	0.82	3.33
Probability	0.83	0.35

¹⁾ Values in parenthesis are expected frequencies

Table 4. Observed and expected segregation of F_2 seeds from the cross between the L-1·L-3-less and L-2-less soybeans for the presence and absence of L-1, L-2 and L-3

L-1	L-2	L-3	Seed number Observed (expected)	Chi-square value (9 : 3 : 3 : 1)	Probability
+	+		92 (88.31)	0.154	10.458 P < 0.01
+	-		29 (29.44)	0.007	
-	+		36 (29.44)	1.462	
-	-		0 (9.81)	8.835 ¹⁾	
	+	+	99 (88.31)	1.294	4.338 P > 0.2
	+	-	29 (29.44)	0.007	
	-	+	20 (29.44)	3.027	
	-	-	9 (9.81)	0.010 ¹⁾	
+		+	89 (88.31)	0.005	1.356 P > 0.5
+		-	32 (29.44)	0.223	
-		+	30 (29.44)	0.011	
-		-	6 (9.81)	1.117 ¹⁾	

¹⁾ Yates correction

L-2·L-3-less seeds, respectively.

These results indicate that the double lacking characteristics of the lipoxxygenase isozymes are stably inherited through generations, and that complete elimination of both L-1 and L-3, and both L-2 and L-3 isozymes from the seed is not critical for the survival of soybean plant.

Discussion

There are some investigations on physiological roles of seed lipoxxygenase enzymes, including the initiation of lipid oxidation in germinating seedling (HOLMAN 1948), involve-

Fig. 1. Resolution of the lipoxxygenase isozymes in crude seed extracts by the improved SDS-PAGE.

Upper panel: lane 1 and 8, Suzuyutaka (normal soybeans); lane 2 to 7 individual L-2·L-3-less F_4 seeds from the cross Tohoku No 74 × PI 86023.

Lower panel: lane 1 and 8, Raiden (normal soybean); lane 2 to 7, individual L-1·L-3-less F_3 seeds from the cross Wasenatsu × PI 408251.

ment in seed aging (HARMAN 1976), pest defence (SHUKLE and MURDOCK 1983) and regulation of metabolism at early germination stage (VERNOOY-GERRISTEN *et al.* 1983), but, the evidence is not conclusive for any investigations. The physiological function of seed lipoxxygenase is far from clear, as yet.

In fact, no physiological problems have been observed with one exception during the life cycles of the three types of lipoxxygenase lacking mutant soybeans, PI 408251 (L-1-less), PI 86023 (L-2-less) and Wasenatsu (L-3-less). The exception was that a percentage of seed damage of PI 86023 soybean by pod borer was about 1.5 times higher than that of Suzuyutaka (normal) soybean during the summer of 1983, but no difference of the seed damage was observed between PI 86023 and Suzuyutaka soybeans in 1984. Whether there exists a relationship between the L-2-less characteristic and the seed feeding by pod borer remains to be clear.

Recently, MATOBA *et al.* (1985) revealed, in the study of the flavor generation from soybean homogenate (soy milk) using the normal and the lipoxxygenase lacking soybeans, that L-2 isozyme of the three lipoxxygenase isozymes is most responsible for the formation of n-hexanal that seems to be a major constituent of the objectionable grassy-beany flavor. The biochemical analysis was consistent with a result of sensory test of soybeans that the L-2-less soybean homogenate developed the lowest level of the grassy-beany flavor among the homogenates of the normal and the lipoxxygenase lacking soybeans. This implies that the incorporation of the L-2-less characteristic into commercial soybean cultivars may be of economical importance for the utilization of soybean.

The results in this investigation have demonstrated that the absence of L-2 isozyme is due to a single recessive gene. This indicates that the L-2-less characteristic as well as the L-1-less and the L-3-less characteristics that were shown to be due to single recessive genes, respectively, can be readily backcrossed into soybean cultivars adapted to any environment. However, in order to lower the undesirable lipoxxygenase actions to an

Fig. 2. DEAE-Sephacel column chromatograms of crude seed extracts of the two types of double mutant soybeans.
 A : 1.5 g of the L-1·L-3-less F₃ seeds
 B : 1.5 g of the L-2·L-3-less F₄ seeds
 A elution profile of Raiden (normal) soybean has been shown by KITAMURA (1984).

acceptable level for the soybean food industry, two to three types of the enzymes should be eliminated at the same time from the seed, because elimination of only one type of the isozymes is not sufficient to attain this level.

In this study, the two types of double mutant F₂ seeds were identified that lacked both L-1 and L-3, and both L-2 and L-3 isozymes, respectively. They germinated, grew and matured normally to produce F₃ seeds in the greenhouse. So long as the L-1·L-3-less and the L-2·L-3-less soybeans were grown at the Iwate University Experimental Farm in the summer of 1984, we observed neither any physiological problems nor any apparent defects negatively affecting the important agricultural traits such as growth, productivity and disease pest resistances in the double mutant soybeans. In addition, it has been shown by the sensory test of soybean homogenates that the grassy-beany flavor level of the L-2·L-3-less seeds was extremely low. These results and observations encourage us to breed

soybean cultivars with very low lipooxygenase levels, therefore having a great economical value for the utilization of soybean.

Preliminary analysis of F_2 and F_3 seeds from the cross between a L-1-less and a L-2-less soybeans indicated that the Lx_1 locus tightly links with the Lx_2 locus (KIKUCHI and KITAMURA 1985). This seems to explain that no double mutant seeds lacking both L-1 and L-2 isozymes have been identified in this study.

A trial is under the way to identify double mutant seeds lacking both L-1 and L-2 or triple mutant seeds lacking all isozymes using a large number of F_2 seeds obtained from crosses between L-1·L-3-less and L-2-less soybeans. Further, more detail studies should be made to clarify whether any defects occur in the important agricultural traits such as plant growth, productivity, seed viability and pathogen-pest resistances in the lipooxygenase lacking soybeans under different conditions.

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ダイズ種子中のリポキシゲナーゼ-2 の遺伝様式、およびリポキシゲナーゼ-1, -2, -3 アイソザイムの遺伝的關係

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ダイズ種子は、不飽和脂質を酸化し、青臭い不快臭を発生する酵素、リポキシゲナーゼを特に多く含有しており、このことがダイズの食品素材としての最大の問題点となっている。これまで、本酵素による不快臭発生を防止するため、種々の試みがなされて来た。現在、食品工業的には主として加熱処理で不快臭発生を防止する方法がとられている。しかし、加熱処理は同時に蛋白質の変性不溶化を招くため、重要な食品加工上の特性であるダイズ蛋白質本来のもつ保水性や粘着性などの低下をもたらす原因となっている。従って現在でも、本酵素作用はダイズを食品素材として有効に利用する上での最大の問題となっている。

ダイズ種子中には性質を異にする3種類のリポキシゲナーゼ(L-1, L-2, L-3)が存在する。1981年から1983年にかけて、L-1欠失、L-3欠失、L-2欠失、各変異体ダイズが相続いて発見され、さらにこれら各欠失特性が遺伝的支配であることが明らかにされたことにより、ダイズ種子中の本酵素活性を遺伝的にコントロールすることが可能となった。

本研究は、L-2欠失特性の遺伝様式およびL-1, L-2, L-3各酵素欠失特性の遺伝的關係を明らかにすることを目的としたものである。

L-2欠失特性の遺伝様式を明らかにするために、スズユタカ(普通ダイズ)×PI 86023(L-2欠失ダイズ)の交雑F₂およびF₃種子中のL-2酵素の有無を免疫2重拡散法および改良したSDS-ゲル電気泳動法で分析した。その結果、L-2欠失特性は単一劣性遺伝子によって支配されていることが示された。この劣性対立遺伝子およびL-2の生産を支配する優性対立遺伝子をそれぞれ lx_2 , Lx_2 の遺伝子記号で表わした。

L-1, L-2, L-3酵素の支配遺伝子間の連鎖の有無を調査するため、以下の交配を行った：1) PI 86023(L-2欠)×東北74号(L-3欠)、および逆交雑；2) PI 86023×L-1・L-3同時欠失系統(早生夏(L-3欠)×PI 408251(L-1欠)の後代種子から得られたF₃植物体)。交配1)から得られたF₂種子474粒のL-2, L-3酵素の有無を改良SDS-ゲル電気泳動(SDS-PAGE)で分析した結果、L-2支配座とL-3支配座は遺伝的に独立であることが示された。また交配2)から得られたF₂種子157粒を分析した結果、L-1支配座とL-3支配座、およびL-2支配座とL-3支配座は各々遺伝的に独立であることが示され、交配1)で得られた結果と一致した。

本研究では、L-1・L-3同時欠失種子およびL-2・L-3同時欠失種子は得られたが、L-1・L-2同時欠失種子は全く得られなかった。L-1・L-3同時欠失F₂種子およびL-2・L-3同時欠失F₂種子とも正常に発芽し、これらのF₂植物体も正常に成長、登熟し、各々L-1・L-3およびL-2・L-3同時欠失F₃種子を生産した。また生ダイズ種子の官能検査の結果は、L-2・L-3同時欠失種子の青臭みの程度が著しく低下していることを示した。