A Gene Encoding a Chloroplast-Targeted Lipoxygenase in Tomato Leaves Is Transiently Induced by Wounding, Systemin, and Methyl Jasmonate

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We investigated the relationship between the expression of lipoxygenase (LOX) genes and the systemin-dependent wound response in tomato (Lycopersicon esculentum) leaves. A polymerase chain reaction-based approach was used to isolate two tomato Lox cDNAs, called TomLoxC and TomLoxD. Both TomLOXC and TomLOXD amino acid sequences possess an N-terminal extension of about 60 residues that were shown by in vitro uptake to function as transit peptides, targeting these proteins into the chloroplast. Within 30 to 50 min following wounding or systemin or methyl jasmonate treatments, the TomLoxD mRNA level increased and reached a maximum between 1 and 2 h. TomLoxC mRNA was not detectable in leaves and was not found following wounding, but it was found in ripening fruits, indicating that the two tomato Lox genes are regulated in different tissues by different processes. The results suggest that the TomLoxD gene is up-regulated in leaves in response to wounding and encodes a chloroplast LOX that may play a role as a component of the octadecanoid defense-signaling pathway.

Damage to leaves of tomato (Lycopersicon esculentum) plants by chewing insects or other mechanical means results in the rapid transcriptional activation of defense genes, both in the wounded leaf and in distant, unwounded leaves (Graham et al., 1986; Hildmann et al., 1992; Schaller et al., 1995). A phloem mobile polypeptide called systemin behaves as a systemic signal released from wounded sites (Pearce et al., 1991; McGurl et al., 1992, 1994; Narvaez-Vasquez et al., 1995), but several other chemicals, including IAA (Thornburg and Li, 1990), ABA (Peña-Cortés et al., 1989), and ethylene (O’Donnell et al., 1996), have been associated with the signaling pathway and with physical forces such as hydraulic effects (Malone and Alarcon, 1995) and action potentials (Herde et al., 1996; Rhodes et al., 1996; Stankovic and Davies, 1996).

The intracellular signaling cascade that is activated in response to wounding and systemin has been shown to involve a lipid-derived pathway leading to the synthesis of PDA and JA (Farmer and Ryan, 1992; Blechert et al., 1995), two powerful activators of defense gene transcription. PDA and JA are derived from linolenic acid, an abundant fatty acid in plant membranes, by cyclization of a LOX-generated hydroperoxide to produce 12-oxo-PDA, with subsequent β-oxidations of PDA (Vick and Zimmerman, 1983) to produce JA.

Several lines of evidence support a role of the octadecanoid pathway in the signaling of the wound response. Application to tomato leaf surfaces of linolenic acid, as well as the biosynthetic intermediates between linolenic acid and JA, results in the induction of defense gene expression (Farmer and Ryan, 1992). Mechanical wounding or supplying systemin to young tomato plants through their cut stems results in a rapid and transient accumulation of linolenic acid (Conconi et al., 1996) and JA (Doares et al., 1995). Inhibitors of the octadecanoid pathway block the induction of defense genes by systemin and linolenic acid (Farmer et al., 1994; Doares et al., 1995). A tomato mutant impaired in the octadecanoid pathway, called def1 (Howe et al., 1996), only weakly expresses defense genes following wounding or supplying excised plants with systemin, linolenic acid, or carbohydrate elicitors.

LOX (EC 1.13.11.12), a class of ubiquitous enzymes in plants and a key enzyme of the octadecanoid pathway, has been studied for its role in plant development and in response to wounding and pathogen attacks (for review, see Siedow [1991]), but only recently could a physiological function be assigned to a specific LOX isoform. In transgenic Arabidopsis thaliana plants having reduced levels of synthesis of the chloroplast AtLOX2, the wound-induced accumulation of JA was suppressed, and the induction of the AtVsp gene by wounding was reduced (Bell et al., 1995). As the research reported herein was being completed, three Lox cDNAs were characterized from potato tubers and leaves that were organ-specific; Lox1 was expressed in tubers and roots, Lox2 was expressed in leaves, and Lox3 was expressed in leaves and roots (Royo et al., 1996). Both LOX2- and LOX3-predicted proteins exhibited a putative chloroplast leader sequence, and their mRNAs accumulated in leaves in response to wounding. In tomato, a*

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Abbreviations: JA, jasmonic acid; LOX, lipoxygenase; MJ, methyl jasmonate; PDA, phytodienoic acid.
membrane-associated LOX was purified and its cDNA was cloned from breaker-stage fruits (Bowscher et al., 1992; Ferrie et al., 1994), but this isoform was not found in leaves. An induction of Lox mRNA and enzyme activity was reported in tomato leaves in response to bacterial infection, but the specific isoforms were not isolated (Koch et al., 1992).

As a first step in evaluating the requirements for the components of the octadecanoid pathway for a functional wound response, we investigated the presence and regulation of Lox isoforms in tomato leaves. A PCR-based approach was used to generate homologous Lox probes, which were used to isolate two tomato Lox cDNAs, TomLoxC and TomLoxD. Although the protein products of both TomLoxC and TomLoxD mRNAs were shown to be transported into the chloroplast, only TomLoxD mRNA accumulated rapidly and transiently in wounded plants, whereas TomLoxC mRNA was not wound-inducible and was found only in ripening fruits. The expression profile of TomLoxD and the properties of the TomLOXD enzyme suggest that it may be a component of the octadecanoid pathway in tomato leaves leading to the activation of defense genes in response to wounding.

MATERIALS AND METHODS

Growth of Plants and Treatments

Tomato (Lycopersicon esculentum cv Castlemart) plants were grown in peat pots and maintained under 17 h of light (30 μEm⁻²s⁻¹) at 28°C and 7 h of dark at 18°C. Tomato (cv Better Boy) plants expressing a transgene consisting of a prosystemin gene were grown in peat pots and maintained under 17 h of light for the duration of the experiment. Each time point indicated periods in sealed Plexiglas boxes (approximately 1-10 L) containing MJ vapors (1.5 μL of MJ placed on a cotton wick). Plants were replaced daily and MJ vapors were refreshed for 24 h by a procedure combining phenol extraction and lithium chloride precipitation as described by Robinson and Barnett (1988), except that Suc was replaced with sorbitol and two-layer (80%/20%) Percoll gradients were used. Radiolabeled TomLOXC and TomLOXD were synthesized with a coupled transcription/translation system using rabbit reticulocyte lysate (TNT, Promega). Protein import experiments were performed at 25°C under illumination for 45 min in 300 μL containing 25 mM Hepes-KOH, pH 8.0, 0.33 M sorbitol, 2 mM EDTA, 8.3 mM Met, 40 μL of translation mixture, and chloroplasts corresponding to 100 μg of chlorophyll. After uptake, one-half of the sample was treated with 30 μg of thermolysin for 30 min on ice. Chloroplasts were washed once and the pellet was resuspended in 25 mM EDTA and boiled in SDS-PAGE buffer. Proteins were analyzed by SDS-PAGE (8% gel) and fluorography.

RNA-Blot Analysis

Total RNA was extracted from tomato leaves, flower parts, and fruit pericarp and analyzed as described by Heitz et al. (1993). Blots were hybridized with the follow-
ing probes: a 2.1-kb EcoRI-HindIII fragment of the TomLoxC clone, a 1.9-kb XbaI-XbaI fragment of the TomLoxD clone, a 0.4-kb EcoRI-HindIII fragment of proteinase Inhibitor 1 cDNA (Graham et al., 1985), and a 1.8-kb EcoRI fragment of a ubiquitin cDNA (a gift from Dr. A. Conconi, Washington State University, Pullman).

RESULTS

Isolation of TomLoxC and TomLoxD cDNAs

Total RNA extracted from the leaves of young tomato plants that had been incubated in the presence of MJ vapors for 8 h was transcribed with reverse transcriptase. These templates were used for PCR amplification with an oligo(dT) primer and a primer derived from the consensus amino acid sequence HAAVNFGQY, which is present in the C-terminal part of nearly all known plant LOX sequences (Peng et al., 1994). A product of the expected size (0.6 kb) was obtained and cloned. These fell into three distinct groups of sequences, which were called LOX 6, LOX 18, and LOX 19. The LOX 19 amino acid sequence exhibited 81% identity to the fruit-specific TomLOXA (Ferrie et al., 1994) and was not studied further. On the basis of sequence similarities with plant LOX sequences (see below), the Lox 6 and Lox 18 clones were chosen as probes to screen a cDNA library constructed from plants overexpressing a prosystemin transgene and overexpressing several defense genes (McGurl et al., 1994; Schaller et al., 1995). We isolated full-length clones of 2807 and 3034 bp corresponding to Lox 6 and Lox 18, respectively, and these clones were called TomLoxC and TomLoxD, respectively. These cDNAs do not resemble two other Lox cDNAs, TomLoxA and TomLoxB, that are already known in tomato (Ferrie et al., 1994).

Sequence Analysis

TomLoxC cDNA has a single in-frame ATG at its 5′ end and encodes a protein of 896 amino acids with a 101.7-kD predicted mass. TomLoxD cDNA possesses four ATG codons at its 5′ end, in frame with the longest open reading frame. Based on the observation that in most plant genes the 5′ proximal ATG is used as the initiation codon (Joshi, 1987; Kozak, 1989) and that all except the third ATG codons in the 5′ end of the TomLoxD cDNA are in a good nucleotide context for initiation of translation (with A in the −3 and G in the +4 positions [Lütcke et al., 1987; Kozak, 1989]), we assume that the first ATG codon serves as the initiation codon in this gene. In this case, the predicted TomLoXD protein has 908 amino acids and a mass of 102.3 kD. The identity between the TomLOXC and TomLOXD proteins is only 46%. Database searches first showed that the TomLoxC gene product presents the highest identity to two members of chloroplast-localized plant LOXs that are thought to be components of the octadecanoid pathway, i.e. AtLOX2 from Arabidopsis thaliana (Bell and Mullet, 1993) and RLL from rice (Oryza sativa) (Peng et al., 1994). The TomLOXD sequence showed 47% identity at most to other known LOX proteins. More recently, additional Lox genes were cloned, and TomLoxC and TomLoxD were found to be highly similar to Lox2 and Lox3, respectively (Royo et al., 1996), two potato Lox genes that appear to be the homologs of the tomato genes described here. The overall identity of TomLOXC and TomLOXD with the previously cloned TomLOXA and TomLOXB (Ferrie et al., 1994) is relatively low. A comparison of the percentage of identity/similarity between cDNA-deduced amino acid sequences of several plant LOXs is presented in Table I. Despite their divergence, both TomLOXC and TomLOXD sequences (Fig. 1) contain the conserved amino acids found in plant and mammalian LOXs that are thought to be important for enzyme activity (Siedow, 1991; Yamamoto, 1992; Peng et al., 1994).

Table 1. Percentage identity/similarity between cDNA-deduced amino acid sequences of plant LOXs

<table>
<thead>
<tr>
<th>LOX</th>
<th>TomLoxA</th>
<th>TomLoxC</th>
<th>TomLoxD</th>
<th>PotLOX2</th>
<th>PotLOX3</th>
<th>AtLOX2</th>
<th>RLL</th>
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<tbody>
<tr>
<td>TomLOXC</td>
<td>42/62</td>
<td></td>
<td></td>
<td>46/65</td>
<td>92/95</td>
<td>47/66</td>
<td>47/66</td>
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<tr>
<td>TomLOXD</td>
<td>42/63</td>
<td>47/66</td>
<td>92/95</td>
<td>96/99</td>
<td>57/73</td>
<td>57/73</td>
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<td>47/66</td>
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<td>47/66</td>
<td>46/63</td>
<td>55/71</td>
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isolated pea chloroplasts with 35S-labeled TomLOXC and TomLOXD translation products. One-half of the chloroplasts were treated with thermolysin to degrade unimported proteins associated with the organelle envelope but not imported proteins. As shown in Figure 2, TomLOXC is synthesized as a 108-kD precursor that is processed to a 104-kD mature protein in the presence of chloroplasts in the absence of thermolysin. In the presence of thermolysin, a species of the protein that is smaller than the precursor survives proteolysis, which indicates that a form of LOXC has been imported into the chloroplasts and protected from the enzyme. TomLOXD appears as a 107-kD precursor yielding a 103-kD mature protein, and a species near 103 kD is also protected from protease action after incubation with chloroplasts. The appearance of processed forms was inhibited when the experiments were performed in the presence of thermolysin,
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LOXC and LOXD

Figure 2. Import of TomLOXC and TomLOXD proteins into isolated pea chloroplasts. Radiolabeled translation products were incubated with isolated chloroplasts as described in "Materials and Methods." One-half of the sample was treated with thermolysin to degrade unimported proteins. Proteins were analyzed by SDS-PAGE (8% gel) and fluorography.

dark (not shown). These results are consistent with the amino acid sequence data that suggest that both TomLoxC and TomLoxD encode chloroplast LOXs.

LOX Gene Expression in Response to Wounding, Systemin, and MJ

We examined the expression pattern of TomLoxC and TomLoxD in response to wounding. Figure 3 shows a time-course analysis of the expression of the genes in lower, wounded leaves and in upper, unwounded leaves of young tomato plants. We found no TomLoxC mRNA in either wounded or unwounded leaves. This was in contrast to AtLox2 from Arabidopsis, the closest relative of TomLoxC, which is constitutively expressed in leaves and is up-regulated after wounding (Bell and Mullet, 1993). The TomLoxD probe, however, hybridized to an mRNA of approximately 3 kb that was just detectable in control leaves. The levels of this mRNA were induced by wounding within 30 min and reached a maximum level 1 to 2 h following wounding and then declined to control levels within 8 h. A similar kinetic profile was observed in unwounded leaves, although the induction did not begin until about 1 h after wounding, and the level of induction was lower than in wounded leaves. The accumulation of proteinase Inhibitor I mRNA was detected about 4 h after wounding, when TomLoxD mRNA levels were already declining.

The wound response in tomato was shown previously to be dependent on the synthesis of prosystemin, the polypeptide precursor of systemin (McGurl et al., 1992). The prosystemin gene was also shown to be activated by wounding, apparently to amplify the signal during continued herbivore attacks. We therefore addressed the question of whether TomLoxC and TomLoxD were also activated by systemin. Young tomato plants were excised and supplied through their cut stem with buffer or systemin for 30 min and then incubated for increasing times. Total RNA was extracted and analyzed for gene expression (Fig. 4). As with wounding, TomLoxC mRNA was undetectable in the leaves, with or without systemin treatment. TomLoxD mRNA slightly increased in plants supplied with buffer, probably due to excision. In plants supplied with systemin, a rapid accumulation of TomLoxD mRNA occurred, peak-

Figure 3. Time-course analysis of Lox and proteinase Inhibitor I (PI-I) genes in leaves of young tomato plants in response to wounding. Leaves were collected from unwounded plants (control, lane C), and from plants at times shown following wounding (hours), when the lower, wounded leaves and the upper, unwounded leaves were collected from six plants. Total RNA was extracted from the leaves and 15 μg was subjected to RNA-blot analysis. The specific mRNAs were hybridized with cDNA probes for TomLoxC, TomLoxD, and Inhibitor I. Ubi, Ubiquitin probe used as an internal control.

Figure 4. Time course of systemin-dependent expression of TomLoxC, TomLoxD, and proteinase Inhibitor I (PI-I) in leaves of young tomato plants. Tomato plants were excised and supplied with buffer alone or systemin in buffer through the cut stem. After transfer to water, the leaves were collected at the times shown (hours). Lane C, Leaves from intact (nonexcised) wild-type plants; and proSys, leaves from intact (nonexcised) plants transformed with a prosystemin gene (McGurl et al., 1994). Total RNA was extracted and a 15-μg sample was subjected to RNA-blot analysis. The specific mRNAs were hybridized with probes as in Figure 3. Ubi, Ubiquitin probe used as an internal control.
ing at about 1 h following systemin treatment. As was observed after wounding, the increase in mRNA was transient, and the signal was undetectable by 4 h, in contrast to *Inhibitor I* mRNA, which began to accumulate at 4 h, similar to wounding, and continued for at least 24 h. Tomato plants overexpressing a prosystemin transgene were shown previously to accumulate proteinase inhibitor proteins constitutively (McGurl et al., 1994). Consistent with *TomLoxD* induction by exogenous systemin, these transgenic plants exhibited a higher constitutive *TomLoxD* mRNA level than wild-type plants (Fig. 4, compare lane C with lane proSys).

* MJ is a strong activator of wound-induced defense genes (Farmer and Ryan, 1990; Farmer et al., 1994) and has also been described as inducing *Lox* gene expression in soybean (Grimes et al., 1992; Sarawitz and Siedow, 1996), Arabidopsis (Bell and Mullet, 1993; Melan et al., 1993), and barley (Feussner et al., 1995). In intact tomato plants exposed to *MJ* vapors, *TomLoxD* mRNA exhibited an early response to *MJ* compared with the *Inhibitor I* mRNA (Fig. 5), as found with wounding and systemin induction (Figs. 3 and 4). However, in contrast to the induction by wounding or by systemin, the levels of *TomLoxD* mRNA remained elevated throughout the experiment. This extended accumulation of mRNA likely reflected the continuous exposure of the plants to *MJ* vapors. *TomLoxC* mRNA was undetectable in the control plants and was only faintly detected at the later stages of the experiment.

Proteinase inhibitor genes have been reported to be activated in different plant organs at specific steps of plant development, such as flowering or fruit ripening in cultivated or wild tomato species (Wingate et al., 1989; Peña-Cortés et al., 1991). We examined the possibility of a correlation between either *TomLoxC* or *TomLoxD* and *Inhibitor I* expression in tomato flower organs or upon fruit ripening. As shown in Figure 6, *TomLoxC* mRNA was detected in only very low levels in the pistil and ovary and not in other dissected flower organs. However, *TomLoxD* mRNA was found in sepals, petals, and female organs, whereas *Inhibitor I* mRNA was found at high levels in petals and the anther cone. Thus, no strict correlation was found among *TomLoxC*, *TomLoxD*, and *Inhibitor I* mRNAs in flowers. In fruits analyzed at three successive stages, mature, green-orange (breaker stage), and red, *TomLoxD* mRNA was detectable at very low levels in green and breaker fruit but not at all in red fruit. *Inhibitor I* mRNA was not detected at any stage. However, *TomLoxC* mRNA was present in breaker stage and red fruit, indicating that *TomLoxC* is a novel fruit-ripening-specific *Lox* gene in tomato. The lack of correlation of *TomLoxD* expression with *Inhibitor I* expression in petals and anthers (Fig. 5) suggests that the regulation of these two genes is coordinated differently in different tissues.

**DISCUSSION**

Several lines of evidence have suggested that defense gene activation by systemin in tomato plants is mediated through the octadecanoid biosynthetic pathway (Farmer and Ryan, 1992; Farmer et al., 1994; Doares et al., 1995; Howe et al., 1996). To further characterize the role of various enzymes of this pathway in tomato, we have investigated the possible role of a specific *Lox* gene in this
RNA from tomato leaves exposed to MJ was used as starting material for PCR cloning. MJ is a potent inducer of wound-responsive genes and has been described previously as up-regulating Lox gene expression in several plant species (Bell and Mullet, 1991; Grimes et al., 1992; Feussner et al., 1995). Copy DNA fragments derived from three Lox genes were cloned and, based on sequence analysis, full-length cDNAs for two were isolated. The proteins encoded by the two cDNAs, TomLOXC and TomLOXD, are relatively divergent and show limited sequence similarity with the Lox1 class of plant LOXs defined by Peng et al. (1994).

The current picture of the LOX gene family in tomato thus resembles the situation in potato described by Royo et al. (1996), with three classes identified to date on the basis of sequence similarity: the Lox1 class includes TomLoxA and TomLoxB (Ferrie et al., 1994), the Lox2 class is represented by TomLoxC, and Lox3 is defined by TomLoxD. As shown for AtLOX2 (Bell et al., 1995) and suggested for rice RLL and for potato LOX2 and LOX3, the N-terminal extensions on both TomLOXC and TomLOXD might function as chloroplast transit peptides. A similar N-terminal extension is not found in TomLoxA and TomLoxB products from tomato (Fig. 1; Ferrie et al., 1994), a characteristic shared with other members of the Lox1 class in various plant species.

We demonstrated that both TomLOXC and TomLOXD are targeted to the chloroplasts (Fig. 2), and this likely applies to potato LOX2 and LOX3. Isoforms of several enzymes of this pathway have been detected in leaf plastids (Douillard and Bergeron, 1981; Vick and Zimmerman, 1987; Song et al., 1993; Feussner et al., 1995; Harms et al., 1995; Blée and Joyard, 1996). Bell et al. (1995) showed that the expression of AtLOX2 is required for wound-induced JA accumulation in Arabidopsis, and Harms et al. (1995) showed that a constitutive increase in JA resulted from the expression of a flax allene oxide synthase in transgenic potato plants (Harms et al., 1995). The increased endogenous JA levels in the latter experiments did not lead to a corresponding increase in levels of proteinase Inhibitor I mRNA. The induction of Lox genes by wounding has been reported previously (Bell and Mullet, 1993; Geerts et al., 1994; Royo et al., 1996; Sarawitz and Siedow, 1996). The kinetics of TomLoxD mRNA accumulation presented here paralleled the kinetics of JA induction by wounding and systemin, which were described in tomato leaves (Doares et al., 1995). Moreover, Royo et al. (1996) showed that the JA precursor 13-hydroperoxylinolenic acid is the major product of the action of potato LOX2 and LOX3 enzymes on linolenic acid. On the basis of the very high sequence similarity between LOX2 and TomLOXC, and between LOX3 and TomLOXD, we predict that the catalytic properties of the two tomato enzymes are likely to be identical. However, the expression patterns of the two tomato genes are clearly distinct and suggest that TomLOXD rather than TomLOXC could be involved in the wound- and systemin-induced JA synthesis. Royo et al. (1996) described a steady increase of Lox2 mRNA in wounded potato leaves, whereas we did not detect mRNA for TomLoXC, the equivalent gene in wounded tomato leaves. The reason for this discrepancy is unclear, but the two Solanaceae may have evolved different regulation mechanisms for fatty acid hydroperoxide metabolism.

The wound-inducible expression of TomLoxD occurred well before the accumulation of proteinase Inhibitor I mRNA (Figs. 3 and 4), and this difference in the timing of the responses might be of physiological relevance. The transient nature of the TomLoxD mRNA suggests that it has a relatively short half-life compared with that of Inhibitor I mRNA (approximately 10 h; Graham et al., 1986) and that systemin is likely degraded with time as well. The different timing of the responses indicates that the expression of TomLoxD, which we term an "early responsive gene," is first detected within 0.5 h following wounding or elicitation (compare Figs. 3-5). Other early responsive genes in tomato leaves include prosystemin (McGurl et al., 1992) and allene oxide synthase (G.A. Howe and C.A. Ryan, unpublished data), both of which are components of the wound-signaling pathway and have mRNAs that also appear 1 to 2 h earlier than the mRNAs coding for the defensive proteinase inhibitors and polyphenol oxidase (Constabel et al., 1995), which we term "late responsive genes." The early responsive genes may be induced rapidly in response to herbivore attacks (wounding) to up-regulate the signaling pathway to enhance the activation of the defensive genes. Thus, although several genes are regulated by wounding, there appears to exist a mechanism that differentially up-regulates the activation of genes of the signal transduction pathway more rapidly than the defensive proteins that interact directly with attacking herbivores.

Taken together, the data presented here demonstrate that TomLoxD is a wound-inducible, early responsive gene in tomato leaves and that the encoded LOX enzyme is targeted to the chloroplast. The characteristics of the gene and its product indicate that it is a strong candidate as a component of the octadecanoid pathway and may play a role in defense signaling in tomato plants in response to herbivore and pathogen attacks.
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LITERATURE CITED


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