Lipid Peroxidation during the Hypersensitive Response in Potato in the Absence of 9-Lipoxygenases*

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Hypersensitive cell death is an important defense reaction of plants to pathogen infection and is accompanied by lipid peroxidation processes. These may occur non-enzymatically by the action of reactive oxygen species or may be catalyzed by enzymes such as α-dioxigenases, lipoxygenases, or peroxidases. Correlative data showing increases in 9-lipoxygenase products in hypersensitively reacting cells have so far suggested that a large part of lipid peroxidation is mediated by a specific set of 9-lipoxygenases. To address the significance of 9-lipoxygenases for this type of pathogen response in potato, RNA interference constructs of a specific pathogen-induced potato 9-lipoxygenase were transferred to normal hypersensitive response in potato, lipid peroxidation transcript levels were observed in transgenic plants after pathogen treatment. In addition, 9-lipoxygenase activity was hardly detectable, and levels of 9-lipoxygenase-derived oxylipins were reduced up to 12-fold after pathogen infection. In contrast to wild type plants, high levels of non-enzymatically as well as 13-lipoxygenase-derived oxylipins were present in 9-lipoxygenase-deficient plants. From this we conclude that during the normal hypersensitive response in potato, lipid peroxidation may occur as a controlled and directed process that is facilitated by the action of a specific 9-lipoxygenase. If 9-lipoxygenase-mediated formation of hydroperoxides is repressed, autoxidative lipid peroxidation processes and 13-lipoxygenase-mediated oxylipins synthesis become prominent. The unaltered timing and extent of necrosis formation suggests that the origin of lipid hydroperoxides does not influence pathogen-induced cell death in potato.

Lipid peroxidation in plants is an important feature of the hypersensitive cell death, a typical defense reaction displayed during incompatible or non-host interactions of plants with pathogens (1). Membrane damage by peroxidation of polyunsaturated fatty acids can be initiated by reactive oxygen species (ROS), a lipid radicals, or enzymatically by the action of lipoxygenases (LOXs) (2). In plants, LOXs introduce molecular oxygen into linoleic (LA) and linolenic acid (LnA) at either the C-9 or the C-13, leading to the formation of the corresponding hydroperoxides. Apart from free fatty acid derivatives, LOXs can also catalyze the oxygenation of membrane lipids (3, 4), leading to altered fluidity and permeability of biomembranes.

In plants, correlative data suggest that 9-LOXs are crucial for lipid peroxidation during the hypersensitive response. Thus, 9-LOX-derived oxylipins accumulate in tobacco leaves undergoing a hypersensitive reaction in response to treatment with the elicitor cryptogein (5) and in cotton leaves infected with Xanthomonas campestris pv. malvacearum (6).

In addition to their proposed role in lipid peroxidation, LOXs may also contribute to defense responses in plants by synthesizing antimicrobial compounds and signal molecules. The products of the LOX reaction in plants, 9- and 13-hydroperoxylinolenic acid (9- and 13-HPOD/HPOT), are substrates for at least seven LOX pathway enzymes (7) that catalyze the synthesis of hydroxyoctadecadienoic acid (HOD) or hydroxyoctadecatrienoic acid (HOT), divinyl ether containing fatty acids such as colnele(n)ic and etherole(n)ic acid, 12-oxo-phytodienoic acid (OPDA), and jasmonic acid (JA), w-oxo fatty acids, aldehydes, and trihydroxy fatty acids.

For several of these oxylipins, antimicrobial activity has been demonstrated (8–11), suggesting that LOXs function in plant defense by contributing to pathogen growth inhibition. On the other hand, oxylipins of both the 9- and the 13-LOX pathway have been identified as signal molecules that activate defense responses. JA, its biosynthetic precursor OPDA, and their derivatives are well studied signaling compounds in the response of the plant to wounding (12, 13), whereas 13-HOT is able to induce PR gene expression in barley (14). Moreover, the products of the LOX reaction, 9- and 13-HPOD, as well as the corresponding hydroxides, 9- and 13-HOD, induce cell death in protoplasts (15, 16).

In solanaceous plants, oxylipins derived from the 9-LOX pathway are of importance for pathogen defense. Thus, pathogen-induced 9-LOX transcript accumulation was reported in a number of plants, for example in tobacco after infection with Phytophthora parasitica var. nicotianae (17) and in potato infected by Phytophthora infestans (18–20). The pathogen-induced accumulation of the 9-LOX products in potato cells (21) and plants (11, 20) suggests a role of these compounds for defense responses. More importantly, a functional analysis of dionioic acid; HOT, hydroxyoctadecatrienoic acid; HPOD, hydroperoxyoctadecadienoic acid; HPOT, hydroperoxyoctadecatrienoic acid; JA, jasmonic acid; LA, linoleic acid; LnA, linolenic acid; LOX, lipoxygenase; OPDA, 12-oxo-phytodienoic acid; PR1/PR10, pathogenesis-related protein A1 and 10, respectively; RNA, RNA interference; GC, gas chromatography; FID, flame ionization detector; HPLC, high pressure liquid chromatography; RT-PCR, reverse transcriptase-PCR.

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the elicitor-induced 9-LOX of tobacco revealed its crucial role for the establishment of resistance (17). However, how 9-LOXs exert their effect in the response to pathogens and, in particular, which of the 9-LOX-derived products are of importance have not been clarified yet.

Here we set out to functionally analyze the role of 9-LOXs for the response of potato to pathogen attack. After identification of the pathogen-induced 9-LOX isoform in our system, RNA interference (RNAi) was used to down-regulate its expression. Transgenic plants have a significant reduction in pathogen-induced 9-LOX activity, and the accumulation of enzymatically produced oxylipins was strongly reduced, but lipid peroxides of autoxidative origin increased. Cell death, however, is not significantly affected suggesting that potato plants can undergo hypersensitive cell death irrespective of the origin of lipid hydroperoxides.

EXPERIMENTAL PROCEDURES

RNA Analyses and Generation of 9-LOX cDNA Fragments by RT-PCR—RNA was isolated from pathogen-infected potato plants and subjected to Northern analyses as described (20). Reverse transcription was carried out using DNase-treated RNA with SuperScript™ II RNase H−Reverse Transcriptase (Invitrogen). Subsequent PCR was performed using the primers 5′-ACAAACAGGCAACTAAGTGTGCTTCACC-3′ and 5′-GCGATTTGGGAGGTAACCTGCATAAGG-3′ under standard conditions.

Cloning of RNA Constructs—A β-glucuronidase (GUS) gene cloned in a pUC19 vector was partially deleted at the EcoRV site by cutting the plasmid with EcoRV and SmaI and subsequent re-ligation. The 374-bp

![Fig. 1. POTLX-3 is the predominant LOX transcript in P. infestans-infected potato leaves.](image)

RNA isolated from P. infestans-infected potato leaves at the time points indicated was subjected to RT-PCR using 9-LOX-specific primers. The PCR products were separated on agarose gels either directly (−) or after restriction digestion with BglII (B) or ClaI (C). dpi, days post-infection.

![Fig. 2. Loss of POTLX-3 expression in RNAi transgenic plants.](image)

RNA was isolated from potato leaves 48 h after infiltration of P. syringae pv. maculicola (A) or 4 days after infection with P. infestans (B) and subjected to Northern analyses. Filters were hybridized with radioactively labeled cDNAs encoding POTLX-3 (19), PR1, PR10, or rRNA (21). A, 11 transgenic RNAi plants (RNAi, 1–11) and four control plants (control, 1–4) were infiltrated with P. syringae pv. maculicola (Pseudomonas) or 10 mM MgCl₂. B, 10 transgenic RNAi plants (RNAi, 1–10), were drop-inoculated with P. infestans (+) or water (−).

![Fig. 3. Loss of 9-LOX activity in transgenic RNAi plants.](image)

9-LOX activity was determined in protein extracts of potato leaves infiltrated with P. syringae pv. maculicola (filled bars) or 10 mM MgCl₂ (dotted bars) 0, 6, 12, 24, 36, and 48 h hours post-infiltration. Results shown were obtained in two independent experiments. Data shown combine the values determined for plants R4 and R6 (black bars), R8 and R9 (gray bars), and empty vector and wild type plants (white bars). 100% activity corresponds to 50 nmol of 9-HOD produced per g fresh weight.
POTLX-3-specific fragment was amplified from RNA of pathogen-infected plants via RT-PCR using the primers 5'-TATGTTGCAGTGAATTGACTCG-3' and 5'-GGATAGTCTTGAATTAGCAAG-3' with sequences for SstI restriction sites at each end. After cloning of the RT-PCR product into the vector pCR2.1 (Invitrogen), the fragment was inserted into an SstI site at the 3'-end of the truncated GUS gene upstream of the nopaline synthase polyadenylation signal. The 374-bp POTLX-3-specific fragment was then amplified from the pCR2.1 clone using primers with BamHI and PstI-KpnI sites at either end. This fragment was inserted upstream of the truncated GUS gene via BamHI/PstI in inverse orientation to the SstI fragment. The RNAi construct was inserted into a binary vector carrying the 35S promoter (22) as a KpnI-EcoRI fragment, and the resulting plasmid was transferred to Agrobacterium tumefaciens GV3101.

**Fig. 4.** 9-LOX products in RNAi plants after infiltration of *P. syringae pv. maculicola*. The amounts of 9-LOX products 9-HPOD, 9-HPOT, 9-HOD, 9-HOT, colneleic acid (CA), and colnelenic acid (CnA) were determined in extracts from leaves after infiltration of *P. syringae pv. maculicola*. Data shown were obtained in two independent experiments and combine the values for plants R4 and R6 (black bars), R8 and R9 (gray bars), and wild type and empty vector control plants (white bars). Data obtained from MgCl₂-infiltrated leaves are shown to the left of the bars. hpi, hours post-infiltration.
Potato plants were transformed with recombinant agrobacteria, and transgenic plants were regenerated as described (23). Infection of Plants and Analysis of Defense Responses—Potato plants (*Solanum tuberosum* L. cv. *De sire*) were grown as sterile plants in a phytochamber with 16 h of light (200 microeinsteins) at 22 °C. After transfer to soil, plants were kept in a phytochamber with 16 h of light (200 microeinsteins), 18 °C, and 60% humidity for 4 weeks. Lower leaves were infiltrated with a suspension of *Pseudomonas syringae pv. maculicola* at a concentration of 10⁸ colony-forming units/ml in 10 mM MgCl₂ or, as a control, 10 mM MgCl₂ solution, and the infiltrated leaves were used for subsequent analyses. For *P. infestans* infections, the lower leaves were inoculated by pipetting 10–20 droplets of 10 μl of a *P. infestans* zoospore suspension in water (2 × 10⁵ zoospores/ml) onto the abaxial leaf surface and keeping the plants at 100% humidity for

**Fig. 5.** 13-LOX products and autoxidative lipid peroxides in RNAi plants after infiltration of *P. syringae pv. maculicola*. JA, OPDA, 13-HOD, 13-HOT as well as 16-HOT and 12-HOT were measured in extracts from leaves after infiltration of *P. syringae pv. maculicola*. Data shown were obtained in two independent experiments and combine the values for plants R4 and R6 (black bars), R8 and R9 (gray bars), and wild type and empty vector control plants (white bars). Data obtained from MgCl₂-infiltrated leaves are shown to the left of the bars. hpi, hours post-infiltration.
the duration of the experiment. As controls, water was pipetted onto the leaves.

Cell death was determined by staining whole leaves with trypan blue as described (24). Hydrogen peroxide formation was visualized by staining with diaminobenzidine as described (25).

**Determination of LOX Activity and LOX Products**—LOX activity was determined using crude protein extracts from plants as described (20). Determination of hydroxy and hydroperoxy fatty acids as well as divinyl ethers was performed by HPLC-based and GC-based analyses. About 0.5 g of frozen leaf tissue was added to 10 ml of extraction medium (isoamyl/isopropyl alcohol, 3:2 (v/v) with 0.0025% (v/v) butylated hydroxytoluene) and immediately homogenized with an Ultra Turrax under a stream of argon for 30 s. As an internal standard, (E)-(+)-13,15-octadecadienoic acid and heptadecanoic acid were added. The extract was centrifuged at 4,500 × g at 4°C for 10 min. The clear upper phase was collected, and the pellet was extracted three times with 3 ml each of extraction medium. To the combined organic phases, a 6.7% (w/v) solution of potassium sulfate was added to a volume of 47 ml. After vigorous shaking, the upper hexane-rich layer was removed. The upper organic phase containing the oxylinps and fatty acids was dried under nitrogen and redissolved in 200 µl of methanol.

For the analysis of fatty acids, 10 µl of an EDAC solution (1 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/10 µl of methanol) and 370 µl of methanol were added to 20 µl of the methanol solution and incubated for 2 h. After adding 200 µl of 0.1 M Tris-HCl, pH 7.5, the fatty acid methyl esters (FAMEs) were extracted twice each with 1 ml of hexane. The combined organic phases were evaporated to dryness under nitrogen. The FAMEs were redissolved in 10 µl of acetonitrile and analyzed using an gas chromatograph/flame ionization detector (GC/FID).

Analysis of oxylipins was carried out by HPLC on an Agilent 1100 HPLC system coupled to a diode array detector. Prior to analysis, the solvent was removed, and the sample was reconstituted in 50 µl of methanol/water/acetic acid (85:15:0.1, v/v). At first, oxylipins were purified on reversed phase-HPLC. This was carried out on a ET250/2 Nucleosil 120–5 C18 column (Macherey-Nagel, 2.1 × 250 mm, 5-µm particle size) with a solvent system of methanol/water/acetic acid (85:15:0.1, v/v), and a flow rate of 0.18 ml min⁻¹. Straight phase-HPLC of the hydro(pero)xy derivatives of linoleic and linolenic acid was carried out on a Zorbax Rx-SIL column (Agilent, 2.1 × 150 mm, 5-µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100:0.1, v/v) and a flow rate of 0.2 ml min⁻¹. The absorbance at 234 nm was recorded indicating the conjugated double bond. Chiral phase-HPLC of the hydro(pero)xy fatty acids was carried out on a Chiralcel OD-H column (Daicel, 2.1 × 150 mm, 5-µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100:5:0.1, v/v) and a flow rate of 0.1 ml min⁻¹. Absorbance at 234 nm was monitored. Reversed phase-HPLC of the divinyl ethers was carried out on a ET250/2 Nucleosil 120–5 C18 column (Macherey-Nagel, 2.1 × 250 mm, 5-µm particle size) with a solvent system of methanol/water/acetic acid (90:10:0.1, v/v) and a flow rate of 0.18 ml min⁻¹. The absorbance at 250 nm was recorded indicating a conjugated diene system in conjugation with an ether bond of the fatty acids.

For the analysis of esterified oxylipins and fatty acids, tricirinolene and triheptadecanoate were used as internal standards, and the extraction was performed as described above. After removing of the solvent, 333 µl of a mixture of toluene and methanol (1:1, v/v) and 167 µl of 0.5 mM sodium methoxide were added. After incubation for 20 min, 0.5 ml of 1 M NaCl and 50 µl of HCl (37%, v/v) were added, and the FAMEs and oxidized derivatives were extracted twice each with 0.75 ml of hexane. The combined organic phases were evaporated to dryness under nitrogen and dissolved in 200 µl of methanol. 20 µl of this solution was dried under nitrogen and dissolved in 10 µl of acetonitrile for the analysis of the FAMEs by GC/FID-based analysis. The methyl esters of the oxylipins and fatty acids was dried under nitrogen and redissolved in 200 µl of methanol.

For determination of JA and OPDA, a GC/mass spectrometry-based analysis was used as described (20).

**RESULTS**

**Identification of POTLX3 as the Pathogen-induced 9-LOX in Potato**—To reduce the pathogen-induced expression of 9-LOXs (7). By using cDNAs of both groups as probes, pathogen-induced accumulation of 9-LOX transcripts in potato has been reported (18–20). However, based on the high sequence similarity of these cDNAs, cross-hybridization might conceal which of these genes are specifically expressed after pathogen attack. Therefore, RNA from plants infected with *P. infestans* was reverse-transcribed and subjected to PCR using primers designed to amplify a 582-bp fragment from all 9-LOX genes reported in the data base. The mixture of PCR products was digested with either BglII or ClaI, enzymes that cut specifically in fragments derived from POTLX-3 mRNA (19) or from tuber-specific 9-LOX mRNA (26, 27), respectively. Subsequently, the restriction fragments were separated on agarose gels (Fig. 1). Starting 1 day after infection with *P. infestans*, cDNA fragments digestable by BglIII but not by ClaI were present, indicating that the majority of 9-LOX-derived cDNA fragments were derived from POTLX-3 transcripts. The portion of cDNA fragments that was not digestable by BglIII decreased and was not detectable after 5 days. Thus, POTLX-3 transcripts represent the majority of pathogen-inducible 9-LOX transcripts. Moreover, our results show that transcripts encoding the tuber-specific 9-LOXs, whose cDNA fragments should have been cut by ClaI, cannot be detected in either untreated or pathogen-infected leaves by this method. Interestingly, in cDNA derived from leaves of uninfected plants, neither BglIII nor ClaI was able to cut the mixture of PCR fragments, indicating that mRNA encoding a so far unidentified 9-LOX isoform had been amplified in the RT-PCR. This LOX cDNA fragment (SIL0X1–4) was subcloned and sequenced (data not shown). Highest sequence similarity was found to a toxin-induced tomato LOX (97%, GenBank™ accession number AY008278) and to the elicitor-induced 9-LOX from tobacco (90%, 28). StIL0X1–4 shared less than 85% homology at the nucleotide level with LOX isoforms from tubers.

**FIG. 6.** Hypersensitive response in RNAi plants induced by infiltration of *P. syringae pv. maculicola*. 24 h after infiltration of *P. syringae pv. maculicola*, leaves of the RNAi plants R6, R8, and R9 as well as a wild type (wt) control plant were photographed (upper panel), stained for cell death with trypan blue (middle panel), or stained for hydrogen peroxide with diaminobenzidine (lower panel).

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**DISCUSSION**

Direct peroxidation of membrane lipids as well as the synthesis of cell death-inducing oxylipins are the proposed contributions of LOXs to programmed cell death in animals (30). Animal 15-LOX can initiate programmed organelle disruption by dioxygenating mitochondrial membranes leading to formation of pore-like structures (31). Treatment of mitochondria with the soybean 15-LOX results in disruption of membrane integrity and release of cytochrome c, usually a trigger of apoptosis (30).

Whether lipid peroxidation during the pathogen-induced hypersensitive cell death in plants is catalyzed by LOXs or is the
result of processes mediated by reactive oxygen species so far has been addressed by the determination of levels and nature of the accumulating lipid peroxides. It is generally assumed that the preponderance of S-enantiomeric hydroperoxides of polyunsaturated fatty acids represents the products of enzymatic reactions and that racemic mixtures result in almost all cases from non-enzymatic reactions, e.g. the autoxidative processes. Thus, the accumulation of large amounts of (9S)-HPPOD and (9S)-HPOT in pathogen-infected plant tissue has led to the view that 9-LOXs are the enzymes responsible for lipid peroxidation (5, 6). Furthermore, the timing of oxylipin accumulation and the onset of cell death which, in potato, occur concomitantly 6–12 h after bacterial infection (20) support the notion that 9-LOX-mediated lipid oxygenation is the cause for membrane damage during the hypersensitive response. In tobacco treated with the hypersensitive response-inducing elicitor cryptogein, LOX activity starts to increase between 8 and 12 h after elicitor treatment, which correlates with cell death formation between 12 and 24 h (5, 32).

However, only functional analyses can prove or disprove a role for LOXs in lipid peroxidation. Gain-of-function experiments using products of the LOX pathway were performed to demonstrate a role for LOXs in inducing cell death. In animals, various lipid hydroperoxides, as well as the substrate of animal LOXs, arachidonic acid, are known to induce apoptosis (30, 33–35). In plants, both the hydroperoxides and the hydroxides of 9- and 13-LOX products of LA and LnA are able to induce programmed cell death in tomato protoplasts (36) and lentil root protoplasts (30). Loss-of-function approaches have included inhibitor studies, expression of LOX antibodies in plant cells, and deprivation of oxygen. In lentil root protoplasts, application of different LOX inhibitors resulted in reduction of H2O2-induced cell death (37). However, LOX inhibitors are generally considered to be problematic because of their unspecific activity on other enzymes. Eicosatetraynoic acid, for example, is considered to be an irreversible inhibitor for LOXs but is also used as a specific inhibitor of phospholipase A2 (38). In addition to their possible effect on other enzymes, most of the LOX inhibitors can also act as general radical scavengers and may thus interfere with cell death via a LOX-independent mechanism.

Electroporation of anti-LOX monoclonal antibodies into lentil root protoplasts decreased the H2O2-induced programmed cell death—2-fold, suggesting that the lentil LOX is important for cell death (30). Finally, the failure of cryptogein to induce necrosis in tobacco under low oxygen pressure was interpreted as specific antimicrobial compounds. Being non-toxic for the plant, they might thus enhance the defense capacity of the plant in addition to the initial hypersensitive cell death reaction.

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fatty acids by ROS. Presumably, in the presence of 9-LOXs, lipid peroxidation proceeds via enzymatic reactions, possibly by the activation of the specific 9-LOX isoform by ROS. The enzymatic formation of lipid peroxides by LOXs during induction of hypersensitive cell death might be of dual advantage to the response of the plants to pathogens as follows: (i) because LOX-derived lipid peroxides are less toxic substances for the plant, because they are rapidly metabolized by enzymes of the LOX pathway; and (ii) several of these metabolites act as specific antimicrobial compounds. Being non-toxic for the plant, they might thus enhance the defense capacity of the plant in addition to the initial hypersensitive cell death reaction.