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An *Arabidopsis thaliana* Lipoxygenase Gene Can Be Induced by Pathogens, Abscisic Acid, and Methyl Jasmonate[1]

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We isolated and characterized a 2.8-kb, full-length, *Arabidopsis thaliana* cDNA clone encoding a lipoxygenase. DNA sequence analysis showed that the deduced amino acid sequence of the *Arabidopsis* protein is 72 to 78% similar to that of legume seed lipoxygenases. DNA blot analysis indicated that *Arabidopsis* contains a single gene, *LOX1*, with appreciable homology to the cDNA clone. RNA blot analysis showed that the *LOX1* gene is expressed in *Arabidopsis* leaves, roots, inflorescences, and young seedlings. *LOX1* expression levels were highest in roots and young seedlings. In mature plants, *LOX1* mRNA levels increased upon treatment with the stress-related hormones abscisic acid and methyl jasmonate and remained high for at least 96 h. Expression of the *LOX1* gene was examined following infiltration of leaves with virulent (*Psm* ES4326) and avirulent (*Pst* MM1065) strains of *Pseudomonas syringae*. *LOX1* mRNA levels were induced approximately 6-fold by both virulent and avirulent strains; however, the response to avirulent strains was much more rapid. Infiltration of leaves with *Pst* MM1065 resulted in maximal induction within 12 h, whereas maximal induction by *Psm* ES4326 did not occur until 48 h. When a cloned *avr* gene, *avrRpt2*, was transferred to *Psm* ES4326, *LOX1* mRNA accumulated in a pattern similar to that observed for the avirulent strain *Pst* MM1065.

LOXs (EC 1.13.11.12), dioxygenases that catalyze the hydroperoxidation of polyunsaturated fatty acids containing a *cis,cis*-1,4-pentadiene-conjugated double-bond system, are present in many if not all eukaryotes (Hildebrand et al., 1988; Siedow, 1991). The primary products of LOX-catalyzed reactions, fatty acid hydroperoxides, are typically metabolized into molecules with known or suspected regulatory activity. In mammals, fatty acid hydroperoxides are precursors for the synthesis of bioregulatory molecules including prostaglandins, prostacyclins, leukotrienes, lipoxins, and thromboxanes. These so-called eicosanoids function in inflammation and vasoconstriction/vasodilation, as well as in a number of other mammalian stress responses induced by trauma, disease, or allergy (Schewe et al., 1986; Samuelsson et al., 1987). In higher plants, LOX-derived fatty acid hydroperoxides are also precursors to molecules such as traumatin and jasmonic acid, which may serve regulatory roles (Anderson, 1989; Siedow, 1991). Traumatin induces cell division and may be involved in the response to wounding (Zimmerman and Coudron, 1979). Jasmonates (jasmonic acid and/or its methyl ester) have been shown to affect a variety of physiological processes (Koda, 1992; Staswick, 1992) including the induction of genes encoding specific leaf polypeptides (Wiedhase et al., 1987), phenylalanine ammonia lyase (Gundlach et al., 1992), protease inhibitors (Farmer and Ryan, 1990, 1992), vegetative storage proteins (Anderson, 1989; Staswick, 1990; Mason and Mullet, 1990), chalcone synthase, Pro-rich cell wall protein (Creelman et al., 1992b), and LOX (Bell and Mullet, 1991; Grimes et al., 1992). In addition, jasmonates have been implicated as signal transduction molecules in the response of plants to stress, particularly to wounding and pathogen attack (Farmer and Ryan, 1990, 1992; Creelman et al., 1992b).

LOX-derived fatty acid hydroperoxides and free radical species, which are cytotoxic and capable of damaging membranes, proteins, and DNA, may also play a role in degradative processes (Vick and Zimmerman, 1987). For example, in mammalian reticulocytes, LOX is involved in the degradation of mitochondrial membranes during red blood cell maturation (Schewe et al., 1986). Similarly, it has been suggested that plant LOXs may play a role in membrane degradation observed during senescence, wounding, and the hypersensitive response to pathogen attack (Hildebrand et al., 1988; Siedow, 1991).

Biochemical studies have indicated that LOX protein and activity levels are modulated in response to both biotic and abiotic stresses. Increases in LOX activity in response to mechanical wounding have been reported (Galliard, 1978; Hildebrand et al., 1988; Ievinsh, 1992). In addition, increases in LOX activity have been reported for a number of plant/pathogen systems (Lupu et al., 1980; Ruzicska et al., 1983; Ocampo et al., 1986; Yamamoto and Tani, 1986; Keppler and Novacky, 1987; Peever and Higgins, 1989; Croft et al., 1990; Rickauer et al., 1990; Ohta et al., 1991; Koch et al., 1992). In those cases in which both virulent and avirulent pathogens were used, LOX activity was found to increase only upon infection by the avirulent pathogen (Keppler and Novacky,

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Abbreviations: *DHS1*, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; LOX, lipoxygenase; MJ, methyl jasmonate; MS, Murashige and Skoog.

1987; Ohta et al., 1991) or to be induced rapidly with the avirulent pathogen and more slowly, if at all, with the virulent strain (Ocampo et al., 1986; Croft et al., 1990). LOX activity is also induced by treatment of cell cultures (Rickauer et al., 1990) or plants (Peever and Higgins, 1989) with elicitors. These results suggest that the rapid induction of LOX is involved in the defense response to pathogen attack.

LOX may also play an important role in plant growth and development. The highest levels of LOX activity have been observed in rapidly growing tissues. Furthermore, there is a positive correlation between LOX activity levels within an organ and its rate of elongation (Mack et al., 1987; Hildebrand et al., 1988; Siedow, 1991). For example, increases in LOX activity during early seedling growth have been observed in a wide variety of plant species (Siedow, 1991). It is interesting that the increase in LOX activity observed during germination is coincident with an increase in hydroperoxide lyase and fatty acid dehydrase activities (Vick and Zimmerman, 1987). Hydroperoxide lyase catalyzes the first reaction in the biosynthesis of traumatin, whereas fatty acid dehydrase initiates jasmonic acid production (Vick and Zimmerman, 1987). The concomitant increase in LOX and the enzymes involved in the metabolism of LOX-derived fatty hydroperoxides is consistent with a role for LOX in generating lipid-derived growth regulators.

Although the physiological function of LOX in higher plants is not well defined, it is clear that the enzyme is involved in a number of important processes. The plants in which LOXs have been studied to date have large numbers of LOX genes and isozymes that have complicated the analysis of the physiological function of LOX. In the hope of finding a simpler more facile system, we have initiated a study of the physiological role of LOX in the model crucifer, *Arabidopsis thaliana*. We report the isolation and characterization of an *Arabidopsis LOX1* cDNA clone. The *Arabidopsis LOX1* gene is differentially regulated in plant organs and is induced in response to the hormones ABA and MJ. Furthermore, the *Arabidopsis LOX1* gene is induced in response to attack by pseudomonad pathogens, suggesting a role in plant defense.

MATERIALS AND METHODS

Growth and Treatment of *Arabidopsis* Plants

Plants of *Arabidopsis thaliana* (Columbia) for total DNA isolation, root RNA isolation, and the ABA and MJ experiments were grown in sterile liquid culture in 250-mL Erlenmeyer flasks with 50 mL of MS basal medium containing sucrose and vitamins (Murashige and Skoog, 1962). Approximately 10 to 20 Clorox-sterilized seeds were placed into each flask and grown under low light with constant shaking (150 rpm). The MS medium was changed weekly. Some cultures were treated 3 weeks after sowing with either 100 μM ABA (Sigma, St. Louis, MO) or MJ (Serva, Heidelberg, Germany) solubilized in ethanol. Control cultures were treated with only ethanol.

Seedlings were obtained from *Arabidopsis* seeds germinated between wet filter papers. Approximately 0.25 g of seed were spread between Whatman No. 1 filter paper moistened with 15 mL of distilled H_2O in Petri dishes and placed in the light at 4°C for 48 h. The dishes were then placed in light at 25°C for 72 h before tissues were collected. To obtain leaf and flower bolt tissues, *Arabidopsis* seeds were sown in Metromix (W.R. Grace, Cambridge, MA) moistened with 0.5× MS basal salts (Murashige and Skoog, 1962). The plants were grown for 3 to 4 weeks under constant light (a mixture of fluorescent and incandescent lamps) at 25°C. Tissues were collected at the indicated intervals, frozen with liquid nitrogen, and stored at −80°C for subsequent RNA isolation. Plants for the pathogen induction experiment were grown and infiltrated with *Pseudomonas syringae* strains as previously described (Dong et al., 1991).

Isolation and Sequencing of *LOX1* cDNAs

Arabidopsis LOX1 cDNA clones were isolated from a λgt11 library constructed from RNA isolated from mature root tissue (Peterman and Goodman, 1991). Approximately 5×10^5 recombinant phage were screened using standard plaque lift methods (Ausubel et al., 1987) and HybondN$^+$ filters (Amersham, Arlington Heights, IL). Filters were hybridized at 65°C with cDNA fragments gel purified from soybean *LOX-1* (978-bp *Eco*RI/*Sal*I 3′-end fragment of pAL134) (Start et al., 1986) and *LOX-3* (1.1- and 1.5-kb *Pst*I fragments of the full-length cDNA) (Yenofsky et al., 1988) and labeled with [α-^{32}P]dCTP by random priming (Multiprime Kit, Amersham) (Feinberg and Vogelstein, 1983). The hybridization solution contained 0.9 M NaCl, 50 mM Na_2HPO_4 (pH 7.7), 5 mM EDTA, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) PVP, 0.5% (w/v) SDS, and 0.02 mg/mL of denatured salmon sperm DNA. The filters were washed at 25°C in 2× SSPE (0.36 M NaCl, 0.02 M NaH_2PO_4 [pH 7.7], 2 mM EDTA, 0.1% [w/v] SDS) and then at 37°C in 1× SSPE. Six positive clones were identified and isolated by plaque purification (Ausubel et al., 1987). *Eco*RI insert fragments from the positive clones were subcloned into Bluescript+ (Stratagene, La Jolla, CA). Partial sequences were determined for two of the smaller clones, and the inserts from the two largest clones, λAtLox1–1 and λAtLox1–2, were completely sequenced on both strands using either the thermal cycling procedure with [γ-^{32}P]dATP end-labeled primers (ds Cycle Sequencing System; Gibco/Bethesda Research Laboratories, Grand Island, NY) or the dideoxy method (Sanger et al., 1977) using Sequenase DNA polymerase (United States Biochemical, Cleveland, OH) and [α-^{35}S]dATP. Sequence analysis was performed using the University of Wisconsin Genetics Computer Group software package.

Genomic Blot Analysis

Total genomic DNA was isolated from roots of plants grown in sterile culture using the procedure for plant tissue described by Ausubel et al. (1987). Two-microgram samples of DNA were digested with either *Pst*I, *Eco*RI, or *Hin*dIII, separated by electrophoresis on 0.8% agarose gels, transferred to GeneScreen Plus membranes (New England Nuclear-Dupont, Wilmington, DE) by capillary transfer, and cross-linked to the filter by UV irradiation (200 mW/cm^2 for 12–15 min). The filters were hybridized with either coding

region- or 3′-untranslated region-specific probes at 65°C. They were washed at 25°C in 0.5% BSA, 1 mM EDTA, 80 mM sodium phosphate (pH 7.2), and 5% SDS and then at 25°C (low stringency) or 65°C (high stringency) in 1 mM EDTA, 80 mM sodium phosphate, and 1% SDS (Church and Gilbert, 1984). The second high-stringency wash for filters probed with the 166-bp 3′-end fragment was carried out at 45°C because of the high A-T content of this probe. Probes were made by random primed labeling of gel-purified *Eco*RI fragments of λAtLox1–1 or λAtLox1–2, containing primarily coding sequences, or of the primarily 3′-untranslated sequence of λAtLox1–1 (166 bp). Filters were exposed to Kodak X-AR5 film (Eastman Kodak, Rochester, NY) with a Dupont Cronex intensifying screen (Dupont, Wilmington, DE).

RNA Isolation and Analysis

Total RNA was isolated using either phenol/SDS extraction and LiCl precipitation (Ausubel et al., 1987; Davis et al., 1991) or guanidinium isothiocyanate extraction (Chomczynski and Sacchi, 1987). RNA samples (5–10 μg) were separated by electrophoresis on formaldehyde-agarose gels (Ausubel et al., 1987). A parallel set of samples was run and stained with 1 μg/mL of ethidium bromide to verify even loading of samples. After the gel was washed with two changes of distilled H_2O at 60°C and three changes of 25 mM phosphate buffer, pH 6.5, at 25°C, the RNA was transferred to GeneScreen Plus membranes by capillary transfer, UV cross-linked, hybridized, and washed as described above for the genomic Southern blots. Probes were generated by random primed labeling with [α-^{32}P]dCTP of a 1.4-kb *Eco*RI-coding sequence fragment from λAtLox1–1 or by polymerase chain reaction priming using oligonucleotides derived from the λAtLox1–1 sequence. Relative levels of expression were determined by densitometry of the autoradiograms using an LKB Ultroscan laser densitometer (LKB, Bromma, Sweden) or by scanning with the Betascope 603 blot analyzer (Betagen).

RESULTS

Isolation and Sequence Analysis of *Arabidopsis LOX1* cDNA Clones

Putative *Arabidopsis* LOX cDNA clones were isolated from a λgt11 library constructed from RNA isolated from mature roots (Peterman and Goodman, 1991). All of the isolated clones fell into a single class based on restriction and hybridization analysis. The sequences of the two largest clones, λAtLox1–1 and λAtLox1–2, were identical except that λAtLox1–2 contained a 97-bp insert, which disrupted the open reading frame, in the region of overlap between the two clones. This insert was judged to be an unspliced intron because the junction sequences were homologous to the consensus sequence for exon-intron junctions (Lewin, 1990). Furthermore, deletion of the intron sequence restored the open reading frame. Partial sequences of two of the other cDNAs were determined and found to be identical with that of λAtLox1–1 and λAtLox1–2. Therefore, all of the cDNAs isolated belong to a single class corresponding to the locus we have designated *LOX1*.

The nucleotide and derived amino acid sequences of the *LOX1* cDNA clone, λAtLox1–2, are presented in Figure 1. (The unspliced intron sequence is not shown.) The 2801-nucleotide sequence consists of an 80-nucleotide, AT-rich (62.5%) 5′-untranslated region that precedes the first ATG, a 2577-bp open reading frame, a stop codon, and a 141-bp 3′-untranslated region. The presence of 5′-untranslated sequences before the open reading frame indicated that λAtLox1–2 contains the entire coding sequence of the *LOX1* gene. The derived amino acid sequence of 859 amino acids has a predicted molecular mass of 98 kD. Two putative poly(A^+) signals (AAUAAA) (Proudfoot and Brownlee, 1976) are found 27 and 40 nucleotides upstream from the poly(A^+) tail. Neither signal nor transit peptide sequences were found in the *LOX1* sequence, suggesting that the *Arabidopsis* LOX1 protein is cytoplasmic in location.

Comparison of the *LOX1* cDNA sequence to that of other plant LOXs confirmed the identity of the clone. The nucleic acid and derived amino acid sequences of *LOX1* were compared to the eight plant LOX sequences on deposit in the GenBank data base using the GAP function of the GCG software package. The sequences available for comparison were from the leguminous plants, soybean (Shibata et al., 1987, 1988, 1991; Yenofsky et al., 1988), pea (Casey and Ealing, 1988; Ealing and Casey, 1989), and bean (A.J. Slusarenko and B.M. Meier, unpublished; A.J. Slusarenko, unpublished). Nucleic acid sequence identity values ranged from 61 to 64%. Amino acid percentage similarity and percentage identity values ranged from 72 to 78% and 56 to 63%, respectively. The *Arabidopsis LOX1* sequence is most similar to the sequence of soybean seed *LOX-3*, exhibiting 63.2% nucleic acid sequence identity, 77.9% amino acid sequence similarity, and 62.7% amino acid sequence identity.

A comparison of the derived amino acid sequences of *LOX1* and soybean *LOX-3* are shown in Figure 2. The deduced amino acid sequence of *LOX1* contains four conserved regions found in other plant LOXs (Siedow, 1991). The shaded boxes in Figure 2 denote these conserved regions. The first conserved region begins at residue 359 in the *Arabidopsis* deduced amino acid sequence. The second region, which begins at residue 514 in the *LOX1* sequence, contains six conserved His residues in soybean. Five of the six His observed in the soybean LOXs are found in the *Arabidopsis* sequence. These same five His residues are also conserved in the mammalian and tobacco LOXs (Siedow, 1991) and have been shown by site-directed mutagenesis of the human 5-LOX to be important for enzymic activity (Nguyen et al., 1991; Zhang et al., 1992).

The next conserved region, which begins at residue 707 on the *Arabidopsis* sequence, is completely identical with the same region of all the legume LOXs. Also, the C-terminal nine amino acids of the *LOX1* sequence are identical with those of the legume LOXs with the exception of a single conservative substitution (Ile to Val) at residue 857. Sequence comparisons were also made to mammalian LOXs, but similarities were much lower (48–52% amino acid similarity) than those for the plant sequences.

The identity of the *LOX1* cDNA was also confirmed by protein expression studies. *Arabidopsis* LOX protein was expressed in *Escherichia coli* using λAtLox1–1 and used for polyclonal antibody production. The resulting antibodies rec-

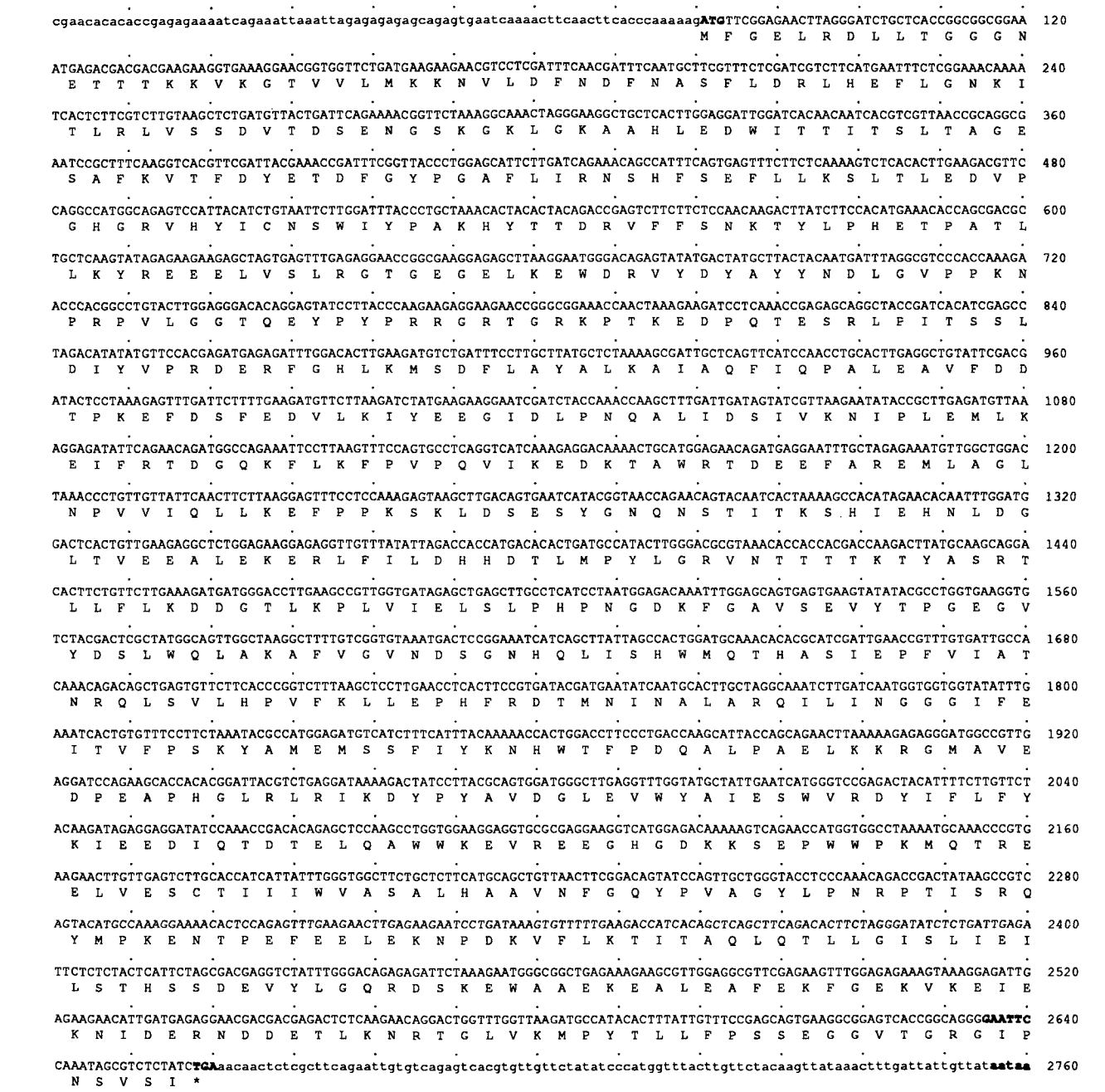

Figure 1. The nucleotide and deduced amino acid sequences of *LOX1*. The nucleic acid sequence is presented on the top line with the derived amino acid sequence below. Uppercase boldface type indicates the positions of the first ATG, the *Eco*RI site, and the stop codon. The two poly(A$^+$) signals are shown in lowercase bold type.

ognized an approximately 100-kD protein in *Arabidopsis* protein extracts and cross-reacted with purified soybean lipoxygenase (data not shown).

Genomic Blot Analysis of the *Arabidopsis LOX1* Gene

Genomic Southern blot analysis was performed to estimate the number of LOX genes in the *Arabidopsis* genome. Total *Arabidopsis* (Columbia) genomic DNA was digested to completion with *Pst*I, *Eco*RI, or *Hin*dIII, separated on a 0.8% agarose gel, and transferred to a nylon membrane. The blots were hybridized with either coding region (Fig. 3, A and B) or 3′-untranslated region-specific (Fig. 3C) probes from the *Arabidopsis LOX1* cDNAs. In all cases the number of fragments detected was consistent with a single *Arabidopsis LOX1* gene, given the known restriction map of the corresponding

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AtLox    MFGELRDLLTGGGNETTTKKVKGTVVLMKKNVLDFN.............D  37
Soylox-3 MLGGLLH.........RGHKIKGTVVLMRKNVLDVNSVTSVGGIIGQGLD  41

         FNASFLDRLHEFLGNKITLRLVSSDVTDSENGSKGKLGKAAHLEDWITTI  87
         LVGSTLDTLTAFLGRPVSLQLISA..TKADANGKGKLGKATFLEGIITSL  89

         TSLTAGESAFKVTFDYETDFGYPGAFLIRNSHFSEFLLKSLTLEDVPGHG 137
         PTLGAGQSAFKINFEWDDGSGILGAFYIKNFMQTEFFLVSLTLEDIPNHG 139

         RVHYICNSWIYPAKHYTTDRVFFSNKTYLPHETPATLLKYREEELVSLRG 187
         SIHFVCNSWIYNAKLFKSDRIFFANQTYLPSETPAPLVKYREEELHNLRG 189

         TGEGELKEWDRVYDYAYYNDLGVPPK...NPRPVLGGTQEYPYPRRGRTG 234
         DGTGERKEWERVYDYDVYNDLGDPDKGENHARPVLGGNDTFPYPRRGRTG 239

         RKPTKEDPQTESRLPITSSLDIYVPRDERFGHLKMSDFLAYALKAIAQFI 284
         RKPTRKDPNSESR.....SNDVYLPRDEAFGHLKSSDFLTYGLKSVSQNV 284

         QPALEAVFDD..TPKEFDSFEDVLKIYEEGIDLPNQALIDSIVKNIPLEM 332
         LPLLQSAFDLNFTPREFDSFDEVHGLYSGGIKLP....TDIISKISPLPV 330

         LKEIFRTDGQKFLKFPVPQVIKEDKTAWRTDEEFAREMLAGLNPVVIQLL 382
         LKEIFRTDGEQALKFPPPKVIQVSKSAWMTDEEFAREMLAGVNPNLIRCL 380

         KEFPPKSKLDSESYGNQNSTITKSHIEHNLDGLTVEEALEKERLFILDHH 432
         KEFPPRSKLDSQVYGDHTSQITKEHLEPNLEGLTVDEAIQNKRLFLLGHH 430

         DTLMPYLGRVNTTTTKTYASRTLLFLKDDGTLKPLVIELSLPHPNGDKFG 482
         DPIMPYLRRINATSTKAYATRTILFLKNDGTLRPLAIELSLPHPQGDQSG 480

         AVSEVYTP.GEGVYDSLWQLAKAFVGVNDSGNHQLISHWMQTHASIEPFV 531
         AFSQVFLPADEGVESSIWLLAKAYVVVNDSCYHQLVSHWLNTHAVVEPFI 530

         IATNRQLSVLHPVFKLLEPHFRDTMNINALARQILINGGGIFEITVFPSK 581
         IATNRHLSVVHPIYKLLHPHYRDTMNINGLARLSLVNDGGVIEQTFLWGR 580

         YAMEMSSFIYKNHWTFPDQALPAELKKRGMAVEDPEAPHGLRLRIKDYPY 631
         YSVEMSAVVYKD.WVFTDQALPADLIKRGMAIEDPSCPHGIRLVIEDYPY 629

         AVDGLEVWYAIESWVRDYIFLFYKIEEDIQTDTELQAWWKEVREEGHGDK 681
         AVDGLEIWDAIKTWVHEYVFLYYKSDDTLREDPELQACWKELVEVGHGDK 679

         KSEPWWPKMQTREELVESCTIIIWVASALHAAVNFGQYPVAGYLPNRPTI 731
         KNEPWWPKMQTREELVEACAIIIWTASALHAAVNFGQYPYGGLILNRPTL 729

         SRQYMPKENTPEFEELEKNPDKVFLKTITAQLQTLLGISLIEILSTHSSD 781
         SRRFMPEKGSAEYEELRKNPQKAYLKTITPKFQTLIDLSVIEILSRHASD 779

         EVYLGQRDSKEWAAEKEALEAFEKFGEKVKEIEKNIDERNDDETLKNRTG 831
         EVYLGERDNPNWTSDTRALEAFKRFGNKLAQIENKLSERNNDEKLRNRCG 829

         LVKMPYTLLFPSSEGGVTGRGIPNSVSI* 860
         PVQMPYTLLLPSSKEGLTFRGIPNSISI* 858
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Figure 2. Comparison of the derived amino acid sequences of *LOX1* and soybean *LOX-3*. Sequence comparisons were made using the GAP function of the GCG software package. The soybean *LOX-3* translation was derived from the cDNA sequence deposited under Genbank accession No. X13302 (Yenofsky et al., 1988). Shaded boxes denote regions of amino acid conservation common to other LOXs (Siedow, 1991).

cDNA clone (Fig. 3D). The 2.6-kb coding sequence probe, which contains two *Hin*dIII and no *Pst*I or *Eco*RI sites, hybridized to a 25-kb *Pst*I fragment, a 6.4-kb *Eco*RI fragment, and three *Hin*dIII fragments of 16, 2.5, and 1.5 kb (Fig. 3A). The 1.4-kb coding region probe hybridized to the 25-kb *Pst*I, 6.4-kb Eco*RI*, and 2.5-kb *Hin*dIII fragments (Fig. 3B). The 3′-untranslated region-specific probe also hybridized to single fragments in each of the three digests. The 25-kb *Pst*I and 2.5-kb *Hin*dIII fragments were the same as those detected by the coding region probe, whereas, as predicted, a different 1.9-kb *Eco*RI fragment was identified (Fig. 3C). Identical results were obtained from blots washed at low stringency (25–45°C) (data not shown). These results indicate that the cDNA probe hybridized only to *LOX1* genomic sequences and did not detect other LOX genes. It is possible, however,

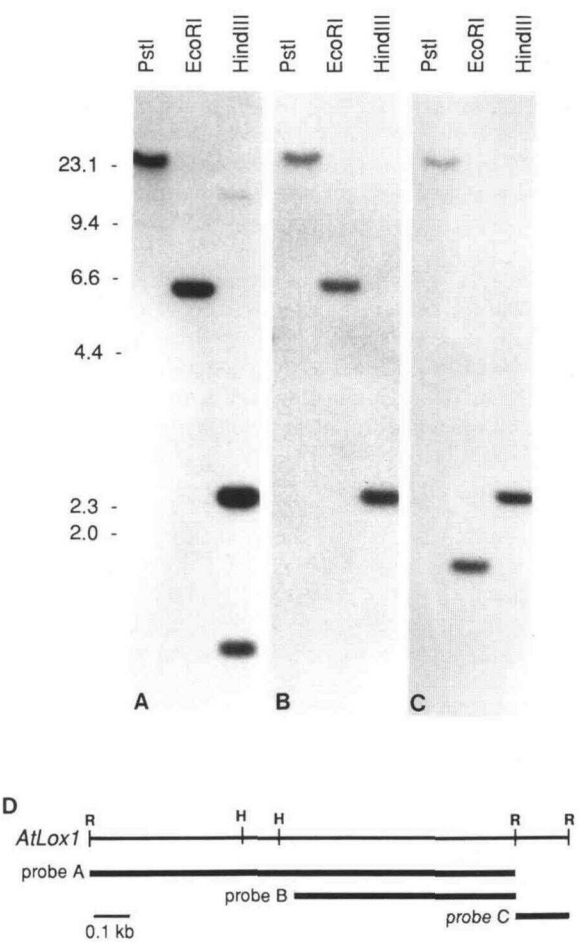

Figure 3. Genomic Southern analysis of *LOX1*. Total genomic DNA (2 μg) was digested with either *Pst*I, *Eco*RI, or *Hin*dIII, separated by electrophoresis on a 0.8% agarose gel, transferred to a GeneScreen membrane, and hybridized with a 2.6-kb coding region Eco*RI* fragment of λAtLox1-2 (A), a 1.4-kb coding region Eco*RI* fragment of λAtLox1-1 (B), or the 166-bp Eco*RI* fragment of λAtLox1-1 containing the 3′-untranslated region and 22 nucleotides of coding sequence (C). Size markers (kb) generated from a *Hin*dIII digest of λ DNA are indicated to the left. A restriction map of the *LOX1* cDNA and the probes used for the genomic blots is shown in D.

that LOX genes with <65 to 70% homology to *LOX1* were not detected under these hybridization conditions. In fact, another *Arabidopsis* LOX cDNA that is distinct from *LOX1* has been isolated from a leaf library (E. Bell and J. Mullet, personal communication). The *LOX1* cDNA did not cross-hybridize with this other gene under the conditions used here.

Expression of *LOX1* in *Arabidopsis* Organs

To determine the expression patterns of *LOX1*, northern blot analysis of total RNA isolated from *Arabidopsis* leaf, root, bolt, and young seedlings was performed. Leaf and bolt samples were collected from 4- to 6-week-old plants. Fully expanded rosette leaves that were 1 to 2 cm in length and bolts that were <2 cm tall and possessed unopened flower buds were chosen. Roots were collected from plants grown in liquid culture for 3 weeks. Seedlings were grown for 72 h between wet filter papers. Ethidium bromide staining of rRNAs was used to confirm that equal amounts of RNA were loaded in each lane. Figure 4 shows a blot that was probed with a 1.4-kb *LOX1*-coding region probe. In all samples, a single band of approximately 2.8 kb was seen. The size of the *LOX1* mRNA predicts a protein size of approximately 100 kD, which is similar to the sizes of other plant LOX proteins (Siedow, 1991) and is in agreement with the molecular mass predicted for the *Arabidopsis* protein from the deduced amino acid sequence. *LOX1* is expressed in different organs of the plant. The lowest levels of expression were seen in leaves and bolts. Expression in roots was approximately 2-fold higher. The highest levels of expression were seen in young seedlings in which expression was approximately 2.5-fold more than the levels in leaf and bolt tissues.

LOX1 Is Induced by ABA and MJ

LOX1 mRNA levels were determined by northern analysis in plants treated with the stress-related hormones ABA and MJ. *Arabidopsis* plants grown in liquid culture were treated with either 100 μM ABA or MJ. Control cultures were treated with an equivalent amount of the ethanol solvent. Figure 5 shows the *LOX1* mRNA levels in roots during the course of

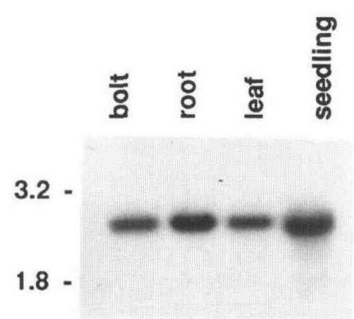

Figure 4. Differential expression of *LOX1* in *Arabidopsis* organs. Total RNA (10 μg) was separated on formaldehyde-agarose gels, transferred to a GeneScreen membrane, and hybridized with a 1.4-kb Eco*RI*-coding region fragment of λAtLox1–1. Ribosomal RNA bands were used as size markers (left).

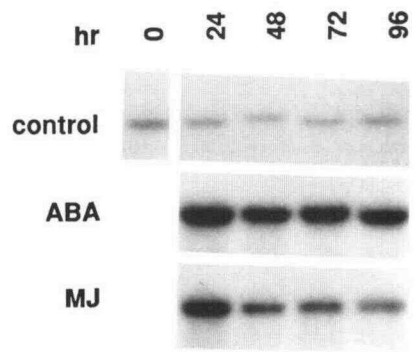

Figure 5. RNA gel blot analysis of *LOX1* mRNA levels in *Arabidopsis* roots following application of ABA and MJ. *Arabidopsis* plants were grown in liquid culture for 3 weeks before treatment with either 100 μM ABA or MJ. Total RNA samples (10 μg) were prepared, separated on formaldehyde-agarose gels, and transferred to a nylon membrane. The blot was hybridized with the 1.4-kb coding region *Eco*RI fragment of λAtLox1–1.

96 h. High levels of *LOX1* induction were seen with both ABA and MJ treatments. The ABA-treated roots showed an 8-fold increase in *LOX1* mRNA levels over the controls. This level of expression remained constant during the entire time course. Expression of *LOX1* in MJ-treated roots was approximately 6-fold more than the controls at 24 h and then decreased to 3-fold greater from 48 to 96 h. The levels of RNA expression in root tissues for these treatments was approximately 3-fold greater than that seen in leaf tissue samples from similar experiments (data not shown). It has been suggested that high levels of jasmonic acid may be toxic (Anderson, 1989). We, however, saw no visible evidence of senescence or toxicity with either of these treatments.

LOX1 Is Induced by *Pseudomonas* Pathogen Attack

Increases in LOX activity have been reported for plants that are under pathogen attack, and it has been suggested that LOX may play an important role in the pathogen defense response (Lupu et al., 1980; Ruzicska et al., 1983; Ocampo et al., 1986; Yamamoto and Tani, 1986; Keppler and Novacky, 1987; Peever and Higgins, 1989; Croft et al., 1990; Rickauer et al., 1990; Ohta et al., 1991; Koch et al., 1992). Northern analysis of total RNA isolated from plants infected with either virulent or avirulent *Pseudomonas* strains revealed that the *Arabidopsis LOX1* gene is dramatically induced in response to pathogen attack (Fig. 6). Leaves of *Arabidopsis* (Columbia) plants were infiltrated with either virulent (*Psm* ES4326) or avirulent (*Pst* MM1065) strains of *P. syringae*. In addition, the strain *Psm* ES4326/pMMXR1, which was produced by transfer of the *avr* gene, *avrRpt2*, isolated from *Pst* MM1065 into the virulent strain *Psm* ES4326 (Dong et al., 1991) was used. Two bacterial concentrations, high (10^7 cfu/mL) and low (10^6 cfu/mL), were used in the experiments. The control treatment consisted of infiltration with 10 mM $MgCl_2$. The virulent strain, *Psm* ES4326, multiplies rapidly and induces large water-soaked lesions after 48 h, whereas the avirulent strain, *Pst* MM1065, does not multiply extensively and induces dry, necrotic, hypersensitive lesions at the point of infection

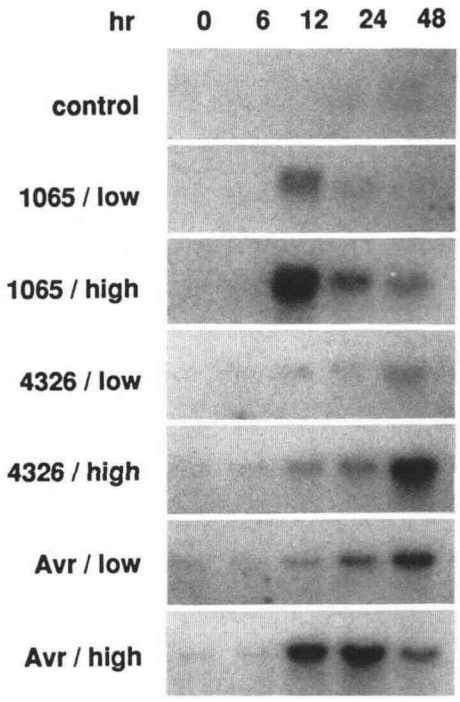

Figure 6. RNA gel blot analysis of *LOX1* mRNA levels in *Arabidopsis* leaves infiltrated with *P. syringae* strains at high (10^7 cfu/mL) or low (10^6 cfu/mL) titer. The *Pseudomonas* strains used were *Pst* MM1065 (1065), *Psm* ES4326 (4326), and *Psm* ES4326 carrying the *avr* gene, *avrRpt2*, on the plasmid pMMXR1 (Avr). Total RNA (5 μg) was loaded in each lane, and blots were hybridized with the 1.4-kb coding region λAtLox1–1 *Eco*RI fragment.

within 12 to 24 h (Davis et al., 1991; Dong, et al., 1991; Whalen et al., 1991). *LOX1* mRNA levels increased approximately 6-fold within 12 h after infiltration with the high titer of the avirulent strain *Pst* MM1065. Within 48 h, *LOX1* mRNA levels had decreased to near those of the control. A similar, albeit lower level, induction was observed with the low titer of *Pst* MM1065 and the high titer of *Psm* ES4326/pMMXR1. Infiltration of leaves with the virulent strain (*Psm* ES4326) also resulted in a 6-fold induction of *LOX1* mRNA levels. Induction by the virulent strain, however, did not occur until 48 h after infiltration. The induction of the *Arabidopsis LOX1* gene, like that of many host-defense genes (Dixon and Harrison, 1990), is rapid in an incompatible interaction and slow in a compatible one.

DISCUSSION

Cloning of an *Arabidopsis LOX1* cDNA

We have isolated and sequenced a full-length *LOX1* cDNA clone from *Arabidopsis thaliana*. Three lines of evidence were used to confirm the identity of this clone. First, the overall nucleotide and amino acid similarity to other plant LOX sequences is very high. For example, the *Arabidopsis LOX1* nucleotide and derived amino acid sequences are 63.2 and 77.9% similar, respectively, to those of soybean seed *LOX-3*. Second, four regions conserved in the plant LOX sequences described to date were identified in the *Arabidopsis LOX1* sequence. Finally, antibodies raised against *Arabidopsis* LOX1 protein expressed in *E. coli* from λAtLox1–1 cross-reacted with purified soybean seed LOXs and recognized an approximately 100-kD polypeptide in *Arabidopsis* protein extracts (data not shown). This molecular mass is in good agreement with the predicted size of the *Arabidopsis* LOX1 protein deduced from the nucleotide sequence of the full-length clone, λAtLox1–2.

Analysis of genomic sequences indicated that *Arabidopsis* contains a single gene with >65 to 70% homology to the *LOX1* cDNA. A second *Arabidopsis* LOX cDNA has been isolated, which does not cross-hybridize with *LOX1* under the conditions used here (E. Bell and J. Mullet, personal communication). Thus, two LOX genes have been identified in *Arabidopsis*. The possibility exists that there is another LOX gene in *Arabidopsis* that is not significantly homologous to the genes that have been identified. This is the case for mammalian cells in which the *5-LOX* gene exhibits only limited sequence homology to the *12-LOX* and *15-LOX*. The *12-LOX* and *15-LOX* exhibit 89% nucleotide identity, whereas there is only 39% amino acid identity between the *5-LOX* and *12-LOX* or *15-LOX* (Sigal et al., 1988).

The apparent simplicity of the *Arabidopsis* LOX gene family is in marked contrast to the genomic complexity of LOX in soybean. In soybean, there is direct evidence, from protein and/or nucleic acid sequences, for five distinct LOX genes (Start et al., 1986; Shibata et al., 1987, 1988, 1991; Yenofsky et al., 1988; Kato et al., 1992). Furthermore, DNA blot analysis of soybean genomic DNA probed with cloned LOX sequences suggests that there are many homologous, yet undescribed, soybean LOX genes (E. Bell and J. Mullet, personal communication). The simplicity of the *Arabidopsis* LOX gene family should greatly facilitate determining the physiological requirements for LOX in higher plants.

Expression of *LOX1* in *Arabidopsis*

The simplicity of the *Arabidopsis* LOX gene family implies that individual genes may serve multiple functions throughout the plant's life cycle, given the diversity of physiological functions suggested by enzyme activity data. If the *Arabidopsis LOX1* gene is meeting a number of physiological demands, then expression of the gene throughout the plant and during development is expected. Northern analysis results presented in Figure 4 show that *LOX1* is expressed in various organs of the *Arabidopsis* plant. Expression levels of the *LOX1* gene are greater in root and seedling tissues than in leaf or inflorescence tissues. Increases in LOX content and activity have previously been reported in several plant species during germination and early seedling growth (Park and Polacco, 1989; Siedow, 1991). The expression of the *LOX1* gene that we observe in roots and seedlings is consistent with a role for LOX in germination and seedling development. LOX in young seedlings could also serve a protective function at this stage of development, which is particularly vulnerable to infection by fungal and bacterial pathogens. The expression of the *Arabidopsis LOX1* gene in inflorescences is of interest in light of the observation that other genes involved in pathogen defense and wounding are also expressed in flow-

ers. It has been suggested that defense-related genes may serve a protective function in flowers as well (Gasser, 1991).

Regulation of *LOX1* Expression by ABA and MJ

Expression of the *Arabidopsis LOX1* gene increased in response to treatment with the hormones ABA and MJ. *LOX1* mRNA levels were elevated in *Arabidopsis* roots treated with MJ within 24 h and remained above control levels for at least 96 h. This is the first report of an effect of MJ on gene expression in roots. LOX mRNA levels are also induced by MJ in soybean suspension culture cells (Bell and Mullett, 1991). In addition, the 94-kD vegetative storage protein LOX is induced by atmospheric MJ (Grimes et al., 1992). A likely role for LOX in plants is as a first step in the synthesis of the putative signal-transducing compound jasmonic acid (Vick and Zimmerman, 1987). The induction of LOX genes by MJ suggests that jasmonate may be a positive regulator of its own synthesis. The *Arabidopsis LOX1* gene is also induced by ABA in roots. In a study of LOX gene expression in soybean, RNAs for LOX were seen to accumulate in the elongating hypocotyl region under conditions of low water potential and in leaves upon wounding (Bell and Mullet, 1991). Osmotic stress and wounding are conditions known to increase the levels of ABA in plants. Induction of LOX was not seen, however, in soybean suspension-cell cultures 6 h after the application of ABA, and the time course was not followed for longer times (Bell and Mullet, 1991). We, on the other hand, see a substantial increase in *LOX1* expression in *Arabidopsis* roots with the application of ABA that continues up to 96 h after treatment. It has been suggested that LOX may play a role in ABA biosynthesis (Vick and Zimmerman, 1987; Creelman et al., 1992a).

LOX1 Induction by Virulent and Avirulent *Pseudomonas* Strains

Our results suggest that LOX may play a role in the pathogen-defense response. Plants, in response to attack by microbial pathogens, activate a complex, highly coordinated set of disease-defense reactions (Dixon and Harrison, 1990). In a successful defense reaction, plants typically undergo a hypersensitive response. Small necrotic lesions develop at the site of infection, and growth of the pathogen is checked (Slusarenko et al., 1991). This hypersensitive response is initiated by a specific recognition event between a pathogen-derived elicitor and a host receptor that triggers the induction of a variety of defense-response genes (Keen, 1990).

A major difference between resistance and susceptibility to pathogens is in the timing of induction of these host-defense genes. Typically, defense-response genes are induced rapidly during a hypersensitive response and more slowly, if at all, in a disease reaction (Dixon and Harrison, 1990). For example, in *Arabidopsis* the phenylalanine ammonia lyase (Dong et al., 1991) and *DHS1* (Keith et al., 1991) genes were induced rapidly upon infection with the avirulent *Pseudomonas* strain *Pst* MM1065. Infiltration with the virulent strain, *Psm* ES4326, resulted in a late induction of the *DHS1* gene (Keith et al., 1991) and no significant induction of the phenylalanine ammonia lyase gene (Dong et al., 1991).

The *Arabidopsis LOX1* gene is dramatically induced upon infiltration with pathogenic pseudomonads. *LOX1* mRNA levels were induced to approximately the same extent with both virulent and avirulent *Pseudomonas* strains, although the kinetics of induction were different. Infiltration of leaves with the avirulent strain *Pst* MM1065 resulted in maximal induction of the *LOX1* gene within 12 h, whereas induction by the virulent strain *Psm* ES4326 was not maximal until 48 h after infiltration. These results are similar to those obtained with other host-defense genes and suggest that the rapid induction of the *Arabidopsis LOX1* gene during a hypersensitive response is important for disease resistance. Furthermore, the induction of *LOX1* by infiltration with *Psm* ES4326/pMMXR1, in a pattern similar to that observed with the avirulent strain *Pst* MM1065, suggests that a signal generated by the *avr* gene carried on pMMXR1, *avrRpt2*, specifically affects *LOX1* expression during the hypersensitive response.

LOX enzyme activity has also been shown to be induced rapidly during a disease-resistance response and more slowly in a susceptible interaction. This association of increased LOX activity and an effective defense response has been observed for tobacco mosaic virus infection (Ruzicska et al., 1983), *Puccinia graminis tritici* infection of wheat (Ocampo et al., 1986), *Puccinia coronata* infection of oats (Yamamoto and Tani, 1986), *P. syringae* infection of cucumber (Keppler and Novacky, 1987), bean (Croft et al., 1990), and tomato (Koch et al., 1992), and *Magnaporthe grisea* infection of rice (Ohta et al., 1991). Differential kinetics of accumulation of LOX mRNA in compatible versus incompatible interactions were also reported for *P. syringae* infection of tomato (Koch et al., 1992).

Jasmonates have been implicated as signal transduction molecules in the response of plants to wounding and pathogen attack (Farmer and Ryan, 1992; Creelman et al., 1992b). Farmer and Ryan (1992) have proposed a model for an octadecanoid signal transduction pathway in plants that is activated by wounding or pathogen attack. According to the model, perception of an extracellular signal results in the activation of a plasma membrane-bound lipase that releases linolenic acid from membrane phospholipids. The linolenic acid is then converted by constitutive enzymes (LOX and others) into the octadecanoid signal molecule, jasmonic acid, which results in gene activation. Our results suggest that induction of the *LOX1* gene may be a regulatory point in the synthesis of jasmonic acid or other octadecanoids in response to pathogen attack. The molecular analysis of *LOX1* in *Arabidopsis* presented here has laid the groundwork for future studies of the role of this gene product in pathogen-induced signal transduction. Furthermore, the sophisticated genetic approaches that are feasible in *Arabidopsis* should allow for the isolation of mutants with lesions in the signal transduction pathway leading to the induction of the *LOX1* gene.

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