Oxylipin Profiling Reveals the Preferential Stimulation of the 9-Lipoxygenase Pathway in Elicitor-treated Potato Cells*

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Cornelia Göbel‡, Ivo Feussner§, Axel Schmidt§§, Dierk Scheel‡, Jose Sanchez-Serrano**, Mats Hamberg‡‡, and Sabine Rosahl §§§

From the ‡Department of Stress and Developmental Biology, §Department of Secondary Metabolism, Institute of Plant Biochemistry, Weinberg 3, Halle/Saale D-06120, Germany, §§Department of Molecular Cell Biology, Institute of Plant Genetics and Crop Plant Research, Correnstrassee 3, Gatersleben D-06466, Germany, **Centro Nacional de Biotecnologia CSIC, Universidad Autonoma de Madrid, Campus Cantoblanco, Madrid E-28049, Spain, and the ‡‡Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, Stockholm S-171 77, Sweden

Lipoxygenases are key enzymes in the synthesis of oxylipins and play an important role in the response of plants to wounding and pathogen attack. In cultured potato cells treated with elicitor from Phytophthora infestans, the causal agent of late blight disease, transcripts encoding a linoleate 9-lipoxygenase and a linoleate 13-lipoxygenase accumulate. However, lipoxygenase activity assays and oxylipin profiling revealed only increased 9-lipoxygenase activity and formation of products derived therefrom, such as 9-hydroxy octadecadienoic acid and coneleic acid. Furthermore, the 9-lipoxygenase products 9(S),10(S),11(R)-tri-hydroxy-12(Z)-octadecenoic and 9(S),10(S),11(R)-trihydroxy-12(Z),15(Z)-octadecadienoic acid were identified as novel, elicitor-inducible oxylipins in potato, suggesting a role of these compounds in the defense response against pathogen attack. Neither 13-lipoxygenase activity nor 13-lipoxygenase products were detected in higher amounts in potato cells after elicitation. Thus, formation of products by the 9-lipoxygenase pathway, including the enzymes hydroperoxide reductase, divinyl ether synthase, and epoxy alcohol synthase, is preferentially stimulated in cultured potato cells in response to treatment with P. infestans elicitor. Moreover, elicitor-induced accumulation of desaturase transcripts and increased phospholipase A2 activity after elicitor treatment suggest that substrates for the lipoxygenase pathway might be provided by de novo synthesis and subsequent release from lipids of the endomembrane system.

The formation of hydroperoxy derivatives of polyunsaturated fatty acids (PUFAs) represents the first step in the synthesis of oxidized PUFAs, the oxylipins. Their formation in plants may occur either by autoxidation or by the action of enzymes. Enzymatic formation of fatty acid hydroperoxides in plants is catalyzed by nonheme iron-containing lipoxygenases (LOXs), whereas hydroperoxide reductase catalyzes the synthesis of hydroxy octadecadienoic acid (HPOD) and hydroperoxy octadecatrienoic acid (HPOT), are substrates of different enzymes within the LOX pathway (see Fig. 1). A peroxysome and a reductase catalyze the synthesis of hydroxy octadecadienoic acid (HOD) or hydroxy octadecatrienoic acid (HOT), whereas the activity of divinyl ether synthase (DES) leads to formation of vinyl ether containing PUFA such as coneleinic and ethereonic acids (5). The synthesis of the signaling compound jasmonic acid originates from 13-HPOT by the activity of an allene oxide synthase (EC 4.2.1.92), whereas a hydroperoxide lyase (HPL) catalyzes the formation of ω-oxo fatty acids and aldehydes (6). Trihydroxy octadecenoate are synthesized from linoleic acid-derived epoxy alcohols via epoxy alcohol synthase (EAS) and epoxy alcohol hydratase (7). Finally, LOX itself can catalyze the synthesis of keto PUFAs.

Functional analyses in transgenic plants have shown the importance of LOXs, the downstream enzymes, and the products of the LOX pathway in the plant’s response to wounding and pathogen attack. Thus, transgenic Arabidopsis plants with decreased levels of 13-LOX do not show the usual rise in jasmonic acid in response to wounding and are deficient in wound-induced csp transcript accumulation (8). Increased susceptibility to insect attack was observed in transgenic potato plants with reduced 13-LOX levels (9) and in Arabidopsis plants that were deficient in the LOX substrate linolenic acid (10).

In addition to altered responses to wounding and insect attack, defense against the fungal root pathogen Phytophthora parasitica was also impaired in plants with decreased levels of linolenic acid (11) as well as in the Arabidopsis jar1 mutant, which is insensitive to jasmonate (12). Similarly, the jasmonate-response mutant coi1 exhibits a higher susceptibility to fungal pathogens such as Alternaria brassicicola and Botrytis cinerea (13).

In contrast to the rather well studied products of the 13-LOX pathway, the products of 9-LOX are less well understood. However, 9-lipoxygenase activity is preferentially stimulated in cultured potato cells in response to treatment with elicitor from P. infestans, which is insensitive to jasmonate (12). Similarly, the jasmonate-response mutant coi1 exhibits a higher susceptibility to fungal pathogens such as Alternaria brassicicola and Botrytis cinerea (13).
reaction, such as hexenals, traumatin, and jasmonic acid (14, 15, 4), 9-LOX products have only recently become the focus of attention. A possible role in the establishment of resistance of potato against late blight, caused by the oomycete Phytophthora infestans, has been suggested for the 9-LOX-derived divinyl ethers colneleic and colnelenic acids. This was based on the observation that they accumulate in potato leaves after fungal infection and that they exhibit antimicrobial activity (5). Recently, the efficient synthesis of isomeric trihydroxy octadecenoates and octadecadienoates via epoxy alcohols derived from the 9-LOX reaction has been shown to occur in potato leaves (7). The accumulation of 9-hydroperoxy PUFAs in tobacco after initiation of the hypersensitive response by cryptogein points to a role of 9-LOXs in lipid peroxidation during the hypersensitive response (16). Apart from these correlative data, the importance of 9-LOXs for resistance was demonstrated in tobacco plants in which the elicitor-induced increase in 9-LOX activity was inhibited by expression of antisense constructs (17). In contrast to the resistant wild type plants, transgenic plants were susceptible to infection with Phytophthora parasitica var. nicotianae. Such a conversion of an incompatible interaction into a compatible one suggests a crucial role for the tobacco 9-LOX in conferring the resistance phenotype.

In potato, three distinct classes of LOX cDNAs have been described, which encode a tuber-specific 9-LOX (stlox1), possibly located in the cytoplasm, and two wound-inducible, probably chloroplastic 13-LOXs (stlox2 and stlox3 (18, 19)). Although expression of stlox1 has been shown to be induced in potato leaves in response to infection by P. infestans or after application of the elicitor arachidonic acid (20), no detailed analysis has been performed addressing the question whether individual LOX transcripts and other products of the LOX pathway apart from the divinyl ethers (5) occur specifically in response to pathogen attack in potato. We therefore used our model system of cultured potato cells to determine the expression pattern of the three LOX isoforms in response to treatment with an elicitor from P. infestans. Furthermore, we performed oxylipin profiling in elicitor-treated potato cells to obtain insight into enzymatic properties of distinct LOX isoforms during conditions of pathogen attack. In addition, enzymes and metabolites upstream from the LOX pathway (Fig. 1) were studied and are discussed with respect to possible functions in plant pathogen interactions.

**EXPERIMENTAL PROCEDURES**

**Infection Experiments**—Culturing of potato cells (cv. Desiree), preparation of crude elicitor from P. infestans, and treatment of suspension-cultured potato cells with elicitor was performed as described previously (21, 22).

**Northern Analyses**—RNA from suspension-cultured potato cells was isolated as described (22). Hybridizations to radioactively labeled fragments of the different cDNA clones were carried out in 5× SSPE, 5× Denhardt’s solution, 0.1% SDS, 50% formamide, 100 μg/ml denatured salmon sperm DNA. Filters were washed three times at 60 °C with 3× SSC, 0.1% SDS. As probes, the following cDNA fragments were used: a 1.4-kb EcoRI fragment from stlox1 (18), a 2.2-kb BamHI fragment from stlox2 (19), a 1.8-kb PsI fragment from stlox3 (19), a 0.9-kb EcoRI

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**Fig. 1. Metabolic routes for LOX-dependent catabolism of PUFAs in plants.** The preferential route described here for elicitor treatment of potato cell cultures is indicated by bold arrows.
Elicitor Inducibility of 9-Lipoxygenase Pathway

Gene expression in cultured potato cells after treatment with P. infestans elicitor. RNA was isolated from cultured potato cells treated with a crude elicitor preparation of P. infestans (P.i.-cf) or H₂O at the time points indicated (h), separated on formaldehyde gels, blotted onto nylon membranes, and hybridized with radioactively labeled probes derived from the cDNA encoding LOX1, LOX3, PR1, PR10, PAL, 4CL, TIT, and, for standardization, with a radioactively labeled rRNA probe.

RESULTS

stlox1 and stlox3 Expression Is Induced in Potato Cells in Response to P. infestans Elicitor—Potato cells grown in suspension respond to elicitation by a crude preparation of P. infestans culture filtrate with the activation of defense genes, for example, those encoding enzymes of the phenylpropanoid pathway (33). To determine the expression pattern of three stlox genes from potato, RNA was isolated from cultured potato cells at different time points after elicitor treatment and subjected to Northern analyses (Fig. 2). Both stlox1 and stlox3 mRNA levels increased transiently in response to elicitor treatment, whereas the level of stlox2 transcripts was below the detection limit both in control and elicitor-treated cultures (data not shown). In different experiments, stlox1 transcripts were first detected 1 to 2.5 h after elicitation, reaching maximal levels after 5 to 10 h. In contrast, stlox3 mRNAs started to accumulate earlier, being detectable already 30 min after initiation of treatment and declining after 5 h. The time point of induction of stlox3 gene expression is similar to that of the activation of speed. The mixture was extracted with two portions of diethyl ether, and the material obtained was loaded onto an amionopropyl Sepeluent tube (0.5 g; Supelco, Bellefonte, PA). Elution was performed with 2-propanol-chloroform (1:2, v/v), diethyl ether-acetic acid (98:2, v/v), and methanol-acetic acid (98:2, v/v). The last-mentioned eluate was taken to dryness, and the residue was treated with diazomethane and trimethylsilylation.

Analysis by gas chromatography/mass spectrometry was carried out using a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph fitted with a SPB-1701 capillary column (length, 15 m; film thickness, 0.25 mm). The initial column temperature was 120 °C and raised at 10 °C/min until 240 °C. Under these conditions, the retention times of the methyl ester/trimethylsilyl ether derivatives of 9,10,11-trihydroxyoctadecadienoic acid, 9,10,11-trihydroxyoctadecenoic acid, and deuterium-labeled standard were 14.3, 14.2, and 14.5 min, respectively. The mass spectrometer was operated in the selected ion-monitoring mode using the ions m/z 278 for the deuterium-labeled standard and m/z 271 for the two 9,10,11-trihydroxy derivatives. Standard curves were constructed by plotting the intensities of m/z 271/278 versus the molar ratios of known mixtures of 9,10,11-trihydroxy acids and deuterium-labeled standard and used to calculate the amounts of 9,10,11-trihydroxy acids present in samples analyzed.

For the analysis of jasmonic acid, 0.5 g of tissue was essentially treated as described (31). To determine the expression pattern of three stlo genes from potato, RNA was isolated from cultured potato cells at different time points after elicitor treatment and subjected to Northern analyses (Fig. 2). Both stlox1 and stlox3 mRNA levels increased transiently in response to elicitor treatment, whereas the level of stlox2 transcripts was below the detection limit both in control and elicitor-treated cultures (data not shown). In different experiments, stlox1 transcripts were first detected 1 to 2.5 h after elicitation, reaching maximal levels after 5 to 10 h. In contrast, stlox3 mRNAs started to accumulate earlier, being detectable already 30 min after initiation of treatment and declining after 5 h. The time point of induction of stlox3 gene expression is similar to that of the activation of speed. The mixture was extracted with two portions of diethyl ether, and the material obtained was loaded onto an amionopropyl Sepeluent tube (0.5 g; Supelco, Bellefonte, PA). Elution was performed with 2-propanol-chloroform (1:2, v/v), diethyl ether-acetic acid (98:2, v/v), and methanol-acetic acid (98:2, v/v). The last-mentioned eluate was taken to dryness, and the residue was treated with diazomethane and trimethylsilylation.

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genes encoding enzymes of the phenylpropanoid pathway, such as PAL and 4-CL as well as THT, as has been shown previously (33, 22). Hybridization to cDNAs encoding PR1 or PR10 showed that expression of the corresponding genes is induced later, i.e. after 5 h.

To determine if the increase in stlox transcript levels correlates with higher protein levels, crude protein extracts were prepared from elicitor-treated as well as water-treated cultured potato cells. Immunoochemical analyses using a polyclonal antiserum against StLOX1 showed higher levels of LOX protein 10 and 20 h after elicitor treatment compared with water-treated controls (Fig. 3). Extracts prepared from cells treated with elicitor for shorter periods, i.e. for 0, 1, 2.5, and 5 h, did not contain detectable amounts of LOX protein (Fig. 3 and data not shown).

**LOX Activity Increases in Elicitor-treated Potato Cells—**A LOX enzyme activity assay performed with a Clark oxygen electrode revealed higher linoleic acid-dependent oxygen consumption in extracts of elicitor-treated cells (data not shown). In a second approach using HPLC analyses, both 13-HOD and 9-HOD were detected, but only 9-HOD was measured to significantly higher amounts in extracts from elicited cells. As shown in Fig. 4, 9-LOX activity started to increase 2.5 h after initiation of treatment and reached five times higher levels after 5 and 10 h. A specific increase in 9-LOX activity upon elicitor treatment is also indicated by a corresponding shift in the ratio of 13- to 9-HOD from 33:67 in extracts of control cells to 7:93 after 5 and 10 h of elicitor treatment (Table I). Chiral phase HPLC revealed that more than 90% of 9-HOD was the enantiomer, indicating that this compound originated from enzymatic conversion, whereas the racemic nature of the 13-HOD analyzed suggested a nonenzymatic origin (data not shown).

**Analysis of Elicitor-induced Changes in Oxylipin Pattern—**The specific induction of a 9-LOX activity in elicitor-treated potato cells was expected to be reflected in elevated levels of metabolites of the oxylipin profile specific for corresponding downstream enzymes. Therefore, this profile was recorded in elicitor-treated and nontreated cells.

Although hydroperoxy PUFAs survive the work-up procedure to a significant extent (29), HPD and HPOT levels were below the detection limit. Analysis of the amounts of HOD, the representative of the reductase branch, showed a 5-fold increase in 9-HOD levels in elicitor-treated cells (Fig. 5A). 9-HOD started to accumulate between 10 and 20 h after initiation of treatment. Neither 9-HOT nor the 13-LOX-derived metabolites 13-HOD and 13-HOT could be detected.

Among the divinyl ethers derived from the DES reaction, colneleic and colnelenic acid, the derivatives of 9-HPOD and 9-HPOT, respectively, have been reported to accumulate in potato leaves after infection with *P. infestans* (5). In extracts of cultured potato cells, colneleic acid was detectable as well. In addition to the conventional HPLC analysis determining its characteristic UV spectrum, the identification of colneleic acid was confirmed by acid hydrolysis of the collected substance and the subsequent detection of (2E)-nonenal as its dinitrophenylhydrazone derivative as one fragment (data not shown). Colneleic acid started to accumulate 5 h after initiation of treatment and reached maximal levels of about 680 pmol/g of fresh weight after 20 h (Fig. 5B). Colnelenic acid, the divinyl ether derived from linolenic acid, and the corresponding derivatives from 13-HPOD or 13-HPOT, etheroleic and etherolenic acid, could not be detected.

In potato leaves, a new branch within the LOX pathway, the EAS pathway, has recently been described, which leads to the synthesis of trihydroxy octadecenoates, the trihydroxy derivatives of linoleic acid (7). The analysis of elicitor-treated cultured potato cells revealed the elicitor-inducible accumulation of 9(S),10(S),11(R)-trihydroxy-12(Z)-octadecenoate, together with a smaller increase in 9(S),10(S),11(R)-trihydroxy-12(Z),15(Z)-octadecadienoate, the corresponding derivative of linolenic acid (Fig. 5, C and D). Only small amounts of trihydroxy octadecenoates and trihydroxy octadecadienoic acid were measured in extracts of untreated or water-treated cells. In elicitor-treated cells, accumulation started between 2.5 and 5 h after addition of the elicitor, and levels increased up to 30 h. For the trihydroxy derivative of linoleic acid, at least 10-fold higher levels were measured after 30 h (about 240 pmol/g of fresh weight), whereas the amounts of the trihydroxy derivative of linolenic acid (46 pmol/g of fresh weight) were significantly lower.

Changes in the level of other 9-LOX-derived products such as products from the HPLC branch were not detected, and, in accordance with the absence of 13-LOX activity in elicited potato cells, no changes in levels of 13-LOX-derived products were found. Neither jasmonic acid nor 13-HOT or 13-HPOT were detected at increased levels of elicitor treatment. Similarly, no C8-aldehydes such as (3Z)-hexenal were detectable. Oxylipins derived from autoxidation such as 12- and 16-HOT (31, 16) could also not be found in our model system. In summary, maximal formation of the oxylipins colneleic acid, 9(S),10(S),11(R)-trihydroxy-12(Z)-octadecenoate, 9-HOD, and 9(S),10(S),11(R)-trihydroxy-12(Z),15(Z)-octadecadienoate was detected.

**Origin of the Substrates for the LOX Reaction—**Because oxylipin profiling showed that the major elicitor-inducible oxylip-
ins in potato cells are 9-LOX-derived metabolites of linoleic acid, studies addressing the origin of LOX substrates were performed. Because SLOX1 appears to be a cytosolic enzyme, a microsomal origin of the substrates was assumed. Therefore, a partial cDNA clone exhibiting high similarity to Δ12-acyl lipid desaturase-homologous (PLA2) activity than extracts from control cells as measured by the release of radioactively labeled linoleic acid from the sn2 position of PC (Fig. 7). PLA2 activity started to increase as early as 2.5 h after elicitation and reached its maximum after 20 h. Thus, both a desaturase, as concluded from its transcript accumulation, and the specific action of a PLA2 appear to contribute to the provision of substrates for the LOX pathway.

**DISCUSSION**

Oxylipins are important signaling and defense compounds in plants whose synthesis may occur either enzymatically via the LOX pathway or by autoxidation. In the present study, the role of the LOX pathway in plant-pathogen interactions was analyzed by parallel recording of levels of mRNAs, proteins, enzyme activities, and metabolites in potato cells in response to elicitor treatment. By using this strategy of complex analyses it was shown that the 9-LOX reaction is preferentially induced in elicitor-treated potato cells, leading to accumulation of compounds derived from the reductase, DES and EAS branches of the LOX pathway (Fig. 1, bold arrows).

Although 13-LOX expression is induced upon pathogen infection in a number of plants (*i.e.*, in rice (35), wheat (36, 37), or broad bean (38)), accumulation of transcripts corresponding to 9-LOX-encoding cDNAs has so far only been reported to take place in the solanaceous plants tobacco and potato in response to infection with oomycetes or to treatment with oomycete-derived elicitors (39, 40, 20). Here, we show further that, concomitant with the accumulation of transcripts corresponding to the 9-LOX-encoding cDNA *stlox1*, higher 9-LOX activity and increased levels of 9-LOX products can be measured after elicitor treatment in potato cells.

In contrast to the large amount of data available for 13-LOX products, until recently not much was known about the products of the 9-LOX reaction. Weber et al. (5) reported the pathogen-induced accumulation of colneleic and colnelenic acid, di-vinyl ethers derived from 9-HPOD and 9-HPOT. Here, we show that linoleic acid also accumulates in suspension-cultured potato cells upon treatment with *P. infestans* elicitor, reaching maximal levels of 600 pmol/g of fresh weight and being the most prominent oxylipin in the present study. Thus, with respect to 9-LOX products, cultured potato cells react to *P. infestans*-derived elicitors qualitatively similar as *P. infestans*-infected leaves.
For LOXs from potato, so far only data on the expression of genes corresponding to the tuber-specifically expressed stlox1 sequences are available. Fidantsef and Bostock (20) demonstrated the inducibility of stlox1 gene expression in potato leaves after infection with P. infestans. The stlox2 mRNA accumulation, shown to occur specifically after elicitor treatment, was not accompanied by a corresponding increase in 13-LOX activity or 13-LOX products. This strengthens the notion that more than the profile of mRNA accumulation is necessary to describe altered metabolic activities.

In contrast to the potato cells described here, cultured cells of a number of other plant species respond to treatment with pathogen-derived elicitors with the accumulation of jasmonic acid and its precursor, 12-oxo-phytodienoic acid (48). In particular, treatment of tobacco cells with an elicitor derived from P. parasitica var. nicotianae results in a rapid and transient increase in jasmonic acid levels (49). Because we could not measure 13-LOX activity in extracts of two independently generated potato suspension cultures, we do not think that the failure to detect 13-LOX activity or products is an artifact of the specific cell culture. Interestingly, no significant changes in jasmonic acid levels were detected in P. infestans-infected potato leaves 3 days postinfection (5), raising the possibility that infection with the oomycete P. infestans or treatment with P. infestans-derived elicitor does not activate the 13-LOX pathway.

The availability of sufficient amounts of substrate seems to play a crucial role in the activation of the LOX pathway as tobacco plants overexpressing different LOX genes show no or only moderately elevated levels of LOX-derived metabolites (50, 51). Because linoleic acid-derived oxylipins were the major derivatives detected in elicitor-treated potato cells, the linoleic acid pool was analyzed in two different ways: (i) by studying mRNA accumulation of Δ12-desaturases, which catalyze the formation of linoleic acid from oleic acid at the sn2 position of PC within the endoplasmic reticulum, and (ii) by measuring activity of PLA2, the major enzyme responsible for release of these PUFAs into the cytosol. Indeed, RNA analyses revealed an induction of Δ12-desaturase transcript accumulation, suggesting that de novo synthesis of linoleic acid might occur upon elicitation in potato as has been suggested for elicitor-induced linoleic and linolenic acid formation in parsley (52, 53). Although we did not detect significant changes in the levels of free PUFAAs in elicitor-treated potato cells (data not shown), the activation of a PLA2 upon elicitation suggests that the substrate for the synthesis of linoleic acid-derived oxylipins in potato might be liberated from PC to serve as a substrate for StLOX1.

Taken together, our results suggest that, in cultured potato cells, elicitor treatment leads to activation of PUFA-generating enzymes, stimulation of the 9-LOX pathway, and subsequently, to increased formation of products of the DES, EAS, and reduc
tase branch of the LOX pathway. The function of these compounds for the response of potato to pathogen attack will be analyzed by gain- and loss-of-function studies in transgenic plants.

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Cornelia Göbel, Ivo Feussner, Axel Schmidt, Dierk Scheel, Jose Sanchez-Serrano, Mats
Hamberg and Sabine Rosahl

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