The Cloning of Two Tomato Lipoxygenase Genes and Their Differential Expression during Fruit Ripening

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A membrane-associated lipoxygenase from breaker-stage fruit of tomato (Lycopersicon esculentum Mill.) was purified and partially sequenced. Using degenerate oligonucleotides corresponding to portions of this sequence, a cDNA was amplified by PCR and used to screen a breaker fruit cDNA library. Two clones, tomloxA and tomloxB, were isolated and one of these (tomloxA) corresponded to the isolated protein. Genomic clones were isolated and used to screen a breaker fruit cDNA library. Two clones, that encode lipoxygenase. RNA blot analysis showed that tomloxA is expressed in germinating seeds as well as in ripening fruit, where it reached its peak during breaker stage. tomloxB appears to be fruit specific and is at its highest level in ripe fruit.

Lipoxygenases (EC 1.13.11.12) comprise a class of iron-containing enzymes that use molecular oxygen in the dioxygenation of fatty acids containing a 1,4-pentadiene structure such as linoleic and linolenic acid in plants and arachidonic acid in mammalian cells. The resulting lipid hydroperoxides are further metabolized into physiologically active compounds. In mammals, some of these bioregulatory molecules produced are leukotrienes, prostaglandins, lipoxins, and thromboxanes (Yamamoto, 1992). The lipoxygenase products in higher plants are metabolized by one of two major pathways leading to the formation of jasmonic acid or traumatin (wound hormone), which are known to have regulatory effects on membranes and proteins and may contribute to the loss of membrane function accompanying fruit ripening and senescence (Todd et al., 1990).

To determine the physiological functions of lipoxygenases, more must be learned about their cellular and subcellular localization. Although most subcellular compartments of higher plant cells have been identified at one time or another as lipoxygenase sites, the results have been inconclusive and often contradictory (Mack et al., 1987). Most lipoxygenases that have been well characterized are soluble enzymes found in the cytoplasm (Siedow, 1991). Immunchemical labeling and fractionation studies have revealed membrane association of lipoxygenase in plant and animal cells (Vick and Zimmerman, 1987; Yamamoto, 1992). In mammalian cells, one type of lipoxygenase is known to oxidize esterified polyenoic fatty acids that include those that are part of complex substrates such as biomembranes and lipoproteins (Schewe and Kuhn, 1991). Another form of mammalian lipoxygenase is found in the cytosol but is rapidly translocated to a membrane compartment when activated. The lipoxygenase associates with an integral membrane protein, FLAP, which has recently been shown to bind arachidonic acid (Mancini et al., 1993). It is thought that after release from membrane phospholipids by phospholipases, this substrate is transferred by FLAP to the enzyme.

In plant cells, membrane-associated lipoxygenases have recently been reported for senescing carnation petals (Rouet-Mayer et al., 1992), tomato fruit (Todd et al., 1990; Bowsher et al., 1992), and cucumber and soybean cotyledons (Feu"{e}ner and Kindl, 1992). The cucumber and soybean proteins were found to be localized in the lipid body membranes, where previously soybean lipoxygenase had not been found. It is not known whether plant lipoxygenases can directly attack membrane fatty acids (Hildebrand, 1989), but most plant lipoxygenases, including a membrane-associated tomato (Lycopersicon esculentum Mill.) fruit lipoxygenase (Todd et al., 1990), prefer free fatty acid substrate. It is proposed that after the release of free fatty acids from the membrane by lipases, lipoxygenases catalyze lipid peroxidation and loss of membrane phospholipid and fatty acid (Thompson, 1988). During the lipoxygenase reaction, free radicals are formed (Lynch and Thompson, 1984; Paliyath and Droillard, 1992). These derivatives and the lipid hydroperoxides can exert deleterious effects on membranes and proteins and may contribute to the loss of membrane function accompanying fruit ripening and senescence (Todd et al., 1990).

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Abbreviations: FLAP, 5-lipoxygenase-activating protein; RACE, rapid amplification of cDNA ends.
interesting that this lipoxygenase is found in lipid bodies that contain fatty acid in the form of triglycerides and are known to lack lipase activity (Feuβner and Kindl, 1992).

To investigate the role of lipoxygenase in ripening, senescence, and membrane turnover, we have purified a membrane-associated lipoxygenase from tomato fruit (Bowsher et al., 1992). In this paper we report the partial amino acid sequencing of this protein and the isolation of the corresponding gene. We also discuss another isolated tomato fruit lipoxygenase gene and the characterization and expression of both.

MATERIALS AND METHODS

Plant Material

Tomato (Lycopersicon esculentum Mill.) plants were grown under standard greenhouse conditions. Pericarp tissue from breaker-stage fruit (cv Caruso) was used for protein purification and isolation of RNA for construction of the cDNA library and PCR amplification. RNA for blot analysis of four stages of fruit ripening was isolated from the pericarp of cv Rutgers. Root, leaf, and seed RNA was isolated from cv Jumbo. Leaf DNA for genomic blot analysis and library construction was isolated from cvs Ailsa Craig and Caruso, respectively. For RNA extraction, seeds were surface sterilized after harvest or frozen in liquid nitrogen and stored at -80°C.

Amino Acid Sequencing

Membrane-associated lipoxygenase was purified as described by Bowsher et al. (1992). Protein digestion with endoproteinase Lys-C (Wako Chemicals, Dallas, TX) was performed in 0.1 M Tris-HCl, pH 8.5, containing 2 M guanidine- HCl for 18 h at room temperature with an enzyme:substrate ratio of 1:50. The protein was incubated at 50°C for 30 min in 6 M guanidine-HCl before the addition of protease. The Lys-C peptides were isolated on an Aquapore OD300 (1 x 250 mm; Brownlee) column employing a linear gradient of 0 to 48% acetonitrile in 0.1% TFA over 90 min with a flow rate of 100 μL/min. Automated Edman sequencing was performed using the ABI 477A liquid-pulse sequencer, and phenylthiohydantoin amino acids were identified on an ABI 120A (Applied Biosystems, Inc., Santa Clara, CA) phenylthiohydantoin analyzer. The amino acid sequences were compared to known plant lipoxygenase sequences (GenBank) and this information was used to construct two degenerate oligonucleotides.

PCR Amplification of cDNA Probe

Following the mixed oligonucleotide primed amplification of cDNA procedure of Lee and Caskey (1990), total RNA (20 μg) from breaker fruit and the antisense oligonucleotide primer were used to synthesize first-strand cDNA. This was then used in a PCR with both degenerate primers following the recommended conditions except for an annealing temperature of 37°C. The PCR product was cloned into pBluescript (Stratagene, La Jolla, CA).

Construction and Screening of cDNA Library

Poly(A)+ RNA was purified from breaker-stage fruit using an odligo(dT) column (Sambrook et al., 1989). Five micrograms was used to construct a cDNA library in λgt11 using the λ Librarian System (Invitrogen, San Diego, CA) and Gigapack (Stratagene). Approximately 56,000 phage were screened using the plaque lift methods of Sambrook et al. (1989) and the 830-bp lipoxygenase PCR fragment as a probe, which was gel purified and labeled by random priming (Feinberg and Vogelstein, 1983). Filters were washed with increasing stringency to 0.2X SSC, 0.1% SDS at 65°C. DNA was isolated from three positive clones using either Lambdaorb (Promega, Madison, WI) or PCR amplification with primers specific to the λ vector. The inserts were released by digestion with NotI and cloned into pBluescript.

Construction and Screening of Genomic Library

Genomic DNA was extracted from tomato leaves as described by Goring et al. (1992) and used to make a genomic library in λ FixII using the Stratagene λ FixII and Gigapack II Gold kits. More than 300,000 phage were transferred to nylon membranes according to Sambrook et al. (1989). Membranes were prewashed in 5X SSC, 0.5% SDS, and 1 mM EDTA (pH 8.0) for 30 min at 42°C and screened with random-primed labeled probes consisting of either a 1.4-kb fragment from the 3’ end of tomatloxA cDNA or a 0.8-kb fragment from tomatloxB cDNA. Prehybridization of the membranes was carried out at 42°C in 5X SSPE, 10X Denhardt’s solution, and 0.5% SDS for 3 h. Hybridization was overnight at 42°C in 50% formamide, 5X SSPE, and 0.5% SDS. Filters were washed twice for 15 min in 2X SSC, 0.1% SDS at room temperature and twice for 30 min in 0.1X SSC, 0.1% SDS at 60°C before autoradiography. Positive plaques were picked and purified and DNA was extracted using the plate lysate method of Sambrook et al. (1989). Clones were analyzed by restriction mapping and several restriction fragments or whole inserts were subcloned into pBluescript and sequenced.

PCR Amplification of 5’ cDNAs

The 5’ end of tomatloxA was isolated using the RACE procedure (Frohman, 1990) with modifications and adapter primers as outlined by Goring et al. (1992). First-strand cDNA was synthesized and polyadenylated according to Harvey and Darlison (1991) using 20 μg of total RNA from breaker-stage fruit. Of the 100 μL of tailed cDNA, 1 μL was amplified in a 100-μL reaction using 50 nm each of the dT17 adapter primer and a gene-specific primer for 4 cycles of 94°C for 1 min, 34°C for 30 s, and 72°C for 30 s. After 4 cycles the adapter primer and a gene-specific primer 5’ to the first one were added to 250 nm at 72°C. The amplification was continued for 45 cycles with the annealing temperature increased to 62°C. One-half of the product was electrophoresed on a 1.7% low-melting-point agarose gel and five plugs were removed from the resulting smears with Pasteur pipettes (Zintz and Beebe, 1991). The plugs were heated to 72°C for 10 min and subjected to 40 cycles of PCR using 300 nm each of the adapter primer and the second specific primer and an annealing temperature of 62°C. The resulting single
band was purified and cloned into pBluescript. The 5' end of *tomloxB* was amplified using an antisense primer specific to the partial cDNA clone (and downstream of several introns) and a 5' sense primer deduced from the corresponding genomic clone. The cDNA (1 μL) prepared for 5' RACE was used in a 100-μL reaction containing 200 nM of each primer, 200 μM each dNTP, and 2.5 units of Taq polymerase. The PCR conditions were 94°C for 1 min, 54°C for 30 s, and 72°C for 1 min for a total of 30 cycles. The product was cloned into pBluescript.

**DNA Extraction and Blot Analysis**

Genomic DNA was extracted from tomato leaves as for the library. Approximately 10 μg of DNA was cleaved with EcoRI and HindIII (BRL). After fractionation on a 0.7% agarose gel, DNA was transferred to Zetabind membrane (Cuno Inc., Meriden, CT) in 20X SSC. The dry membrane was prewashed in 0.1X SSC, 0.5% SDS at 60°C for 30 min. Prehybridization of the membrane was as for genomic plaque lifts. Hybridization was overnight at 42°C in 50% formamide, 5X SSPE, 10% dextran sulfate, 0.5% SDS, and 50 μg L⁻¹ salmon sperm DNA. The first probe used was a 1.5-kb gel-purified fragment from the 3' end of *tomloxB* cDNA. After hybridization, the membrane was washed twice for 30 min in 2X SSC, 0.1% SDS at room temperature and twice for 30 min in 2X SSC, 0.1% SDS at 60°C after autoradiography. Higher-stringency washes were also performed for 30 min in 0.1X SSC, 0.1% SDS at 60°C and at 65°C, with each wash being followed by autoradiography of the membrane. The membrane was stripped of probe according to the Zetabind manufacturer and prehybridized and hybridized as above using as probe a 1.4-kb fragment from the 3' end of *tomloxA* cDNA. Washes were performed as described.

**RNA Extraction and Blot Analysis**

RNA for blot analysis of fruit stages was extracted from pericarp tissue as outlined by Rastogi et al. (1993). The method of Chang et al. (1993) was used to extract RNA from other tissues. RNA (20 μg) was fractionated through a 1.2% formaldehyde gel (Sambrook et al., 1989) and transferred to Zetabind membrane. The membranes were baked at 80°C for 1 to 2 h and prewashed in 0.1X SSC, 0.5% SDS at 60°C for 1 h. Prehybridization was in 5X SSPE, 10X Denhardt's solution, 0.5% SDS at 42°C for 3 h, and hybridization was in 5X SSPE, 0.5% SDS, 50% formamide, 10% dextran sulfate, and 50 μg L⁻¹ salmon sperm DNA at 42°C overnight. Membranes were probed with full-length cDNAs of *tomloxA* and *tomloxB* and washed under high-stringency conditions of 0.1X SSC, 0.1% SDS at 65°C. Equal loading of RNA samples was determined by probing with p318, which contains rRNA genes from wheat (S. Rothstein, unpublished data), and washing in 0.2X SSC, 0.1% SDS at 55°C. The blots were stripped of probe between hybridizations.

**DNA Sequencing and Analysis**

Nucleotide sequences were determined by dideoxy sequencing using the Sequenase enzyme (United States Biochemical, Cleveland, OH). To avoid errors that may have occurred during PCR amplification of the 5' cDNA clones, either three clones from separate PCR reactions were sequenced or the sequence was compared to the genomic DNA, where it was available. The cDNA clones were sequenced using restriction fragments and specific primers. The genomic clones were deleted using exonuclease III and mung bean nuclease using the procedure recommended in the Stratagene kit. The softwares DNASIS and PROSIS (Hitachi Software Engineering America Ltd., Brisbane, CA) were used for nucleic acid and protein sequence analysis, respectively.

**RESULTS**

**Protein Sequencing and Isolation of cDNA Probe**

A membrane-associated lipoxygenase was isolated from breaker-stage tomato fruit. This enzyme was kinetically distinguishable from the soluble form and Triton X-100 (2%, v/v) was required to solubilize its activity after sedimentation with the microsomal membranes (Bowsher et al., 1992). This enzyme was subsequently digested with endoproteinase and the resulting fragments were purified and sequenced. Amino acid sequences of the four resulting peptides (underlined in Fig. 1a) were compared to the plant lipoxygenase sequences (pea and soybean) available from GenBank and to a partial tobacco sequence (Bookjans et al., 1988). Three of the peptides showed a high level of similarity to the legume (68-89% identity) and tobacco (78-100% identity) sequences. The fourth peptide (amino acids 245-260, Fig. 1a) showed 94% identity with the tobacco sequence but no similarity to pea or soybean lipoxygenases. N-terminal sequencing of the tomato lipoxygenase was not possible due to blockage of the N-terminal amino acid.

Two regions of low degeneracy were chosen to design two 17-bp degenerate oligonucleotides (shaded in Fig. 1a). These were used as primers to amplify cDNA synthesized from RNA of breaker tomato fruit. A single band of around 830 bp was produced and, based on the known plant sequences, a band of approximately 800 bp was expected. This product was cloned and partially sequenced. The DNA sequence obtained showed 57 to 63% identity with the legume lipoxygenase sequences and the translation of the ends of the clone predicted amino acid sequences with 100% identity to the isolated peptides. This result indicated that it was a partial cDNA for tomato lipoxygenase.

**Isolation of Lipoxygenase cDNAs**

A breaker-stage tomato pericarp cDNA library was constructed in λgt11 and screened with the 830-bp PCR product. Of the three clones isolated, two were found to be identical after partial sequencing. The larger of the two (1.8 kb) and the third clone (2 kb) were fully sequenced. Both of these clones were found to be similar to other plant lipoxygenase genes, with overall nucleic acid sequence identity values ranging from approximately 60 to 70%. The 1.8-kb clone was found to code for amino acids that corresponded exactly with sequence from the purified membrane-associated protein. This clone was designated *tomloxA* and the second clone was named *tomloxB*.
Figure A1. (Legend on facing page.)
Figure 1. Nucleotide sequence and deduced amino acid sequence of tomato lipoxygenase during tomato ripening.
SDS-PAGE of the purified membrane-associated protein indicated an apparent molecular mass of 97 kD (Bowsher et al., 1992). Together with preliminary RNA blot analysis, this suggested that the lipoxygenase transcripts are around 3 kb. The library was rescreened with a 5′ fragment of the tomlolxA clone, but a larger cDNA was not detected.

To obtain the 5′ ends of tomlolxA and tomlolxB, genomic clones (see below) were used. The genomic clone corresponding to tomlolxB contained the 5′ end of the gene, whereas the genomic clone of tomlolxA was found to be missing approximately 250 bp of coding region. Because the RACE procedure (Frohman, 1990) was unsuccessful using primers specific to the cDNA clones, antisense primers closer to the 5′ end of the mRNA were designed based on exon sequence within the tomlolxA genomic clone. First-strand fruit cDNA was polyadenylated and amplified with these primers and the RACE adapter and poly(T)-containing primers to produce a 330-bp PCR fragment. A sense primer specific to the 5′ end of this fragment was then used with a tomlolxA antisense primer to amplify from the fruit cDNA a fragment that overlapped with tomlolxA. These fragments were cloned together to produce a full-length tomlolxA cDNA (Fig. 1a).

The start codon for tomlolxB was deduced from the corresponding genomic clone. There is an in-frame stop codon upstream of this ATG, and all other prospective ATG codons were followed by in-frame stop codons. Also, the deduced amino acid sequence within this region is similar to that of the cDNA clones, antisense primers closer to the 5′ end of the mRNA were designed based on exon sequence within the tomlolxA genomic clone. First-strand fruit cDNA was polyadenylated and amplified with these primers and the RACE adapter and poly(T)-containing primers to produce a 330-bp PCR fragment. A sense primer specific to the 5′ end of this fragment was then used with a tomlolxA antisense primer to amplify from the fruit cDNA a fragment that overlapped with tomlolxA. These fragments were cloned together to produce a full-length tomlolxA cDNA (Fig. 1a).

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Analysis of Genomic Clones

A λ FixII tomato genomic library was screened with fragments of the two cDNAs. Six positive clones were isolated and restriction fragments were identified in the genomic clones that corresponded to those identified in the genomic blots. One of these clones was partially sequenced and found to contain the complete sequence of the gene corresponding to tomlolxB as well as the 3′ end of a closely related gene. Preliminary results from the analysis of a second genomic clone indicated the presence of portions of these same two genes. The inserts of three other genomic clones were similarly analyzed to obtain the complete sequence of the gene corresponding to tomlolxA.

Sequence Analysis of Lipoxygenase cDNAs

The nucleotide and derived amino acid sequences of the two tomato lipoxygenase cDNAs are presented in Figure 1. The 2860-nucleotide sequence of tomlolxA (Fig. 1a) consists of a 58-bp 5′ untranslated region, a 2580-bp open reading frame, a stop codon, and a 219-bp 3′ untranslated region including an 18-bp poly(A) tail. The derived amino acid sequence of 860 amino acids has a predicted molecular mass of 96.8 kD. The amino acid sequences of the four purified peptides were found within this sequence (underlined in Fig. 1a). In Figure 1b, the 2759-nucleotide sequence of tomlolxB is shown. The open reading frame of 2577 bp predicts an 859-amino acid protein with a molecular mass of 97.1 kD. The 3′ untranslated region is 162 bp including a 15-bp poly(A) tail. The amino acid sequence of tomlolxB was found to differ at several residues from the purified peptides. Although the purified lipoxygenase was associated with thylakoid membranes, there was no evidence of transit peptide sequence in either cDNA.

At the DNA level the two tomato lipoxygenase genes show 74% homology, and at the amino acid level they show 72% identity. The sequences were compared to nine plant lipoxygenase sequences available from GenBank (refs. in Fig. 2 legend) and a partial tobacco amino acid sequence (Bokojans et al., 1988). Nucleic acid sequence homology values ranged from 54 to 65%. At the amino acid level, identity values for tomlolxA ranged from 56% with rice to 81% with tobacco (values on right in Fig. 2b). Values were similar but slightly lower (by 1–3%) for tomlolxB and the other plant sequences except for the tobacco sequence, which shared 72% identity. There was little difference with either tomato sequence compared to the various lipoxygenase forms of soybean or pea; therefore, a representative sequence was used for each in Figure 2b.

The sequences of N termini of lipoxygenases differ markedly (Siedow, 1991). Soybean lipoxygenase-1 has about 30 fewer residues than other plant lipoxygenases in this region. The comparison of the first 100 residues in Figure 2b demonstrates this dissimilarity in contrast to the remaining regions. However, following the N-terminal 7 to 20 residues, there is a 17-amino acid region within the N termini that is highly conserved, with most substitutions being conservative (Fig. 2a).

There are three important regions of sequence similarity in lipoxygenases. The C-terminal 8 amino acids are highly conserved (GIPNISI for all the legumes and rice and GIPNVS1 for Arabidopsis and both tomato lipoxygenases). Tobacco is an exception to this (Bokojans et al., 1988). All of the plant and mammalian lipoxygenase sequences share a highly conserved region of 38 amino acids (region III in Fig. 2b). This region spans amino acids 516 to 553 in tomlolxA and tomlolxB. There are 13 completely conserved residues in this region (Steczko et al., 1992) that are also found in both tomato sequences. The five conserved His’s were suggested as possible iron ligands. There is a sixth conserved His about 160 residues downstream in all sequences (712 in tomlolxA, 711 in tomlolxB). This His is situated in a region of about 13 residues that are highly conserved in plant lipoxygenases and mammalian 5-lipoxygenases (Siedow, 1991), and it has also been suggested as a putative iron binding site. Site-specific mutations of the six His’s in soybean lipoxygenase-1 showed that H499, H504, and H690 (H521, H526, H712 in tomlolxA) were necessary for enzyme activity and iron binding (Steczko et al., 1992; Steczko and Axelrod, 1992). Recent crystallographic determinations have revealed the three-dimensional structure of lipoxygenase (soybean lipoxygenase-1) and suggest the importance of many of the conserved residues in iron binding and enzyme activity (Boyington et al., 1993a, 1993b; Minor et al., 1993). Minor et al. (1993) identified five ligands: H499, H504, H690, the C-terminal carboxylate group of 1839, and N694. The latter residue is conserved in all of
Lipoxygenase Expression during Tomato Ripening

Figure 2. Lipoxygenase amino acid sequence comparisons. a, Comparison of sequences of a 17-amino acid region from N termini of plant lipoxygenases. LOX1, Soybean lipoxygenase-1 (Shibata et al., 1987); LOX2, soybean lipoxygenase-2 (Shibata et al., 1988); LOX3, soybean lipoxygenase-3 (Yenofsky et al., 1988); SC5, soybean cotyledon lipoxygenase (Shibata et al., 1991); PEA2, pea seed lipoxygenase-2 (Ealing and Casey, 1989); PEA3, pea seed lipoxygenase-3 (Ealing and Casey, 1988); BEAN, French bean lipoxygenase (A.J. Slusarenko, unpublished data); RICE, rice seed lipoxygenase-1 (Ohta et al., 1992); ARAB, Arabidopsis lipoxygenase (Melan et al., 1993); TOMA, TOMB, this paper. Boldface letters refer to invariant amino acids; numbers indicate distance from N-terminal M. b, Comparison of deduced amino acid sequence identity of lipoxygenase genes with *tomloxA*. Region I, first 100 residues; region II, from amino acid 100 to C terminal; region III, the 38-residue conserved region (Steczko et al., 1992)—amino acids 516-553 in *tomloxA*. Numbers on the right represent overall identity.

Figure 3. Genomic blot analysis of *tomloxA* and *tomloxB*. Genomic DNA (10 μg) was digested with either EcoRI or HindIII. The same blot was hybridized with a 1.4-kb fragment of *tomloxA* cDNA (A) or a 1.5-kb fragment of *tomloxB* cDNA (B). Washes were in 0.1X SSC at 60°C. Size markers (kb) are indicated on the right.

The plant sequences including *tomloxA* (N716) but is substituted for by His in *tomloxB* (H715) and some of the mammalian lipoxygenases. This is assumed to be a functional substitute (Minor et al., 1993). These residues have been marked in Figure 1. Boyington et al. (1993a, 1993b) determined that the iron was coordinated to four ligands (the three His's and the C-terminal Ile) and that there were two adjacent, unoccupied positions, with N694 being very close to an unoccupied coordination position.

Genomic Blot Analysis

To evaluate the number of lipoxygenase genes in the tomato genome, Southern hybridization of tomato genomic DNA was performed with the 3' ends of *tomloxA* and *tomloxB* cDNAs. At low stringency (2X SSC, 60°C), both probes hybridized with various intensities to a common set of 8 and 13 fragments for the EcoRI and HindIII digests, respectively, with sizes ranging from 0.8 to 12 kb (data not shown). At higher stringencies (0.1X SSC, 60 and 65°C), the probe from *tomloxA* hybridized most strongly to single EcoRI and HindIII fragments and hybridized moderately to two other HindIII fragments (Fig. 3). A different set of bands was observed with the probe from *tomloxB*. When washed in 0.1X SSC at 60°C, it hybridized most strongly to two EcoRI fragments and moderately to a third fragment. It also hybridized strongly to two HindIII fragments and moderately to three other fragments (Fig. 3). When the stringency was increased further (0.1X SSC, 65°C), *tomloxA* hybridized most strongly.
to a single band and moderately to a second band for the EcoRI digest and hybridized moderately to two bands for the HindIII digest (data not shown).

These results suggest that tomato lipoxygenase is encoded by a family of at least three genes. Two of these genes can be associated with the tomloxA and tomloxB cDNAs characterized in this paper. In addition, a putative third gene highly related to the tomloxB cDNA was detected when washing was at a moderate stringency (Fig. 3). Extra bands observed at lower stringencies also suggest the presence of similar sequences that could putatively encode a fourth, less closely related gene. The genomic clones confirm the presence of at least three genes in the genome.

**Expression of Lipoygenase Genes**

To determine the expression patterns of the two tomato lipoygenase genes during fruit ripening, total RNA was isolated from pericarp at four ripening stages (immature green, mature green, breaker, and ripe) and subjected to RNA blot analysis. This was repeated for other tissue (seed, germinated seed, leaf, and root) to determine whether the genes were fruit specific. As seen in Figure 4a, during fruit ripening tomloxA mRNA first appeared in mature green fruit, increased to a maximum at breaker stage, and was not evident in ripe fruit. The pattern was different for the tomloxB message, which was first seen in breaker fruit but reached its highest level in ripe fruit. In the course of several experiments the patterns of expression did not vary. However, considering differences in probe-specific activities and exposure times, the levels of tomloxA and tomloxB are probably similar at their respective peaks. In other tissues transcript for tomloxB was undetectable (Fig. 4b). tomloxA message was faintly expressed in seeds that had imbied, it was more strongly expressed in germinating seeds, and it was undetectable in leaf or root. Neither transcript was seen in pistils, anthers, or senescing leaves (data not shown). In each case the transcript observed was approximately 3 kb.

**DISCUSSION**

We have isolated and sequenced two full-length lipoygenase cDNA clones from tomato. As evidenced by amino acid sequence data, one of these (tomloxA) encodes a lipoygenase that was purified from breaker-stage fruit. This enzyme had been found through several lines of evidence to be associated with thylakoid membranes (Bowsher et al., 1992). The deduced amino acid sequences for the two genes are very similar (72% identity) to each other and to other plant lipoygenases. Although the lipoygenases are most divergent at the N termini, tomloxA displays enough similarity to the others to indicate the lack of a chloroplast transit peptide. Analysis of the genomic clones confirmed the translation start codon for tomloxA, thus excluding the possibility of peptide sequence beginning farther upstream.

Nuclear-encoded chloroplast proteins are thought to require a cleavable, N-terminal transit peptide for transport into the chloroplast (Berry-Lowe and Schmidt, 1991; De Boer and Weisbeek, 1991). At least three exceptions to this have been published. A heat-shock protein from *Chlamydomonas* (Grinn et al., 1989), a spinach chloroplast membrane protein (Salomon et al., 1990), and most recently, a stromal betaine aldehyde dehydrogenase from spinach and sugar beet (Rathinasabapathi et al., 1994) all lack typical transit peptides. There is a possibility that the tomloxA protein is targeted to the chloroplast using information in the mature protein.

During ripening of tomato fruit, chloroplasts develop into chromoplasts and at an early stage in this process a massive reordering of plastid structure occurs. This is characterized by the disappearance of Chl pigments, the disintegration of the intricate thylakoid membrane system, and the accumulation of the carotenoid lycopene in less elaborate membranous structures and within the chromoplast envelope (Cheung et al., 1993; Lawrence et al., 1993). Thylakoid membranes contain a high amount of polyunsaturated fatty acids. In some species up to 95% of the total fatty acids in these membranes is linolenic acid (Douce and Joyard, 1991). Although the cues for the chloroplast-chromoplast transition are unknown, the nuclear genome is known to be involved and there is recent evidence that there is synthesis and import of new proteins during the transition (Lawrence et al., 1993). Perhaps lipoygenase is involved in this transition.

There also exists the possibility that tomloxA lipoygenase is not associated with thylakoid membranes in vivo, given nuclear-targeted experiments, although...
thylakoids isolated from intact chloroplasts were used in the immunological identification of the enzyme (Bowsher et al., 1992). Lipoxygenases could be associated with membranes (other than thylakoids) in other ways, including through the action of another protein, as was outlined for the mammalian 5-lipoxygenase and FLAP (Mancini et al., 1993). The recent crystallographic studies have shown the structure of soybean lipoxygenase-1 to contain two domains (Boyington et al., 1993). The first domain comprises the 146 N-terminal residues, which form a barrel that has an interior of densely packed hydrophobic side chains. It is interesting to note that this domain is identical in connectivity to a similar barrel found in human pancreatic lipase, which is involved in colipase binding. Domain I is separate from the rest of the molecule, making only loose contact, and is not present in mammalian lipoxygenases. This region could possibly be involved in lipoxygenase binding to a membrane or to a membrane protein.

The two tomato lipoxygenase genes display different expression patterns during development and fruit ripening. *tomloxA* is expressed in germinating seeds (found to be a site of high lipoxygenase activity in other studies [Siedow, 1991]). In fruit, *tomloxA* is expressed most strongly in the breaker stage, whereas *tomloxB* mRNA is at its highest level in ripe fruit. *tomloxB* appears to be fruit specific, whereas *tomloxA* may have different functions in different tissues. The significance of these expression patterns is unknown. As mentioned, ripening and senescence involve the loss of membrane integrity, and it has been proposed that lipoxygenase is involved in this process. Perhaps the product of *tomloxA* contributes to changes in membrane structure that allow changes in metabolite distribution during ripening and germination. The lipoxygenase isoform of *tomloxB* may be more specific to the degradative process of fruit senescence. Another possible mechanism for the involvement of lipoxygenase in tomato ripening is its role in the biosynthesis of jasmonic acid. This growth regulator has been shown to play a role in senescence with exogenous applications.

Lipoxygenase is also thought to be important in the formation of flavors and odors (Mack et al., 1987; Hildebrand, 1989). The fatty acid hydroperoxides that are the primary products of the lipoxygenase reaction and that can be damaging to membranes can be converted to less damaging products. The characteristic odor of tomato is in part due to the volatile aldehyde hexenal, which results from the cleavage of the 13-linoleic acid hydroperoxide (Mack et al., 1987). *tomloxB* may be more specifically involved in this reaction.

Our findings further implicate lipoxygenase in the process of ripening/senescence. Further study is necessary to determine the nature of the membrane association of the lipoxygenase encoded by *tomloxA* and to definitively demonstrate its subcellular localization. This information would help elucidate the specific role of lipoxygenase in ripening and in various other physiological functions in which it has been implicated.

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cation into the organelle without a processing step. Eur J Biochem 182: 539–546