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Accumulation of chloroplast-targeted lipoxygenase in passion fruit leaves in response to methyl jasmonate

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Abstract

Wounding caused local and systemic induction of lipoxygenase (LOX) activity in passion fruit (*Passiflora edulis f. flavicarpa*) leaves, while exposing intact plants to methyl jasmonate (MJ) vapor provoked a much stronger response. Western blot analysis of these leaf protein extracts using polyclonal antibodies against cucumber LOX, revealed an accumulation of a 90 kDa protein, consistent with LOX enzymatic assays. The inducible LOX was purified to apparent homogeneity, and in vitro analysis of LOX-activity using linoleic acid as substrate showed that it possesses C-13 specificity. Immunocytochemical localization studies using leaf tissue from MJ-treated plants demonstrated that the inducible LOX was compartmented in large quantities in the chloroplasts of mesophyll cells, associated with the stroma. The results suggest that the wound response in passion fruit plants may be mediated by a chloroplast 13-LOX, a key enzyme of the octadecanoid defense-signaling pathway.

Keywords: *Passiflora flavicarpa*; Passifloraceae; Lipoxygenase; Chloroplast; Wound response; Methyl jasmonate

1. Introduction

Lipoxygenases (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) are non-heme iron-containing dioxygenases that catalyze hydroperoxidation of fatty acids by introduction of molecular oxygen into the *cis*,*cis*-1,4-pentadiene structure of its substrate (Siedow, 1991). In plants, the hydroperoxide fatty acids, (mainly derived from linolenic and linoleic acids) are further metabolized into physiologically active lipid-breakdown products, such as growth regulators and signal transduction molecules like, for example, traumatin abscissic acid and jasmonic acid (JA) (Farmer and Ryan, 1992a; Sembdner and Parthier, 1993; Vick, 1993). Therefore, LOXs are implicated in different aspects of plant physiology, although their biological functions are not yet fully understood. Additionally, in different plant species LOXs are present as multiple isoforms or isozymes, suggesting that each one may play distinct functions within the plant (Royo et al., 1996; Heitz et al., 1997; Smith et al., 1997; Fischer et al., 1999).

Plants have evolved inducible defenses against insect and pathogen attacks, which involve a variety of signaling molecules released in complex temporal patterns following initial damage to tissues (Ryan, 2000; Leon et al., 2001). The wound response activates defense gene expression by triggering a lipid-derived pathway leading to the synthesis of JA (Farmer and Ryan, 1990, 1992b; Wasternack and Parthier, 1997). In response to specific signals, linolenic acid is enzymatically released from the membrane lipid pool, then oxygenated by LOX, and subsequently converted to JA via the octadecanoid pathway. JA itself and derivatives, collectively called jasmonates, are powerful mediators of physiological and developmental responses in plants (Wasternack and Parthier, 1997).

LOX expression and/or activity are modulated by a variety of stimuli. In rye seedlings LOX activity increases in response to wounding (Ievinsh, 1992). In potato and tomato plants LOX transcripts are induced by wounding and treatment with MJ (Royo et al., 1996; Heitz et al., 1997). Similar results were previously reported for

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Arabidopsis thaliana (Bell and Mullet, 1991). MJ is also a potent inducer of LOX accumulation in barley leaves, as demonstrated by Western blot analysis (Feussner et al., 1995). Pathogen- or elicitor-induced LOXs were reported in monocots and dicots, indicating a role for LOX in plant disease resistance (Fournier et al., 1993; Peng et al., 1994). Abiotic stress, such as water deficit, can also modulate LOX expression in *Arabidopsis* (Bell and Mullet, 1991). A LOX present in lipid bodies was shown to be involved in mobilization of lipid reserves in cotyledons of cucumber seedlings (Feussner et al., 1997), and a specific LOX isoform (VLXD) was shown to play a major role as a vegetative storage protein in soybean pod walls (Dubbs and Grimes, 2000). A correlation between LOX activity and senescence of detached maize leaves was suggested (Hung and Kao, 1996) and, more recently, northern blot analysis showed that senescing rose petals accumulate high levels of *Rlox1* transcripts. (Fukuchi-Mizutani et al., 2000). Altogether, these results indicate that LOX can regulate a wide range of environmental and developmental processes in plants.

Passion fruit juice is an important commodity in the Brazilian agroindustry, and during the last decade, due to specific agronomic programs, the area cultivated with passion fruit has significantly increased. Therefore, this fruit became an attractive model for the study of plant defense mechanisms. In the present study we investigated whether passion fruit LOX activity could be modulated in response to wounding and exogenous MJ.

2. Results and discussion

2.1. Induction of LOX activity by wounding and MJ exposure

Fig. 1 shows an increase in LOX activity in passion fruit leaves in response to wounding and MJ treatment. Intact (control) plants showed no detectable amount of LOX activity under the assay conditions employed. However, upon wounding, LOX activity was induced throughout the plant since both wounded and undamaged leaves exhibited an increase of LOX activity. At both sites (local and distal) LOX activity reached similar levels in 24 h, and was maintained for the next 24 h. The LOX activity induced by continuous exposure to MJ was substantially higher than that observed in response to wounding, corroborating previous findings that plant LOXs are strongly induced by exogenous MJ/JA (Feussner et al., 1995; Avdiushko et al., 1995; Royo et al., 1996; Heitz et al., 1997).

The systemic induction of LOX activity observed in passion fruit plants suggested the participation of a mobile signal molecule that was produced in wounded leaves and reached distal undamaged leaves. At the distal site this signal molecule leads to an increase in LOX activity. Time course experiments (Fig. 2) showed that the LOX activity was clearly detectable in the wounded leaves 8 h after injury and increased continuously for the next 16 h. During the entire experiment a short delayed response was observed in undamaged leaves. The temporal increase in LOX activity suggested that, as in other plants, the gene may be involved in the defense signaling transduction pathway early during the wound response.

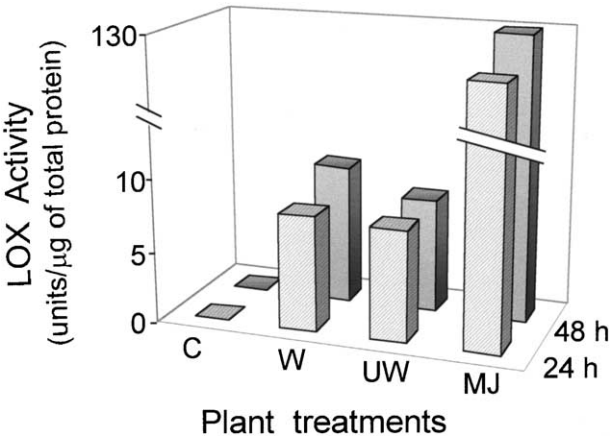

Fig. 1. Increase of lypoxygenase activity in passion fruit leaves in response to wounding and exogenous methyl jasmonate. C: control (intact) plants; W: wounded leaves of experimental plants; UW: unwounded leaves from the wounded plants; MJ: leaves from methyl jasmonate treated plants. At 24 and 48 h after stimuli samples were collected for analysis. In all cases three plants were pooled for each protein extraction to obtain an average response. Data are a mean of three independent experiments, ratios of standard error to mean were 0.03% or less. Enzymatic assays were carried out using linoleic acid as substrate (see Experimental).

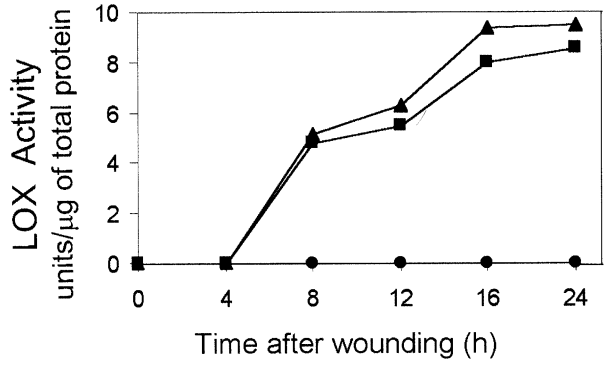

Fig. 2. Time course of lipoxygenase activity in passion fruit leaves in reponse to wounding. Extracts from intact plants were assayed as controls (●). Wounded leaves from experimental plants (▲); unwounded leaves from the wounded plants (■). At specific intervals over a 24 h period samples were collected for analysis. In all cases three plants were pooled for each protein extraction to obtain an average response. Data are a mean of three independent experiments, ratios of standard error to mean were 0.1% or less. Enymatic assays were carried out using linoleic acid as substrate (see Experimental).

2.2. Wounding and MJ induce protein(s) antigenically related to cucumber LOX

In order to identify induced proteins in response to wounding and MJ, crude leaves extracts were analyzed by SDS–PAGE. Fig. 3A shows that a major protein of 90 kDa markedly accumulated in MJ treated plants. This protein band was weakly induced by wounding, both in local and distal leaves.

Plant LOXs with sizes ranging from 90 to 100 kDa have been found to accumulate in various plants in response to exogenous MJ, JA and elicitor (Fournier et al., 1993; Feussner et al., 1995; Jensen et al., 1997). Based on these data, Western blot analysis using polyclonal antibodies against cucumber LOX (Feussner et al., 1995) were performed. As shown in Fig. 3B, an immuno-reactive protein band was not observed in extracts from control plants. As expected, the inducible 90 kDa protein band cross-reacted very specifically with these antibodies. This result corroborates the enzymatic assays showing that the LOX activity was systemically induced upon wounding and highly induced by MJ (Fig. 1). In addition, two slightly smaller immuno-reactive protein bands were also observed only in extracts from MJ treated leaves (Fig. 3B), suggesting either the induction of LOX isoforms, differing in size, or the presence of degradation products from the major protein band.

2.3. Purification of the MJ-induced LOX and functional analysis

A purification procedure was established using crude leaves extract from MJ treated plants as enzyme source. The LOX activity was precipitated between 20 and 55% ammonium sulfate saturation. To eliminate salt and reduce the amount of small proteins the ammonium sulfate fraction was subjected to two rounds of gel filtration chromatography on a Sephadex G-200 column. The resulting fraction with LOX activity was subjected to two runs of DEAE-HPLC (data not shown). The 90 kDa protein band enrichment during the purification steps was assessed by SDS–PAGE and Western blot analyses (Fig. 4A and B).

When the MJ-induced LOX purified to apparent homogeneity was assayed for its positional specificity in adding molecular oxygen at either C-9 or C-13 of linoleic acid, only 13-hydroperoxide products were detectable (Fig. 5). Similar results were obtained when hydroperoxides generated using freshly prepared crude protein extracts from wounded and MJ-treated leaves were analyzed (data not shown), indicating that only 13-LOX isoform(s) were induced. These findings support a putative defensive role of the inducible LOX, since the 13-hydroperoxy-derivatives of polyunsaturated C18 fatty acids can be converted to oxylipins in vivo (Blée,

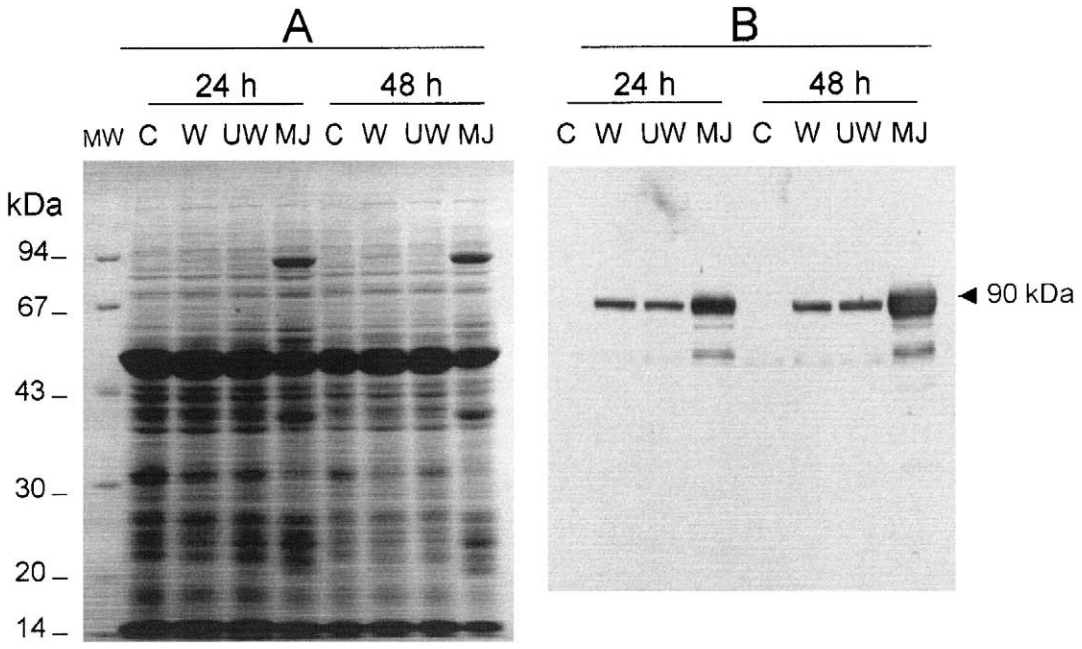

Fig. 3. A. Electrophoretic analysis (10%) SDS–PAGE) of crude protein extracts from passion fruit leaves. C: leaves from control (intact) plants; W: wounded leaves of experimental plants; UW: unwounded plants; MJ: leaves from methyl jasmonate treated plants. At 24 and 48 h after stimuli samples were collected for analysis. For each lane, 80 μg of protein were loaded. In all cases leaves from three plants were pooled for each protein extraction to obtain an average protein pattern. The gel was stained with Coomassie brilliant blue and molecular weight (MW) markers are shown on the left. B. Western blot analysis of a replica gel shown in A. Proteins were probed with 5000-fold diluted immune serum against cucumber lipoxygenase.

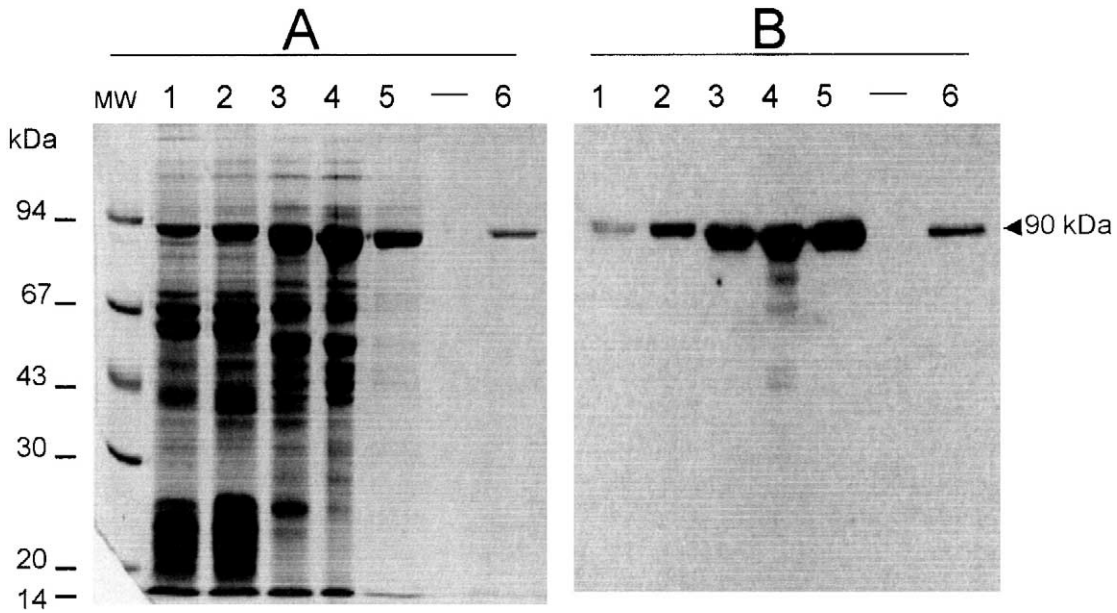

Fig. 4. A. Electrophoretic analysis (10% SDS–PAGE) of fractions from different stages of partial lipoxygenase purification. Line: crude leaf extract (20 μg of protein); lane 2: ammonium sulfate (20–55%) fraction (20 μg of protein); lane 3: active fraction obtained by chromatography on G 200 Sephadex (20 μg of protein); lane 4: active fraction obtained by rechromatography on Sephadex G-200 (20 μg of protein); lane 5: active fraction obtained by DEAE-HPLC (5 μg of protein); lane 6: active fraction obtained by rechromatography on DEAE-HPLC (2 μg of protein). The gel was stained with Coomassie brilliant blue and molecular weight (MW) markers are shown on the left. B. Western blot analysis of a replica gel shown in A. Proteins were probed with 5000-fold diluted immune serum against cucumber lipoxygenase.

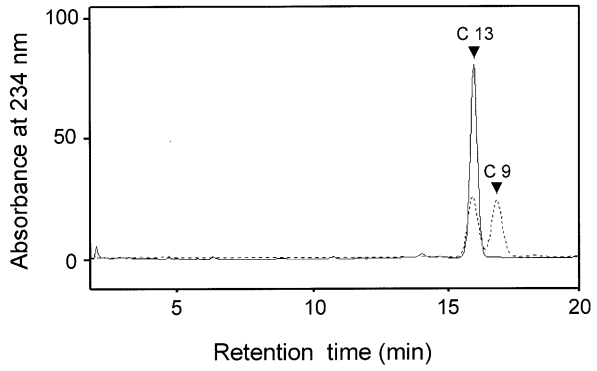

Fig. 5. Analysis of the intramolecular activity of methyl jasmonate-induced lipoxygenase. Linoleic acid was incubated with purified enzyme and the reaction products were separated by RP-HPLC. The elution profile of authentic standards is represented by a dashed line (1 nmol of each: 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid and 9(*S*)-hydroperoxy-10(*E*),12(*Z*),octadecadienoic acid indicated in the figure as C 13 and C 9, respectively). The black line shows the elution profile of reaction products. This figure represents an individual result representative of 3 independent experiments.

1998). Further investigation of the stereospecificity of the inducible LOX will help to understand the possible fate of the hydroperoxide products.

2.4. MJ-induced LOX are chloroplast-targeted proteins

Intracellular localization of the inducible LOX was investigated at the subcellular level. Leaf tissue sections from control plants showed low background-type labelling (Fig. 6A), but the antibodies labelled the chloroplasts of palisade and spongy parenchyma cells of MJ-treated leaves, confirming that LOX accumulation had occurred (Fig. 6B). Within the chloroplast, labelling occurred mainly in the stroma, and only occasionally at the thylakoid membranes. The minor labelling observed in the cytosol may represent ongoing synthesis and sorting of the LOX protein. Additional experiments using isolated chloroplasts will be required to understand the mechanism of import of the MJ-induced LOX, as well as the sorting of this protein into different sub-organellar structures. We also observed minor labelling in the vacuole (data not shown). MJ-induced accumulation of vacuolar LOX in soybean primary leaves has been previously reported by Grimes et al., (1992).

We noted that exposure to MJ caused modification in the chloroplasts, since the stroma and grana thylakoid membranes became disorganized (compare Fig. 6A and B). These results are compatible with previous data from the literature, since MJ is known to repress chloroplastic protein synthesis, compromising the photosynthetic apparatus (Reinbothe et al., 1994), and to promote degradation of plastid membranes (Hause et al., 1994).

Currently, chloroplasts are thought to be the primary site of the initial steps of JA formation. In various plants species, different enzymes related to JA biosynthesis have been identified biochemically in chloroplast fractions or visualized via immunolocalization (Blée and Joyard, 1996; Feussner et al., 1995; Ziegler et

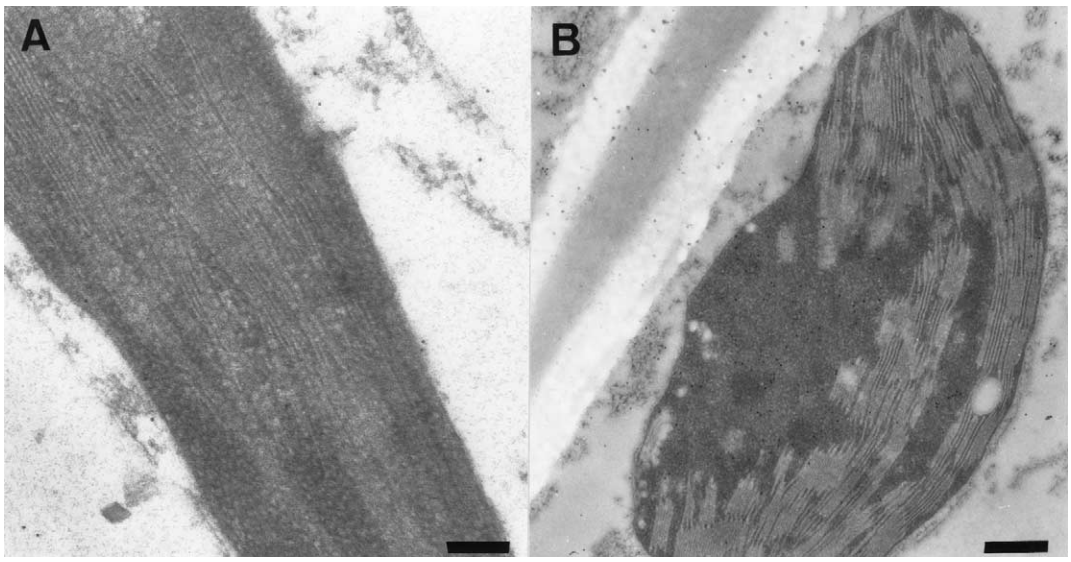

Fig. 6. Immunocytochemical localization of the methyl jasmonate induced lipoxygenase protein in passion fruit leaves. At 24 h after stimuli samples were collected for analysis. A: Cross-section of control leaf (×50,000, bar = 150 nm); B: cross-section of MJ-treated leaf(× 20,000, bar = 400 nm).

al., 2000; Froehlich et al., 2001). In addition chloroplast membrane lipids contain high levels of linolenic acid and could possibly fuel an activated octadecanoid pathway (Douce and Joyard, 1980). During the last few years, several reports emphasized a role of choroplast-localized LOX in the regulation of plant defense responses. In *Arabidopsis,* via antisense strategy, a specific LOX isoform (LOX2) was shown to be implicated in JA synthesis, and to be a chloroplast-localized protein (Bell et al., 1995). A gene coding a chloroplast-targeted LOX in tomato leaves, *TomLoxD*, was shown to be up-regulated in response to wounding and exogenous MJ (Heitz et al., 1997). A wound-inducible 13-LOX from potato (LOX3) was predicted to be targeted to the chloroplasts (Royo et al., 1996), and in barley leaves a MJ-induced 13-LOX was shown to be confined within chloroplasts (Vörös et al., 1998).

The results presented here from a combination of enzymatic assays, Western blot and immunocytochemical analysis suggest that a chloroplast-targeted 13-LOX may play an important function in MJ-signaling of passion fruit leaves to protect against chewing insect attack. Additionally, the data presented further support the importance of plastids as a component of the defense signal transduction pathway leading to plant resistance against predators and pathogens.

3. Experimental

3.1. Plant material

Passion fruit (*Passiflora edulis f. flavicarpa*) were purchased at the local market, seeds were collected, dried at room temperature and stored at 4 °C in the dark until used. Plants were grown in peat pots and maintained in environmental chambers for 17 h under 300 mE m^{-2} s^{-1} light at 28 °C and for 7 h in the dark at 18 °C.

3.2. Plant treatments

Three-week-old plants with two developed leaves and a small apical leaf were used for all experiments. The lower leaves were wounded multiple times (3–4) with a hemostat. Four hours later the same leaves were wounded again as repeated mechanical wounding causes an increase in the defense response. For the time course experiments, the second wounding was inflicted 1 h after the first one. Intact plants were exposed continuously to MJ vapor in closed Plexiglas boxes essentially as described by Farmer and Ryan (1990). For all treatments plants were maintained in environmental chambers under constant light for the first 24 h. Plants that would be assayed 48 h following treatment had the normal day light period returned.

3.3. Crude leaf extract and LOX enzymatic assay

Leaf tissue was ground to a fine powder in liquid N_2, 10% (w/w) insoluble polyvinylpolypyrolidone (PVPP) was added and total protein was extracted by the addition of 3 ml of ice-cold extraction buffer (50 mM sodium phosphate, pH 6.5) to 1 g of powdered tissue. The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C, and the supernatant was used as enzyme source. LOX activity was determined spectrophotometrically at 234 nm, using linoleic acid as substrate according to the method of Axerold et al. (1981). Briefly, using 1 ml of reaction buffer (50 mM Tris–HCl, pH 8.0) containing 400 μM of linoleic acid, the reaction was started by

addition of 1–5 μl of crude leaf extract. One unit of LOX activity was defined as the amount of enzyme that generates 1 μmol of the hydroperoxide per min at 25 °C. Controls were run in which the protein extract was omitted. To determine the pH value to be used in the enzymatic assays, the inducible LOX activity was studied at various pH values (data not shown), and for both treatments (wounding and MJ exposure) the inducible LOX displayed alkaline optimum pH. Protein concentration was determined using the Bradford assay kit (BioRad) and bovine serum albumin (BSA) as standard protein according to the manufacturer's instructions.

3.4. Gel electrophoresis and immunoblotting analysis

Proteins were analyzed in 10% SDS-polyacrylamide gel according to Laemmli (1970). The transfer of proteins to nitrocellulose membranes was carried out as described by Towbin et al. (1979). Blots were developed using chemiluminescent detection (Amersham ECL reagent) according to the manufacturer's instructions. Polyclonal antibodies raised in rabbit against cucumber LOX were a gift from Dr. Claus Wasternack, Germany.

3.5. Partial LOX purification

Leaf tissue (10 g) was ground to a fine powder in liquid N_2, 10% (w/w) insoluble PVPP was added and total protein was extracted by addition of 30 ml of ice-cold extraction buffer (50 mM sodium phosphate, pH 6.5). The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C, and the supernatant was used as enzyme source. Powdered $(NH_4)_2SO_4$ was added to the crude extract to achieve 20% of saturation. The solution was gently stirred for 3 h at 4 °C and then centrifuged as described above. $(NH_4)_2SO_4$ was added to the resulting supernatant to achieve 55% saturation. The solution was gently stirred for 3 h at 4 °C and then centrifuged as describe above. The precipitate was collected and suspended in a minimum volume of 50 mM Tris, pH 8.0. The suspension was subjected to chromatography through a Sephadex G-200 column (1.5×45 cm) previously equilibrated with 50 mM Tris, pH 8.0. Fractions with LOX activity were combined and concentrated by ultrafiltration using a Centriprep-50 apparatus (cut-off 50,000 MW, Amicon). The resulting solution was subjected to a second run on a Sephadex G-200 column, essentially as described above. Fractions with LOX activity were combined and concentrated by ultrafiltration as described above. The resulting solution was injected onto a DEAE HPLC column (Vydac, weak anion, 7.5×50 mm, 5 μm), previously equilibrated with 10 mM Tris, pH 8.0. Proteins were eluted with a linear gradient of 0–0.5 M of NaCl at the flow rate of 0.7 ml/min. The active fractions were pooled, concentrated and desalted by ultrafiltration, using a Centricon-50 apparatus (cut-off 50,000 MW, Amicon). The resulting solution was rechromatographed at a flow rate of 0.7 ml/min with 100% solvent A (10 mM Tris, pH 8.0) for 10 min, 30% solvent B (solvent A plus 0.5 M NaCl) over 12 min, 40% solvent B over 15 min and finally 100% solvent B over 24 min. The active fractions were pooled, concentrated and desalted by ultrafiltration as described above.

3.6. Analysis of the intramolecular activity of the MJ-inducible LOX

For product analysis, 0.25 μg of the purified enzyme (~700 units) was incubated with 1 ml of reaction buffer (50 mM Tris–HCl, pH 8.0) containing 400 μM of linoleic acid, with a constant flow of oxygen for 15 min. The reaction was stopped by adjusting the incubation mixture to pH 3.0 with glacial acetic acid. The resulting hydroperoxides were extracted with $CHCl_3$ (2×0.5 ml). The solvent was evaporated and the remaining lipids were dissolved in $CH_3CN/H_2O/CH_3COOH$ (55:45:0.02, v/v). The reaction products were analyzed by RP-HPLC (Shimadzu HPLC system coupled to a diode array detector), using a Wakosil PTH column (Wakopak, 4.6×250 mm). A solvent system of $CH_3CN/H_2O/CH_3COOH$ (55:45:0.02, v/v) with a flow rate of 1.4 ml/min was used, and the absorbance at 234 nm was recorded. The retention times of the reaction products were compared with those of authentic 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid and 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid isomers (Cayman Chemical).

3.7. Immunocytochemical analysis

Leaf samples were fixed in 50 mM cacodylate buffer, pH 7.4 containing 0.1% glutaraldehyde and 4% paraformaldehyde for 2 h at room temperature. Samples were washed (3×10 min) with 50 mM cacodylate buffer, pH 7.4, and tissues were dehydrated with a MeOH series (from 30 to 90%) and embedded in LR GOLD resin. Ultrathin sections (60 nm) were mounted on nickel grids and submitted to the immunocytochemistry assay as follows: sections were bathed for 30 min with PBS buffer (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.3) containing 1% (v/v) Tween 20 and 2 h with 50 mM NH_4Cl, pH 5.25. To block nonspecific protein-binding sites, sections were incubated with PBS buffer +1% BSA for 2 h. Subsequently, sections were incubated for 2 h with polyclonal antibodies against cucumber LOX diluted (1:200) with PBS +1% BSA. Grids were washed with PBS +1% BSA (8×5 min) and PBS alone (4×5 min). Sections were incubated for 2 h with protein A-gold (10 nm, Sigma Chemical Co.) diluted (1:100) with PBS + 1% BSA, followed by washes with PBS +1% BSA (8×5 min), PBS alone (4×5 min) and bidistilled water (2×5 min). Sections were post-stained 5 min with 5%

aqueous uranyl acetate, followed by 1 min with lead citrate. The sections were examined and photographed with a Zeiss TEM 900 transmission electron microscope.

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