Activation of the Fatty Acid α-Dioxygenase Pathway during Bacterial Infection of Tobacco Leaves

FORMAION OF OXYLIPINS PROTECTING AGAINST CELL DEATH

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A pathogen-induced oxygenase showing homology to prostaglandin endoperoxide synthases-1 and -2 was recently characterized in vitro experiments as a fatty acid α-dioxygenase catalyzing formation of unstable 2(R)-hydroperoxy fatty acids. To study the activity of this enzyme under in vivo conditions and to elucidate the fate of enzymatically produced 2-hydroperoxides, leaves of tobacco were analyzed for the presence of α-dioxygenase-generated compounds as well as for lipoxygenase (LOX) products and free fatty acids. Low basal levels of 2-hydroxylinolenic acid (0.4 nmol/g leaves fresh weight) and 8,11,14-heptadecatrienoic acid (0.1 nmol/g) could be demonstrated. These levels increased strongly upon infection with the bacterium Pseudomonas syringae pv syringae (548 and 47 nmol/g, respectively). Transgenic tobacco plants overexpressing α-dioxygenase were developed, and incompatible infection of such plants led to a dramatic elevation of 2-hydroxylinolenic acid (1778 nmol/g) and 8,11,14-heptadecatrienoic acid (86 nmol/g), whereas the levels of LOX products were strongly decreased. Further analysis of oxylipins in infected leaves revealed the presence of a number of 2-hydroxy fatty acids differing with respect to chain length and degree of unsaturation as well as two new doubly oxygenated oxylipids identified as 2(R),9(S)-dihydroxy-10(E),12(Z),15(Z)-octadecatrienoic acid and 2(R),9(S)-dihydroxy-10(E),12(Z)-octadecadienoic acid. α-Dioxygenase-generated 2-hydroxylinolenic acid, and to a lesser extent lipoxypgene-generated 9-hydroxyoctadecadienoic acid, exerted a tissue-protective effect in bacterially infected tobacco leaves.

Lipoxygenase (LOX) catalyzes primary oxygenation of polyunsaturated fatty acids into reactive hydroperoxide derivatives which can be further transformed into leukotrienes and other biologically active compounds. In plants, linoleic and linolenic acids are the most important substrates for LOX catalysis, and secondary modification of hydroperoxides is mainly effected by the enzymes allene oxide synthase, hydroperoxide lyase, and divinyl ether synthase (1). The oxygenated derivatives thus formed include the phytotonic jasmonic acid as well as reactive oxylipins possessing epoxide, conjugated carbonyl, or aldehyde functionalities (2). It is well recognized that LOX-derived oxylipins are involved in physiological processes in plants such as growth and fertility (3). Furthermore, several lines of evidence indicate that oxylipins have important roles to fulfill in the defense reactions that take place as a consequence of infection of plants with bacteria, insects, fungi, and other pathogens (4). Thus, aldehydes generated through the LOX-hydroperoxide lyase pathway can exert toxic effects against bacteria and fungi, and coelomic acid and other divinyl ether fatty acids formed by the 9-LOX-divinyl ether synthase pathway inhibit mycelial growth of the potato pathogen Phytophthora infestans (5). Other oxylipins, notably those belonging to the jasmonate family of compounds, can act as signaling molecules leading to activation of specific defensive genes in the host plant. In agreement with the proposed role of oxylipins in plant defense, several studies have demonstrated that phytopathogens can activate phospholipases in plant leaves (6) resulting in liberation of free fatty acids, a prerequisite for the formation of oxylipins by LOX catalysis.

It was recently discovered that there exists in plant leaves, besides the lipoxygenases, another group of dioxygenases attacking fatty acids, i.e. α-dioxygenases. α-Dox1, the major α-dioxygenase in tobacco, possessed hem-binding motifs and showed homology to mammalian prostaglandin endoperoxide synthases but was structurally unrelated to lipoxygenases (7–9). In addition to tobacco, several other plants, including Arabidopsis (7–9), cucumber (8, 10), pea (11), rice (12), and the green alga Ulva pertusa (13), have been reported to possess α-Doxes, a few of which have been partially purified. In vivo studies using recombinant α-Dox1 from tobacco demonstrated that this enzyme catalyzes the stereospecific introduction of oxygen at the C-2 (α-) position of saturated and unsaturated fatty acids to form unstable 2-hydroperoxide derivatives (8).

13-HOT, 13(S)-hydroxy-9(Z),11(E),15(Z)-octadecatrienoic acid; 9,10,11-TrifHOD, 9(S),10(S),11(R)-trihydroxy-12(Z),15(Z)-octadecadienoic acid; 9,12,13-TrifHOD, 9(S),12(S),13(S)-trihydroxy-10(E),15(Z)-octadecadienoic acid; GC-MS, gas-liquid chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; Me3Si, trimethylsilyl; hpl, hours after inoculation; dpl, days after inoculation; HR, hypersensitive reaction.

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The role of α-dioxynogenase in higher plants is not well established, although its participation in the plant α-oxidation pathway has been suggested (8, 11). Bacterial infection of tobacco leaves leads to accumulation of the α-DOX1 protein (7), and the levels are higher and appear earlier when the infection results in a hypersensitive reaction (HR) (7, 14). This finding and results obtained with α-DOX1 transgenic Arabidopsis plants suggest that the pathway protect plant tissues from undergoing excessive necrosis during the HR response (14). Further studies in this area were hampered by the lack of information regarding α-DOX activity in intact plant tissues and the metabolic fate of the putative 2-hydroperoxides. The present study provides qualitative and quantitative information about the oxylipins biosynthesized by α-DOX in tobacco leaves under basal conditions and during infection with compatible or incompatible strains of Pseudomonas. The incompatible infection caused a massive production of 2(8)-hydroxy fatty acids, and these compounds emerge as the prime candidates for α-DOX-generated signaling molecules. Additionally, experimental evidence for the antinecrotic activity of two of the oxylipins accumulating in leaves is provided.

EXPERIMENTAL PROCEDURES

Plants—Tobacco plants used in this study were wild type or transgenic Nicotiana tabacum cv Petit Havana SR1 grown on soil under a 14-h-light and 10-h-dark photoperiod. Plants were treated and examined 8 weeks after seed germination.

Oxylipins—2(8)-Hydroxystearic Acid (2(8)-HOT) was isolated from seeds of Thymus vulgaris as previously described (8). These seeds also contained low levels of 2(8)-hydroxystearic acid as well as 8(2,11,14z)-2,6,9,12,13,16,19-Hexadecahexaenol (8,11,14z)-heptadecatrienonic acid. 2-Hydroxy derivatives of oleic, stearic, and palmitic acids were prepared by chemical oxygenation (8). All other oxylipins were purchased from Larodan Fine Chemicals, Malmö, Sweden.

2,9-Dihydroxystearic Acid—Methyl 2(8,11,14z)-9,12,13-trihydroxystearate (2(8,11,14z)-HOT) was synthesized by catalytic deuteration of 7,10,13-hexadecatriynol acid was prepared by catalytic deuteration of 7,10,13-hexadecatriynol with the 9300

Deuterium-labeled 9-HOT (200 μg) was stirred at 23 °C for 20 min with the 100,000 g particulate fraction from homogenate of cucumber (8), and the extract was subjected to reverse phase-HPLC. The main peak of radioactive material was due to 9(8)-hydroxy-9,12,13,16,19-pentadecahexaenonic acid, which was identified by its mass spectrum (MeSi derivative) showing prominent ions at m/z 336 (M+), 267 (M+ - 69; loss of CH3-CH-CH=CH-CH3), 239 (M+ - 90; loss of CH3-CH=CH=CH=CH3), and 221 (M+ - 104; loss of CH3-CH=CH=CH=CH3). The mass spectrum of the methyl ester derivative of the compound showed prominent ions at m/z 339 (M+), 363 (M+ - 36; loss of CH3-CH=CH=CH=CH3), and 345 (M+ - 54; loss of CH3-CH=CH=CH=CH3). The mass spectrum of the 2(8,11,14z)-9,12,13-trihydroxystearic acid was characterized by mass spectral comparison with the authentic compound. An additional product formed in these incubations was the one-carbon shortened homolog of 9-HOT, i.e. 8-hydroxy-9,11,14-octadecatrienonic acid.

Deuterium-labeled 2-HOT—Methyl-2(8)-oxo-9,12,13-trihydroxy stearate (2(8)-HOT) was isolated by extraction with diethyl ether and purified by reverse phase-HPLC. The [2,3,3-2H3]-HOT obtained (27 mg) had the following isotope composition: 98.4% trideuterated, 2.6% dideuterated, and 0.4% undeuterated molecules.

α-Dioxynogenase in Infected Tobacco

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α-Dioxygenase in Infected Tobacco

After electrophoresis, Protein extracts of plant tissues for immunoblot analyses were prepared by grinding leaves to a fine powder in liquid nitrogen. Extraction buffer was then added at a ratio of 2 ml/g fresh tissue. Supernatants were clarified by centrifugation for 10 min at 3000 × g, and 25 µg of protein was separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Immunoblot assays were performed as previously described (7) using anti-tobacco-α-DOX1 antibodies. Bacterial inoculation was done by injecting leaves in the apoplast with a late-logarithmic culture using a syringe. The bacteria were grown in King’s medium (2% protease peptone, 2% glycerol, 6.5 mM K2HPO4, and 6 mM MgSO4·7H2O) at 27 °C. After centrifugation, pellets were resuspended in sterile water to reach a final concentration of 106 bacteria per milliliter. An incompatible bacterium (*P. syringae*) was added to leaves or powdered leaves were carried out on dry ice or using liquid nitrogen. Aliquots of powdered leaves were rapidly (30 s) weighed at sub-zero temperature, and the sample (100–200 mg) was immediately thereafter added to ice-cold ethanol (10 ml) containing 9,12,13-TriHOD, 9,10,11-TriHOD, and 2-HOT. Material present in the two fractions containing acidic lipids were treated with diazomethane and subsequently derivatized with trimethylchlorosilane/hexamethyldisilazane/pyridine prior to GC-MS analysis.

**RESULTS**

**Generation of Transgenic Tobacco Plants with Altered Levels of α-DOX1 Expression**—Transgenic plants containing the entire α-DOX1 cDNA fused to the CaMV35S promoter, in both sense and antisense orientation, were generated and used to study the activity of α-dioxygenase *in vivo*. Selection of appropriate transgenics was based on the level of α-DOX1 expression found in independent lines responding to inoculation with the incompatible bacterium *P. syringae*. Results from these analyses are shown in Fig. 1. In wild type plants, untreated leaves gave a weak hybridization signal that increased in RNA

- **Linoleic acid**: 1.5 ± 0.3
- **Linolenic acid**: 2.3 ± 0.1
- **8,11,14-Heptadecatrienoic acid**: 0.1 ± 0.05
- **2-HOT**: 0.4 ± 0.1
- **9-HOT**: 0.1 ± 0.05
- **9,12,13-TriHOD**: 0.1 ± 0.05

In vivo dioxygenase levels in infected tobacco leaves.

**TABLE I**

| Compound analyzed | Wild type plants | α-DOX1 sense plants |
|-------------------|------------------|---------------------|
| Amount            | nmol/g           | nmol/g              |
| Linoleic acid     | 1.5 ± 0.3        | 1.7 ± 0.2           |
| Linolenic acid    | 2.3 ± 0.1        | 3.0 ± 0.1           |
| 8,11,14-Heptadecatrienoic acid | 0.1 ± 0.05 | 0.6 ± 0.1 |
| 2-HOT             | 0.4 ± 0.1        | 0.6 ± 0.1           |
| 9-HOT             | 0.1 ± 0.05       | <0.02               |
| 9,12,13-TriHOD    | 0.1 ± 0.05       | <0.02               |

**Antibacterial Activity Assay**—In vitro antibacterial activity of oxylipins was evaluated according to Broekart et al. (21). Bacteria were grown in wells of microplates and growth was monitored by measuring the absorbance of the microcultures after 24 h of incubation. A culture volume of 100 µl per well was used. Bacterial species examined were: *P. syringae* pv *syringae* (NCPBP2686), *P. syringae* pv tabaci (NCPBP1427), *P. syringae* pv tomato DC3000 (22), *P. syringae* pv maculicola (NCPBP 1820), Xanthomonas campestris pv. campestris 8004, X. campestris pv campestris 147 (23), Erwinia carotovora sp. carotovora (NCPB 550), and E. carotovora sp. carotovora SCC3193 (24). Bacterial cultures were grown at 28 °C in the presence of the test products and ~2000 bacteria per well. Erwinia was grown in LPG medium (0.3% yeast extract, 0.5% Bacto-peptone, and 0.5% glucose), *Pseudomonas* in King’s medium (see above), and Xanthomonas in Kado medium (1% yeast extract, 0.8% casamino acids, 1% saccharose, 6.5 mM K2HPO4, and 6 mM MgSO4·7H2O). Absorbance measurements were carried out at 595 nm with a Bio-Rad microplate reader. Reported data are the means and standard deviations of the values obtained in at least three independent experiments.

**Examination of Cell Viability in Response to Oxylipin Treatment**—50 µl of pure oxylipin solutions, at concentrations of 50 or 100 µM, were injected into the apoplast of tobacco leaves using a syringe. Test products were applied alone or with suspensions (5 × 106 bacteria per ml) of the incompatible strain *P. syringae* pv *syringae* (NCPBP2686) or the compatible bacterium *P. syringae* pv tabaci (NCPBP1427). Visible symptoms were observed daily, and cell death of infiltrated areas was examined by lactophenol-trypsin blue staining (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, 20 mg of trypan blue, dissolved in 10 ml of distilled water) at 2 and 4 days after inoculation. Leaf discs from infiltrated areas were boiled for ~1 min in the stain solution and then decolorized with chloral hydrate (2.5 g of chloral hydrate dissolved in 1 ml of distilled water) for at least 30 min. They were mounted in chloral hydrate and viewed under a light microscope. At least three independent plants were used for each treatment, and a minimum of 10 infiltrated areas were examined per plant.

**RESULTS**

**Generation of Transgenic Tobacco Plants with Altered Levels of α-DOX1 Expression**—Transgenic plants containing the entire α-DOX1 cDNA fused to the CaMV35S promoter, in both sense and antisense orientation, were generated and used to study the activity of α-dioxygenase *in vivo*. Selection of appropriate transgenics was based on the level of α-DOX1 expression found in independent lines responding to inoculation with the incompatible bacterium *P. syringae*. Results from these analyses are shown in Fig. 1. In wild type plants, untreated leaves gave a weak hybridization signal that increased in RNA...
samples extracted 8 h after bacterial inoculation (8 hpi). The expression of \(\alpha\)-DOX1 reached maximum levels 1 day after inoculation (1 dpi) and decreased at later intervals of time. A clearly different situation was found in sense line 16, which had the highest level of \(\alpha\)-DOX1 expression. In this line, high amounts of transcripts were detected in leaves prior to bacterial inoculation, and RNA content was little affected by bacterial treatment. In contrast, the amount of \(\alpha\)-DOX1 transcripts in the antisense line 5 was severely reduced compared with wild type plants, and only a weak hybridization signal was detected in RNA samples extracted at 8 hpi.

These results correlated well with the accumulation of \(\alpha\)-DOX1. Gel blot analyses with an antiserum raised against the \(\alpha\)-DOX1 protein revealed the presence of a protein band of \(\pm 75\) kDa, i.e., the size predicted for the \(\alpha\)-DOX1 protein, in leaves of control plants responding to bacterial infiltration. The amount of \(\alpha\)-DOX1 protein increased significantly at 24 hpi and decreased to low level at the last time point examined, i.e., at 14 dpi. In accordance with the expression found in sense line 16, a high level of \(\alpha\)-DOX1 protein was detected in overexpressing plants at all the time intervals examined, and a slight transient increase in protein content could be observed at 4 dpi. In contrast to these results, accumulation of \(\alpha\)-DOX1 protein was almost undetectable in the antisense plants, and only a very small amount of protein was found at 1 dpi.

Analyses of plants responding to the compatible bacterium \(P.\) \(pv\) \(tabaci\) revealed similar results to those described above. As previously reported (7), \(\alpha\)-DOX1 expression was induced in wild plants after \(P.\) \(pv\) \(tabaci\) inoculation, although the accumulation of both RNA and protein showed a slight delay with respect to that found in the incompatible interaction. Results with sense line 16 revealed high levels of \(\alpha\)-DOX1 expression, whereas the presence of \(\alpha\)-DOX1 RNA and \(\alpha\)-DOX1 protein was almost undetectable in antisense line 5 treated with \(P.\) \(pv\) \(tabaci\) (data not shown).

**Identification of \(\alpha\)-DOX and LOX Products in Tobacco Leaves**—GC-MS analysis of derivatized extracts of wild type tobacco leaves revealed the presence of low levels of fatty acids, including linoleic and linolenic acids, the LOX products 9-HOT and 9,12,13-TriHOD, and the \(\alpha\)-DOX products 8,11,14-heptadecatrienoic acid and 2-HOT (Table I). On the other hand, 8,11,14-heptadecatrienal (earlier found to be the main product formed during the in vitro oxygenation of linolenic acid by \(\alpha\)-DOX1), and 13-HOT (13-LOX product of linolenic acid) were undetectable. A similar pattern was observed in tobacco leaves overexpressing \(\alpha\)-DOX1. Despite the considerable variability associated with determination of these low levels, the results indicated that resting tobacco leaves possess weak \(\alpha\)-DOX activity and that 2-HOT is the main \(\alpha\)-DOX product in vivo. The structures of the LOX products indicated that tobacco leaves metabolized linolenic acid in a similar way as recently recorded for leaves of potato (17, 25), i.e., by sequential action of 9-LOX, epoxy alcohol synthase and epoxide hydrolase. Notably, potato leaves produced mainly the trihydroxy oxylipin 9,10,11-TriHOD, whereas 9,12,13-TriHOD was the major trihydroxy isomer formed in tobacco leaves (Fig. 2).

**Synthesis of \(\alpha\)-DOX and LOX Products in Infected Tobacco Leaves**—Infection of tobacco leaves with \(P.\) \(pv\) \(syringae\) led to a rapid release of linoleic and linolenic acids. The peak values were observed at 8–24 hpi and amounted to 244 and 469 nmol/g leaves, respectively. A strong induction of \(\alpha\)-DOX products was also observed, i.e., 2-HOT (548 nmol/g at 1 dpi) and 8,11,14-heptadecatrienoic acid (47 nmol/g at 1 dpi) (Fig. 3). The main LOX products measured were 9-HOT (285 nmol/g at 1 dpi) and 9,12,13-TriHOD (359 nmol/g at 1 dpi). This product pattern was qualitatively similar to that observed in uninfected leaves (Table I and Fig. 2). Use of the bacterial strain \(P.\) \(pv\) \(tabaci\) led to a release of fatty acids that was about 7-fold lower than that observed with \(P.\) \(pv\) \(syringae\). Also the accumulation of dioxygenase products was weaker with \(P.\) \(pv\) \(tabaci\), i.e., a 4-fold lower yield of \(\alpha\)-DOX products and a more than 60-fold lower yield of LOX products (Fig. 4). Furthermore, the responses observed during infection with \(P.\) \(pv\) \(tabaci\) were delayed to 2–3 dpi.

**Fig. 2.** Major pathways in dioxygenase-catalyzed metabolism of linolenic acid in tobacco leaves.
Synthesis of α-DOX and LOX Products in Transgenic Tobacco Lines—Infection of tobacco plants overexpressing α-DOX1 with *P. pv syringae* led to a massive accumulation of α-DOX products, i.e. 1778 nmol/g 2-HOT and 86 nmol/g 8,11,14-heptadecatrienoic acid, corresponding to a 3-fold elevation compared with infected wild type plants. Notably, the yield of LOX products (120 nmol/g) were reduced 5.5-fold compared with wild type plants (Fig. 4). The data obtained with overexpressing α-DOX1 plants infected with *P. pv tabaci* showed the same trend, i.e. a 4-fold elevation of α-DOX products and a 2-fold lowering of LOX products (Fig. 4).

The amounts of α-DOX products produced in α-DOX1 antisense plants in response to *P. pv syringae* or *P. pv tabaci* were reduced 4-fold compared with wild type plants, however, zero levels were never observed (Fig. 4). The persisting α-DOX products were qualitatively the same as those in wild type plants, i.e. 2-HOT and 8,11,14-heptadecatrienoic acid in proportions ranging from 10–20:1 at most time points. Presumably, these remaining levels of α-DOX products were due to other α-DOX activity in tobacco leaves. A 1.3- to 3.6-fold elevation of LOX products was noted in α-DOX1 antisense plants compared with wild type plants.

Identification of 2-Hydroxy Acids in Leaves of Infected Tobacco Plants—As mentioned above, when wild type tobacco plants or plants overexpressing α-DOX1 were infected with the incompatible strain *P. pv syringae*, a strong accumulation of 2-HOT took place. Extracts of such leaves were analyzed by GC-MS for the presence of other endogenously produced 2-hydroxy acids. Authentic samples of 2-HOT, 2-hydroxyoleic acid, 2-hydroxystearic acid, and 2-hydroxypalmitic acid were used to allow unequivocal identification. Eleven 2-hydroxy acids differing with respect to chain length and number of double bonds could be identified, i.e. 2-hydroxy-18:3, 2-hydroxy-18:2, 2-hydroxy-18:1, 2-hydroxy-18:0, 2-hydroxy-17:3, 2-hydroxy-17:2, 2-hydroxy-17:0, 2-hydroxy-16:3, 2-hydroxy-16:1, 2-hydroxy-16:0, and 2-hydroxy-15:0. Of these compounds, 2-hydroxy-18:3 and 2-hydroxy-18:0 were the most abundant.

Identification of Doubly Dioxygenated Oxylipins—Deriva-
tized extracts of infected tobacco leaves were analyzed by GC-MS operated in the scan mode in search for new oxylipins. Peaks due to comparable amounts of two unknown compounds designated as Compound 1 and Compound 2 (Me₃Si ether/methyl ester derivatives) were observed in extracts from leaves of wild type or overexpressing plants infected with *P. pv syringae* but were undetectable in *α*-DOX antisense plants and in all plants infected with *P. pv tabaci*. The compounds were present in the fraction of polar acidic lipids isolated by solid phase extraction, and their GC retention times (Compound 1, 13.6 min; Compound 2, 13.8 min) were intermediary to those of extraction, and their GC retention times (Compound 1, 13.6 min; Compound 2, 13.8 min) were intermediary to those of monohydroxy C₁₈ oxylipins (11.5 min; Compound 2, 13.8 min). The time points in Fig. 3, which gave the highest level for each category, were selected for the calculation. *SR1*, wild type plants; *Sense*, *α*-DOX sense transgenic plants; *Antisense*, *α*-DOX antisense transgenic plants; *syringae*, *P. syringae pv syringae*; *tabaci*, *P. syringae pv tabaci*.

Because of the unavailability of the required deuterium-labeled standards, precise quantification of the doubly oxygenated oxylipins could not be performed by the GC-MS technique. However, using the intensities of the ions *m/z* 399, 363, and 223, the peak levels of 2(*R*),9(*S*)-dihydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid in wild type or *α*-DOX sense tobacco leaves infected with *P. pv syringae* could be estimated to 20–35 and 60–100 nmol/g, respectively.

**Sequences of the Biosynthesis of *α*-DOX-LOX Double Oxygenation Products**—Two pathways were conceivable for the biosynthesis of 2(*R*),9(*S*)-dihydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid from linolenic acid in tobacco leaves, *i.e.* initial oxygenation at C-9 by 9-LOX to produce 9-HPOT followed by *α*-DOX-catalyzed oxygenation at C-2, or the reversal of this sequence. Although it was not possible to distinguish between these possibilities in the *in planta* system, *in vitro* experiments were conducted to clarify the preferred pathways of the formation of doubly oxygenated oxylipins. In one set of experiments, 2(*R*)-hydroxylinolenic acid was incubated with tomato fruit 9-LOX or recombinant potato tuber 9-LOX using conditions under which linolenic acid was efficiently oxygenated. No conversion into 2-hydroxy-9-hydroperoxoctadecatrienoic acid (or other polar products) could be detected. On the other hand, incubation of 9-HPOT (200 μM) with membrane-bound *α*-DOX from cucumber fruit (8) for 20 min at 23 °C led to a mixture of 8(*S*)-hydroxy-9(*E*),11(*Z*),14(*Z*)-heptadecatrienal and 2(*R*),9(*S*)-dihydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (see “Experimental Procedures”). In other experiments, 2(*R*)-hydroxylinolenic acid was found to serve as an excellent substrate for...
Fig. 5. Sequences established for the \textit{in vitro} formation of $\alpha$-DOX-LOX double dioxygenation products from linolenic acid.
soybean lipoxygenase-1 to produce a 2-hydroxy-13-hydroperoxyocta-decatrienyl alcohol that was identified following reduction as 2(R),13(S)-dihydroxy-9(Z),11(E),15(Z)-octadecatrienoic acid. Furthermore, the linolenic acid-derived α-DOX product 8(Z),11(Z),14(Z)-heptadecatrienal was oxygenated into 12(S)-hydroperoxy-8(Z),10(E),14(Z)-heptadecatrienal by soybean lipoxygenase (see “Experimental Procedures”). On the other hand, using cucumber fruit material(s) was detectable. That 9-HOT, but not 13-HOT, prevented the growth of all bacterial strains examined.

**Lack of Antibacterial Activity**—The potential antibacterial activity of the major oxylipins measured in tobacco leaves, i.e., 2-HOT, 9-HOT, and 9,12,13-TriHOD, was assayed in *vitro* by growing bacterial cultures in microtiter plates in the presence of varying concentrations of oxylipins and monitoring the absorbance with a microplate reader. As seen in Fig. 6, no detectable alteration of the growth of *P. syringae* or *P. tabaci* was seen after addition of 11–170 μM oxylipin. Additional results (data not shown) revealed that none of the products inhibited the growth of *P. syringae*, *P. tabaci*, *X. campesiris* pv *campesiris*, or *E. carotovora* sp. *carotovora*. In contrast, a low concentration (11 μM) of 2(E)-hexenal, a short-chain hydroperoxide lyase-derived oxylipin, prevented the growth of all bacterial strains examined.

**Protective Effect against Cell Death**—The necrotic lesions produced in response to infiltration of bacterial suspensions into leaves were assessed in the absence or in the presence of varying concentrations of 2-HOT, 9-HOT, or 9,12,13-TriHOD added to the bacterial inoculum. As seen in Fig. 7, leaves developed necrotic lesions characteristic of an HR after inoculation with an incompatible strain of *P. syringae*. Thus, a weak necrotic response was observed at 24 hpi, whereas a strong necrotic aspect was apparent at 48 hpi (Fig. 7A). Inoculation of the compatible bacterium *P. tabaci* provoked symptoms characteristic of this infection, i.e., the appearance of small areas of disease necrosis and tissue chlorosis at 3–4 dpi (Fig. 7F). The extent of necrosis was strongly reduced when the bacterial suspensions contained 2-HOT or 9-HOT. The strongest effect was exerted by 2-HOT, which produced a clear reduction of cell death already at 50 μM concentration (Fig. 7, B and G), whereas smaller but still visible necrotic HR lesions were observed in leaves injected with *P. syringae* and 9-HOT (Fig. 7, D and E). Analyses of cell death by trypan blue staining was in accordance with visible symptoms and thus, the intensity of the coloration correlated precisely with the intensity of the necrosis formed (Fig. 7, K–U). In contrast to the results with 2-HOT and 9-HOT, addition of 9,12,13-TriHOD or linolenic acid to the bacterial inoculum did not cause any apparent alteration in the appearance of symptoms (data not shown). In other experiments, oxylipins alone were infiltrated (concentrations, 50 or 100 μM) and found to produce neither visible damage nor cell death in the corresponding area.

**DISCUSSION**

*In vitro* studies have shown that the dioxygenase reaction catalyzed by recombinant α-DOX1 consists of stereospecific removal of the pro-R hydrogen from carbon-2 of the fatty acid chain followed by stereospecific introduction of molecular oxygen to provide a 2(R)-hydroperoxyderivative (8, 9). Because of their inherent chemical instability (t½ in aqueous buffer at 23 °C, about 30 min), these hydroperoxides are further converted by non-enzymatic reactions to provide a one-carbon-shortened fatty aldehyde (83%) accompanied by smaller amounts of the hydroxy acid (15%), and the next lower fatty acid homolog (2%). In the present study, lipids from tobacco leaves were fractionated by a solid-phase extraction procedure, and the materials obtained were assayed for the presence of α-DOX products. In contrast to the *in vitro* results, the main linolenic acid-derived α-DOX products in tobacco leaves were identified as 2-HOT (90–95%) and 8,11,14-heptadecatrienoic acid (5–10%), whereas aldehydes, including 8,11,14-heptadecatrienal were not detected. Apparently, in leaves the major metabolic fate of enzymatically generated fatty acid 2-hydroperoxides is reduction (Fig. 2). The nature of this reduction step is unknown but may involve peroxiredoxin (26) or other peroxidase activity. In this context it should be noted that the α-DOX purified from pea seeds possesses an inherent peroxi-dase activity (11). Such activity is absent from recombinant tobacco α-DOX1 (8), however, it cannot be excluded that it is exhibited by native α-DOX1 in leaves. Irrespective of which peroxidase catalyzes reduction of 2-hydroperoxylinolenic acid and other 2-hydroperoxy acids, and of 9-HPOT and other LOX-derived hydroperoxides, it is apparent that this process will lead to consumption of considerable quantities of reducing equivalents (up to 1–2 μmol/g) in infected leaves.
Infection of tobacco leaves with Pseudomonas led to release of linoleic and linolenic acids and a strong accumulation of γ9251-DOX and 9-LOX products (Fig. 3 and 4). 9-LOX-derived divinyl ethers and trihydroxy fatty acids have earlier been found to accumulate in leaves of potato in response to infection with the pathogenic fungus P. infestans, and at least the divinyl...

**Fig. 7.** Analysis of symptoms developed with oxylipin-containing bacterial suspensions. Bacterial suspensions (5 × 10^6 bacteria per ml) spiked with 2-HOT or 9-HOT at concentrations indicated were inoculated in the apoplast of tobacco leaves. The two upper panels (A–J) show images of leaves infiltrated with bacterial suspensions of *P. pv syringae* (2 dpi) or *P. pv tabaci* (4 dpi), and the two lower panels (K–U) show corresponding images of infiltrated areas subjected to trypan blue staining.

**Fig. 8.** α-Oxidation of linolenic acid (18:3) in intact tobacco leaves. Part of the 2-hydroperoxylinolenic acid escapes reduction and undergoes spontaneous decarboxylation to 8,11,14-heptadecatrienal, which is enzymatically dehydrogenated into 8,11,14-heptadecatrienoic acid (17:3). The sequence can then be repeated.
ether fatty acids are believed to play a role in the defense against Phytophthora and other pathogens (5, 25). The pathogen-induced formation of α-DOX products in amounts comparable to those of 9-LOX products observed in the present study makes it necessary to take into account also the α-DOX-generated compounds, notably 2-hydroperoxy and 2-hydroxy fatty acids, as additional factors of potential importance in plant-pathogen interactions. In this context, the finding, that infection with the incompatible, HR-inducing bacterial strain P. pv syringae led to a 4-fold higher accumulation of α-DOX products compared with the compatible strain P. pv tabaci (Fig. 4), is of interest in view of the proposed role of the α-DOX pathway in protecting plant tissue from undergoing excessive necrosis during the HR response (14).

Tobacco leaves infected with P. pv syringae accumulated not only linolenic acid-derived α-DOX products but also 2-hydroxy derivatives of other fatty acids, including odd-numbered 2-hydroxy acids. The abundance of such compounds particularly in leaves from plants overexpressing α-DOX gave strong support for the previously postulated involvement of α-DOX in the plant α-oxidation pathway for one-carbon shortening of fatty acids (8, 11). Formation of the main odd-numbered hydroxy acid, i.e. 2-hydroxyheptadecatrienoic acid, will require two steps of α-DOX-catalyzed oxygenation, the first of which will be followed by decarboxylation and the second by reduction (Fig. 8). It seems likely that the α-oxidation pathway in plants is controlled in part by peroxidase activity, which diverts the 2-hydroperoxides from the pathway.

Sense or antisense α-DOX1 transgenic plants were developed inter alia to examine the possible interplay between the α-DOX and 9-LOX pathways during bacterial infection. Overexpressing plants responded to infection with P. pv syringae or P. pv tabaci with the accumulation of a major linolenic acid-derived oxylipin, i.e. 2-HOT, whereas this compound was a minor metabolite in infected antisense plants (Figs. 3 and 4). Interestingly, the amounts of α-DOX and LOX products varied reciprocally in transgenic plants, i.e. the increased formation of α-DOX products in overexpressing plants was accompanied by a reduced production of LOX products, whereas the opposite was true in α-DOX antisense plants (Fig. 4). This finding indicated that α-DOX and LOX compete for a common pool of free fatty acids liberated in the course of the infection. Such a shunting effect would be expected to limit production of specific dioxygenase products, however, whether this is of biological relevance remains to be determined.

Cooperation of α-DOX and LOX resulting in the formation of doubly oxygenated fatty acid derivatives was observed during infection with the incompatible strain P. pv syringae. Thus, linolenic acid provided 2(R),9(S)-dihydroxy-10,12,15-octadecatrienoic acid, the structure of which was confirmed by e.g. mass spectrometry, and linoleic acid was analogously transformed into 2(R),9(S)-dihydroxy-10,12-octadecadienoic acid. Judging from in vitro experiments, these compounds were formed by α-DOX-catalyzed oxygenation of 9-oxygenated fatty acids, i.e. 9-LOX catalysis preceding α-DOX (Fig. 5).

Using α-DOX transgenic Arabidopsis plants we recently obtained evidence suggesting that one or more products generated through the α-DOX pathway protect hypersensitively reacting leaf tissue from undergoing excessive necrosis (14). Identification in the present study of 2-HOT as the major α-DOX product in infected leaf tissue prompted us to determine whether this oxylipin can act as a cell-protecting factor in infected leaves. Interestingly, administration of 50–100 μM 2-HOT together with the bacterial inoculum strongly reduced the extent of cell death (Fig. 7). A qualitatively similar but weaker effect was observed for 9-HOT, whereas the trihydroxy oxylipin 9,12,13-TriHOD was inactive. The mechanism behind this protective effect of 2-HOT and 9-HOT in infected leaves is unknown, although it could be excluded from in vitro experiments that a direct bacteriostatic or bacteriocidal effect was involved (Fig. 6). The striking parallel between the cell-protecting effect observed in infected tobacco leaves to the anti-inflammatory and protecting effect exerted by the lipoxins, a group of lipoxigenase-generated eicosanoids in animal tissue (27), deserves mentioning.

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