Lipid peroxidation was investigated in relation with the hypersensitive reaction in cryptogeen-elicited tobacco leaves. A massive production of free polyunsaturated fatty acid (PUFA) hydroperoxides dependent on a 9-lipoxygenase (LOX) activity was characterized during the development of leaf necrosis. The process occurred after a lag phase of 12 h, was accompanied by the concomitant increase of 9-LOX activity, and preceded by a transient accumulation of LOX transcripts. Free radical-mediated lipid peroxidation represented 10% of the process. Inhibition and activation of the LOX pathway was shown to inhibit or to activate cell death, and evidence was provided that fatty acid hydroperoxides are able to mimic leaf necrotic symptoms. Within 24 h, about 50% of leaf PUFAs were consumed, chloroplast lipids being the major source of PUFAs. The results minimize the direct participation of active oxygen species from the oxidative burst in membrane lipid peroxidation. They suggest, furthermore, the involvement of lipase activity to provide the free PUFA substrates for LOX. The LOX-dependent peroxidative pathway, responsible for tissue necrosis, appears as being one of the features of hypersensitive programmed cell death.

In plant-pathogen interactions, a typical feature of plant resistance is hypersensitive reaction (HR), characterized by the induction of rapid cell death at the site of an attempted attack by either an avirulent strain of a pathogen or a non-pathogen. The collapse of challenged cells, occurring during incompatible interactions, was shown in most cases to be dependent on a gene for gene plant pathogen interaction (1, 2). HR is accompanied by a battery of defense mechanisms including de novo synthesis of antimicrobial enzymes and metabolites, strengthening of the cell wall, and the onset of systemic acquired resistance dependent on salicylic acid accumulation (3, 4). HR often leads to dry lesions that are supposed to limit pathogen growth. Other proposed roles is the release in apoplasm of defense-related proteins and toxic metabolites, as well as of signals that activate the defenses of both neighboring and distant cells. Hypersensitive cell death appears to not be the result of the direct action of released pathogenic factors but is rather under the genetic control of the host. Indeed, several observations underline that HR is an example of PCD in plants (1, 2). Furthermore, hypersensitive cell death has morphological and molecular features similar to the mammalian PCD, called apoptosis. These include cytoplasm and chromatin condensation followed by their fragmentation, activation of calcium-dependent endonucleases (5–8) and of cysteine proteases (9–11), and involvement of similar regulation factors (2). Some differences between HR and mammalian apoptosis were observed, however, such as changes in DNA laddering (5, 8) and the lack in HR of the repressor role of Bcl-xL (12). One ultimate characteristic of HR is the loss of membrane integrity, and thus HR is often characterized by an associated electrolyte leakage (5, 13). This feature is not encountered in mammalian apoptosis but is one characteristic of the catastrophic cell death called necrosis, which is not dependent on gene activation (14). In this way, the use of the term “necrosis” assumes different meanings when referring to mammalian cell death or to pathogen-associated plant cell death.

Membrane damage during HR is in close correlation with lipid peroxide production and with AOS generation (12, 15–17). AOS can initiate lipid peroxidation in membranes by fatty acid free radical production, and the process can be propagated by autoxidation (18). The generation of AOS during the oxidative decatrienoic acid; 12-HOTE, 12-hydroxy-9,13,15(Z,E,Z)-octadecatrienoic acid; 13-HOTE, 13-hydroxy-9,11,15(Z,E,Z)-octadecatrienoic acid; 16-HOTE, 16-hydroxy-9,12,14(Z,E)-octadecatrienoic acid; 9-HPODE, 9-hydroperoxy-10,12(Z,E)-octadecadienoic acid; 13-HPODE, 13-hydroperoxy-9,11(E,Z)-octadecatrienoic acid; 9-HPOTE, 9-hydroperoxy-10,12,15(E,Z,Z)-octadecatrienoic acid; tert-ButOOH, tert-butyl hydroperoxide.
burst is an important early event during the course of plant-pathogen interactions and is well documented (19, 20). In the HR, lipid peroxidation is often a late process occurring at the same time as the appearance of necrosis. Since AOS production preceded lipid peroxidation, it is generally admitted that AOS are implicated in the initiation of membrane damage, and hence hypersensitive cell death. Indeed, inhibition of oxidative burst by exogenous supplied enzymes, scavengers, or inhibitors of AOS generator systems suppresses or delays both lipid peroxidation and hypersensitive cell death (15, 21–23).

Lipid peroxidation might also be due to LOX (EC 1.13.11.12) activity (24, 25). Initiation of HR membrane damage by LOXs has been suggested as an alternative hypothesis to free radical action, and the process might be propagated by autoxidation (26, 27). Indeed, induction of LOX activity has been observed in several plants during incompatible interaction and occurs after a lag phase of few hours (28). The observation that an incompatible interaction can be suppressed in transgenic tobacco plants expressing antisense LOX clearly demonstrates the role of LOX in plant resistance to pathogens (29). Finally, since (i) the LOX pathway leads to products, such as hydroperoxides, alkenals, and aldehydes, that may kill plant cells and pathogen (30, 31) and (ii) HR triggering is an example of PCD, the induction of a LOX pathway could be considered as an active process of membrane degradation leading to plant cell death.

Thus, it is not clear at present whether lipid peroxidation during HR is induced by AOS and free radicals or is the result of a LOX action. Both mechanisms could operate in parallel or be exclusive. Furthermore, the question of whether membrane lipid peroxidation induces cell death or is the consequence of cell death is still open.

The HR induced in tobacco (Nicotiana tabacum) leaves by cryptogeen, a purified protein from the fungus Phytophthora cryptoptega (32), was investigated in this work. Cryptogeen leads also to defense gene activation (33) and systemic acquired resistance (34). Features of PCD were observed, as assessed by plasma membrane blebbing, cell shrinkage, and cyttoplasmic condensation (6), and the expression of har 203J, a gene proposed as the hallmark of HR-inducing pathogens or elicitors (35). On tobacco cells cryptogeen induces an early oxidative burst (36) and late LOX activity (37). An early AOS production was also characterized on leaves (38). The AOS production and lipid peroxidation induced by cryptogeen are in close correlation with the intensity of necrosis (17). Applied to detached leaves cryptogeen causes total leaf necrosis, and this model appears perfectly suited to biochemical analyses of necrotic associated processes. Molecular insights on the peroxidation regiospecificity and enantioselectivity of PUFAs were expected to discriminate between a free radical mediated process, or a LOX pathway, i.e., nonspecific versus specific peroxidation, respectively. Thus, lipid peroxidation was analyzed in this microarray analysis project using a previously established hydroxy fatty acid HPLC assay (39) and was further investigated by chiral phase HPLC (40).

Our results demonstrated the involvement of an induced 9-LOX-dependent lipid peroxidation pathway using free fatty acids as substrates. Evidence was provided that PUFA hydroperoxides are available for membrane lipids, the activation of the LOX pathway, leading to a massive production of fatty acid hydroperoxides from membrane lipids, appears as being an active process in plant-hypersensitive cell death. The involvement of this pathway to pathogen growth limitation is also discussed.

**Experimental Procedures**

**Chemicals—**Cryptogeen, prepared according to Bonnet et al. (34) was purified by H. Ponchet (INRA, Antibes, France). FUFAs and fatty acid standards were purchased from Fluka (Buchs) or Sigma and MeJa from TCI (Interchim, Montluçon, France). The hydroxy fatty acid chromatographic standards have been previously described (41). In addition, 15-HEDE, used as an internal standard for HPLC quantification, was prepared from eicosadienoic acid, according to the previously described procedure (42), and the chemical structure was assessed by 1H and 13C NMR spectroscopy. An enriched fraction containing 16:3 (16:3/18:3/18:2) composition (26/57/7) was prepared from parsley leaf lipid extract, by TLC on galactolipid hydrolysis, as described below.

**Plant Growth and Treatments—**Tobacco plants (N. tabacum var. Petit Havana) were grown for 8 to 9 weeks in a greenhouse, at 100–120 μmol/m2/s light radiance (HQI-BT 400 watts-D OSRAM lamps, München, Germany), with a 14/10 h, 25/20 °C light/dark cycle and 60% relative humidity. Leaves (about 5 g) were selected in the middle of the stem, dried, and treated with 10 μl of an aqueous solution of cryptogeen (0.2 μg/μl) for water, orcontrol, as described previously (17). For MeJa treatment, tobacco plants were placed into an airtight 25-liter chamber for 5 days, and MeJa (5 μl) was applied on a piece of filter paper (43). Chemicals, in a 0.5% Tween 80 aqueous solution, were infiltrated between two secondary leaf veins, applying the syringe tip to the epidermis of excised leaves. Leaf petioles were then dipped into water and the leaves kept in the dark at room temperature. Necrosis was assessed from changes with time of leaf water content, and expressed as % of initial FW.

**Hydroxy Fatty Acid and Hydroxy fatty acid Analysis—**Free and bonded hydroxy and hydroperoxy fatty acids were analyzed by HPLC as free hydroxy fatty acids, after NaBH4, reduction and hydrolysis. Tobacco leaf sample (80 mg FW) was determined according to Miquel (34) and was further investigated by chiral phase HPLC as described above. In order to assess for free fatty acid hydroxy fatty acid hydroperoxides from membrane lipids, appears as being an active process in plant-hypersensitive cell death. The involvement of this pathway to pathogen growth limitation is also discussed.
transferred into a screw-capped centrifuge tube with 6 ml of 10/10/1 (v/v/v) chloroform/methanol/formic acid and stored overnight at −20 °C. After centrifugation (2,000 × g, 5 min), the supernatant was collected and the tissue pellet re-extracted with 2.2 ml of 5/5/1 (v/v/v) chloroform/methanol/water. Both extracts were combined and washed with 3 ml of 0.2 m NaCl and 1 ml KCl. Lipids were recovered in the chloroform phase, dried under N2, and dissolved in 0.5 ml of 2/1 (v/v) chloroform/methanol.

Individual lipids were purified from the extracts by monodimensional TLC using either 25/25/25/10/9 (v/v/v/v/v) chloroform/methylene chloride/propyl alcohol/0.25% aqueous KCl (v/v) for polar lipids or 90/15/2 (v/v/v) hexane/diethyl ether/acetic acid for neutral lipids. Lipids were then located by spraying the plates with a solution of 0.001% (v/v) primuline in 80% acetone, followed by visualization under UV light. The silica gel zones corresponding to individual lipids were scraped from the plates, and fatty acid methyl esters were prepared and analyzed as described above.

LOX Activity Determination—Frozen leaf tissue (2 g) was ground in ice-cold 100 mM, pH 6, sodium phosphate buffer (4 ml), containing 2% (w/v) of polyvinyl polypyrrolidone, and a protease inhibitor mixture (Roche Molecular Biochemicals CompleteTM). The mixture was centrifuged for 20 min at 16,000 × g, and the pellet was discarded. This crude extract was used for the LOX assay and protein quantification. The enzyme extract (0.5 ml) was incubated for 20 min at 25 °C, with 0.25 M sodium phosphate buffer, pH 7, at a final volume of 1.5 ml, and 5 μl of 18:2 (0.1% in ethanol). The reaction was stopped by adding 200 μl of 1 v NaOH. In addition, the internal reference (100 nmol of 15-HEDE), hydroxy fatty acids were extracted in 1.5 ml of 70/30 (v/v) hexane/diethyl ether and analyzed by straight phase HPLC and chiral phase as above. Protein content was determined using Pierce BCA protein assay reagent, following the enhanced protocol of the manufacturer’s instructions (Pierce), with bovine serum albumin as standard.

Preparation of the LOX-specific Probe and RNA Analysis—Total RNA fraction was extracted from control and treated leaves at various time points, RNA extraction was performed using the Plant RNA easy mini-kit, and poly(A) RNA extraction was carried out using an oligotex mRNA kit (both from Qiagen, Courtaboeuf, France) following the manufacturer’s instructions. The LOX-specific probe was prepared by RACE amplification using the MarathonTM cDNA Amplification Kit (CLonTECH, Ozyme, St. Quentin-Yvelines, France) with 1 μg of poly(A) RNA extracted from tobacco cells treated with cryptogein for 60 min. The two primers used for the 5'-RACE reactions were a gene-specific primer deduced from the sequence of the LOX1 of N. tabacum (45) (GenBankTM accession number X84040), 5'-GAGGAGTTAGCTGTT-GAGGACTGGAGCTCCC-3' (30-mers), and a primer corresponding to the Marathon adapter 5'-CCATCCTAATACGACTCACTATAGGGC-3' (27-mers). Briefly, poly(A) RNA were reverse-transcribed with reverse transcriptase with Moloney murine leukemia virus reverse transcriptase using (T30N-NN) as primer. The second strand performed with a mixture of Escherichia coli DNA polymerase I, RNase H, and E. coli DNA ligase was monitored by addition of [32P]dCTP. Following the creation of blunt ends with T4 DNA polymerase, the double strand cDNA was ligated to the Marathon cDNA-Adapter. The 5'-RACE reaction was performed on this cDNA population with the ExpandTM Long Template PCR System (Roche Molecular Biochemicals) for 30 cycles using the following steps: 3 °C for 90 s, 55 °C for 30 s, and 68 °C for 4 min. The PCR products were analyzed by electrophoresis on 1.2% agarose gel in TAE buffer. One unique band of almost 1 kilobase pair was extracted from the gel and cloned in pGEM-Teasy vector (Promega, Charbonnieres, France). Fluorescent sequencing was done by Genome Express S.A. (Grenoble, France) using SP6 as the downstream primer and a custom-designed upstream primer. Sequence analysis was carried out with FASTA, NCBI, and the Wisconsin Sequence Analysis Package (Genetics Computer Group, WI). The obtained sequence (998 base pairs) showed 98% identity with the tobacco LOX1. This cDNA was used as specific LOX probe for RNA analysis.

Northern blots were carried out according to standard protocols using 15 μg of total RNA per lane. After electrophoresis, RNA samples were transferred to nylon membranes using a cross-linker (UV) filters. The blot was hybridized with a cDNA probe, 32P-labeled by random priming (rediprim, Amersham Pharmacia Biotech.) at 42 °C overnight as described previously (33). Filters were washed with 2 × SSC, 0.1% SDS at room temperature, 4 × 10 min, then with 0.2 × SSC, 0.1% SDS at 60 °C 2 × 10 min, and analyzed with a PhosphorImager (Molecular Dynamics, Les Bordes, France).

RESULTS

LOX-mediated Lipid Peroxidation in Cryptogein-induced HR—Lipid peroxidation was investigated in tobacco leaves using straight phase HPLC analysis of hydroxy fatty acids obtained after NaBH4 reduction and lipid hydrolysis. Control and cryptogein-treated tobacco leaves were kept in the dark, and after 24 h, total hydroxy acids from free and bonded fatty acids were extracted, using the NaBH4, reduction-hydrolysis procedure and submitted to straight phase HPLC, as described under “Experimental Procedures.” A, HPLC traces of cryptogein-treated leaf extract (lower trace) and of control leaf extract (upper trace), using a Zorbax rtx-SIL column. The various hydroxy fatty acids isomers of 18:2 and 18:3 fatty acids were identified, as described previously (41), and quantified with reference to the internal standard 15-HEDE. In addition, among the four isomers of 16:3, two were separated from partly purified 16:3, using soybean tobacco LOX activities (13- and 9-specific on 18:2, respectively), and were not detected in the chromatograms of both control and elicited leaves (respective retention times 21.1 and 30.9 min). In the chromatogram of cryptogein-treated leaf extract, two early eluting compounds appeared just before 15-HEDE, and were designated X and Y. According to their UV spectra, both compounds were not identified as hydroxy fatty acids. Hydroxy fatty acids were collected from the preceding HPLC analyses and submitted to chiral phase HPLC, using a Chiralcel OD column, as described under “Experimental Procedures.” B, separation of the (9R)-HOTE and (9S)-HOTE enantiomers from control leaf extract. C, separation of the (9R)-HOTE and (9S)-HOTE enantiomers from cryptogein-treated leaf extract.
TABLE I

Enantiomer composition of the hydroxy fatty acids obtained by the NaBH₄/hydrolysis procedure from control and cryptogein-treated leaves

| Hydroxy fatty acids | Metabolite enantioselectivity S/R ratio |
|---------------------|---------------------------------------|
|                     | Control, 0 h   | Control, 24 h | Cryptogein, 24 h |
| 9-HODE              | 51/49 ± 5     | 51/49 ± 8     | 88/12 ± 2       |
| 9-HOTE              | 49/51 ± 3     | 48/52 ± 1     | 91/09 ± 1       |
| 13-HODE             | 80/20 ± 7     | 79/21 ± 5     | 72/28 ± 2       |
| 13-HOTE             | 90/10 ± 6     | 87/13 ± 6     | 84/16 ± 1       |
| 12-HOTE             | 52/47 ± 4     | 52/47 ± 5     | 57/43 ± 3       |
| 16-HOTE             | 54/46 ± 4     | 51/49 ± 5     | 54/46 ± 2       |
| 9-HODE              | 51/49 ± 5     | 51/49 ± 8     | 88/12 ± 2       |
| 9-HOTE              | 49/51 ± 3     | 48/52 ± 1     | 91/09 ± 1       |
| 13-HODE             | 80/20 ± 7     | 79/21 ± 5     | 72/28 ± 2       |
| 13-HOTE             | 90/10 ± 6     | 87/13 ± 6     | 84/16 ± 1       |
| 12-HOTE             | 52/47 ± 4     | 52/47 ± 5     | 57/43 ± 3       |
| 16-HOTE             | 54/46 ± 4     | 51/49 ± 5     | 54/46 ± 2       |

characterized in plants, positional 9 and 13 isomers can arise from either LOX activity or autoxidation, whereas 12-HOTE and 16-HOTE can be considered to be specific of fatty acid autoxidation. In addition, if peroxidation products are chiral, they necessarily arise from LOX activity, whereas racemic products can be obtained either by autoxidation or by a non-specific LOX. Each chromatographic peak was collected and submitted to chiral phase HPLC, the example of (9R)- and (9S)-HOTE enantiomer separation in control and treated samples being described in Fig. 1. Results are expressed as mean ± S.D. of three independent analyses.

FIG. 2. Time course of LOX-dependent peroxidation and autoxidation in control and cryptogein-treated leaves; relationship with tissue necrosis. Hydroxy fatty acids were extracted from control and cryptogein-treated leaves at different times following application, using the NaBH₄ reduction-hydrolysis procedure and analyzed by HPLC as described in Fig. 1. A, open and closed symbols represented the level of hydroxy fatty acids for water-treated and cryptogein-treated leaves, respectively. ▲, total hydroxy fatty acids; ●, 9-HOTE + 9-HODE representative of 9-LOX activity; ■, 13-HODE + 13-HOTE representative of 13-LOX activity; inset, ●, 12-HOTE + 16-HOTE, representative of PUFA autoxidation. B, relationship between leaf necrosis and total lipid peroxidation; tissue necrosis was evaluated by measurement of leaf dehydration and total lipid peroxidation by total hydroxy fatty acid level. Mean and S.D. from three independent experiments are given.

Fatty Acid Hydroperoxides in Plant-hypersensitive Cell Death

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The steady-state level of mRNA encoding for LOX was analyzed using Northern blot experiments at different times following cryptogein application to tobacco leaves. A specific probe corresponding to tobacco LOX 1, described by Véronési et al. (45), was prepared by RACE-PCR and recognized a 2.9-kilobase pair mRNA band. The accumulation of LOX mRNAs monitored in excised tobacco leaves infiltrated with cryptogein or with water is described in Fig. 3, A and B. A high transitory accumulation occurred between 6 and 8 h after the treatment with cryptogein, whereas no accumulation was observed in the water-treated leaves. Changes in 9-LOX activity were analyzed in the same experiment and are described in Fig. 3C. In addition, in both elicited and control leaves, attempts to characterize 13-LOX activity, as suggested by the previous metabolite analyses, were not successful (see below). In elicited leaves, the time course of 9-LOX activity level followed a similar transitory increase, reaching an apparent maximum (27 picokatals/mg protein) after 16 h after a lag phase of 8 h. An important decrease was then observed (7 picokatals/mg protein after 28 h). In control leaves the activity was at the limit of the detection level (<2 picokatals/mg protein) for at least 72 h.

Product Specificity and Substrates of Elicited LOX—Analyses of LOX metabolites were conducted to assess free fatty acid hydroperoxide formation by cryptogein action. Since the NaBH₄/hydrolysis extraction procedure leads to the global analysis of free and esterified hydroperoxide and hydroxy fatty acids, in a first type of experiment, reductive conditions were designed, in which the hydrolytic step was omitted. Lipids were extracted from 24-h cryptogein-treated leaves, by grinding at pH 4.5, in the presence of iron chelators to avoid hydroperoxide formation.
degradation, and with triphenyl phosphine in the chloroform/methanol mixture to reduce the hydroperoxides under mild conditions (42). Although HPLC of these extracts appeared to be more complex, as compared with the NaBH₄/hydrolysis procedure, free hydroxy fatty acids could, however, be analyzed by straight phase HPLC, after a silica Sep-Pak column enrichment. Under such experimental conditions, only the free 9-hydroxy fatty acid isomers were observed, and the 9-HODE/9-HOTE ratio was 20/80 (results not shown). The 9-hydroxy fatty acids recovered under these conditions represented 60% of the total 9-hydroxy fatty acids obtained by the NaBH₄/hydrolysis extraction procedure. In a second type of experiment, the triphenyl phosphine was not present in the extraction buffer. Free fatty acid hydroperoxides as well as free hydroxy fatty acids could then be identified and quantified by straight phase HPLC analysis. The HPLC analyses of free fatty acid hydroperoxides described in Fig. 4 clearly show that, among the various possible positional hydroperoxide isomers, only 9-HPODE and 9-HPOTE were observed and, as above for free 9-hydroxy fatty acids, in a 20/80 ratio. By using the latter extraction procedure, the free 9-hydroperoxides represented 51 ± 7% (n = 2) of the sum of free 9-hydroperoxide and 9-hydroxy fatty acids.

The preceding results suggested that free PUFAs should be the substrates of cryptogein-induced LOX. Thus, LOX activity was measured using 18:2 as a substrate and by HPLC to assess for 9/13 specificity. The activity showed a maximum value at pH 7. Cryptogein-induced LOX was shown as being regio-specific producing 98% of 9-HODE, and the specificity was similar between pH 5 and 9. Substrate specificity was analyzed by competition in a mixture of 16:3/18:3/18:2 (37/56/7, respectively). All the PUFAs were substrates and shown to be peroxidized with relative reaction rates of 16/79/5, for 16:3/18:3/18:2, respectively. In addition, since MeJA has been described to induce transcriptional activation of the 9-LOX gene and accumulation of 9-LOX activity in tobacco (46), the MeJA-induced LOX was analyzed similarly. Tobacco plants were treated with MeJA for 5 days, under the conditions described by Avdiushko et al. (43). The LOX activity analyzed on leaves was shown to be at the same level as in leaves treated with cryptogein for 16 h and to exert the same regio-specificity, 9-HODE representing over 96% of the products. The enantioselectivity of MeJA- and cryptogein-induced LOXs was determined on 9-HODE production, either in vitro, with an enzymatic extract, or in planta, after 18:2 substrate infiltration into leaf and metabolite analysis (Table II). Both enzymatic extracts possessed the same 9S-enantioselectivity (around 92%). The 18:2 infiltration experiments led to similar increased levels of (9S)-HODE and similar enantioselectivity (around 86%). In both cases, although the induced LOX appeared very specific at the 9-position, the infiltration experiments led, in addition, to increased levels of (13S)-HODE (around 85% enantioselectivity; Table II). This result suggested again the presence of a (13S)-LOX, which could not be characterized in vitro in the present experiments.

**PUFA Consumption in Cryptogein-elicited Leaves**—To investigate the potential source of free fatty acids as substrates for the induced LOX, global changes in the fatty acid composition of control and elicited leaves were compared after treatment, when the symptoms were not fully developed (18 h), and also compared with the composition of control leaves at the beginning of the treatment (Fig. 5A). First, the fatty acid composition of control leaves remained unchanged after leaf excision. Second, the composition of all fatty acids did not change significantly in elicited leaves with the exception of 18:2 and 18:3, the major fatty acid in leaves, which decreased to 63 ± 8% and 70 ± 9% (n = 3) of initial levels, respectively. The different classes of lipids were analyzed for their fatty acid composition, and the decrease was confirmed for 18:2 and 18:3. As shown in Fig. 5B,
The enantioselective transformation of 18:2 into hydroperoxides was investigated in vitro using enzymatic extracts, and in planta, after 18:2 infiltration into leaves, by the NaBH₄/hydrolysis extraction procedure. Results are expressed as mean ± S.D. of three independent experiments.

|                   | 9-HODE (S/R) level increase | 13-HODE (S/R) level increase |
|-------------------|-----------------------------|-------------------------------|
|                   | in Vitro³                  | in Planta³                    | in Vitro³                  | in Planta³                    |
| Cryptogin-induced (16 h) | 92/8 ± 1                  | 85/15 ± 1 × 4.0 ± 1.1         | 93/7 ± 1                  | 88/12 ± 3 × 4.9 ± 1.3         |
| MeJA-induced (5 days)     | ND⁴                        | 83/17 ± 3 × 1.7 ± 0.5         | ND⁴                        | 87/13 ± 2.8 ± 0.9             |

a Leaf protein extract was incubated with 18:2, and after NaBH₄, reduction, the reaction products were separated by straight phase HPLC and analyzed by chiral phase HPLC, as described under “Experimental Procedures.”
b 18:2 (5 mM) was infiltrated into half-leaf, and metabolites were analyzed in both parts by the NaBH₄/hydrolysis procedure, as described under “Experimental Procedures”; incubation was for 30 min for MeJA-treated plants and 1 h for cryptogin-elicited leaves.
c HODE level increase due to 18:2 infiltration, as compared with the non-infiltrated part of the leaf.
d ND, not determined; the 9-LOX specificity was greater than 98 and 96% for cryptogin- and MeJA-induced LOX, respectively.

FIG. 5. Changes in fatty acid composition of tobacco leaves after cryptogin treatment. Total lipids were extracted from control leaves before treatment and 18 h after water or cryptogein application. Fatty acid composition of total lipids, or of the various classes of lipids, separated by TLC, was evaluated by GPC, as described under “Experimental Procedures.” A, total fatty acid composition. B, respective composition in 18:3 and 18:2 fatty acids, of galactolipids (GL), phosphatidylcholine (PC), phosphatidylglycerol and ethanolamine (PG-PE), neutral lipids (NL), and free fatty acids (FA). Cross-hatched bars, control, time 0; white bars, control, time 18 h; black bars, cryptogein treatment, time 18 h. In order to compare results, are expressed in μmol/g DW. Mean and S.D. from three analyses of the same experiment are given, in which fatty acid composition of total lipids (A) and of the various classes of lipids (B) were analyzed simultaneously.

Significantly (15–20% of consumption), whereas 18:2 and 18:3 reached 53 ± 8 and 54 ± 9% (n = 3) of initial levels, respectively. In accordance with the observation that 16:3 hydroperoxides do not accumulate in elicited leaves, it is worth mentioning that the decrease in 16:3 level was low, similar to fatty acids that are not LOX substrates. With reference to the control, the material balance calculation carried out on 18:3 indicated that, 24 h after cryptogein treatment, the steady-state level of 9-LOX-dependent peroxidation, as measured by 9-HOTE level according to the NaBH₄/hydrolysis procedure, represented 10 ± 6% (n = 3) of 18:3 consumption. Taken together, these results suggest the involvement of specific lipase(s) in the process and demonstrate the participation of the chloroplastic lipids.

Inhibition of Cryptogein-induced LOX Metabolism and Cell Death by Limiting Oxygen Availability—HR can be inhibited in the absence of oxygen (12). In a first type of experiment, cryptogein-treated leaves were kept under normal atmosphere for 1 h, allowing the initiation of the oxidative burst, and then transferred at low oxygen pressure (0.6%) for a further 25 h. As compared with leaves under normal atmosphere, the leaves did not develop the necrotic symptoms. LOX activity was shown to be induced at low oxygen pressure at comparable level as in leaves under normal conditions, but metabolites were not synthesized (results not shown). In a second type of experiments, oxygen availability was limited by water-dipping experiments. Cryptogein-treated and control leaves were kept under normal atmosphere for 1 h and then half-dipped into water for the next 23 h in order to compare symptom development on the same leaf. As described in Fig. 6A, necrotic symptoms developed in the upper aerial leaf part of cryptogein-treated leaves but not in the lower immersed part. Although LOX activity was induced in both parts at similar levels, metabolite analysis showed increase of lipid peroxidation in the upper part but not in the lower (Fig. 6B). Control leaves did not show any symptom or activation of LOX metabolism. In both types of experiments, the inhibition of HR was reversed, as soon as leaves were transferred under normal oxygen conditions. These results showed that LOX activity can be induced under low oxygen conditions. However, since oxygen is the PUFA co-substrate of LOX, LOX-dependent peroxidation is inhibited when oxygen is limiting, and consequently cell death.

Activation of Cell Death by LOX Substrates and Metabolites—Substrates and products of the LOX pathway were tested in the induction of tobacco leaf tissue cell death. Although PUFA 9-hydroperoxides could not be prepared with sufficient purity to be tested (47), 13-HPODE can be conveniently prepared with soybean LOX (42). The effects of 18:2 and 13-HPODE, at 1, 2, 5, and 10 mM concentration in 0.5% of a Tween 80 aqueous solution, were first compared by infiltration between two secondary veins of leaves. As shown in Fig. 7A, after 4 h of incubation, 13-HPODE infiltration induced necrotic ar-
mental Procedures."

Leaf sampling and analysis

Leaves were analyzed at different times to determine the evolution of the 9-LOX activity level and of 9-hydroperoxide metabolite level. Leaf tissue was half-dipped into water 1 h after the treatment and left to dry. Necrosis symptoms after 24 h of cryptogein-treated leaf infiltration were further observed in water-dipping experiments (see above) indicating that oxygen was then not necessary to induce the necrotic lesions (results not shown). In leaves of MoJA-treated plants, for which 9-LOX activity accumulated (see above), and of the corresponding control, necrotic symptoms were not observed. Infiltration of 18:2 and 18:3 between two secondary leaf veins was carried out at 1, 2, 5, and 10 mM concentration. Leaf tissue was more sensitive to PUFA infiltration, as compared with the preceding experiments. In control leaves necrotic areas were induced, after 4 h, for 5 mM concentration and above (results not shown). In the MoJA-treated material, necrotic lesions appeared within 15 min in the 5–10 mM 18:3-infiltrated parts (results not shown), and within 4 h for all concentrations of both PUFAs (see Fig. 7C for 18:2).

The above efficient hydroperoxide or PUFA concentrations appear consistent with the decrease of the PUFA level observed in cryptogein-treated leaves. On the one hand, taking into account the volume of compound infiltration (0.75 ml/g FW), a final level of 3.75 μmol/g of leaf initial FW can be calculated from a 5 mM product infiltration. On the other hand, assuming that 18:2 + 18:3 fatty acids represent 65% of total fatty acids in leaf lipids (17 ± 3 μmol/g FW; n = 3), the PUFA decrease in the cryptogein-treated leaves (around 50%, after 24 h) can be estimated to be around 5.5 μmol/g FW, which is a value close to the preceding one. Taken together, these results confirmed that free PUFAs are the LOX substrates in vivo and demonstrated that application or in planta production of PUFA hydroperoxides, at relevant physiological levels, induce leaf necrosis.

DISCUSSION

The involvement of a LOX pathway causing the development of tissue necrosis during HR is shown for the first time in this work as follows: (i) free fatty acid hydroperoxides are produced massively in tobacco leaves, in response to cryptogein; (ii) the intensity of leaf necrosis is correlated to the level of fatty acid peroxidation, dependent on cryptogein-induced 9-LOX; (iii) since oxygen is the PUFA co-substrate of LOX, LOX metabolism and cell death are both blocked when oxygen availability is limited; (iv) finally, leaf necrosis can be induced either by indirect production of hydroperoxides in planta, infiltrating PUFAs into leaves where LOX activity has been induced by MoJA, or by direct infiltration of physiological relevant levels of fatty acid hydroperoxides.

The production of fatty acid hydroperoxides by a plant LOX was initially described in potato-Phytophthora infestans interaction, but arachidonate, the LOX substrate, was released from the fungus (48). In our model, it is worth emphasizing that plant lipids are the precursors for hydroperoxide production. Thus, our results showed that cryptogein elicits a plant program to provide adequate substrates and enzymes for free fatty acid hydroperoxide production leading to cell death. Increasing LOX activity during the development of HR was described for many plants. Enzyme specificity appears to be, however, dependent on the plant model. For instance, 13-LOX was described in rice (49) and soybean (27), whereas 9-LOX was characterized in tomato (50) and tobacco (Ref. 51 and this work). Since it has been further shown that infiltration of 13-HPDE as well as in planta production of 9-HPDE were able to induce tobacco leaf necrosis, these data taken together suggest that, dependent on the plant species, 13- and (or) 9-hydroperoxide isomers of fatty acids can be produced as potent effectors of hypersensitive cell death. Surprisingly, infiltration in the tobacco leaves of tert-BuOOH up to 20 mM concentration did not induce necrotic lesions. Thus, it can be proposed that, in addition to the hydroperoxides, fatty acid hydroperoxide metabolites and degradation products would also play an important role in toxicity. Autoxidation of esteri-
fied fatty acids was also observed and tentatively attributed to membrane lipid peroxidation. This process, representing around 10% of the total lipid peroxidation level, occurs late after elicitation and is induced simultaneously with the enzymatic process. Therefore, it can be proposed that this additional membrane lipid peroxidation is likely initiated by free fatty acid hydroperoxides produced massively via LOX action, rather than AOS generated early during the oxidative burst (38).

Anyway, the hypothesis that massive lipid peroxidation occurring in cryptogein-induced HR is the result of free radical production by the oxidative burst can be ruled out. Additional experiments, including the study of other models, are needed, however, to demonstrate the systematic occurrence of the LOX pathway in hypersensitive cell death, and the involvement of AOS as effectors or (and) as signaling components in the process (22, 52, 53).

Analysis of changes in the fatty acid composition of cryptogein-treated leaves was assessed for the importance of the peroxidative pathway. During the first 18 h, whereas the level of the other fatty acids did not change significantly, about 30% of the 18:2 and 18:3 were consumed specifically, reaching 50% after 24 h (5.5 μmol/l FW). Despite the high reactivity of hydroperoxides, the steady-state level of PUFA hydroperoxides after 24 h was shown to represent still 10% of PUFA consumption. In addition, infiltration of 18:2 into leaves, 16 h after cryptogein treatment, increased the 9-HODE level, indicating that 9-LOX activity was not substrate-saturated in vivo. Overall, these results strongly suggest that massive PUFA consumption occurs mainly via the LOX pathway. Among the lipid changes following tobacco elicitation, previous investigations described a late decrease in galactolipid levels (15). In the present model, the analysis of the fatty acid composition of the lipid classes showed further that PUFAs originated mainly from galactolipids. Thus, chloroplasts appear as being the main source of PUFAs in the process. The key role of chloroplast lipids in synthesis of products derived from fatty acid hydroperoxides, the so-called oxylipin pathway, was first established using mutants of PUFAs for freezing-induced volatile aldehyde production (54). Furthermore, the metabolism site of fatty acid hydroperoxides was shown to be located on the envelope membranes of chloroplasts (55), and it appeared that some pathogen- or MeJA-induced LOX genes are chloroplast-targeted (49, 56). In addition, morphological changes on chloroplasts were previously observed in various models of plant-pathogen interaction (6–8) and in cryptogein-treated tobacco leaves (57). The present results highlighted the major role of

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FIG. 7. LOX substrate and product infiltration on the development of tobacco leaf necrosis. Leaves from control plants were infiltrated between 2 secondary leaf veins with various concentrations of compounds in 0.5% Tween 80. A, infiltration of increased concentrations of 13-hydroperoxide of 18:2 (0, 1, 2, 5, and 10 mM). B, comparison of 5 mM substrate reactivity was carried out on the same leaf by infiltration of 18:2 free fatty acid (18:2), tert-butyl hydroperoxide (tert-BuOOH), 13-hydroperoxide of 18:2 (HPODE), and the corresponding 13-hydroxy 18:2 fatty acid (HODE). C, leaves from 5 days MeJA-treated tobacco plants, as described under “Experimental Procedures”, infiltrated with 1, 2, 5, or 10 mM 18:2 fatty acid. The symptoms presented in A–C were those observed after 4 h of incubation. Typical photographs from triplicate experiments leading to the same results were shown.

A tobacco 9-LOX was previously purified and characterized on cultured cells treated by an elicitor from the pathogen Phytophthora parasitica var. nicotianae (51). The 9/13-LOX specificity (87/13 and 93/7 on 18:2 and 18:3 substrates, respectively) is consistent with our results on cryptogein-induced (9S)-LOX in leaves (98/2 on 18:2). The expression of the gene was shown to occur in elicited cell cultures and, furthermore, in plants upon infection (46). In the present work, a cryptogein-mediated expression of the same gene was demonstrated. From the chiral analyses, a low constitutive (13S)-LOX metabolism can be proposed in control leaves. Upon cryptogein treatment, the metabolism is shifted toward a massive production of (9S)-fatty acid hydroperoxides. The initial low constitutive (13S)-LOX activity could be sufficient for the synthesis of JA, an early plant defense signaling compound considered to operate in plant pathogen interactions (25, 30, 31). The enzymes required for JA synthesis have been proposed as being expressed constitutively (58), and the 18.3 precursor might be provided by the induction of phospholipases, known to occur rapidly after elicitation (19, 59). JA was mentioned as being produced in elicited tobacco cells before 9-LOX activity induction and shown to mediate 9-LOX gene expression (46). In the present work, cryptogein- and MeJA-induced LOX activities were shown to exhibit the same 9S specificity. Thus, participation of JA in the signaling cascade leading to cryptogein-induced 9-LOX gene expression appears likely. In addition, early accumulation of JA was described in infected tobacco leaves undergoing HR (60). These results, taken together, suggest that at least two different LOXs, with 13 and 9 specificity, operating in signaling and in production of cell death effectors, respectively, should be involved in tobacco leaf HR.

During the development of cryptogein-elicited leaf necrosis, free fatty acid hydroperoxides were produced from membrane lipids, indicating the involvement of hydrolase activity. Indeed, a late induction of acyl hydrolases or lipase activities was observed in various models of plant pathogen interactions (26, 61). In general, LOX substrates are free fatty acids. However, in the early stages of seed germination, lipid body-associated LOX led to lipid peroxidation prior to hydrolase action (40). In addition, in elicited soybean seedlings, the induced LOX was shown to act in vitro on phospholipids (27). Thus, in the cryptogein-induced tobacco HR, the question whether LOX acts before or after the hydrolase action was addressed. First, all hydroxy fatty acid isomers were characterized in esterified lipids whereas, consistent with the 9-LOX activity determined
in vitro on free fatty acids, only 9-positional isomers were analyzed as free fatty acid hydroperoxides, suggesting that hydrolysis occurs before peroxidation. Second, free 16:3 fatty acid was shown to be peroxidized by elicited 9-LOX in vitro but 16:3 was not consumed and apparently not peroxidized in elicited leaves. Since 16:3 is exclusively located on the sn-2 position of chloroplast lipids (62), this last result could be explained assuming lipase(s) specific of the sn-1 position act before 9-LOX. Finally, the observation that MeJA-induced 9-LOX activity did not lead to either metabolite accumulation or to HR symptoms, in the absence of PUFA infiltration, also argues in favor of that hypothesis. This last result further suggests that lipase(s) might be induced by a signaling pathway different from JA. The characterization of elicited lipases, their product specificity, and the signaling pathway leading to their induction are currently under investigation.

The events leading to cryptogein-induced HR are tentatively summarized in Fig. 8. The induction of LOX pathway is an active process of membrane degradation leading to hypersensitive cell death. Lipase activity appears to be involved upstream from 9-LOX action. JA was proposed as an early signal for 9-LOX gene induction. In the light of our results, further peroxidation of membrane lipids was considered as a consequence of massive production of fatty acid hydroperoxides, rather than AOS production during the early oxidative burst. The induction of the LOX peroxidative pathway was not described in mammalian PCD (63) and can be considered as a characteristic of plant HR-PCD. Apoptosis in mammalian cells and lipid peroxidation were correlated, both induced by oxidative agents, and both inhibited in transfected cells overexpressing the oncogene bcl-2 (64). Apoptosis was shown to be induced by free fatty acid hydroperoxides but was not repressed by the Bcl-2 protein, suggesting that the protein acts before lipid peroxidation (65). In plants, HR induction appears not dependent on the Bcl-2 family (12), and lipid peroxidation was shown in this work as being an active process.

In many aspects, the production of free fatty acid hydroperoxides must be considered as an important part of the plant defense response to limit pathogen invasion. Products from the LOX pathway include hydroxy fatty acids, observed in this work, which are substrates for cutin biosynthesis, reinforcing tissue defenses (66). Lipid hydroperoxides and derived products are toxic to microorganisms (25, 30, 31, 50, 67). Finally, fatty acid hydroperoxides were also proposed as acting as signaling compounds to neighboring cells (25) and eliciting phytoalexin synthesis (48, 66). A recent investigation (29), showing that tobacco plants exhibiting HR and a resistance toward the pathogen P. parasitica displayed susceptibility when antisense 9-LOX plants were infected, is in favor of that assumption.

The massive production of free fatty acid hydroperoxides observed in this work was shown to induce plant cell death and can also be considered as a defense mechanism against the invading pathogen. This metabolism has been observed during the development of leaf HR, and chloroplast lipids appear essential for the response. Among the questions arising from these results, further investigations must be focused to address the occurrence of this pathway in other plant-pathogen interactions, other tissues (i.e. non-photosynthetic tissues), and in the cultured cell model. The active process of membrane degradation described in this work, together with the induction of nuclease (5–8) and proteases (9–11), can be considered as one of the important features leading to the hypersensitive programmed cell death.

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