Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5828–5832, June 1994
Genetics

Molecular basis of a null mutation in soybean lipoxygenase 2: Substitution of glutamine for an iron-ligand histidine

(*Glycine max*/flavor/missense mutation/protein degradation)

WAN HENG WANG*, TETSUO TAKANO*, DAISUKE SHIBATA†, KEISUKE KITAMURA‡, AND GENKICHI TAKEDA*§

*Laboratory of Plant Breeding, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan; †Mitsui Plant Biotechnology Research Institute, TCI-A10, Sengen 2-1-6, Tsukuba, Ibaraki 305, Japan; and ‡National Agriculture Research Center, Tsukuba, Ibaraki 305, Japan

Communicated by Oliver E. Nelson, January 27, 1994

ABSTRACT **We have investigated the nucleotide sequence and expression of *lox2*, a mutant form of the gene encoding lipoxygenase 2, an enzyme responsible for unpleasant flavors in soybean [*Glycine max* (L) Merr.] seeds. Although *lox2* transcripts accumulate normally, there are no detectable transcripts for lipoxygenase 1 or 3 in mutant lines that display similar phenotypes characterized by the lack of corresponding lipoxygenase activity and protein in mature seeds. The enzymatically inactive *lox2* gene product is readily detectable in mid-maturation-stage seeds but is apparently unstable, since it is absent from mature seed. The protein sequence deduced from the cDNA and genomic DNA sequences of *lox2* differs from that of the wild-type gene, *Lox2*, in the substitution of glutamine for His-532. It is known that His-504 in soybean lipoxygenase 1, which corresponds to His-532 in lipoxygenase 2, is one of the iron-binding ligands essential for lipoxygenase activity. Here we present evidence that the missense mutation substituting Gln for His-532 results in the loss of lipoxygenase 2 from mature soybean seeds.**

The discovery of null mutations has paved the way for investigating relationships between phenotypes and genes and for studies of the types of genetic defects that can affect gene expression. A number of null alleles have been identified in plants, and some have been characterized at the molecular level. These mutations are due to insertions (1, 2), deletions of DNA fragments (3, 4), and changes in the initiation codon (5). Some null alleles result from single-base mutations producing frameshifts (6), premature stop codons (6), or intron/exon junction alterations leading to incorrect RNA processing (7).

Soybean seeds contain at least three lipoxygenase isozymes, lipoxygenases 1, 2, and 3 (L-1, L-2, and L-3) (8). These isozymes are responsible for the production of unpleasant grassy and beany flavors in soybean seeds that have limited the development of soybean protein products for human consumption (9–11). These isozymes are well characterized biochemically (8, 12), and their primary amino acid sequences have been deduced from the DNA sequences of the corresponding genes (13–15). In the early 1980s, three soybean lines each deficient in one of the three lipoxygenase isozymes in mature seed (16–18) were identified. Genetic studies of these lines demonstrated that the absence of L-1, L-2, and L-3 in mature seeds was due to single recessive alleles—*lx1*, *lx2*, and *lx3*, respectively. The loci defined by *lx1* and *lx2* are closely linked and are not genetically linked to the *lx3* locus (12, 18–20). Two double mutant lines, one deficient in L-1 and L-3 and the other lacking L-2 and L-3, have been constructed (18). More recently, lines deficient in all three isozymes as well as a line lacking L-1 and L-2 were obtained (19, 21, 22). The double and triple mutants are physiologically normal (22, 40). The undesirable flavors are absent or less pronounced in food products made from seeds deficient in these isozymes, particularly those lacking L-2 (9, 23).

Although a complete series of null mutants of soybean seed lipoxygenases has been obtained, the molecular basis of these mutations remains to be clarified. We have determined the molecular basis for L-2 deficiency. A single T → A transversion results in a null L-2 phenotype in mature soybean seeds.¶

MATERIALS AND METHODS

Plant Material. Suzuyutaka, a soybean cultivar widely grown in Japan, was used as the wild-type or parental genotype. Three single-mutant lines lacking L-1, L-2, or L-3—hereafter designated as L-1 null, L-2 null, and L-3 null, respectively—were near-isogenic lines of Suzuyutaka (22), in which *lx1*, *lx2*, and *lx3* were derived originally from soybean lines PI408251 (16), PI86023 (12), and PI417458 (17), respectively. Three double-mutant lines were also analyzed: Kanto 101, lacking L-2 and L-3 (L-2/3 null); Kanto 102, lacking L-1 and L-3 (L-1/3 null) (22); and a third double-mutant line lacking L-1 and L-2 (L-1/2 null) (19). Two triple-mutant lines lacking L-1, L-2, and L-3 (L-1/2/3 null) were also studied: Kyushu 111 (21) and a line hereafter designated as K (22). All plants were field grown at the Japanese National Agricultural Research Center in Tsukuba. Mid-maturation-stage seeds, harvested 35–40 days after flowering, and mature seeds were used in this study.

Nomenclature. The symbols *Lx1*, *Lx2*, and *Lx3* have been used to designate the alleles coding for the presence of L-1, L-2, and L-3, respectively, and *lx1*, *lx2*, and *lx3* refer to the alleles coding for the lack of detectable L-1, L-2, and L-3 proteins in mature seeds (17, 18, 20). It has not been determined whether the *Lx1*/*lx1*, *Lx2*/*lx2* and *Lx3*/*lx3* alleles in the above soybean lines are themselves the structural genes of the corresponding lipoxygenase isozymes. The structural genes encoding L-1, L-2, and L-3 have been cloned (13–15) and designated *Lox1*, *Lox2*, and *Lox3*, respectively. The allele of *Lox2* present in the mutant lines lacking L-2 is referred to as *lox2*.

Isolation of Poly(A)$^+$ RNA. One gram of mid-maturation-stage seeds stored at −80°C was used in isolating total RNA (24). Poly(A)$^+$ RNA was isolated from the total RNA with an oligo(dT)-cellulose Spun column (Pharmacia) to minimize contamination with rRNA.

Northern Blotting. Two micrograms of poly(A)$^+$ RNA was used for electrophoresis in a 0.8% agarose gel. Blotting to Hybond N$^+$ membranes and hybridization were carried out

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: L-1, lipoxygenase 1; L-2, lipoxygenase 2; L-3, lipoxygenase 3.

§To whom reprint requests should be addressed.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. D13949).

according to the procedure provided by the manufacturer (Amersham). Probes specific for the *Lox1*, *Lox2*, and *Lox3* genes were obtained by PCR amplification of their highly divergent 3′ noncoding regions. The *Lox1* probe was generated with primers 5′-GGAGCCTGTG-3′ and 5′-ACTAATAGTG-3′ and spanned nt 2605–2800 (13), the *Lox2* probe required primers 5′-GGGAGCTATG-3′ and 5′-GGATCTCTAG-3′ and spanned nt 2680–2752 (14), and the *Lox3* probe was obtained with primers 5′-AGAGGTTTGT-3′ and 5′-GCACTCAAAC-3′ and spanned nt 4955–5062 (15). Probes were labeled with [α-^{32}P]dCTP by use of a random-primer DNA labeling kit (Takara, Otsu, Japan).

Construction and Screening of cDNA Library. Poly(A)$^+$ RNA from the mid-maturation-stage seeds of the Kyushu 111 line was used as a template for cDNA synthesis. cDNA was prepared (cDNA synthesis kit from Pharmacia), ligated to bacteriophage λgt11 by using *Eco*RI linkers, and then packaged by Gigapack II Gold (Stratagene). Recombinant cDNA clones were screened with the 72-bp *Lox2*-specific fragment labeled with [α-^{32}P]dCTP and with a full-length cDNA of *Lox2*, designated 3C76 (14), labeled with the ECL gene detection system (Amersham). Selected positive clones from the cDNA library were digested with *Eco*RI and the restriction fragments were subcloned into the pUC19 plasmid vector with a DNA ligation kit (Takara, Otsu, Japan).

Nucleotide Sequencing. Double-stranded DNA for sequencing was purified by equilibrium gradient centrifugation in CsCl/ethidium bromide or by precipitation in polyethylene glycol (25). Both strands of the DNA were sequenced by *Taq* dye primer cycle sequencing on a 373A DNA Sequencer (Applied Biosystems).

L-2 Purification and Antibody Preparation. The L-2 protein was purified from mature seeds of the L-1/3 null line. Seven milligrams of the purified L-2 protein was used for preparation of rabbit antibody as described (12).

Western Blotting. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE, 7% resolving gel) was performed as described (12). The gel was analyzed by Western blotting on poly(vinylidene difluoride) transfer membranes (Immobilon; Millipore).

Enzyme Assay. Lipoxygenases were extracted by grinding 100 mg of mature seeds or 300 mg of mid-maturation-stage seeds, which had been separated from the embryonic axes and seed coats, in 2 ml of 0.05 M sodium phosphate (pH 6.8). The extracts were centrifuged at 16,000 × *g* at 4°C for 5 min. The supernatant was immediately assayed for enzyme activity. The activity assay was performed with an oxygen electrode as described (26). Protein concentration was estimated by the Bradford method (27).

DNA Amplification. Genomic DNA was prepared by the plant DNA minipreparation method (28) using 2 g of mid-maturation-stage seeds. The DNA was further purified by equilibrium centrifugation in CsCl/ethidium bromide gradients (25). PCR amplification was conducted at an annealing temperature of 50°C (25). The sequences of the primers used for amplification of specific regions of lipoxygenase genomic DNA are underlined in Fig. 3. The PCR products were inserted into a pCR vector using the TA cloning system (Invitrogen) and sequenced.

RESULTS

Expression of Lipoxygenase Transcripts in the Mutants. Northern blot analysis of poly(A)$^+$ RNA from mid-maturation-stage soybean seeds of single-, double-, and triple-mutant lines indicated that the presence of L-1 or L-3 was invariably accompanied by detectable levels of the corresponding transcript and that lines lacking either isozyme also lacked the appropriate transcript. In contrast, the *Lox2*-specific probe elicited a positive signal of similar strength whether or not L-2 was present (Fig. 1). These results demonstrate that, unlike the genes encoding L-1 and L-3 in the corresponding null lines, the structural gene for L-2 in the lines lacking L-2, *lox2*, is transcribed.

Isolation and Sequencing of *lox2* cDNAs. With a short DNA fragment specific to *Lox2* or the full-length *Lox2* cDNA as a probe, 8 positive clones were identified after screening of

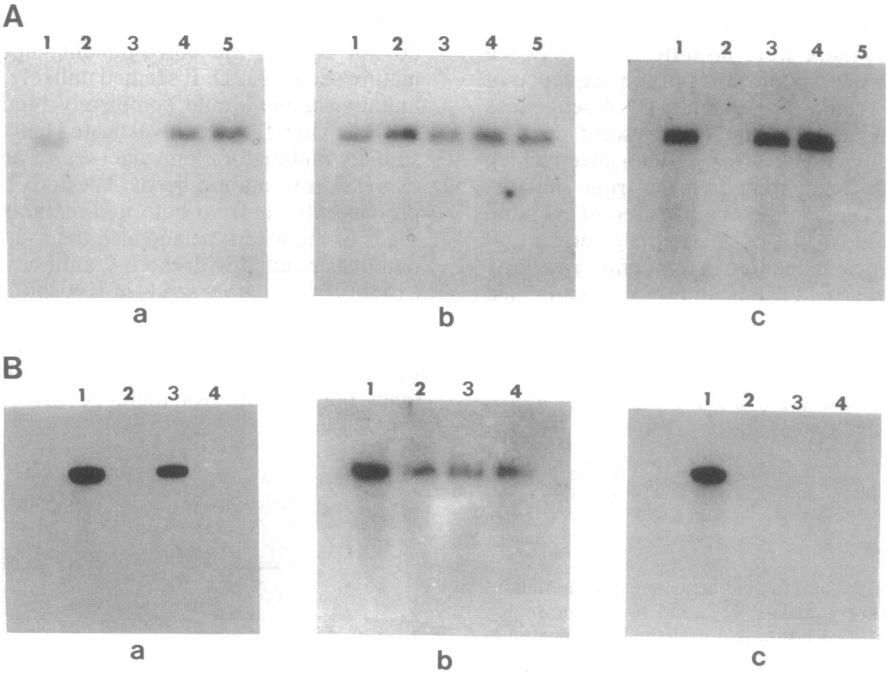

FIG. 1. Northern hybridization patterns of lipoxygenase transcripts in mid-maturation-stage soybean seeds probed with DNA fragments specific for *Lox1* (*a*), *Lox2* (*b*), and *Lox3* (*c*). (*A*) Lane 1, Suzuyutaka (wild type); lane 2, K line (L-1/L-2/L-3 null); lane 3, L-1 null; lane 4, L-2 null; lane 5, L-3 null. (*B*) Lane 1, Suzuyutaka; lane 2, Kanto 102 (L-1/L-3 null); lane 3, Kanto 101 (L-2/L-3 null); lane 4, Kyushu 111 (L-1/L-2/L-3 null).

28,000 recombinants of a cDNA library of Kyushu 111. Restriction analysis indicated that these clones consisted of overlapping segments covering the coding region of the *Lox2* cDNA (14). Nucleotide sequencing revealed that these clones encompassed 2905 bp with 55 bp of the 5′ flanking region and 252 bp of the 3′ flanking region. The open reading frame encodes 866 aa comprising a protein with an estimated molecular weight of 97,261.

Comparison of *lox2* with Other Lipoxygenase Genes. A comparison of the *lox2* sequence with the *Lox2* sequence revealed two distinct differences in the coding region of *lox2*. One was a T → A transversion at nt 1596 of the *lox2* cDNA (position 1 was assigned to the first nucleotide in the coding region and the first methionine in the amino acid sequence), which resulted in a missense mutation, the substitution of glutamine for His-532. The other discrepancy was an insertion of GAA at nt 2557–2559, which resulted in the introduction of an additional Glu-852 residue without altering the reading frame. Since the histidine corresponding to His-532 of L-2 is conserved in L-1 and L-3, its substitution in *lox2* is striking. In contrast, the additional glutamic codon appears to constitute the norm, as L-3 contains a glutamic residue and L-1 contains a lysine residue at the equivalent position (Fig. 2).

Confirmation of the Missense Mutation in *lox2* by Genomic DNA Sequencing. To determine whether the two differences detected in the *lox2* sequence were really due to point mutations causing the lack of L-2, to differences in the plant materials used in this (Kyushu 111) and previous (Century variety; ref. 14) studies, or to RNA editing, we analyzed the genomic DNA sequence. Using *Lox2*-specific primers (Fig. 3), we determined partial genomic DNA sequences from the triple mutants (Kyushu 111 and the K line) and the wild-type cultivar, Suzuyutaka, which contained the two regions of interest and covered the histidine-rich motif found in all lipoxygenases (29). Nucleotide sequencing of four independent PCR products revealed that His-532 was present in the genomic DNA of Suzuyutaka but was replaced by glutamine in both Kyushu 111 and the K line. The inserted amino acid residue, Glu-853, was present in Suzuyutaka as well as in the Kyushu 111 and K lines. Aside from the replacement of His-532 with glutamine, there were no differences between the wild-type and the triple-mutant lines in the sequence of the histidine-rich-motif region. The genomic DNA sequences in these regions of the Kyushu 111 and K lines were identical to that of the *lox2* cDNA sequence except for an intron which was slightly different in Suzuyutaka and the triple mutants (Fig. 3). Glu-852 of L-2 is present in several different lines and the absence of this residue in L-2 of the Century variety (14) most likely reflects an ancient deletion in the Century variety. Therefore, we believe that Glu-852 is an original amino acid

```
        L-2   527 H W L N T H A V I E P F I I A T N R H L 546
lox2 protein  527 * * * * * Q * * * * * * * * * * * * * * 546
        L-1   499 * * * * * * A M * * * V * * * H * * * 509
        L-3   518 * * * * * * * V * * * * * * * * * * * 528

        L-2   847 L H P N S   E G L T C R G I P N S I S I 865
lox2 protein  847 * * * * * E * * * * * * * * * * * * * 866
        L-1   820 * Y * S * K * * * * F * * * * * * * * * 839
        L-3   838 * L * S * E * * * * F * * * * * * * * * 857
```

FIG. 2. Comparison of the amino acid sequences of the mutant L-2 protein encoded by *lox2* with L-1, L-2, and L-3 in the region which differs in sequence. The proteins are aligned for maximum homology. Stars indicate identity with L-2. Shaded boxes indicate the position of the mutations in the protein encoded by *lox2*. Numbers in the margins indicate amino acid residue number. A blank space indicates a single-residue gap inserted into the L-2 sequence in order to maximize the match with the other lipoxygenase protein sequences.

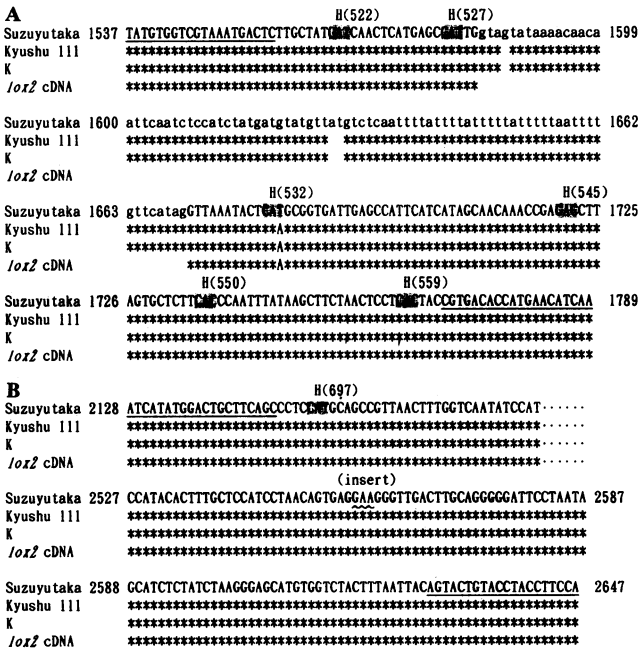

FIG. 3. Comparison of histidine-rich motifs deduced from genomic DNA sequences of three soybean lines with the corresponding regions of the *lox2* cDNA. Shaded boxes indicate conserved histidine residues in plants and mammals, and the numbers in parentheses indicate the position of the amino acids in the *lox2*-encoded protein. Numbers in the margins correspond to the nucleotides in the *lox2* cDNA sequence. Introns are depicted in lowercase letters. Primers are underlined. The primers in *A* and *B* are 253 and 520 nt apart, respectively. Blank spaces represent gaps inserted in order to maximize fit. Asterisks indicate residues which are identical in all four sequences. Ellipses indicate a 345-nt stretch of identical sequences.

residue in the L-2 coding region. These findings confirmed that the replacement of His-532 by glutamine was the only change among 866 aa encoded in *lox2*.

Expression of *lox2* in Mid-Maturation-Stage and Mature Seeds. The mutant lines do not exhibit enzymatic activity and do not contain the corresponding lipoxygenase protein in mature seed (12, 21). It seemed unlikely that a single missense mutation alone would completely block translation of *lox2* mRNA. Therefore, we investigated lipoxygenase gene expression in mid-maturation-stage seeds and compared it with expression in mature seeds. Western blotting indicated that protein extracts from both mid-maturation-stage and mature seeds of the wild-type and also the L-1/L-3 null and L-2/L-3 null lines reacted with the L-2 antibody. In contrast, protein extracts from mature seeds of Kyushu 111 failed to react with the L-2 antibody; however, the extract from mid-maturation-stage seeds reacted with it positively, but more weakly than wild type (Fig. 4). Similar results were obtained with the K line (data not shown). As there is high amino acid sequence

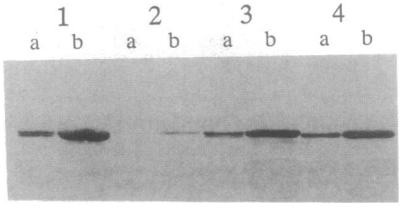

FIG. 4. Western blots showing lipoxygenase expression in extracts from cotyledons of mature (lanes a) and mid-maturation-stage (lanes b) soybean seeds immunodecorated with an L-2 antibody. Lanes 1, Suzuyutaka (wild type); lanes 2, Kyushu 111 (L-1/L-2/L-3 null); lanes 3, L-1/L-3 null; lanes 4, L-2/L-3 null.

homology among L-1, L-2, and L-3 (14) and there is some crossreactivity of both L-1 and L-3 with the L-2 antibody (12, 30), we expected that the double mutants, the L-1/L-3 null and the L-2/L-3 null lines, would produce a positive reaction with the L-2 antibody. Nevertheless, our results clearly indicate that lipoxygenase proteins are expressed in mid-maturation-stage seeds of the Kyushu 111 and K lines. Among the three lipoxygenase structural genes, only the transcript encoding L-2 was detected in mid-maturation-stage seeds of Kyushu 111 and the K line (see Fig. 1). Thus, the lipoxygenase protein expressed in mid-maturation-stage seeds of the triple-mutant lines must be L-2. These results indicate that the lack of L-2 in mature seeds of these lines is not due to a failure of protein synthesis, as is the case in the L-1 and L-3 null lines, but is apparently due to degradation of the L-2 protein before the seed reaches maturity.

Enzymatic Activity of the *lox2*-Encoded Protein. To see whether the protein produced by *lox2* in mid-maturation-stage seeds of the triple mutants had lipoxygenase activity, the extracts from the cotyledons of the mid-maturation-stage and mature seeds were assayed for L-2 and L-3 activities at pH 6.5 and for L-1 activity at pH 9.0. The lipoxygenase activities found in the extracts of the mid-maturation-stage seeds were similar to those seen in mature seeds, for all mutant lines analyzed, as well as the wild type (Table 1), indicating that normal lipoxygenases are stable from mid-maturation stage through maturity. Compared with the wild type, the three double mutants exhibited reduced lipoxygenase activity at pH 6.5, and the lines lacking L-1 also lacked the activity at pH 9.0, whereas neither triple mutant displayed any activity at pH 9.0 and only extremely low oxygen consumption was observed at pH 6.5 regardless of the developmental stage of seed (Table 1). To determine whether the extremely low oxygen consumption observed in assays of the triple mutants reflects actual activity or just background noise, the amount of each extract was increased 5- or 10-fold in the reaction mixture. The activity of Suzuyutaka increased in proportion to the amount of extract included in the reaction mixture, while the oxygen consumption in both triple-mutant extracts was independent of extract dose and remained very low. Extracts from Kyushu 111 and Suzuyutaka boiled for 20 min also had low oxygen consumption, similar to that observed for the native extracts from the triple-mutant lines (data not shown). These results demonstrate that there is no lipoxygenase activity present in mid-maturation-stage or mature seeds of either Kyushu 111 or the K line and that the protein produced by *lox2* in mid-maturation-stage seeds is enzymatically inactive.

Table 1. Lipoxygenase activity of soybean mutant lines

Material	Stage of seed	O_2 consumption, μmol/sec per mg of protein (mean ± SD, n = 3) At pH 6.5	At pH 9.0
Suzuyutaka	Mid-maturation	55.3 ± 8.7	44.2 ± 3.9
	Mature	48.2 ± 3.1	50.3 ± 8.5
L-1/L-3 null	Mid-maturation	31.0 ± 4.6	0.0 ± 0.0
	Mature	38.0 ± 6.2	0.0 ± 0.0
L-1/L-2 null	Mid-maturation	11.8 ± 3.1	0.0 ± 0.0
	Mature	15.4 ± 5.9	0.0 ± 0.0
L-2/L-3 null	Mid-maturation	2.5 ± 0.1	35.0 ± 3.9
	Mature	2.3 ± 0.4	40.1 ± 6.1
Kyushu 111	Mid-maturation	0.4 ± 0.1	0.0 ± 0.0
	Mature	0.5 ± 0.0	0.0 ± 0.0
K line	Mid-maturation	0.4 ± 0.2	0.0 ± 0.0
	Mature	0.2 ± 0.2	0.0 ± 0.0

DISCUSSION

In this study we have shown that the molecular basis for L-2 deficiency in the seeds of two triple-mutant soybean lines is a single missense mutation in which His-532 is replaced by glutamine. The missense mutation in *lox2* does not block the expression of the gene but drastically affects the protein structure and function and culminates in the degradation of L-2. Generally, missense mutations affect gene function less drastically than nonsense (31) and frameshift (6) mutations. Recently, two variants of human lysozyme were reported in which missense mutations conferred high resistance to protein degradation, leading to amyloid deposition and illness (32). To our knowledge, our findings provide the first documentation of a spontaneous missense mutation which results in rapid degradation of the protein product.

Destabilization of Protein Structure Due to a Single Missense Mutation. The histidine residue corresponding to His-532 in lipoxygenase L-2 of soybean is one of highly conserved amino acid residues in the histidine-rich motif found in all 13 lipoxygenase sequences reported to date from both plants and mammalian species (29). Recently, site-directed mutagenesis was used to study the function of each conserved histidine residue in soybean L-1 and in human 5-lipoxygenase *in vitro*. His-499, His-504, and His-690 in soybean L-1 (29) and His-367, His-372, and His-551 in human 5-lipoxygenase (33, 34) were shown to be required for lipoxygenase activity. Mutant genes in which any one of these histidine residues was replaced by glutamine or by serine were able to produce lipoxygenase protein in *E. coli*, but no lipoxygenase activity was detected (29, 33, 34). Our results showing that the replacement of His-532 does not block the production of the L-2 protein but causes a loss of enzyme activity are consistent with the above findings. Moreover, the protein with His-532 replaced by glutamine was unstable since it was not present in mature seeds, suggesting that the His-532 residue in L-2 is crucial not only for the expression of the enzymatic activity but also for preserving the structure of the protein itself.

Lipoxygenases are non-heme iron enzymes. Mutant soybean L-1 proteins with His-499 replaced by glutamine, His-504 replaced by glutamine or serine, and His-690 replaced by glutamine do not contain iron (35). A crystallographic electron density map of L-1 has indicated that His-499, His-504, and His-690 in soybean L-1 are involved in the binding of the active-site iron atom (36, 37). His-532, which is replaced by glutamine in the L-2 null mutant line, corresponds to His-504 of soybean L-1; thus, like His-504 in L-1, His-532 in L-2 appears to be one of the iron-binding ligands. We speculate that the inability of lipoxygenase to bind iron due to the replacement of His-532 by glutamine results in a structural distortion of the L-2 protein. Enzymatic inactivity is a clear consequence of the amino acid substitution, and protein degradation may occur due to structural distortion. It cannot be ruled out that the missense mutation might create an amino acid sequence being more recognized by ubiquitin or another proteolytic degradation system (38), but degradation due to general protein destabilization seems a more probable mechanism.

The absence of L-1, L-2, and L-3 is controlled by the recessive alleles *lx1*, *lx2*, and *lx3*, respectively (17, 18, 20), but previously it was not clear whether these were alleles of regulatory loci or of structural genes encoding the lipoxygenases themselves. Our data indicating that the absence of L-2 results from a single T → A transversion in the structural gene encoding L-2, *Lox2*, suggests that the *lx2* allele identified genetically is a mutant structural gene allele, *lox2*, encoding a defective L-2 protein. Our data do not clarify the relationship between *lx1* and *lx3* and the corresponding lipoxygenase structural genes.

The Molecular Basis for L-2 Deficiency Differs from That of L-1 and L-3 Deficiency. In view of the high degree of homology among the three soybean lipoxygenase isozymes (14), a long DNA fragment of *Lox1* or *Lox3* used as a probe is likely to hybridize with the *Lox2* mRNA. Therefore, it would be difficult to distinguish the mRNA of *lox2* from those of the structural genes for L-1 and L-3 in the triple mutant lines, even if the structural genes for L-1 and L-3 in the triple lines were transcribed. We used short fragments of the 3′ noncoding region of *Lox1* and *Lox3* and our results with these probes indicate that transcription of the structural gene for L-1 or L-3, unlike transcription of *lox2*, does not occur in the mutant lines (Fig. 1). Unfortunately, Northern analysis conducted with these probes did not permit us to rule out the possibility that shorter segments containing the coding regions for L-1 or L-3 might be transcribed, but further evidence suggests they are not. We screened a number of recombinants in the Kyushu 111 cDNA library by using full-length fragments of *Lox2* and *Lox1* cDNA as probes. The only positive clones we obtained were identical to the *lox2* cDNA, suggesting that no part of the mRNA encoding L-1 or L-3 is transcribed in this triple mutant line. An earlier study of lipoxygenase gene expression in L-1 and L-3 null lines suggested that these lines accumulated little mRNA encoding L-1 and L-3, respectively (39). These results demonstrate that there are at least two types of molecular bases which eliminate lipoxygenase expression in soybean mature seeds: one in which the block occurs during transcription or transcript processing (in the L-1 and L-3 null lines) and a second in which protein structure and function are impaired (in L-2 null lines). There is no evidence for a large insertion or deletion mutation in the structural gene for L-1 or L-3 in the corresponding null mutant lines, since Southern blotting did not reveal any differences in lipoxygenase gene organization between those mutants and the wild type (data not shown). Therefore, it seems likely that small-scale alterations in the 5′ regulatory regions of the structural genes for L-1 and L-3 in those mutant lines may have occurred which block transcription of these mRNAs and result in enzyme deficiencies.

We thank Drs. Bernard Axelrod, Yasuo Ukai, and Robert F. Whittier for critical reading of the manuscript and helpful comments. We thank Dr. Li Tao for technical assistance and Tomohiko Kato and Yumiko Shirano for assistance in the preparation of RNA and activity measurement. This work was supported in part by a Grant 03556001 from the Ministry of Education, Science, and Culture, Japan.

1. Goldberg, R. B., Hoschek, G. & Vodkin, L. O. (1983) *Cell* **33**, 465–475.
2. Taylor, L. P. & Walbot, V. (1985) *EMBO J.* **4**, 869–876.
3. Kreis, M., Shewry, P. R., Forde, B. G., Rahman, S. & Miflin, B. J. (1983) *Cell* **34**, 161–167.
4. Ladin, B. F., Doyle, J. J. & Beachy, R. N. (1984) *J. Mol. Appl. Genet.* **2**, 372–380.
5. Scallon, B. J., Dickinson, C. D. & Nielsen, N. C. (1987) *Mol. Gen. Genet.* **208**, 107–113.
6. Sharp, D., Blinderman, L., Combs, K. A., Kienzle, B., Ricci, B., Wager-Smith, K., Gil, C. M., Turck, C. W., Bouma, M. E., Rader, D. J., Aggerbeck, L. P., Gregg, R. E., Gordon, D. A. & Wetteran, J. R. (1993) *Nature (London)* **365**, 65–69.
7. Busslinger, M., Moschonas, N. & Flavell, R. A. (1981) *Cell* **27**, 289–298.
8. Axelrod, B., Cheesbrough, T. M. & Laakso, S. (1981) *Methods Enzymol.* **71**, 411–451.
9. Matoba, T., Hidaka, H., Narida, H., Kitamura, K., Kaizuma, N. & Kito, M. (1985) *J. Agric. Food Chem.* **33**, 852–855.
10. Rackis, J. J., Sessa, D. J. & Honig, D. H. (1979) *J. Am. Oil Chem. Soc.* **56**, 262–271.
11. Sessa, D. J. (1979) *J. Agric. Food Chem.* **27**, 234–239.
12. Kitamura, K. (1984) *Agric. Biol. Chem.* **48**, 2339–2346.
13. Shibata, D., Steczko, J., Dixon, J. E., Hermodson, M., Yazdanparast, R. & Axelrod, B. (1987) *J. Biol. Chem.* **262**, 10080–10085.
14. Shibata, D., Steczko, J., Dixon, J. E., Andrew, P. C., Hermodson, M. & Axelrod, B. (1988) *J. Biol. Chem.* **263**, 6816–6821.
15. Yenofsky, R. L., Fine, M. & Lui, C. (1988) *Mol. Gen. Genet.* **211**, 215–222.
16. Hildebrand, D. F. & Hymowitz, T. (1981) *J. Am. Oil Chem. Soc.* **58**, 583–586.
17. Kitamura, K., Davies, C. S., Kaizuma, N. & Nielsen, N. C. (1983) *Crop Sci.* **23**, 924–927.
18. Kitamura, K., Kumagai, T. & Kikuchi, A. (1985) *Jpn. J. Breed.* **35**, 413–420.
19. Hajika, M., Kitamura, K., Igita, K. & Nakazawa, Y. (1992) *Jpn. J. Breed.* **42**, 787–792.
20. Hildebrand, D. F. & Hymowitz, T. (1982) *Crop Sci.* **22**, 851–853.
21. Hajika, M., Igita, K. & Kitamura, K. (1991) *Jpn. J. Breed.* **41**, 507–509.
22. Kitamura, K. (1991) *Gamma Field Symp.* **30**, 61–69.
23. Kitamura, K., Ishimoto, M., Kikuchi, A. & Kaizuma, N. (1992) *Jpn. J. Breed.* **42**, 905–913.
24. Kato, T., Shirano, Y., Iwamoto, H. & Shibata, D. (1993) *Plant Cell Physiol.* **34**, 1063–1072.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 1.40, 1.46, 14.5.
26. Kato, T., Ohta, H., Tanaka, K. & Shibata, D. (1992) *Plant Physiol.* **98**, 324–330.
27. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
28. Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983) *Plant Mol. Biol. Rep.* **1**, 19–21.
29. Steczko, J., Donoho, G. P., Clemens, J. C., Dixon, J. E. & Axelrod, B. (1992) *Biochemistry* **31**, 4053–4057.
30. Mack, A. J., Peterman, T. K. & Siedow, J. N. (1987) *Isozymes: Curr. Top. Biol. Med. Res.* **13**, 127–154.
31. Baserga, S. J. & Benz, E. T., Jr. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2056–2060.
32. Pepys, M. B., Hawkins, P. N., Booth, D. R., Vigushin, D. M., Tennent, G. A., Soutar, A. K., Totty, N., Nguyen, O., Blake, C. C. F., Terry, C. J., Feest, T. G., Zalin, A. M. & Hsuan, J. J. (1993) *Nature (London)* **362**, 553–557.
33. Nguyen, T., Falgueyret, J. P., Abramovitz, M. & Riendeau, D. (1991) *J. Biol. Chem.* **266**, 22057–22062.
34. Zhang, Y. Y., Radmark, O. & Samuelsson, B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 485–489.
35. Steczko, J. & Axelrod, B. (1992) *Biochem. Biophys. Res. Commun.* **186**, 866–869.
36. Boyington, J. C., Gaffney, B. J. & Amzel, L. M. (1993) *Science* **260**, 1482–1486.
37. Minor, W., Steczko, J., Bolin, J. T., Otwinowski, Z. & Axelrod, B. (1993) *Biochemistry* **32**, 6320–6323.
38. Goldberg, A. L. & Rock, K. L. (1992) *Nature (London)* **357**, 375–377.
39. Start, W. G., Ma, Y., Polacco, J. C., Hildebrand, D. F., Freyer, G. A. & Altschuler, M. (1986) *Plant Mol. Biol.* **7**, 11–23.
40. Kitamura, K., Kikuchi, A. & Harada, K. (1987) *Soybean Genet. Newsl.* **14**, 109–112.