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Soybean Lipoxygenase L-4, a Component of the 94-kilodalton Storage Protein in Vegetative Tissues: Expression and Accumulation in Leaves Induced by Pod Removal and by Methyl Jasmonate

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A lipoxygenase L-4 gene was isolated from a soybean genomic library. The amino acid sequence of lipoxygenase L-4 is highly homologous with the partial amino acid sequence of the 94-kDa vegetative storage protein, vsp94, found in paraveinal mesophyll cells in the leaves of depodded soybean plants. No L-4 expression was observed in maturing seeds. The L-4 gene is highly expressed in the vegetative tissues of young seedlings, including cotyledons, hypocotyls, roots and primary leaves. L-4 expression followed the same pattern as lipoxygenase activity in cotyledons peaking 3 to 5 days after germination, and returning to a basal level by 9 days after germination. L-4 gene expression was low in the roots, stems and leaves of 10-week-old plants. Exposure of 4-week-old plants to atmospheric methyl jasmonate increased L-4 mRNA in leaves. Continuous pod removal from 7-week-old plants over a 2 week period resulted in dramatic accumulation of L-4 mRNA in leaves. Accumulation of the L-4 protein and three other lipoxygenase fractions in the leaves of depodded plants was demonstrated by ion exchange chromatography. These results indicate that lipoxygenase L-4 is a component of vsp94.

Key words: *Glycine max* — Lipoxygenase — Vegetative storage protein — vsp94.

Several classes of polyunsaturated fatty acid derivatives have been isolated from plants and implicated in a variety of physiological events. For example, jasmonate and related compounds, derived from a hydroperoxidation product of linolenic acid (Vick and Zimmermann 1984), are thought to be signal molecules which trigger gene expression during potato tuber formation (Koda and Kikuta 1991) and in response to wounding (Farmer and Ryan 1992). Another class of related compounds, oxygenated unsaturated fatty acids, have been shown to have antifungal activity in rice leaves (Kato et al. 1983). The biosynthesis of these compounds via lipid hydroperoxidation has also been implicated in plant defense (Ohta et al. 1990, 1991).

Abbreviations: VSP, vegetative storage protein; vsp94, the 94-kDa vegetative storage protein, PAGE; polyacrylamide gel electrophoresis.

The nucleotide sequence is available in the DDBJ/EMBL/GenBank data bases under the accession number, D13999.

Lipoxygenases comprise a class of enzymes which catalyze the hydroperoxidation of polyunsaturated fatty acids and are believed to be involved in biosynthesis of regulatory or signal compounds such as jasmonate. Lipoxygenases and related enzymes have been the subject of much investigation and several excellent reviews have recently been written on the subject (Hildebrand 1989, Gardner 1991, Siedow 1991). However, despite the intense study of plant lipoxygenase pathways, no clear function for any plant lipoxygenase has been established.

Among plant lipoxygenases, soybean seed lipoxygenases L-1, L-2 and L-3 have been most thoroughly investigated (Axelrod et al. 1981). cDNAs encoding these three enzymes have been isolated and characterized in detail (Shibata et al. 1987, 1988, Yenofsky et al. 1988). Recently, Hajika et al. (1991) reported a soybean mutant which lacks all three lipoxygenases in seeds, and grows normally throughout the entire life cycle. This suggests that these three lipoxygenases are not essential in soybean growth or development, however they may have roles in secondary

physiological processes such as plant defense.

A class of soybean lipoxygenases distinct from those in seeds have been identified in vegetative tissues (Park and Polacco 1989, Grayburn et al. 1991). Recently, Kato et al. (1992a) characterized a major lipoxygenase, designated L-4, and two minor lipoxygenases, L-5 and L-6, which appears after germination in soybean cotyledons. The genes encoding lipoxygenase L-4 and L-6 differ from those encoding L-1, L-2 and L-3 (Shibata et al. 1987, 1988, Yenofsky et al. 1988). As with the seed lipoxygenases, the physiological functions of lipoxygenases present in vegetative tissues remain to be elucidated.

Recently we reported the molecular cloning of soybean lipoxygenases expressed in vegetative tissues (Shibata et al. 1991). We isolated three classes of lipoxygenase cDNAs from 5-day-old soybean cotyledons, which are distinct from the genes encoding L-1, L-2 and L-3, and sequenced a genomic clone which corresponds to one of these cDNAs, SC514. Bell and Mullet (1991) isolated two classes of lipoxygenase cDNAs, *lox*A and *lox*B, from soybean seedlings. These two cDNAs are expressed at high levels in the roots and non-growing regions of seedling hypocotyls. Identification of the corresponding gene products has not yet been reported.

Tranbarger et al. (1991) reported that a 94-kDa vegetative storage protein, vsp94, located in paraveinal mesophyll cells of soybean is a member of the lipoxygenase family. vsp94 is one of three vegetative storage proteins (VSPs) that accumulate at high levels in soybean seedlings, and depending upon nitrogen status in leaves as well. The latter result has led to the suggestion that VSPs are a storage form of nitrogen in leaves. The genes encoding the other VSPs, vsp27 (or vspα) and vsp29 (or vspβ) have been isolated and their expression characterized in relation to several factors, including pod removal, water deficit, wounding, jasmonic acid, and increases in nitrogen (Staswick 1990, Mason and Mullet 1990). However, no molecular cloning of the vsp94 gene has been reported.

Here, we report genomic cloning of the gene encoding soybean lipoxygenase L-4 and demonstrate that lipoxygenase L-4 is a component of vsp94. We also report expression of the gene in vegetative tissues of young seedlings, and accumulation in leaves induced by pod removal and by atmospheric methyl jasmonate.

Materials and Methods

Plant material—Soybean seeds (*Glycine max* Merr. cv. Enrei) were sterilized by immersion in 0.1% (w/v) benomyl [methyl-(butylcarbamoyl)-2-benzoimidazole-carbamate] (BENLATE, Du Pont) for 6 h at room temperature, then germinated in vermiculite at 28°C under a 16/8 h day/night cycle. Germinated seedlings were sown in soil and grown at 25°C in natural lighting conditions until pod removal. These plants started flowering 6 weeks after germination. Pods were removed from soybean plants continuously for two to four weeks, beginning 1 week after flowering. The leaves were then harvested and stored at −135°C.

(+)-Methyl jasmonate was a gift from Takasago Co. (Tokyo). Methyl jasmonate treatments were conducted in sealed plastic 75 liters boxes. Plants were exposed to methyl jasmonate vapor by placing cotton soaked in methyl jasmonate in the sealed container such that the cotton did not directly contact the plant. These methyl jasmonate concentrations were obtained by diluting it in ethanol, so control plants were exposed to cotton soaked in 100% ethanol. The final amount of methyl jasmonate was 10 nl, 100 nl, or 1,000 nl per liter of box volume. Plants were exposed to methyl jasmonate for 24 h, then the leaves were harvested and RNA was extracted.

Genomic cloning—Genomic hybridization was conducted with part of a cDNA clone, SC501, isolated from a cDNA library made from 5-day-old soybean seedlings (Shibata et al. 1991). An *Eco*R I fragment (179 bp) containing the 3′-untranslated region of SC501 was used to screen a soybean genomic library constructed in the EMBL3 vector (Clontech). The DNA fragment was labeled with [^{32}P]dCTP using a random primer labeling kit (Boehringer Mannheim). Approximately 300,000 recombinant phage plaques were plated and transferred onto nitrocellulose filters. Hybridization with the probe was carried out in 50% (v/v) formamide, 6× SSPE, 5× Denhardt's, 0.5% (w/v) SDS, and 0.1 mg ml^{-1} salmon sperm DNA. The membrane was then washed in 0.1× SSC, 0.1% (w/v) SDS at 65°C for 1 h.

A *Sal* I/*Sal* I fragment (approximately 12 kb) from a positive clone, λSCG501, was subcloned into pBluescript, pSK- (Stratagene), and designated pSS12. From this plasmid, a *Sal* I/*Eco*R I fragment (3.6 kb) and two *Eco*R I fragments (3.1 kb and 1.7 kb) were each subcloned separately into pSK-. Nested deletion constructs of these clones were made in both orientations using a kit (Promega). Single stranded DNA was prepared from the deleted constructs using a helper phage, VCSM13, then sequenced by the dideoxy method using a 7-deaza sequencing kit (Takara Shuzo Co., Ltd., Japan). To determine the locations of these three subcloned fragments in the original clone, pSS12, and to show whether or not are other sequences between these fragments, pSS12 was sequenced in the regions corresponding to the ends of the subfragments using a double stranded template and oligonucleotide primers prepared from the ends of these fragments.

Northern blot analysis—Plant tissue samples (1–3 g) were ground to a powder in liquid nitrogen with a mortar and pestle. The powder was homogenized in 14 ml of 4 M guanidium thiocyanate, 117 mM mercaptoethanol, 25 mM sodium acetate (pH 5.2) in a Polytron homogenizer for 1 min, then centrifuged at 13,000×g for 25 min. The

supernatant was layered on a cushion of 1.5 ml of 5.7 M CsCl in an ultracentrifuge tube (14×95 mm). Centrifugation was carried out at 182,000×*g* for 20 h at 20°C. RNA was dissolved in 1 ml of 10 mM Tris/Cl, 0.5% (w/v) SDS (pH 7.5), then precipitated with ethanol.

Total RNA (25 μg) was electrophoresed on a 1% (w/v) agarose gel containing 0.67 M formaldehyde in 0.02 M Mops buffer (pH 7.0). RNA was transferred onto a nylon membrane (Hybond N+, Amersham) by capillary transfer in 20× SSC. The *Eco*R I fragment of SC501 (corresponding to nucleotides 6,048–6,225 in Fig. 1) was labeled with [^{32}P]dCTP, then incubated with the membrane containing total RNA. The membrane was washed in 0.1× SSC, 0.1% (w/v) SDS at 65°C for 1 h, then exposed to an imaging plate (Fuji Film Co. Ltd.). Using a BIO-IMAGE ANALYZER BAS2000 (Fuji Film Co. Ltd.), the hybridizing bands were visualized, and the absolute ratio of radioactive decay event detection between these bands was calculated.

Primer extension—An oligonucleotide primer, TACTTTCAACCAAACACTCTCAGAAGATTG, was labeled with [γ-^{32}P]ATP using an end labeling kit (Promega). The primer extension reaction was carried out using 30 μg of total RNA prepared from 5-day-old cotyledons and a primer extension kit (Promega) according to the protocol of the supplier.

Purification of lipoxygenases—Lipoxygenases were purified by the method of Kato et al. (1992a). Soybean leaves (5 g) were harvested from pod-bearing and depodded plants, homogenized in 25 ml of 50 mM sodium phosphate and 1.5% (v/v) Triton X-100 (pH 6.8) and centrifuged at 18,000×*g* for 15 min. The supernatant was mixed with 6 g of Amberlite XAD-2 to remove the Triton.

This extract (15 ml) was desalted on a Sephadex G-25 column (2.7×23.5 cm) equilibrated with buffer A: 10 mM sodium phosphate, 10% (v/v) glycerol, 0.1 mM EDTA, and 0.1% (v/v) Tween 20 (pH 6.8). The desalted extract was applied to a DEAE-Toyopearl column (1.0×11 cm) equilibrated with buffer A, then washed with buffer A. The enzyme activity bound to the DEAE-Toyopearl column was eluted with 100 mM sodium phosphate, 10% (v/v) glycerol, 0.1 mM EDTA, and 0.1% (v/v) Tween 20 (pH 6.8). The eluate (20 ml) was desalted on a Sephadex G-25 column with buffer A. The desalted extract from depodded plants (64.1 mg protein) was divided into six fractions (10.6 mg each). The desalted extract from pod-bearing plants (20.2 mg) was divided into two fractions (10.6 and 9.6 mg protein). Each desalted extract was applied to a Mono Q HR5/5 column (Pharmacia) equilibrated with buffer A and the enzyme activities were eluted with a linear gradient of 0 to 0.2 M NaCl in buffer A. Lipoxygenase activity was assayed using 7.5 mM of linoleic acid in 0.1 M sodium phosphate (pH 6.8) at 30°C as described previously (Kato et al. 1992a) and the fractions containing activity were pooled. Oxygen consumption during the lipoxygenase reaction was measured with a Clark oxygen electrode. Protein was estimated using a protein assay kit (BioRad).

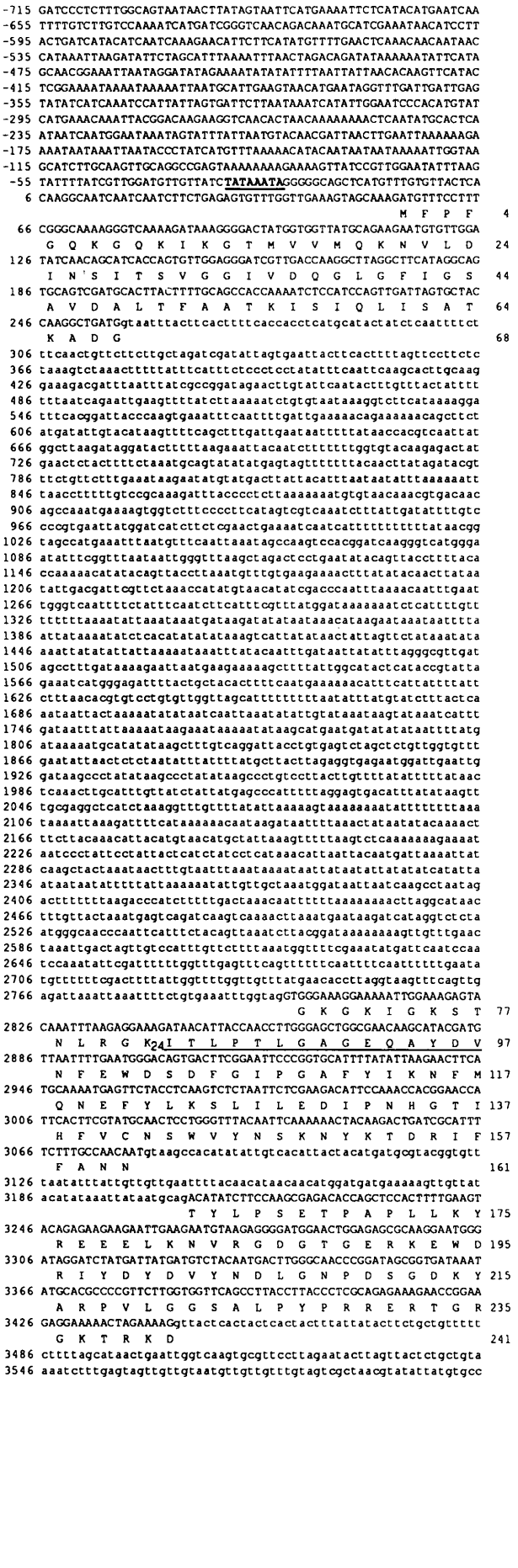
For comparison of enzymatic characteristics, soybean lipoxygenase L-4 was purified from 5-day-old cotyledons as described by Kato et al. (1992a).

Results

Genomic cloning of the gene encoding soybean lipoxygenase L-4—We took advantage of a partial cDNA clone, SC501, which was isolated from a library made from 5-day-old cotyledons of soybean (Shibata et al. 1991) and encodes lipoxygenase L-4, to isolate the genomic counterpart. Sequencing the cDNA (589 bp) revealed a partial amino acid sequence which corresponds to a proteolytic peptide (peptide #27) derived from soybean lipoxygenase L-4 (Kato et al. 1992a). Using the cDNA clone as a probe, a genomic clone, λSCG501, was isolated from a soybean genomic library.

A region of the genomic clone containing sequences homologous to known lipoxygenase genes as well as the DNA flanking the regions of homology were sequenced (7,400 bp, Fig. 1). The SC501 cDNA was identified without mismatches in the genomic sequence (nucleotides 5,637 to 6,225 in Fig. 1). The protein coding region of the gene contains 9 exons and 8 introns. The location of the introns is conserved with respect to soybean lipoxygenase L-3 (Yenofsky et al. 1988) and a lipoxygenase gene which is expressed in soybean cotyledons, SC514 (Shibata et al. 1991). The length of the introns vary between these genes, particularly the first intron, which is the longest reported to date in plant lipoxygenase genes (2,542 bp).

The transcription initiation site was determined by primer extension (Fig. 2). The size of the longest product synthesized from the primer and the mRNA template indicates that transcription begins at an adenine residue located 54 bp upstream of the translation start site, designated as +1 in Figure 1. The sequences around the transcription initiation site are homologous to those of the gene encoding soybean seed lipoxygenase L-3 (Yenofsky et al. 1988) as well as those encoding a lipoxygenase gene expressed in soybean seedlings, SC514 (Shibata et al. 1991). A putative TATA sequence, TATAAATA (−30 to −23) was found upstream of the transcription start site. No obvious sequence homology was found between the upstream sequence of λSCG501 and those of the SC514 gene (Shibata et al. 1991) which is expressed in the cotyledons, hypocotyl and roots of young seedlings at high levels (unpublished data). The translation initiation site was identified by comparing it to those in previously identified lipoxygenases. A sequence near the translation initiation site, GCAAAG-*ATG*TTT (49 to 60) is conserved among soybean lipoxygenase genes. A similar sequence, CAAG*ATG*TTG, is



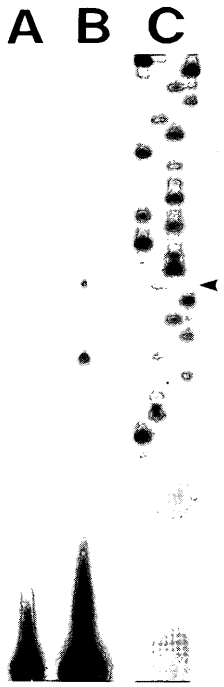


Fig. 2 Identification of the L-4 transcription start site by primer extension. The primer extension products were separated on a sequencing gel. (A) no reaction products, (B) primer extension products, (C) size marker; sequencing reaction products produced with the primer and a DNA template corresponding to the gene.

present near the translation initiation site in the lipoxygenase L-2 gene of rice (Ohta et al. 1992). This sequence increased transient expression of a prokaryotic gene, *uid*A, under control of the 35S promoter of CaMV in tobacco and rice protoplasts, so it may have a role in increasing the efficiency of translation (Kato et al. 1991).

Amino acid sequence homology with lipoxygenases and vsp94—Partial amino acid sequences of lipoxygenases L-4 (Kato et al. 1992a) and L-6 (Kato et al. 1992b), purified from 5-day-old cotyledons, were compared with that of the putative gene product encoded by λSCG501. It is unlikely that the gene product is lipoxygenase L-6 because 7 of 24 amino acid residues identified in 5 proteolytic peptides of lipoxygenase L-6 purified from 5-day-old cotyledons (Kato et al. 1992b) differ from those of the gene product. All 88 amino acid residues identified in 7 proteolytic peptides of lipoxygenase L-4 (Kato et al. 1992a) were found in the putative gene product encoded by λSCG501. These results indicate that the gene contained in λSCG501 encodes lipoxygenase L-4.

The molecular mass of the putative gene product encoded by λSCG501 was calculated to be 96,533 daltons and is consistent with that estimated for lipoxygenase L-4 by SDS-PAGE (Kato et al. 1992a). The amino acid sequence of lipoxygenase L-4 was compared with previously identified plant lipoxygenases and shares 64%, 67%, 74%, 67%, 73% and 67% homology with soybean lipoxygenases L-1 (Shibata et al. 1987), L-2 (Shibata et al. 1988), L-3 (Yenofsky et al. 1988) and the gene products of SC514 (Shibata et al. 1991) in soybean, pPE1036 (Ealing and Casey 1988) in pea and pPE923/320 (Earling and Casey 1989) in pea, respectively. The homology between L-4 and rice lipoxygenase L-2, which is expressed in maturing seeds and young seedlings (Ohta et al. 1992), is 51%. These comparisons and comparisons with mammalian lipoxygenases indicate that lipoxygenase L-4 contains amino acid residues which are conserved among plant and mammalian lipoxygenases (Ohta et al. 1992) (Fig. 1), and suggests that the gene product encoded by λSCG501 is a functional lipoxygenase.

We compared the amino acid sequence of lipoxygenase L-4 with partial amino acid sequences of the 94-kDa vegetative storage protein (vsp94) found in paraveinal mesophyll cells in the leaves of depodded plants (Tranbarger et al. 1991). Tranbarger et al. (1991) isolated vsp94 by excision from an SDS-PAGE gel, subjected to CNBr cleavage, and sequenced the resulting polypeptides. The partial amino acid sequences were found to be homologous with previously identified soybean lipoxygenases, but not identical. They suggested that vsp94 is a member of the lipoxygenase gene family and designated it pvmLOX. Comparison of lipoxygenase L-4 and the partial amino acid sequence of pvmLOX reveals high homology between them; 43 of 47 amino acid residues identified in pvmLOX matched those of lipoxygenase L-4. The slight difference in sequence suggests that pvmLOX may be comprised of several closely related proteins.

Expression of lipoxygenase L-4 in vegetative tissues—The L-4 transcript levels in vegetative tissues were analyzed using a 3′ end probe specific for the L-4 mRNA. The probe was prepared from the 3′ region of the gene (nucleotides 6,048–6,225) containing a 23 bp segment encoding the C-terminus and 156 bp of the 3′ untranslated region. No cross hybridization of the 3′ end probe was seen under stringent conditions with any of four other soybean lipoxygenase cDNAs tested; the distinct SC514 and SC500 cDNAs iso-

Fig. 1 Nucleotide and deduced amino acid sequences of the gene encoding lipoxygenase L-4. Numbers in the left margin refer to nucleotides. Numbers in the right margin refer to amino acid residues. Amino acid sequences identified in proteolytic peptides derived from the purified L-4 enzyme (Kato et al. 1992a) are underlined and the peptide numbers are given (unidentified amino acids are underlined with broken line). Conserved amino acid residues among plant and mammalian lipoxygenases are boxed. Introns are depicted in small letters. The putative TATA box sequence is shown in bold face with underline. The transcription start site is indicated by italic letters. The polyadenylation signal sequences (AATAAA) are underlined.

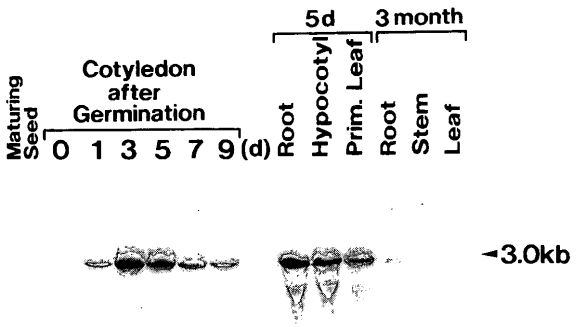


Fig. 3 Accumulation of lipoxygenase L-4 mRNA in vegetative tissues. Total RNA (25 μg) isolated from maturing seeds and various vegetative tissues was separated on a 1% (w/v) agarose gel, transferred to a nylon membrane, then probed with a ^{32}P-labeled gene specific DNA fragment.

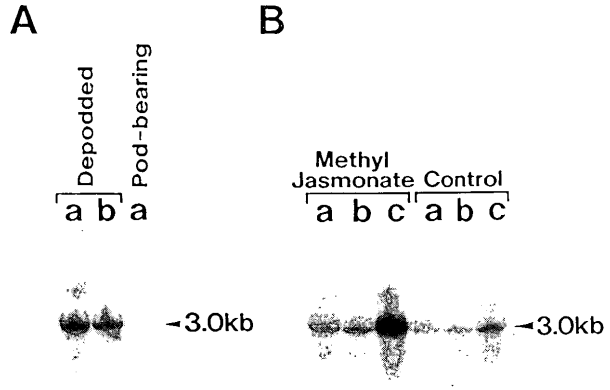


Fig. 4 Accumulation of lipoxygenase L-4 mRNA after pod removal and induced by methyl jasmonate. (A) Pods were continuously removed from four 7-week-old plants for two weeks, then the sixth leaf from the bottom (a) and the lowest leaf (b) were harvested. As a control, the sixth leaf was harvested from each of four 9-week-old pod-bearing plants (the lowest leaves on the pod-bearing plants abscised by 9 weeks of age). (B) Four-week-old plants bearing an unexpanded leaf at the top and four fully expanded leaves (a, upper; b, middle; c, two lower leaves attached to the stem at the same height) were exposed to atmospheric methyl jasmonate (100 nl liter^{-1}) for 24 h in a plastic box. RNA was analyzed as described in Figure 3.

lated from cotyledons of 5-day-old seedlings (Shibata et al. 1991) or cDNA clones of the distinct L-1 and L-2 lipoxygenases isolated from maturing seed (Shibata et al. 1987, Shibata et al. 1988).

High amounts of L-4 mRNA appeared in cotyledons, hypocotyls and roots of 5-day-old seedlings, and in the primary leaves of 7-day-old seedlings. L-4 mRNA increased in cotyledons from 3 to 5 days after germination, then reached a basal by 9 days after germination (Fig. 3). The appearance and disappearance of L-4 mRNA in cotyledons coincided with lipoxygenase activity in the cotyledons of a soybean mutant which lacks lipoxygenases L-2 and L-3 in mature seeds (Kato et al. 1992a). Mature plants (10-week-old) contained lower levels of L-4 mRNA in leaves, stems and roots (Fig. 3). L-4 mRNA was not detected in either maturing or ungerminated seeds (Fig. 3).

L-4 mRNA accumulation after pod removal and exposure to methyl jasmonate—As pvmLOX is induced in leaves by pod removal (Tranbarger et al. 1991), and is highly homologous with lipoxygenase L-4 as shown in this study, we analyzed expression of the L-4 gene in pod-bearing and depodded plants (Fig. 4A). Pods were continuously removed from 7-week-old soybean plants for two weeks and mRNA levels were analyzed using the L-4 specific probe (Fig. 4A). L-4 mRNA in the upper leaves of depodded plants was 50-fold higher than that found in similar leaves on pod-bearing plants. It is of interest that abscission occurred in the lowest leaves of pod-bearing plants during these experiments, but not in depodded plants. The lowest leaves of depodded plants also contained significant levels of L-4 mRNA (Fig. 4A).

We also found that atmospheric methyl jasmonate induces the accumulation of L-4 mRNA in leaves (Fig. 4B). Soybean plants (4 weeks old) bearing one small trifoliate leaf at the top and four fully expanded trifoliate leaves (upper, middle and two lower leaves attached to the stem at the same height) were exposed to atmospheric methyl jasmonate for 24 h in a plastic box, then the upper, middle and lower leaves were harvested and analyzed for L-4 mRNA. The lower leaves of control plants contained the highest amounts of L-4 mRNA, followed by the middle, then upper leaves (Fig. 4B). The L-4 mRNA level in the lowest leaves of control plants was lower than that found in cotyledons. Treatment with 100 nl liter^{-1} of methyl jasmonate significantly increased L-4 mRNA in all leaves (Fig. 4B). Three concentrations of methyl jasmonate (1,000, 100 and 10 nl liter^{-1}) produced the same L-4 mRNA level in the lower leaves (data not shown).

Taken together, the amino acid sequence similarity between lipoxygenase L-4 and pvmLOX, the high level accumulation of the gene product in cotyledons, and induction in leaves by pod removal or methyl jasmonate treatment suggest that lipoxygenase L-4 is a component of vsp94.

Isolation of lipoxygenase L-4 from leaves—To confirm that lipoxygenase L-4 is a component of vsp94 in leaves after removal of pods, we analyzed pod-bearing and depodded plants for lipoxygenase L-4 accumulation. Protein extracts were prepared from leaves of both pod-bear-

ing and depodded plants, and the lipoxygenases from each extract were separated by Mono Q column chromatography (Fig. 5, Table 1). Four lipoxygenase activities were identified in each extract, however the activities in pod-bearing plants were much lower. The lipoxygenase which eluted at the lowest salt concentration eluted in the same fraction from the Mono Q column as did lipoxygenase L-4 purified from cotyledons under the same conditions (Kato et al. 1992a). A mixture of this lipoxygenase fraction from leaves and the lipoxygenase L-4 derived from cotyledons produced a single peak of activity when analyzed chromatographically (data not shown).

We compared the enzymatic characteristics of this leaf lipoxygenase fraction with those of the cotyledon lipoxygenase L-4 fraction. This leaf lipoxygenase fraction showed the same optimum pH at 6.4 as the cotyledon one, and neither enzyme showed any activity at pH 5.0. The measured K_ms of the leaf enzyme fraction toward linoleic and linolenic acids were 0.79 mM and 10.4 mM respectively, and the same values measured for the cotyledon enzyme were 0.92 mM and 8.5 mM respectively. The K_m values for these two fractions are thus indistinguishable from each other within experimental error.

The L-4 enzyme is the major lipoxygenase in the chromatogram. The amount of the L-4 enzyme in the crude extract was calculated based on the specific activity of the purified L-4 enzyme (0.354 μkat mg^{-1}), the ratio of L-4 to total lipoxygenase activity loaded onto the column, and based on the recovery of activities, assuming the recovery of each activity was the same throughout purification. The L-4 enzyme comprises 2.7% (w/w) of the total soluble protein in crude leaf extracts after pod removal. Thus, high levels of lipoxygenase L-4 accompany high L-4 mRNA levels in depodded plants, and the L-4 enzyme and mRNA levels are concomitantly low in pod-bearing plants (Fig. 5), indicating that lipoxygenase L-4 is a component of vsp94.

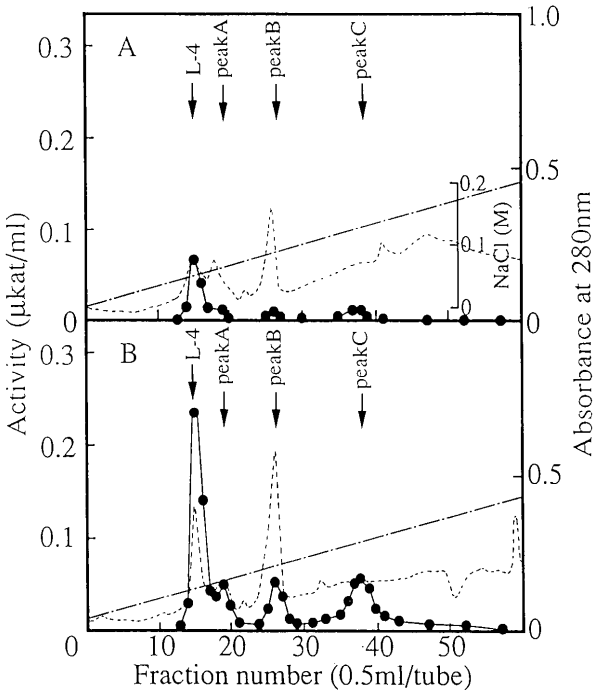


Fig. 5 Mono Q chromatography of lipoxygenases isolated from leaves. Soybean leaves were harvested from pod-bearing plants (A) and depodded plants (B). Extracts (10.6 mg protein) from depodded and pod-bearing plants were loaded onto the column. The fraction which bound DEAE-Toyopearl was 3.1-fold higher in depodded plants than in pod-bearing plants, therefore B represents 3.1-fold more lipoxygenase activity per harvested leaf than A. Lipoxygenases were separated using a linear gradient of NaCl (---). Lipoxygenase L-4 and three uncharacterized lipoxygenase activities (Peaks A, B, and C) obtained in this chromatogram are indicated by arrows. Protein, absorbance at 280 nm (----), and lipoxygenase activity (-●-) are shown.

Table 1 Purification of lipoxygenase activity from leaves of depodded plants

Purification step	Protein (mg)	Activity (μkat)	Specific activity (μkat mg^{-1})
Crude extract	236	5.4	0.023
DEAE-Toyopearl column			
Bound fraction	64.1	5.8	0.090
Unbound fraction	15.1	1.60	0.104
Mono Q column			
Lipoxygenase L-4	3.33	1.18	0.354
Peak A	0.62	0.15	0.242
Peak B	3.11	0.27	0.087
Peak C	1.37	0.34	0.248

Pods were removed from soybean plants continuously for four weeks, beginning 1 week after flowering. The leaves (5 g) were harvested for isolation of lipoxygenases.

Three unidentified lipoxygenases were also present on the Mono Q column (peaks A, B and C in Fig. 5). Interestingly, these lipoxygenase activities also increased after pod removal, but were not further characterized in this study.

Discussion

In this study we report the molecular cloning of lipoxygenase L-4, which was recently characterized as a major lipoxygenase in 5-day-old soybean cotyledons (Kato et al. 1992a). Several lines of evidence indicate that lipoxygenase L-4 is a component of vsp94, a 94-kDa soybean vegetative storage protein. The gene is expressed at high levels in vegetative tissues of young seedlings, but at lower levels in mature plants (Fig. 3). No L-4 expression was observed in developing or mature seeds (Fig. 3). Pod removal (Fig. 4A) and exposure of atmospheric methyl jasmonate (Fig. 4B) result in high levels of L-4 mRNA in leaves. Pod removal induced accumulation of lipoxygenase L-4 up to 2.7% (w/w) of the total soluble protein in leaves (Fig. 5). These lipoxygenase L-4 profiles are identical to those of the VSPs which have been described in relation to nitrogen status in soybeans (Staswick 1990).

Bell and Mullet (1991) isolated partial cDNA clones for soybean lipoxygenases, *lox*B1 (263 bp) and *lox*B2 (333 bp) which are 96% identical to each other in overlapping nucleotide sequences. The nucleotide sequence of *lox*B1 is identical at the appropriate junctures to λSCG501, and thus appears to be derived from the L-4 gene. A nearly full-length cDNA, pTK18 (3,020 bp) which is derived from the L-4 gene was isolated from a seedling cDNA library (J. C. Polacco, personal communication). Bell and Mullet (1991) also reported that methyl jasmonate induced expression of *lox*B in soybean cell cultures. This report did not distinguish between the expression of *lox*B1 and *lox*B2, probably due to high nucleotide sequence homology between these two genes. Expression of *lox*B was also modulated in response to water deficit and wounding, suggesting a role for lipoxygenases in plants responding to such stress. Their finding of two highly similar genes introduces the possibility that the expression of the L-4 gene as detected by the L-4 specific probe is indistinguishable from that of *lox*B2. Crosshybridization of the L-4 specific probe to the *lox*B2 transcript cannot be tested due to the unavailability of the *lox*B2 3′ untranslated region. We are currently working on transgenic plants using upstream regions of the L-4 gene to address *bona fide* expression of the gene in leaves in response to several metabolic conditions.

The amino acid sequence of lipoxygenase L-4 is highly homologous to partial amino acid sequences of vsp94, which was isolated from leaves of depodded plants (Tranbarger et al. 1991). It should be noted that, in their study, vsp94 is defined as an abundant 94-kDa protein which appears on SDS-PAGE and is not necessarily composed of a single 94-kDa polypeptide species. It is also unclear whether or not vsp94 species which appear in various tissues are identical. In this study we have clearly demonstrated that a component of vsp94 is lipoxygenase L-4 and that it accumulates both in vegetative tissues of young plants and in leaves of depodded plants.

Methyl jasmonate induces vsp94 (Franceschi and Grimes 1991) and pvmLOX found in the primary leaves (Grimes et al. 1992). A question related to this observation is whether or not these inductions result in further biosynthesis of jasmonate-related messengers in plant cells (Tranbarger et al. 1991). As the major component of vsp94, lipoxygenase L-4 is also induced by methyl jasmonate (Fig. 4B). To determine whether or not L-4 induced by methyl jasmonate causes further synthesis of jasmonate-related messengers, we are enzymatically characterizing lipoxygenases isolated from leaves after pod removal. Our results indicate that lipoxygenase L-4 has an unusually high K_m value for linolenic acid (10.4 mM). As linolenic acid is the biosynthetic precursor of jasmonate (Vick and Zimmermann 1984), it seems unlikely that the enzyme is involved in the biosynthesis of jasmonate and related compounds. However, we can not exclude the possibility that another lipoxygenase(s) which appears in leaves of depodded plants, e.g. peaks A, B and/or C in Figure 5, are involved in jasmonate biosynthesis. The effects of methyl jasmonate or factors which may induce these lipoxygenases and their specificity for linolenic acid are unknown.

Tranbarger et al. (1991) used a polyclonal antibody raised against a soybean leaf lipoxygenase to show that pvmLOX (vsp94) is localized in the vacuole, and not in ER or Golgi. We analyzed the amino acid sequence of lipoxygenase L-4 to determine whether lipoxygenase L-4 has specific features in the primary structure which might be responsible for translocation of the protein from the cytosol into vacuole. Lipoxygenase L-4 has no signal peptide sequence at the amino terminus, indicating that the protein is probably not translocated into the ER, consistent with the findings of Tranbarger et al. (1991) for pvmLOX. It is unlikely that the carboxy terminal sequence has a function in translocation, since this sequence, RGIPNSISI, is conserved among all plant lipoxygenases and no localization of soybean lipoxygenases in the vacuole have been demonstrated (Vernooy-Gerritsen et al. 1984) except for pvmLOX.

Comparison of a hydropathy profile of lipoxygenase L-4 with those of other plant lipoxygenases suggests that there are no significant differences in secondary structures (data not shown). This result is not surprising because the similarity in amino acid sequence between the L-4 enzyme and known plant lipoxygenases occurs throughout the entire polypeptide. Amino acid sequence comparisons among

plant lipoxygenases indicate that the amino termini vary in both sequence and length, but only to a small extent. This type of analysis may not be useful in defining sequences or elements which function in targeting from the cytosol to the vacuole. It should be noted that pod removal results in increases of at least four lipoxygenase activities (lipoxygenase L-4 and peaks A, B and C in Fig. 5) in leaves. The polyclonal antibody used by Tranbarger et al. (1991) was not shown to be specific for a single lipoxygenase species, so we can not exclude the possibility that lipoxygenases other than L-4 are localized in the vacuole. Analysis of these as yet uncharacterized lipoxygenases should provide a resolution to this question.

A notable enzymatic feature of lipoxygenase L-4 is that it has the lowest specific activity (0.354 μkat mg^{-1}, Table 1) among soybean lipoxygenases characterized to data (3.0 μkat mg^{-1} for L-1, 1.0 μkat mg^{-1} for L-2, and 1.1 μkat mg^{-1} for L-3). In addition to this, our studies show that the K_m value for linoleic acid (0.79 mM) is much higher than those of other soybean lipoxygenases (12 μM linoleic acid for L-1, 16 μM arachidonic acid for L-2, and 0.34 mM linoleic acid for L-3 (Axelrod et al. 1981)). Although these K_m values indicate that the enzyme is a relatively weak lipoxygenase, it could be that the high concentration of the enzyme in the cell compensates for the low specific activity and high K_m values, such that it is functionally active in the hydroperoxidation of fatty acids in vivo. However, if this enzyme is localized in the vacuole as suggested for pvmLOX by Tranbarger et al. (1991), it is unlikely that lipoxygenase L-4 is active in the vacuole because the enzyme is most active at pH 6.4 but has no activity at pH 5.0. If L-4 is an active lipoxygenase which produces hydroperoxides in vivo, there is a question about whether or not it is bifunctional and also serves as a nitrogen depot during vegetative growth. To address the physiological function of lipoxygenase L-4, we must characterize the enzyme in more detail. In particular it is essential to determined its intracellular location using an L-4 specific (perhaps monoclonal) antibody.

During the preparation of this manuscript, Grimes et al. (1992) reported that low levels of atmospheric methyl jasmonate induce the expression and accumulation of lipoxygenase activity in various cell types of soybean seedlings, and that most of the methyl jasmonate-responsive lipoxygenases are associated with vacuoles where the lipoxygenases are present in tubular paracrystalline structures. These results are consistent with our observation of lipoxygenase L-4 expression in young seedlings and induction of L-4 expression by methyl jasmonate.

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