Plant Physiol. (1996) 112: 997–1004

Lipoxygenase Gene Expression in the Tobacco-*Phytophthora parasitica nicotianae* Interaction[1]

Christophe Véronési*, Martina Rickauer, Joëlle Fournier, Marie-Laure Pouénat, and Marie-Thérèse Esquerré-Tugayé

Centre de Physiologie Végétale, Unité Mixte de Recherche Centre National de la Recherche Scientifique Université Paul Sabatier 5546, Université Paul Sabatier, 118, Route de Narbonne, 31062 Toulouse Cedex, France

A recently isolated cDNA clone of tobacco (*Nicotiana tabacum* L.) lipoxygenase (LOX) was used to study LOX gene expression in tobacco cell-suspension cultures and intact plants in response to infection with *Phytophthora parasitica nicotianae* (*Ppn*). Southern blot analysis of tobacco DNA indicated that only a small number of LOX genes hybridize to this probe. These genes were not constitutively expressed to a detectable level in control cells and healthy plants. In contrast, a rapid and transient accumulation of transcripts occurred in cells and plants after treatment with elicitor and inoculation with zoospores of *Ppn*, respectively. In cell cultures LOX gene expression could also be induced by linolenic acid, a LOX substrate, and by methyl jasmonate, one of the products derived from the action of LOX on linolenic acid. In the infection assays, LOX gene expression and enzyme activity were observed earlier when the plants carried a resistance gene against the race of *Ppn* used for inoculation. The differential expression of LOX during the race-cultivar-specific interaction between tobacco and *Ppn*, as well as its regulation by elicitors and jasmonate, suggest a role of LOX in plant resistance and establishment of the defense status against this pathogen.

LOX (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyzes the addition of molecular oxygen to PUFAs, which present a *cis,cis*-1,4-pentadiene site, such as linoleic acid (18:2), linolenic acid (18:3), and AA (20:4). These nonheme-iron-containing dioxygenases are widely distributed in nature among plants and animals (Gardner, 1991; Siedow, 1991; Toh et al., 1992).

In animals hydroperoxides arising from LOX action on AA can be further converted through the so-called AA cascade into leukotrienes or lipoxins. These eicosanoids, together with their precursor hydroperoxides, are involved in the regulation of numerous physiological processes and pathological reactions, including the immune response (Nicolaou et al., 1991). Similarly, plant LOXs have been proposed to form biologically active compounds both during normal developmental stages such as germination or growth and during responses to environmental stress such as wounding or pathogen attack (Gardner, 1991; Siedow, 1991).

The involvement of LOX in plant-microbe interactions was suggested when AA and eicosapentaenoic acid were isolated from the mycelium of *Phytophthora infestans*, a phytopathogenic fungus, and were shown to elicit phytoalexin accumulation and hypersensitive cell death in potato tissues, probably via LOX action (Ricker and Bostock, 1994). Other data also point to a role for LOX in plant pathogenesis. The primary products of LOX, the fatty acid hydroperoxides, are very reactive and may cause oxidative damage to membranes, leading to cell necrosis and death (Hildebrand, 1989). In some cases, lipid pentadienyl, peroxyl free radicals (Gardner, 1991), or other active oxygen species such as the superoxide anion may also be generated by LOX action (Roy et al., 1994). These reactive molecules could take part in the oxidative burst that occurs during the plant defense response and are believed to promote hypersensitive cell death (Levine et al., 1994). PUFA hydroperoxides can also undergo enzymatic conversions that prevent their damaging accumulation in plant cells and generate an array of less-reactive species (Siedow, 1991). These secondary LOX products include PUFA-derived compounds such as monoepoxy fatty acids, mono- and trihydroxy fatty acids or volatile alkenals showing antimicrobial activities, and molecules with regulatory functions, such as traumatic acid or jasmonates (JA and MJ) (Farmer, 1994; Creelman and Mullet, 1995). Along with a variety of other biological activities, jasmonates induce the production of proteinase inhibitors in tomato and tobacco (*Nicotiana tabacum* L.) (Farmer and Ryan, 1990; Rickauer et al., 1992) and regulate other wound-inducible or defense-related genes as well (Farmer, 1994). In plant cell cultures, JA accumulation is stimulated by elicitor treatment and seems to take part in a signaling pathway leading from elicitor perception to secondary metabolite production (Mueller et al., 1993).

Together these observations strengthen the hypothesis that LOX may play a role in pathogenesis due to its involvement in the biosynthesis of compounds that are signal or defense molecules. Indeed, stimulation of LOX activity

[1] Part of this work was supported by Rhône Poulenc Agrochimie and the Bio Avenir program no. 9333016.

* Corresponding author; e-mail esquerre@cict.fr; fax 33–61–55–83–78.

Abbreviations: AA, arachidonic acid; JA, jasmonic acid; LOX, lipoxygenase; MJ, methyl jasmonate; *Ppn*, *Phytophthora parasitica nicotianae*; PUFA, polyunsaturated fatty acid; SA, salicylic acid.

has been reported in dicots and monocots after infection with pathogenic microorganisms and viruses, e.g. in tobacco infected with *Erysiphe cichoracearum* (Lupu et al., 1980), *Ppn* (Fournier et al., 1993), or tobacco mosaic virus (Ruzicska et al., 1983), in *Pseudomonas syringae*-inoculated bean (Croft et al., 1990) and *Arabidopsis thaliana* (Melan et al., 1993), in wheat infected with *Puccinia graminis* (Ocampo et al., 1986), and in rice infected with *Magnaporthe grisea* (Ohta et al., 1991). In most cases, a correlation between induction of LOX activity and resistance of the plant has been shown.

We previously demonstrated that LOX activity is rapidly induced in tobacco cells treated with elicitors prepared from the cell walls of *Ppn*, the causal agent of black shank disease (Rickauer et al., 1990; Fournier et al., 1993). An increase in LOX activity is also observed upon microbial elicitor treatment in other plant-microbe models, e.g. wheat-*P. graminis* (Ocampo et al., 1986) and rice-*M. grisea* (Ohta et al., 1991).

LOX gene expression was recently investigated in several plant-pathogen interactions and LOX transcripts were shown to accumulate in rice plants upon inoculation with the fungus *M. grisea* (Peng et al., 1994), and in tomato, bean, and *A. thaliana* plants inoculated with *Pseudomonas* (Koch et al., 1992; Meier et al., 1993; Melan et al., 1993).

A cDNA clone encoding a LOX has been obtained in our laboratory from elicitor-treated tobacco cells (Véronési et al., 1995a). This clone was used to investigate LOX gene expression in tobacco cells and plants.

MATERIALS AND METHODS

Apple's isolates 1156 (race 0) and 1452 (race 1) of *Ppn* were grown on oatmeal agar at 25°C in the dark (Helgeson et al., 1972). Zoospores were obtained by the method of Gooding and Lucas (1959), as already described (Fournier et al., 1993). Elicitors were extracted with hot water from the cell walls of *Ppn* (Pélissier et al., 1986). The ethanol-soluble fraction was used after dialysis (spectrapor M6, cut-off molecular weight 1000) against deionized water and lyophilization.

Cell-suspension cultures of *Nicotiana tabacum* L. cv Wisconsin 38 were grown in a liquid Murashige-Skoog medium modified by Jouanneau (1973) under constant light (10 μE m^{-2} s^{-1}, 400–700 nm) at 24°C in Erlenmeyer flasks. The cells were subcultured every 14 d by adding 35 mL of cell suspension to 250 mL of fresh medium. Seven-day-old tobacco cell cultures were used for the elicitor treatment and prepared as already described (Rickauer et al., 1990). Elicitors and other compounds were added under sterile conditions at the concentrations indicated in the figure legends; cells were harvested at different times afterward, frozen in liquid nitrogen, and stored at −80°C.

Tobacco plants (*N. tabacum* L. isolines 46–8 and 49–10) (Helgeson et al., 1972) were grown on vermiculite in a greenhouse. Line 46–8 is resistant to race 0 and susceptible to race 1 of *Ppn*, whereas line 49–10 is susceptible to both races of the pathogen. Inoculation of 5-week-old plants with zoospores of *Ppn* was performed as previously described (Fournier et al., 1993).

LOX Assay and Protein Measurement

The enzyme assay was performed using a polarographic method as described elsewhere (Fournier et al., 1993). LOX activity was expressed in nkat mg^{-1} protein.

The protein content of the extracts was determined according to Lowry et al. (1951) after precipitation with TCA. Soybean LOX type I (Sigma) was used as the standard protein.

Isolation of RNA and DNA

Total RNA was extracted from tobacco cells or plants according to Haffner et al. (1978). RNA concentration was measured spectrophotometrically at 260 nm.

Total genomic DNA was isolated from tobacco cells as described by Dellaporta et al. (1983).

LOX Probes

As previously described (Véronési et al., 1995a, 1995b), two tobacco LOX cDNA clones were isolated successively. The first, pTL-35, was obtained after construction of a cDNA library from elicitor-treated cells and screening with a fragment of a pea cDNA clone (Casey et al., 1985) and contained a partial (1.4-kb) LOX insert (Véronési et al., 1995b). Screening of a second cDNA library from elicitor-treated cells with TL-35 allowed the isolation of a full-length clone, pTL-J2 (2.9 kb). Sequencing of these clones showed that TL-35 was an internal *Eco*RI fragment of TL-J2.

Northern Blot Hybridization

Total RNA (10 μg/track) was separated on a denaturing 1.2% agarose, 20% (v/v) formaldehyde gel; rRNA bands were visualized by coloration with ethidium bromide to ensure that equal amounts of intact RNA had been applied. After passive transfer to nitrocellulose membranes (Schleicher & Schuell), the RNA was fixed irreversibly by incubation at 80°C under vacuum for 2 h. The membranes were prehybridized for 4 h at 42°C in 50% (v/v) formamide, 2× SSC, 1× Denhardt's solution, 0.1% (w/v) SDS, and 100 μg/mL denatured calf thymus DNA, then hybridized for 15 h with TL-35 under the same conditions. The probe was labeled with [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983). The membranes were successively washed in 2× SSC, 0.1% SDS at 62°C, and exposed to hyperfilm MP (Amersham) at −80°C. Each experiment, including sample preparation and RNA isolation, was carried out two or three times independently.

Southern Blot Hybridization

Ten micrograms of DNA from tobacco cells was digested with restriction enzymes at 37°C, separated on a 0.8% agarose gel, transferred to a nitrocellulose membrane (Schleicher & Schuell) after alkaline treatment according to Sambrook et al. (1982), and fixed at 80°C for 2 h under

vacuum. Prehybridization was performed in 5× SSPE, 5× Denhardt's solution, 0.1% (w/v) SDS, and 100 μg/mL denatured calf thymus DNA at 65°C for 4 h, and hybridization continued in the same solution containing the radioactive LOX probe. Hybridized membranes were washed with 2× SSC, 0.1% SDS at 65°C, and exposed to hyperfilm MP at −80°C.

RESULTS

Restriction Maps of the LOX Probes

Two tobacco LOX cDNA clones, pTL-35 and pTL-J2, were previously isolated from two independent cDNA libraries prepared from cell cultures treated with an elicitor of *Ppn*, a soil pathogen of this plant. pTL-35 contained a 1.4-kb insert, which covered about one-half of the entire LOX sequence (Véronési et al., 1995b), whereas pTL-J2 was a full-length clone containing 5′ and 3′ nontranslated regions and a poly(A) sequence (Véronési et al., 1995a). Restriction maps of these clones are shown in Figure 1. Sequencing of both clones revealed that TL-35 was an *Eco*RI fragment of TL-J2 (nucleotides 1220–2635), and that the two clones contained the conserved His-binding domain II of LOXs. The LOX cDNA TL-35 was used in all northern blot experiments to avoid problems that might result from a too-large LOX cDNA probe.

Southern Blot Analysis

DNA from tobacco cells that had been digested with different restriction enzymes was analyzed by Southern blot hybridization to the two LOX cDNAs, TL-35 and TL-J2. With both probes, several distinct bands appeared after digestion with *Eco*RI, *Bam*HI, *Hin*dIII, or *Xba*I (an enzyme that does not cut inside TL-J2), suggesting the occurrence of a small LOX gene family in tobacco (Fig. 2). Not much difference was observed between hybridization with the two LOX probes, with the exception of a weakly hybridizing additional band of about 4.4 kb after digestion with *Eco*RI and an additional faint band of about 2 kb after *Bam*HI digestion, when TL-J2 was retained as a probe. The weak hybridization of these additional bands might be due to the presence of large introns such as those that have been found in soybean, bean, and Arabidopsis LOX genes (Kato et al., 1993; Eiben and Slusarenko, 1994; Melan et al., 1994).

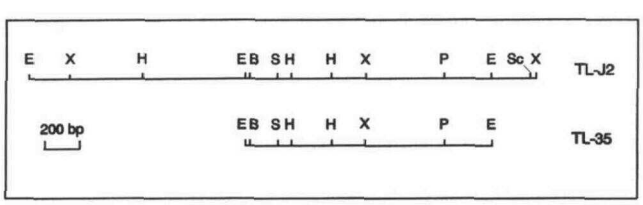

Figure 1. Partial restriction map of tobacco LOX cDNA clones TL-35 and TL-J2. Restriction endonuclease site positions were deduced from double-digestion experiments and confirmed after sequence analysis of both clones. Digestions were performed with the following enzymes: *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Sal*I (S), *Sca*I (Sc), and *Xho*I (X).

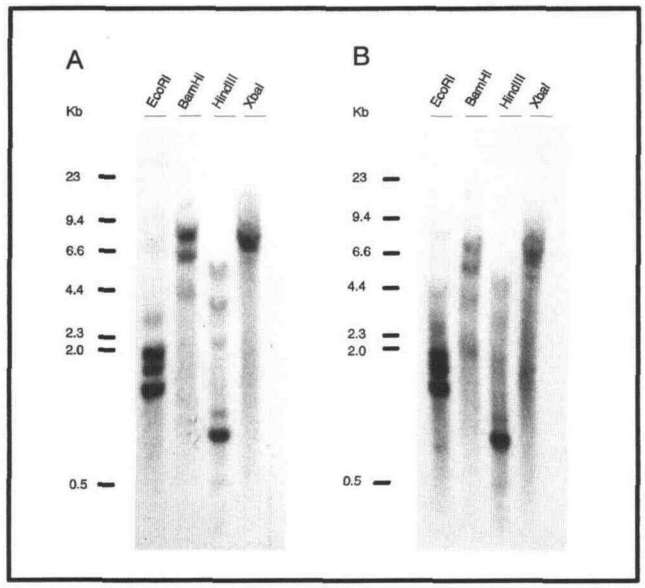

Figure 2. Southern blot analysis of tobacco LOX DNA. Total genomic DNA (10 μg) was digested with either *Eco*RI, *Bam*HI, *Hin*dIII, or *Xba*I. After electrophoresis on a 0.8% agarose gel, denaturation, and transfer to a nitrocellulose membrane, hybridization was carried out with a [α-^{32}P]dCTP-labeled TL-35 (A) or TL-J2 (B) LOX probe. Size markers (kb) generated from *Hin*dIII-digested λ DNA are indicated on the left.

Expression of LOX in Tobacco Cell Cultures

A time-course study of LOX gene expression was performed in control or elicitor-treated tobacco cells. Northern blot hybridization of total RNA to TL-35 cDNA showed that LOX transcripts of about 3 kb began to accumulate in tobacco cells within the first 2 h after addition of elicitor, reached a maximum level between 8 and 24 h, and declined thereafter (Fig. 3). In one experiment LOX gene expression was detected as soon as 1 h after elicitor treatment (data not shown). No transcripts were detectable in control cells during the entire period of the experiment, thereby indicating that TL-35 (and TL-J2) corresponded to an elicitor-induced LOX gene.

LOX Activity and Expression in Healthy or Infected Tobacco Seedlings

We previously reported that LOX activity was induced in tobacco cv Kentucky 15 seedlings upon infection with zoospores of a compatible race of *Ppn* (Fournier et al., 1993). To further investigate whether this stimulation might be related to resistance in tobacco, we measured LOX activity in both compatible and incompatible interactions. Two near-isogenic lines of tobacco, 46–8 and 49–10, were root-inoculated with zoospores of race 0 or 1 of *Ppn*; isoline 46–8 is resistant to race 0 of the fungus, whereas all other combinations result in compatible interactions (Table I). In all interactions, a transient stimulation of LOX activity was observed after inoculation; the enzyme activity remained low in mock-inoculated plants. This stimulation appeared earlier (d 3) in the

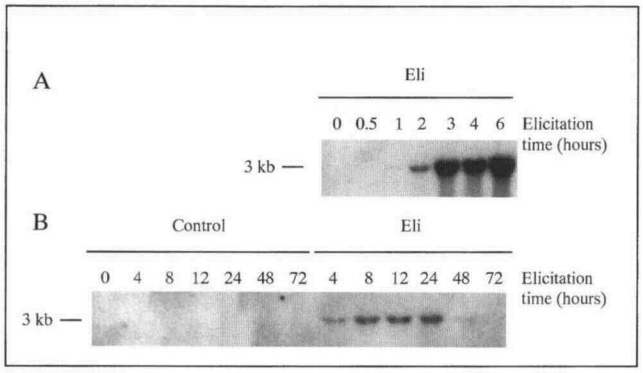

Figure 3. Time-course study of LOX gene expression in tobacco cell-suspension culture. Tobacco cells were treated with water (Control) or *Ppn* elicitor at a final concentration of 30 μg/mL (Eli) during 6 h (A) or up to 72 h (B). After the indicated incubation times total RNA was extracted and samples (10 μg) were subjected to electrophoresis on a formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and hybridized with the radiolabeled TL-35 LOX probe. Gels were stained with ethidium bromide to ensure that equal amounts of intact RNA had been charged. Membranes were exposed to x-ray films for 96 h in A and for 24 h in B.

incompatible interaction (Fig. 4A) than in the three compatible interactions (Fig. 4, A and B), where it peaked at the time of disease symptom appearance (d 4). Black shank disease symptoms developed only in compatible interactions, whereas resistant plants remained devoid of any visible symptoms. Since whole plants were used for this study, it is likely that the recorded values were underestimated at early stages of the interaction. Indeed, at these stages this disease concerns primarily the basal part of tobacco.

We subsequently focused on isoline 46–8, which harbors a resistance locus to race 0 but not to race 1 of the fungus and shows a differential activation of LOX upon inoculation with the two races. LOX gene expression was examined by northern blot analysis of total RNA from roots of healthy or inoculated seedlings. The data included in Figure 5 are representative of two independent experiments and show that LOX transcripts appeared early and transiently in roots of infected seedlings, whereas they were not detected in control, mock-inoculated roots. Moreover, LOX transcripts accumulated earlier in the incompatible interaction, with a maximum level around 8 h after inoculation, than in the compatible interaction, in which maximum intensity was reached 24 to 48 h after inoculation.

Table I. *Diagram of the race-cultivar-specific interaction between tobacco isolines and P. parasitica nicotianae*

I, Incompatible; C, compatible.

P. parasitica nicotianae	*N. tabacum* Isolines	
	46–8	49–10
Race 0	I	C
Race 1	C	C

Expression of LOX in Tobacco Cells upon Treatment with Putative Signal Molecules

To further investigate the mechanism by which fungal elicitors may induce LOX gene expression, several putative plant signal molecules were examined for their potential ability to mimic elicitor activity. Previous results from our laboratory have shown that MJ is able to induce defense proteins in tobacco cells (Rickauer et al., 1992). Although a moderate induction of LOX activity was observed after treatment with 50 μM MJ, the concentration of 800 μM was retained, since it gave a maximal response for LOX (C. Véronési, unpublished results) and proteinase inhibitor induction (Rickauer et al., 1992). This value seems high, but is only a theoretical value, since the commercial solution contains both active and inactive isomers in an undetermined ratio. Furthermore, the lipophilic and volatile nature of this compound makes it probable that the actual quan-

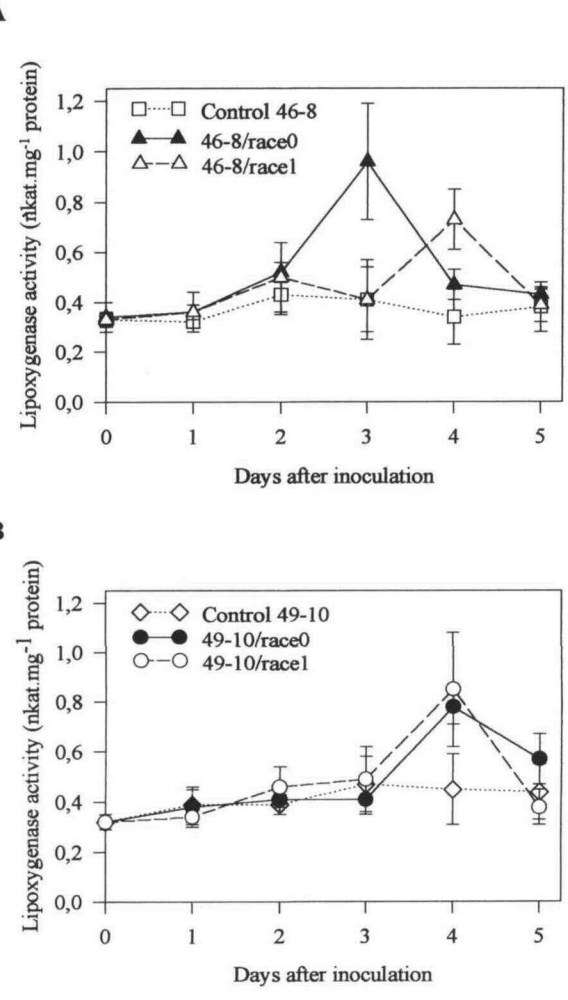

Figure 4. Time course of LOX activity in tobacco plants infected with *Ppn*. LOX activity was measured at the indicated times in seedlings that had been root-inoculated with or without (Control) zoospores from *Ppn* race 0 or race 1. A, Tobacco line 46–8 seedlings; B, tobacco line 49–10 seedlings. Reported values are the means of three independent experiments ± SD.

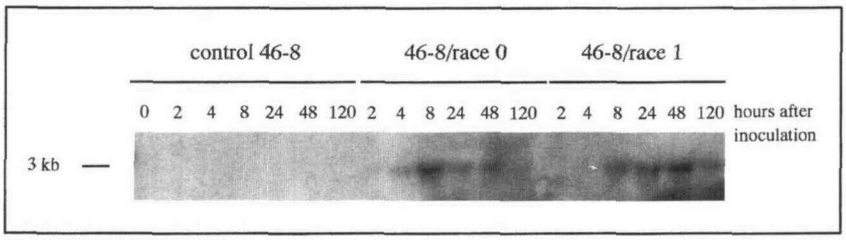

Figure 5. Northern blot analysis of LOX transcripts in tobacco plants after root-inoculation with *Ppn*. Total RNA was extracted from roots of line 46–8 seedlings at the indicated times after mock-inoculation (control) or inoculation with zoospores of *Ppn* race 0 or race 1. Total RNA (10 μg) from roots was separated on a formaldehyde-agarose gel and transferred to a nitrocellulose membrane. Hybridization of the blot was performed with the radiolabeled TL-35 probe.

tity of MJ dissolved in the incubation medium is significantly lower.

SA is involved in pathogenesis-related protein gene induction during the establishment of systemic acquired resistance in tobacco (Vernooij et al., 1994). The effect of MJ and SA on LOX gene expression was studied by northern blot analysis 12 h after the addition of these compounds. At this time, LOX transcripts are fully induced by the fungal elicitor. Figure 6A shows that MJ is also able to induce LOX transcript accumulation, whereas SA had no effect under the conditions of the assay. Linoleic and linolenic acid, the two substrates for LOX in plants, are supposed to be released from membrane phospholipids during elicitation (Mueller et al., 1993; Roy et al., 1995). Linolenic acid, a precursor for JA biosynthesis, stimulated LOX gene expression, although more weakly than the fungal elicitor (Fig. 6B), whereas linoleic acid was inactive. Induction of LOX gene expression and enzyme activity by MJ was more prolonged than stimulation by the fungal elicitor (Fig. 7).

DISCUSSION

Recently we reported the purification of an elicitor-induced LOX from a tobacco cell suspension, with a pI of approximately 5.1 and a molecular weight of about 96,000 (Fournier et al., 1993). To study LOX gene expression in defense-related situations, we isolated two LOX clones (TL-35 and TL-J2) from two independent cDNA libraries of elicitor-treated tobacco cells. The TL-J2 clone covers the TL-35 clone and contains a 2.9-kb cDNA insert, which encodes an 862-amino acid polypeptide with a calculated molecular weight of 97,577 and a pI of around 5.3. A comparison of the deduced amino acid sequence of this tobacco LOX with other plant LOX sequences suggests that TL-J2 cDNA covers the whole protein and includes four

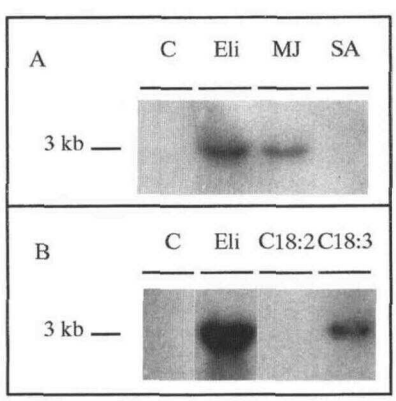

Figure 6. Northern blot analysis of LOX mRNA in tobacco cells upon MJ, SA, or fatty acid treatment. A, Cultured cells were treated with ethanol (C) (1 μL/mL), elicitor (Eli) (30 μg/mL), MJ (800 μM), or SA (100 μM). B, Cell suspensions were treated with water (C), elicitor (Eli) (30 μg/mL), 150 μM linoleic acid (C18:2), or 150 μM linolenic acid (C18:3). Incubation time was 12 h. Total RNA (10 μg) was separated on a formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and hybridized with the radiolabeled TL-35 probe.

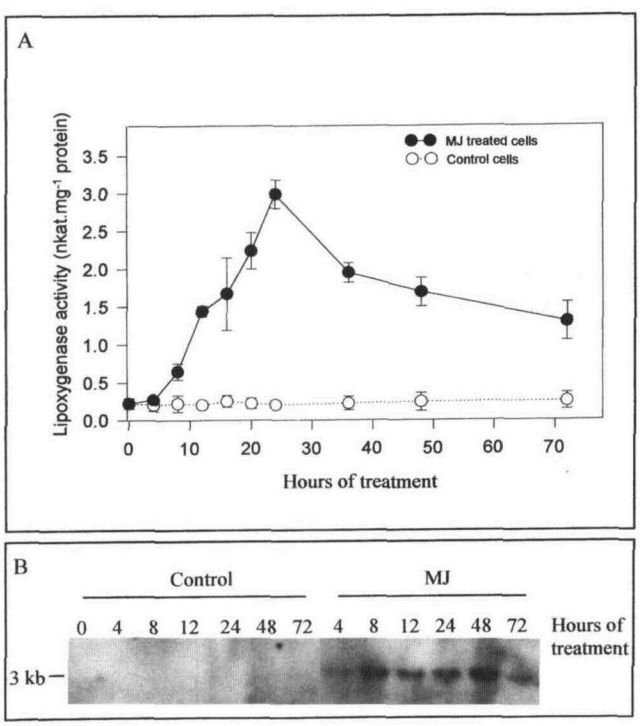

Figure 7. Time course of LOX enzyme activity (A) and LOX gene expression (B) in tobacco cells treated with MJ. Tobacco cell suspensions were incubated with ethanol (control) or MJ (800 μM) for various times. LOX enzyme activity was measured polarographically with linoleic acid as a substrate (A). LOX gene expression was determined by northern blot analysis (B). Ten micrograms of total RNA was separated by denaturing electrophoresis, transferred onto a nitrocellulose membrane, and hybridized with the radiolabeled TL-35 probe.

regions that are highly conserved among LOXs (Siedow, 1991; Véronési et al., 1995a). The primary sequence of this polypeptide shows homology (40–80%) to other animal or plant LOXs, the highest homology (70–80%) being recorded within solanaceous plants and with *A. thaliana* Lox-1 (Melan et al., 1993). It also has 80% identity to a partial tobacco LOX genomic clone (Bookjans et al., 1988). Compared with this clone, differences in amino acid residues are found throughout the sequence and even within the regions of high homology, which leads us to the conclusion that pTL-J2 corresponds to a different tobacco LOX gene, and that at least two LOX genes occur in tobacco. This was confirmed by Southern blot hybridization with TL-J2 and TL-35. The occurrence of a small number of LOX genes in the same species has been reported for a number of plants, including *A. thaliana*, tomato, potato, and rice (Bell and Mullet, 1993; Ferrie et al., 1994; Geerts et al., 1994; Peng et al., 1994).

Northern blot analysis revealed the presence of LOX transcripts in elicitor-treated cells only, but not in water-treated control cells, indicating that LOX is not constitutively expressed and that the cloned cDNA derives from a tobacco LOX gene with an expression induced by a fungal elicitor. However, since TL-J2 detected at least two genes in Southern blot analysis, it cannot be ruled out that the signal obtained by northern blot hybridization corresponds to two transcripts of the same size. The time course of the transient accumulation of LOX transcripts is in agreement with the previously reported transient induction of LOX activity in elicitor-treated tobacco cells (Rickauer et al., 1990).

LOX activity and gene expression were also examined in tobacco seedlings root-inoculated with zoospores of *Ppn*. This mode of inoculation has been retained because it closely reflects the situation occurring during natural infection in the field. Two near-isogenic lines of tobacco were used in combination with two races of the fungus, allowing the comparison of incompatible versus compatible interactions. Inoculation of tobacco plants resulted in increased levels of LOX activity as well as gene expression, as it has also been reported to occur in other plant-microbe systems (Koch et al., 1992; Melan et al., 1993, Peng et al., 1994). The transient increase in total activity, as well as transcript accumulation in the roots, occurred earlier in the incompatible than in the compatible interactions, which is in good agreement with a presumed role of LOX in disease resistance. The difference of 1 d between both interactions is interesting given the time scale of our experiments, since susceptible plants were totally colonized by the pathogen after 6 d and died thereafter. It is highly likely that the same LOX gene is expressed in inoculated seedlings and elicitor-treated cells; however, this cannot be definitively concluded from our experiments and will be investigated in the future.

In contrast to published data obtained with bean, *A. thaliana*, and potato (Meier et al., 1993; Melan et al., 1993; Geerts et al., 1994), LOX transcripts could not be detected in uninoculated tobacco roots, aerial parts (data not shown), and untreated cell suspensions. Only a faint hybridization was observed with the RNA isolated from young leaves, sepals, petals, and germinating seeds. Thus, the transcripts revealed by northern blot analysis are not induced by nonspecific stress, e.g. wounding during the manipulation of cell cultures or seedlings, which makes this clone an interesting probe for studying the tobacco-*Ppn* interaction.

Since jasmonates (JA and MJ) are compounds derived from the action of LOX on polyunsaturated fatty acids and have been associated with wound and defense responses of plants (Farmer, 1994), it was interesting to look for their possible effect on LOX itself. The present work shows that LOX gene expression and activity are also induced by MJ in tobacco, with a time course roughly similar to induction by a fungal elicitor. This is in agreement with the previously reported induction of LOX by MJ in soybean (Grimes et al., 1992), *A. thaliana* (Bell and Mullet, 1993; Melan et al., 1993), and barley (Feussner et al., 1995). JA has been shown to accumulate in cell cultures and plants in response to elicitor treatment (Mueller et al., 1993). A transient production of JA is also observed in tobacco cell cultures treated with *Ppn* elicitor (M. Rickauer, unpublished results). Jasmonates might mediate fungal elicitor effects on tobacco defense responses, including LOX stimulation, via the so-called octadecanoid pathway (Farmer and Ryan, 1992), as was recently suggested for other plant systems (Creelman and Mullet, 1995). This pathway involves linolenic acid release from membrane lipids, followed by conversion of this fatty acid into JA, a reaction catalyzed by several enzymes, notably LOX, allene oxide synthase, and allene oxide cyclase (Vick and Zimmerman, 1984; Farmer and Ryan, 1992). Indeed, an increase in lipid acyl hydrolase activity was recently reported in tobacco cells after treatment with *Ppn* elicitor (Roy et al., 1995), and linolenic acid itself is able to induce LOX gene expression in tobacco (this work) and promote proteinase inhibitor accumulation in tomato (Farmer and Ryan, 1992).

SA had no effect under the retained conditions. This compound has been proposed as an endogenous plant signal leading to the induction of defense molecules and systemic acquired resistance in tobacco (Vernooij et al., 1994). In our system, however, it failed to induce LOX expression or the production of proteinase inhibitors (Rickauer et al., 1990). Similar results have been reported for *A. thaliana* LOX 1 and 2, which were induced by MJ but not by sodium salicylate (Bell and Mullet, 1993). It is interesting that SA and aspirin have been shown to inhibit wound-induced gene expression in tomato, and aspirin also inhibited wound-induced production of JA (Pena-Cortés et al., 1993). Thus, SA seems to exert a negative regulation on the LOX pathway, at least in the wound response. The relationship between the two putative signal compounds JA and SA, and their respective pathways, deserves to be further investigated.

Several roles for LOX in plant-microbe interactions have now been suggested. During the last few years it has turned out that this enzyme is able to generate molecules that can be divided into three groups with different functions: (a) hydroperoxides and free radicals that might be involved in the localized cell death observed during the

hypersensitive response; (b) signal molecules such as JA and its methyl ester that can trigger defense gene expression and amplify the initial response; and (c) antimicrobial compounds such as 2-*trans*-hexenal that constitute a direct defense against pathogen attack (Croft et al., 1993). These putative functions might well be fulfilled by more than one LOX. Indeed, most plants appear to produce several isoforms of this enzyme. Local and systemic plant responses to invasion by pathogens might involve distinct LOXs as well. Whether the LOX transcripts induced by elicitors, MJ, or pathogen infection derive from the same gene or from different genes that are specifically expressed can only be addressed by the use of gene-specific probes. Recently, a specific expression pattern of different 3-hydroxy-3-methylglutaryl-CoA reductase genes has been reported to occur in potato after treatment with MJ and the fungal elicitor AA (Choi et al., 1994). The isolation of probes specific for the different genes is therefore a prerequisite for the investigation of the temporal, spatial, and differential expression of LOX(s).

The isolated tobacco LOX cDNA clone allowed us to study LOX gene expression in tobacco during the interaction with the fungal pathogen *Ppn*. Differential gene expression and activity of LOX in compatible and incompatible interactions reinforce the hypothesis of a relationship between LOX and resistance. Further work will therefore be aimed at engineering tobacco plants with a modified LOX gene expression. Analysis of defense responses and resistance patterns in these plants should help us to gain more insight into the role of LOX during plant-pathogen interactions.

ACKNOWLEDGMENTS

We are indebted to Drs. J.P. Helgeson and E. Farmer for kindly providing tobacco seeds and *Phytophthora* isolates, and to Dr. C. Domoney for her gracious gift of pea LOX cDNA. We thank H. Grimal for cell culture, M. Marolda for DNA sequencing, and P. Sanchez for elicitor preparation.

Received March 20, 1996; accepted June 4, 1996.
Copyright Clearance Center: 0032-0889/96/112/0997/08.

LITERATURE CITED

Bell E, Mullet JE (1993) Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol **103:** 1133–1137

Bookjans G, Altschuler M, Brockman J, Yenofsky R, Polacco J, Dickson R, Collins G, Hildebrand D (1988) Molecular biological studies of plant lipoxygenases. *In* TH Applewhite, ed, Proceedings of the World Conference on Biotechnology for the Fats and Oils Industry. Kraft Inc, Glenview, IL, pp 301–304

Casey R, Domoney C, Nielsen NC (1985) Isolation of a cDNA clone for pea (*Pisum sativum*) seed lipoxygenase. Biochem J **232:** 79–85

Choi D, Bostock RM, Avdiushko S, Hildebrand DF (1994) Lipid-derived signals that discriminate wound- and pathogen-responsive isoprenoid pathways in plants: methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. Proc Natl Acad Sci USA **91:** 2329–2333

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA **92:** 4114–4119

Croft KPC, Jüttner F, Slusarenko AJ (1993) Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. Plant Physiol **101:** 13–24

Croft KPC, Voisey CR, Slusarenko AJ (1990) Mechanism of hypersensitive cell collapse: correlation of increased lipoxygenase activity with membrane damage in leaves of *Phaseolus vulgaris* (L.) inoculated with an avirulent race of *Pseudomonas syringae* pv. *phaseolicola*. Physiol Mol Plant Pathol **36:** 49–62

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Eiben HG, Slusarenko AJ (1994) Complex spatial and temporal expression of lipoxygenase genes during *Phaseolus vulgaris* (L.) development. Plant J **5:** 123–135

Farmer EE (1994) Fatty acid signalling in plants and their associated microorganisms. Plant Mol Biol **26:** 1423–1437

Farmer EE, Ryan C (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA **87:** 7713–7716

Farmer EE, Ryan C (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell **4:** 129–134

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Ferrie BJ, Beaudoin N, Burkhart W, Bowsher CG, Rothstein SJ (1994) The cloning of two tomato lipoxygenase genes and their differential expression during fruit ripening. Plant Physiol **106:** 109–118

Feussner I, Hause B, Vörös K, Parthier R, Wasternak C (1995) Jasmonate-induced lipoxygenase forms are localized in chloroplasts of barley leaves (*Hordeum vulgare* cv. Salome). Plant J **7:** 949–957

Fournier J, Pouénat M-L, Rickauer M, Rabinovitch-Chable H, Rigaud M, Esquerré-Tugayé M-T (1993) Purification and characterization of an elicitor-induced lipoxygenase in tobacco cell. Plant J **3:** 63–70

Gardner HW (1991) Recent investigations into the lipoxygenase pathway of plants. Biochim Biophys Acta **1084:** 221–239

Geerts A, Feltkamp D, Rosahl S (1994) Expression of lipoxygenase in wounded tubers of *Solanum tuberosum* L. Plant Physiol **105:** 269–277

Gooding GV, Lucas GB (1959) Factors influencing sporangial formation and zoospore activity in *Phytophthora parasitica* var. *nicotianae*. Phytopathology **49:** 277–281

Grimes HD, Koetje DS, Franceschi VR (1992) Expression, activity, and cellular accumulation of methyl jasmonate-responsive lipoxygenase in soybean seedlings. Plant Physiol **100:** 433–443

Haffner MH, Chin MB, Lane BG (1978) Wheat embryo ribonucleases. XII. Formal characterization of terminal and penultimate nucleoside residues at 5′ end of "capped" RNA from imbibing wheat embryo. Can J Biochem **56:** 729–733

Helgeson JP, Kemp JD, Haberlach GT, Maxwell DP (1972) A tissue culture system for studying disease resistance: the black shank disease in tobacco callus cultures. Phytopathology **62:** 1439–1443

Hildebrand DF (1989) Lipoxygenase. Physiol Plant **76:** 249–253

Jouanneau JP (1973) Contribution à l'étude du rôle moléculaire des cytokinines dans le contrôle de la mitose chez les cellules de tabac. PhD thesis, Université Paris-Sud, Orsay, France

Koch E, Meier BM, Eiben HG, Slusarenko AJ (1992) A lipoxygenase from leaves of tomato (*Lycopersicon esculentum* Mill) is induced in response to plant pathogen *Pseudomonas*. Plant Physiol **99:** 571–576

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:** 583–593

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275

Lupu R, Grossman S, Cohen Y (1980) The involvement of lipoxygenase and antioxidants in pathogenesis of powdery mildew on tobacco plants. Physiol Plant Pathol **16:** 241–248

Meier B, Shaw N, Slusarenko AJ (1993) Spatial and temporal accumulation of defense gene transcripts in bean (*Phaseolus vulgaris*) leaves in relation to bacteria-induced hypersensitive cell death. Mol Plant Microbe Interact **6:** 453–466

Melan AM, Dong X, Endara ME, Davis KR, Ausubel FM, Peterman TK (1993) An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid and methyl jasmonate. Plant Physiol **101:** 441–450

Melan AM, Nemhauser JL, Peterman TK (1994) Structure and sequence of the *Arabidopsis thaliana* lipoxygenase-1 gene. Biochim Biophys Acta **1210:** 377–380

Mueller MJ, Brodschelm W, Spannagl E, Zenk MH (1993) Signalling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc Natl Acad Sci USA **90:** 7490–7494

Nicolaou KC, Ramphal JY, Petasis NA, Serhan CN (1991) Lipoxins and related eicosanoids: biosynthesis, biological properties, and chemical synthesis. Angew Chem Int Ed Engl **30:** 1100–1116

Ocampo CA, Moerschbacher B, Grambow HJ (1986) Increased lipoxygenase activity is involved in the hypersensitive response of wheat leaf cells infected with avirulent rust fungi or treated with fungal elicitor. Z Naturforsch **41c:** 558–563

Ohta H, Shida K, Peng YL, Furusawa I, Shishiyama J, Aibara S, Morita Y (1991) A lipoxygenase pathway is activated in rice after infection with the rice blast fungus *Magnaporthe grisea*. Plant Physiol **97:** 94–98

Pélissier B, Thibaud JB, Grignon C, Esquerré-Tugayé MT (1986) Cell surfaces in plant-microorganism interactions. VII. Elicitor preparations from fungal pathogens depolarize plant membranes. Plant Sci **46:** 103–109

Pena-Cortés H, Albrecht T, Prat S, Weiler EW, Willmitzer L (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta **191:** 123–128

Peng YL, Shirano Y, Ohta H, Hibiro T, Tanaka K, Shibata D (1994) A novel lipoxygenase from rice. Primary structure and specific expression upon incompatible infection with rice blast fungus. J Biol Chem **269:** 3755–3761

Rickauer M, Bottin A, Esquerré-Tugayé M-T (1992) Regulation of proteinase inhibitor production in tobacco cells by fungal elicitors, hormonal factors and methyl jasmonate. Plant Physiol Biochem **30:** 579–584

Rickauer M, Fournier J, Pouénat M-L, Berthalon E, Bottin A, Esquerré-Tugayé M-T (1990) Early changes in ethylene synthesis and lipoxygenase activity during defense induction in tobacco cells. Plant Physiol Biochem **28:** 647–653

Ricker KE, Bostock RM (1994) Eicosanoids in the *Phytophthora infestans*-potato interaction: lipoxygenase metabolism of arachidonic acid and biological activities of selected lipoxygenase products. Physiol Mol Plant Pathol **44:** 65–80

Roy P, Roy SP, Mitra A, Kulkarni AP (1994) Superoxide generation by lipoxygenase in the presence of NADH and NADPH. Biochim Biophys Acta **1214:** 171–179

Roy S, Pouénat M-L, Caumont C, Cariven C, Prévost M-C, Esquerré-Tugayé M-T (1995) Phospholipase activity and phospholipid patterns in tobacco cells treated with fungal elicitor. Plant Sci **107:** 17–25

Ruzicska P, Gombos Z, Farkas GL (1983) Modification of the fatty acid composition of phospholipids during the hypersensitive reaction in tobacco. Virology **128:** 60–64

Sambrook J, Fritsch EF, Maniatis T (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Siedow JN (1991) Plant lipoxygenases: structure and function. Annu Rev Plant Physiol Plant Mol Biol **42:** 145–188

Toh H, Urade Y, Tanabe T (1992) Molecular evolution of enzymes involved in the arachidonic acid cascade. Mediators of Inflammation **1:** 223–233

Vernooij B, Uknes S, Ward E, Ryals J (1994) Salicylic acid as a signal molecule in plant-pathogen interactions. Curr Opin Cell Biol **6:** 275–279

Véronési C, Fournier J, Rickauer M, Marolda M, Esquerré-Tugayé M-T (1995a) Nucleotide sequence of an elicitor-induced tobacco lipoxygenase cDNA (GenBank X84040). Plant Physiol **108:** 1342

Véronési C, Fournier J, Rickauer M, Pouénat M-L, Esquerré-Tugayé M-T (1995b) Cloning of an elicitor-induced lipoxygenase cDNA from tobacco. *In* J-C Kader, P Mazliak, eds, Plant Lipid Metabolism. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 286–288

Vick BA, Zimmerman DC (1984) Biosynthesis of jasmonic acid by several plant species. Plant Physiol **75:** 458–461