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Envelope Membranes from Spinach Chloroplasts Are a Site of Metabolism of Fatty Acid Hydroperoxides

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Enzymes in envelope membranes from spinach (*Spinacia oleracea* L.) chloroplasts were found to catalyze the rapid breakdown of fatty acid hydroperoxides. In contrast, no such activities were detected in the stroma or in thylakoids. In preparations of envelope membranes, 9*S*-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid, 13*S*-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, or 13*S*-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid were transformed at almost the same rates (1–2 μmol min^{-1} mg^{-1} protein). The products formed were separated by reversed-phase high-pressure liquid chromatography and further characterized by gas chromatography-mass spectrometry. Fatty acid hydroperoxides were cleaved (a) into aldehydes and oxoacid fragments, corresponding to the functioning of a hydroperoxide lyase, (b) into ketols that were spontaneously formed from allene oxide synthesized by a hydroperoxide dehydratase, (c) into hydroxy compounds synthesized enzymatically by a system that has not yet been characterized, and (d) into oxoenes resulting from the hydroperoxidase activity of a lipoxygenase. Chloroplast envelope membranes therefore contain a whole set of enzymes that catalyze the synthesis of a variety of fatty acid derivatives, some of which may act as regulatory molecules. The results presented demonstrate a new role for the plastid envelope within the plant cell.

Signaling cascades involving oxylipins that function in animal cells may also operate in plants (Zimmermann and Coudron, 1979; Hamberg and Fahlstadius, 1990; Song and Brash, 1991; Vick, 1993). Oxylipins are derived from hydroperoxides of polyunsaturated fatty acids, like linoleic and linolenic acids (Fig. 1). The biosynthesis of fatty acid hydroperoxide is catalyzed by lipoxygenase (EC 1.13.11.12), a dioxygenase which produces either 9- or 13-hydroperoxyoctadeca(e)noate or a mixture of both of them, depending on the source of the enzyme. These highly reactive aliphatic molecules are then rapidly metabolized by plant cells into physiologically active lipid-breakdown products (Zimmermann and Coudron, 1979; Hamberg and Fahlstadius, 1990; Song and Brash, 1991; Vick, 1993). For instance, hydroperoxide lyase and hydroperoxide dehydratase (EC 4.2.1.92) were shown to catalyze the breakdown of hydroperoxides into compounds with a possible protective role, for example antibacterial and wound-healing agents, or regulatory substances, such as jasmonic acid, that control gene expression under stress conditions or during embryogenesis. The hydroperoxide lyase cleaves the bond between C-12 and C-13 (α scission of the *trans*-11 double bond) to give C_6 aldehydes and 12-oxo-9(Z)-dodecenoic acid, finally yielding the wound hormone traumatin (Zimmermann and Coudron, 1979). The hydroperoxide dehydratase,[1] a nonclassical Cyt P450-type enzyme (Song and Brash, 1991), catalyzes dehydration of the 13-hydroperoxylinolenic acid producing a very unstable allene oxide, followed either by its enzymatic cyclization to 12-oxo-PDA (Hamberg and Fahlstadius, 1990), the precursor of jasmonic acid, or by its hydrolysis into α- and γ-ketols (Vick, 1993).

Recently, Blée and Schuber (1993) described a new fate for fatty acid hydroperoxides: the peroxygenase cascade, which also leads to products relevant to plant defense mechanisms, since some of them are cutin monomers (cutin is the chief component of cuticle, the first barrier against invasion by pathogens) or possess antifungal properties (Ohta et al., 1990; Namai et al., 1993). This new pathway involves (a) a peroxygenase, which catalyzes an intramolecular transfer of one oxygen atom from fatty acid hydroperoxides, yielding an epoxyalcohol and/or co-oxidation reactions such as epoxidation of double bonds of unsaturated fatty acids (Blée et al., 1993), and (b) an epoxide hydrolase, which preferentially hydrates the epoxides formed by the peroxygenase (Blée and Schuber, 1992a). Lipid-breakdown metabolism is generally enhanced under stress conditions (Ohta et al., 1991; Farmer and Ryan, 1992a; Croft et al., 1993; Choi et al., 1994). At present, it seems that peroxygenase and hydroperoxide lyase pathways are involved in the response to pathogen infection (Ohta et al., 1991; Croft et al., 1993), and the hydroperoxide dehydratase cascade operates in wound-response programs (Farmer and Ryan, 1992a; Choi et al., 1994).

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[1] The nomenclature for hydroperoxide dehydratase has been somewhat confusing and is now clarified. In a recent article, Simpson and Gardner (1995) describe the evolution of this nomenclature.

Abbreviations: 13-HOD, 13*S*-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid; 13-HOT, 13*S*-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 9-HPOD, 9*S*-hydroperoxy-10(E),12(*Z*)-octadecadienoic acid; 13-HPOD, 13*S*-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 13-HPOT, 13*S*-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 12-oxo-PDA, *cis*-12-oxo-10,15(*Z*)-phytodienoic acid; RP-HPLC, reversed-phase high-pressure liquid chromatography; TMS, trimethylsilyl ether.

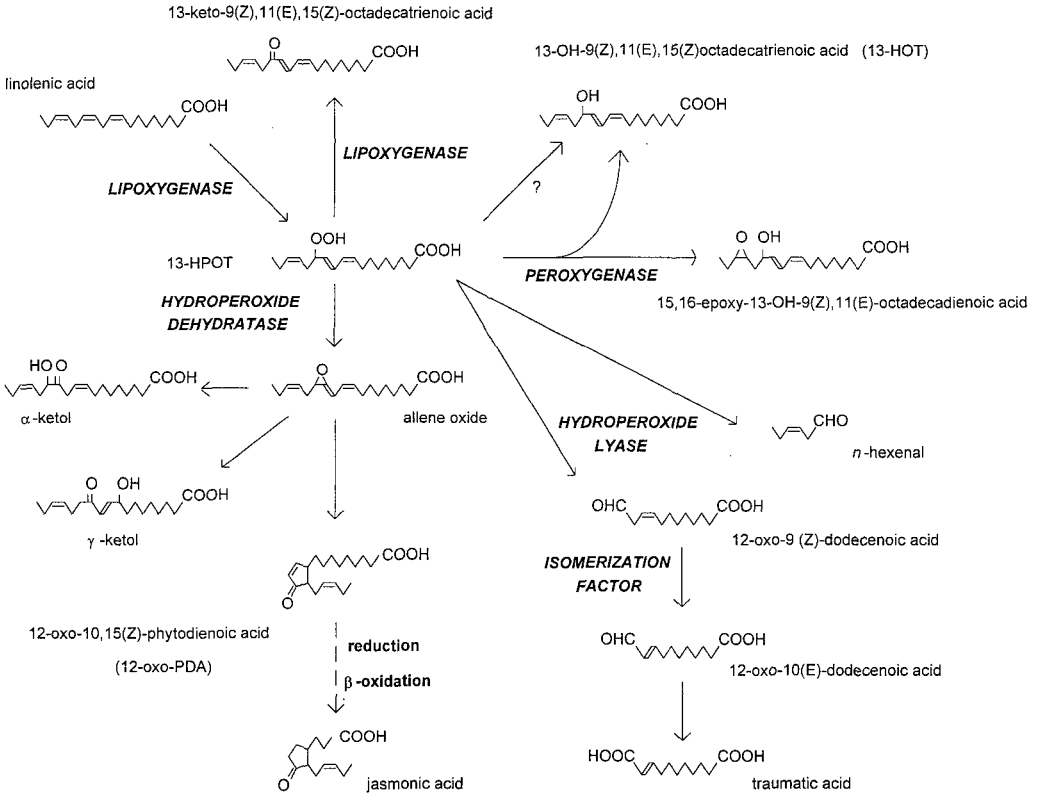

Figure 1. Biosynthetic scheme leading to the formation of oxylipins in plants.

Plant organelles such as chloroplasts may be a site of biosynthesis of regulatory molecules potentially involved in plant-defense reactions, since lipoxygenase, hydroperoxide lyase, and dehydratase activities have been measured in chloroplast preparations (Vick and Zimmerman, 1987; Bowsher et al., 1992; Hatanaka, 1993; Song et al., 1993). In addition, chloroplast membrane lipids contain high levels of linole(n)ic acids (Douce and Joyard, 1980), the substrates for lipoxygenase. In the present work, we have addressed the question of compartmentation of enzymes involved in the metabolism of fatty acid hydroperoxides within the plant cell as a possible element for regulation. Therefore, we have initiated such a study using highly purified chloroplast fractions (envelope, stroma, and thylakoids). The results reported here clearly demonstrate that some of the key enzymes catalyzing the synthesis of a wide variety of potentially essential regulatory molecules are restricted to the envelope membranes of plastids.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Linolenic acid (54 Ci/mol) and [1-^{14}C]linoleic acid (56 Ci/mol) were purchased from DuPont-New England Nuclear. Linoleic and linolenic acids, lipoxygenase (type I), and triphenylphosphine were from Sigma.

Preparation of Products

[1-^{14}C]13-HPOT was obtained by treatment of [1-^{14}C]-linolenic acid by lipoxygenases from soybean and purified as already published (Blée et al., 1993). [1-^{14}C]13-HOT was prepared by reducing the hydroperoxide by triphenylphosphine following procedures already described (Blée et al., 1993). Similar procedures were applied to obtain [1-^{14}C]9-HPOD, [1-^{14}C]13-HPOD, and [1-^{14}C]13-HOD from linoleic acid (Blée and Schuber, 1990, 1992b). Authentic radioactive α-ketol, γ-ketol, and 12-oxo-PDA were formed from [^{14}C]13-HPOT incubated in the presence of flaxseed acetone powder extracts according to Baertschi et al. (1988). The structures of all of these compounds were confirmed by GC-MS analysis.

Preparation of Spinach Chloroplasts and Their Compartments

All the operations were carried out at 0 to 5°C. Chloroplasts were isolated from spinach (*Spinacia oleracea* L.) leaves obtained from local markets. A crude chloroplast pellet was obtained and purified further by isopycnic centrifugation in Percoll gradients (Douce et al., 1973; Douce and Joyard, 1982). The Percoll-purified chloroplasts are devoid of phosphatidylethanolamine, indicating the absence of extraplastidial membranes (Douce and Joyard, 1980, 1982; Dorne et al., 1990). Purified intact chloroplasts were then lysed in hypotonic medium, and the stroma,

envelope membranes, and thylakoids were purified from the lysate by centrifugation through a step Suc gradient (Douce and Joyard, 1982). Envelope membranes prepared according to this procedure were deep yellow due to the presence of carotenoids and the absence of Chl, and were, therefore, devoid of contaminating thylakoid vesicles (Douce and Joyard, 1982; Dorne et al., 1990). In contrast, crude thylakoid fractions obtained from Suc gradients contained some envelope vesicles. A series of washings of this fraction was performed to remove most of the contamination of thylakoids by envelope membranes, as described by Dorne et al. (1990).

Enzyme Assays

Hydroperoxide breakdown was measured at 20°C by following the disappearance of the A_{234} of the conjugated diene. The assay mixture consisted of 0.1 M sodium phosphate (NaH_2PO_4/Na_2HPO_4) buffer (pH 6.5; final volume 1 mL) containing hydroperoxides (14.5 nmol). The reaction was started by addition of envelope membranes (equivalent to about 10 μg of protein).

Hydroperoxide lyase was characterized by following the formation of aldehydes and keto acids. Aldehydes were determined as 2,4-dinitrophenylhydrazone derivatives. 13-HPOD (final concentration 30 μM) was incubated with envelope (10 μg of protein) in potassium phosphate (KH_2PO_4/K_2HPO_4) buffer (0.1 M, pH 6.8; final volume 1 mL). After 5 min of incubation at 26°C, the reaction mixture was acidified to pH 3 with HCl, and 1 μg of *n*-nonenal was added as an internal standard. Hydrazone derivatives of aldehydes formed after addition of 2.5 mL of 0.1% 2,4-dinitrophenylhydrazine were extracted twice with *n*-hexane and concentrated in vacuo. The concentrate was dissolved in methanol and subjected to HPLC analysis (see below). Calibration curves were obtained with authentic *n*-hexanal by using *n*-nonenal as an internal standard.

Hydroperoxide lyase, hydroperoxide dehydratase, and lipoxygenase activities were assayed by analysis of the products formed during the incubation of envelope membranes (10 μg of protein) with [^{14}C]13-HPOD (35 nmol, 19,000 dpm) or [^{14}C]13-HPOT (30 nmol, 17,700 dpm) in KH_2PO_4/K_2HPO_4 (0.1 M, pH 6.5; final volume 1 mL). The reaction was stopped after a given time of incubation (up to 180 min) by addition of two drops of 4 N HCl, and the mixture was extracted twice by 2 volumes of chloroform. The organic phase was recovered and evaporated to dryness. The residue was then redissolved in acetonitrile and analyzed by RP-HPLC as described below.

Linoleic acid metabolism corresponding to lipoxygenase activity was measured at 20°C by following the A_{234}. The assay mixture consisted of 0.01 M acetate buffer (pH 4.5; final volume 1 mL) containing linoleic acid (70 nmol). The reaction was started by the addition of envelope membranes (equivalent to about 5 μg of protein).

Analytical Procedures

Hydrazone derivatives were quantified by RP-HPLC (Shimadzu [Kyoto, Japan] apparatus) under isocratic conditions using a μBondapak C_{18} column (3.9 × 300 mm; Waters) with a solvent mixture of acetonitrile:water:tetrahydrofuran (450:240:10, v/v/v) at 1 mL/min. The compounds were detected at 350 nm. RP-HPLC of ^{14}C-labeled oxidized metabolites of 9- and 13-HPOD or 13-HPOT was performed, at room temperature, using a Lichrospher (Merck) 100-RP-18 (5 μm) column (4.0 × 125 mm) with a solvent mixture of acetonitrile:water:acetic acid (50:50:0.1, v/v/v) at 1 mL min^{-1} for about 25 min, followed by the same system at 80:20:0.1 (v/v/v) to complete the run. Radiochromatograms and peak area integrations were obtained using a Flo-one β-detector with the program Boréal (Flotec, La Queue les Yvelines, France).

GC-MS was performed on a Fisons (Beverly, MA) MD 800 apparatus with ionizing energy of 70 electron volts. The separations were carried out on a DB5 (J&W Scientific) fused column (30 m, diameter 0.1 μm) operated between 80 and 280°C at 5°C min^{-1}. Spectrophotometric measurements were carried out using a Kontron (Montigny le Brettonneux, France) Uvikon model 860 spectrophotometer.

RESULTS

Metabolism of Fatty Acid Hydroperoxides by Envelope Membranes from Spinach Chloroplasts

Addition of a fresh preparation of spinach chloroplast envelope membranes to a phosphate buffer containing 13-HPOD triggered a very rapid disappearance of the conjugated diene absorbing at 234 nm, whereas boiled envelope showed no activity (Fig. 2). Under these experimental conditions, the initial velocity of the reaction was high, since a decrease of 0.2 optical density unit was observed within 12 s when 12 μg of envelope proteins were added to the reaction mixture (not shown). Indeed, hydroperoxide metabolism in envelope membranes was linear with time during the first 30 s and reached a plateau upon total utilization of the substrate after 1 to 2 min of incubation (Fig. 3). Initial rates of the reaction were linearly related to protein concentration up to 10 to 15 μg/mL (Fig. 3). The hydroperoxide-metabolizing activity was maximal around pH 6.5 and displayed Michaelis-Menten-type kinetics with an apparent K_m of about 20 μM for 13-HPOD (Fig. 3). With 9-HPOD or 13-HPOT as substrates, similar rates of disappearance of the respective conjugate dienes were observed (not shown). Importantly, enzymatic transformation of fatty acid hydroperoxides was restricted to the chloroplast envelope membranes, whereas stroma and thylakoids were almost devoid of this activity (Table I).

Hydroperoxide Metabolic Pathways in the Chloroplast Envelope

Fatty acid hydroperoxides are very reactive molecules that are rapidly metabolized by several enzymatic pathways in plants (summarized in Fig. 1 with 13-HPOT as substrate). Therefore, we investigated which pathway was responsible for such rapid metabolism in spinach chloroplast envelope membranes. In an attempt to identify them, we analyzed by RP-HPLC the products resulting from 1-^{14}C-labeled fatty acid hydroperoxides metabolized by

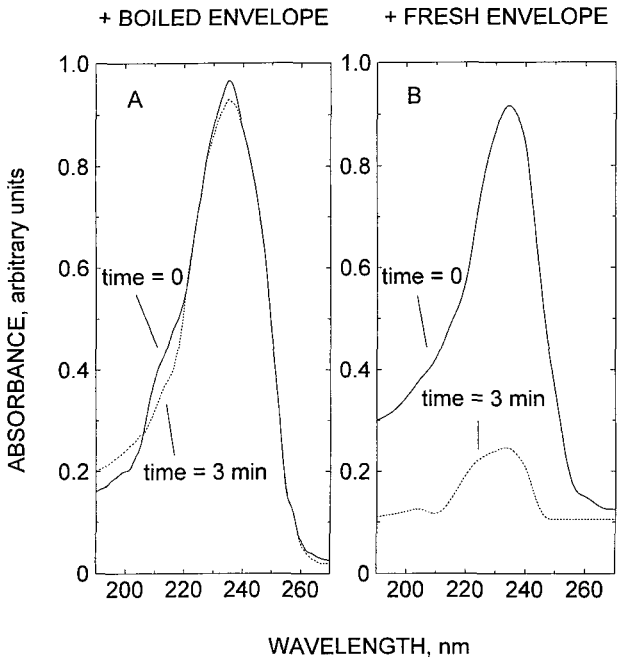

Figure 2. UV absorption profiles of 13-HPOD in the presence of boiled (A) and fresh (B) envelope from spinach chloroplasts. Absorption spectra of the oxygenated fatty acids (38 μM) were recorded at time 0 and 3 min after the addition of the spinach chloroplast envelope (equivalent to 12 μg of protein). Boiled envelope was prepared by heating in boiling water, for 5 min, the tube containing the incubation mixture together with the envelope membranes, prior to the addition of 13-HPOD.

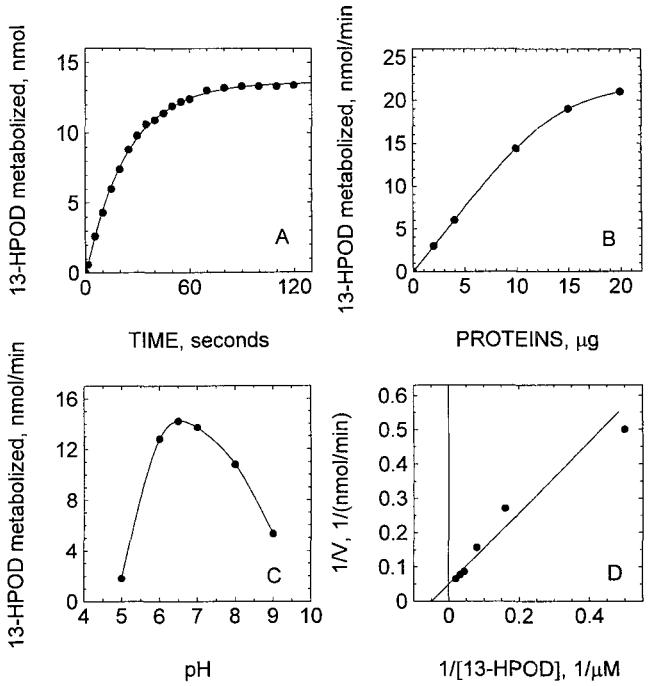

Figure 3. Kinetic properties of hydroperoxide metabolism in spinach chloroplast envelope membranes. Disappearance of the conjugated double bonds of 13-HPOD was followed in function of time (A), proteins (B), and pH (C). D shows the double-reciprocal plot of the initial velocity of hydroperoxide metabolism versus substrate concentration. Hydroperoxide metabolism was measured with a spectrophotometer by following the disappearance of the hydroperoxide conjugated diene at 234 nm.

purified chloroplast envelope membranes. Figure 4 depicts representative experiments with [1-^{14}C]13-HPOT (Fig. 4A) or [1-^{14}C]13-HPOD (Fig. 4B). For instance, after 45 min of incubation of envelope membranes in the presence of [1-^{14}C]13-HPOD, six major peaks were detected by RP-HPLC, which on integration account, respectively, for 11, 5, 19, 6.5, 10, and 44% of the total recovered counts. Residual substrate (when present) was identified and eluted at about 19 min (peak 5).

Hydroperoxide Lyase Pathway

After incubation of envelope membranes in the presence of [1-^{14}C]13-HPOD, the first peak (elution time 2.5 min; Fig. 4B) detected by RP-HPLC was characterized as 12-oxo-10(*E*)-dodecenoic acid by GC-MS. The mass spectrum of its methyl ester derivative was the same as the one published earlier (Zimmerman and Coudron, 1979) and presented peaks at *m/z* 226 (M), 195 (M-31), 194 (M-CH_3OH), and 166 (M-CH_3OOH). This compound is the active component of traumatin, the so-called "wound hormone." This aldehyde is known to result from the rapid isomerization (enzymatic or not) of 12-oxo-9(Z)-dodecenoic acid, the initial product of the hydroperoxide lyase (Vick, 1993). It is interesting that the same compound was formed when [1-^{14}C]13-HPOT was used as a substrate (Fig. 4A).

The presence of hydroperoxide lyase in envelope membranes incubated in the presence of 13-HPOD was further analyzed by measuring the formation of volatile C_6 aldehydes such as hexanal. We observed that hexanal was indeed synthesized by envelope membranes (Fig. 5). At low protein concentration ($\leq$2 μg/mL), the formation of aldehydes almost matched hydroperoxide consumption, whereas the total amount of aldehyde formed at higher protein concentration (>2 μg/mL) never reached more than 45% of the consumed hydroperoxides. No attempt was made to determine whether 2- or 3-hexanal were produced. When 13-HPOT was used as substrate, we observed the formation of the corresponding *n*-hexenal (not shown). In contrast, when 9-HPOD was used as substrate, we still observed the disappearance of the conjugated double bonds from the hydroperoxide, but could not detect any formation of aldehydes such as 2*E*-nonenal (the aldehyde

Table I. *Localization of fatty acid hydroperoxide metabolism within spinach chloroplasts*

The assay mixtures contained 14.5 nmol of 13-HPOD and chloroplast fractions equivalent to 10 μg of envelope proteins and 50 μg of thylakoid or stroma proteins. nd, Not detected.

Chloroplast Fraction	13-HPOD Metabolized
	nmol min^{-1} mg^{-1} protein
Thylakoids	6
Stroma	nd
Envelope	1570

resulting from the cleavage of 9-HPOD), indicating that, as for many other plants, the envelope lyase was quite specific for the 13-hydroperoxide regioisomer. Overall, these results emphasized that, beside lyase activity, other hydroperoxide-metabolizing enzymes exist in envelope membranes.

In some plants, the hydroperoxide lyase pathway involves alcohol dehydrogenases catalyzing the reduction of aldehyde into alcohol. By incubating spinach chloroplast envelope in the presence of [1-^{14}C]13-HPOD and NADPH (1 mM), we noticed slight changes in the radioactive RP-HPLC profile of the products formed: peak 1 disappeared in favor of a new peak, which was eluted at 3.2 min (not shown). Analysis of the methyl ester TMS derivative of this new compound by GC-MS showed predominant peaks at *m/z* 184 (M-CH_2OTMS), 285 (M-15), and 227 (M-CH_2COOCH_3) corresponding to the methyl ester TMS of 12-hydroxy-9(Z)-dodecenoic acid. This alcohol was also synthesized by pea leaf microsomes incubated with [^{14}C]linoleate and NADPH (Grechkin et al., 1990) and revealed the occurrence of an alcohol dehydrogenase in envelope membranes.

Hydroperoxide Dehydratase Pathway

Analysis of 13-HPOD metabolites after incubation with envelope membranes demonstrates (Figs. 4 and 6A) that peaks 2 and 3 in the RP-HPLC had the same elution times, i.e. 7.5 and 10.7 min, respectively, and were present in the same ratio as authentic γ- and α-ketols synthesized by a flaxseed extract, a known source of hydroperoxide dehydratase (Fig. 6B). The identity of peak 3 as 12-keto-13-hydroxy-9(Z)-octadecenoic acid was confirmed by GC-MS analysis, and the mass spectrum of the methyl ester TMS derivative was identical to those already published (Gardner, 1970; Hamberg, 1987). If instead of 13-HPOD, 13-HPOT was incubated with envelope membranes, α-ketol [12-oxo-13-hydroxy-9(Z),15(Z)-octadecadienoic acid] and γ-ketol [12-oxo-9-hydroxy-10(*E*),15(Z)-octadecadienoic acid] were formed (elution time 11 and 7.6 min, respectively), but a minor new peak could also be detected (named 3′ in Fig. 6C; elution time 15 min). The mass spectra of its methyl ester derivative showed prominent ions at *m/z* 306 (M), 275 (M-31), 238 (M-C_5H_8), 206 (M-C_5H_8 + CH_3OH), 149 [M-$(CH_2)_7COOCH_3$], and 95 (base peak, C_6H_70). These fragments were consistent with the structure of 12-oxo-PDA methyl ester (Zimmerman and Feng, 1978). Thus, the formation of ketols from 13-HPOD and ketols and cyclopentenyl metabolites from 13-HPOT strongly suggests the location of hydroperoxide dehydratase activity in envelope membranes from spinach chloroplasts. However, immunodecoration experiments of envelope polypeptides with antibodies raised against purified flaxseed hydroperoxide dehydratase were unsuccessful. This is not surprising, since few of these enzymes show any significant immuno cross-reactivity among plant species.

Spontaneous cyclization of the allene oxide yields the formation of a racemic mixture (9*S*,13*S* and 9*R*,13*R*) of 12-oxo-PDA, but this reaction also occurs stereoselectively catalyzed by an allene oxide cyclase (Hamberg and Fahlstadius, 1990). This enzyme catalyzes the formation of the 9*S*,13*S* enantiomer of 12-oxo-PDA from the 12,13*S* allene oxide, and this enantiomer is the precursor of the phytohormone 7-iso-jasmonic acid (Vick and Zimmerman, 1983). The proposed pathway for this conversion consists of initial reduction of the ring followed by three steps of β oxidation. Although allene oxide cyclase has been conclusively demonstrated as a soluble enzyme (Hamberg and Fahlstadius, 1990), the locations of the reductase and the β-oxidation enzymes are still unknown. We collected [^{14}C]12-oxo-PDA formed by spinach chloroplast envelope

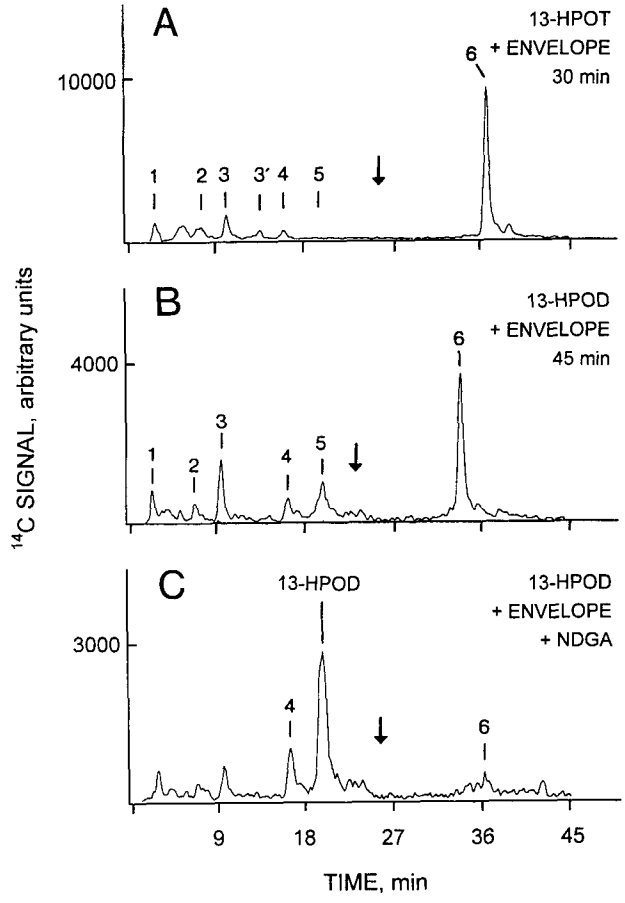

Figure 4. Radiochromatograms of the products formed after incubation of [^{14}C]13-HPOT (A) or [^{14}C]13-HPOD (B and C) in the presence of envelope membranes from spinach chloroplasts. The incubation mixture contained either 30 nmol of [^{14}C]13-HPOT (17,700 dpm) or 35 nmol of [^{14}C]13-HPOD (19,000 dpm) and envelope membranes (equivalent to 10 μg of protein). In C, 1 mM nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, was added to the incubation mixture. The reaction products were extracted after incubation and analyzed by RP-HPLC. The numbers on the chromatograms refer to the following compounds: A, 1, 12-oxo-10(*E*)-dodecenoic acid; 2, γ-ketol; 3, α-ketol; 3′, 12-oxo-PDA; 4, 13-HOT; 5, 13-HPOT (substrate); 6, 9-keto-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid and 13-keto-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid. B and C, 1, 12-oxo-10(*E*)-dodecenoic acid; 2, γ-ketol; 3, α-ketol; 4, 13-HOD; 5, 13-HPOD (substrate); 6, 9-keto-10(*E*),12(*Z*)-octadecadienoic acid and 13-keto-9(*Z*),11(*E*)-octadecadienoic acid. The arrows indicate when the second solvent system was added to elute the compounds in peak 6.

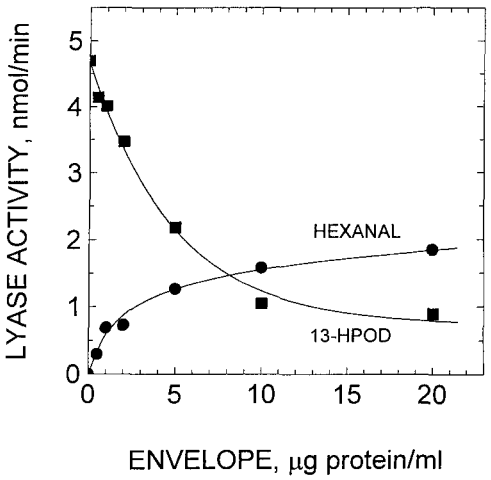

Figure 5. Formation of hexanal by chloroplast envelope membranes during metabolism of 13-HPOD. The incubation mixture contained 35 nmol of 13-HPOD. The aldehydes formed were quantified by HPLC analysis after conversion to 2,4-dinitrophenylhydrazone derivatives.

dehydratase activity and incubated it further in the presence of envelope and NADPH. Even after a 1-h incubation, the cyclopentenone remained unchanged, indicating that under our experimental conditions the last enzymes of the hydroperoxide dehydratase pathway were not active in chloroplast envelope membranes.

Peroxygenase Pathway

When [1-^{14}C]13-HPOT was incubated with chloroplast envelope membranes, peak 4 (elution time 16.4 min) was detected by radio HPLC (Fig. 4A). This peak was identified as 13-HOT because it co-eluted with an authentic sample. Its structure was confirmed by GC-MS. The methyl ester TMS derivative gave the expected molecular ion at *m/z* 380 and prominent ions at 311, base peak (M-69, loss of $\cdot CH_2CH{=}CHCH_2CH_3$) and 365 (M-15, loss of $\cdot CH_3$). In the same manner, when 13-HPOD was the substrate, 13-HOD was formed. These compounds were apparently formed enzymatically, since (a) great care was taken to purify the hydroperoxides used as substrates, and (b) no peak was found around 16 to 17 min in the radiochromatogram obtained from 13-HPOD incubated with boiled envelope (not shown).

Reduction of hydroperoxides occurs during co-oxidation reactions catalyzed by peroxygenase (Hamberg and Hamberg, 1990; Blée et al., 1993). In this case, the resulting 13-HOD and 13-HOT are epoxidized by intra- or intermolecular mechanisms into 9,10-epoxy-13-hydroxy-11(*E*)-octadecenoic acid and 15,16-epoxy-13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid, respectively (Blée et al., 1993). But such epoxyalcohols could not be detected during hydroperoxide metabolism by envelope membranes. Moreover, β-mercaptoethanol, a strong inhibitor of peroxygenase (Blée and Schuber, 1993), did not affect the pattern of the obtained radiochromatogram. Furthermore, envelope membranes were unable to catalyze a typical peroxygenase reaction (Blée and Schuber, 1990): oleic acid epoxidation with either cumene hydroperoxide or fatty acid hydroperoxides as co-substrates. These results suggest that 13-HOD and 13-HOT production in spinach chloroplast envelope membranes was not due to a peroxygenase activity.

13-HOD and 13-HOT were also shown to be produced in rice leaves from hydroperoxides by a peroxidase sensitive to chlorogenic acid (Li et al., 1991). But neither chlorogenic

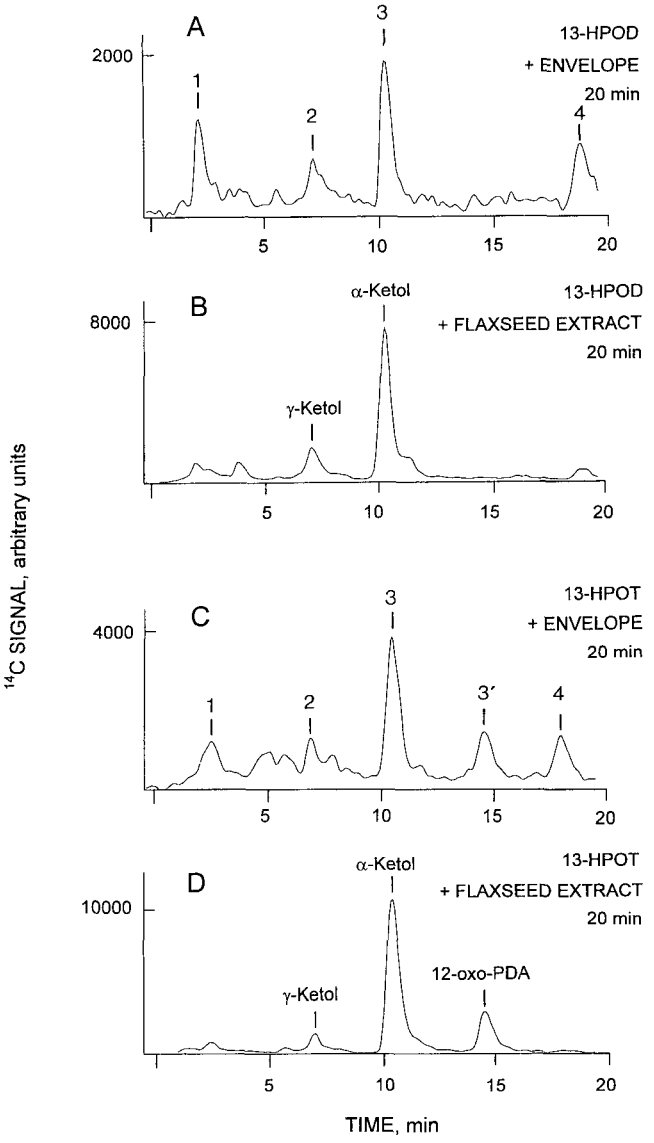

Figure 6. Radiochromatograms of the products formed after incubation of [^{14}C]13-HPOD with the envelope of spinach chloroplast (A) or flaxseed extract (B) and [^{14}C]13-HPOT in the presence of envelope (C) or flaxseed extract (D). The incubation mixture contained 30 nmol of [^{14}C]13-HPOD (16,200 dpm) or 30 nmol of [^{14}C]13-HPOT (17,700 dpm). In A and C, envelope membranes equivalent to 10 μg of protein were added. In B and D, the flaxseed extract was prepared and used as described by Baertschi et al. (1988). The reaction products were extracted after 20 min of incubation and analyzed by RP-HPLC. Only the first 20 min of the radiochromatograms are shown. The numbers on the chromatograms refer to the compounds listed in the legend for Figure 4.

acid (1 mM) nor KCN, a well-known inhibitor of peroxidase activity, had any effect on the formation of hydroxy derivatives by envelope membranes. Thus, the enzymatic system catalyzing hydroperoxide reduction within the chloroplast envelope (indicated by ? on the scheme in Fig. 1) remains undetermined, unless it is a nonclassical peroxidase or peroxygenase activity.

Lipoxygenase Activities

The major product (40–45%) formed by chloroplast envelope from [1-^{14}C]13-HPOD eluted on radio HPLC (Fig. 4B, peak 6; time of elution 37 min, i.e. about 11 min after addition of the second solvent system). In UV spectroscopy, this compound showed an absorbance maximum near 280 nm, characteristic of a conjugated dienone chromophore (Vioque and Holman, 1962). The HPLC fractions containing this labeled compound were collected, treated by diazomethane, and analyzed by GC-MS. Two major peaks that chromatographed with C values of 30.1 and 30.4 were obtained. The mass spectrum of the first peak was characterized by ions of high intensity at *m/z* 99 and 237 (α cleavage on either side of the keto group), 151 [M-157, loss of $(CH{=}CH)_2(CH_2)_7COOCH_3$], 277 (M-31, loss of $CH_3O\cdot$), and 308 (M). This spectrum was very similar to that already published (Kühn et al., 1991) and corresponded to 13-keto-9(*Z*),11(*E*)-octadecadienoic acid. The mass spectrum of the second peak presented major fragment ions at *m/z* 185 and 151 (α cleavage on either side of the keto group), 166 (loss of methyl 6-heptenoate due to a McLafferty rearrangement), 185 [$CO(CH_2)_7COOCH_3$], 237 (M-71, loss of terminal pentyl group), 277 (M-31), and 308 (M), indicating that this compound was 9-keto-10(*E*),12(*Z*)-octadecadienoic acid (Funk and Powell, 1983; Kühn et al., 1991).

Figure 7, A and B, represents the mass spectrum of methyl esters from 9-keto-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid and its 13-keto-regioisomer, which chromatographed with C values of 44.3 and 45.6, respectively. The mass spectrum of the first peak was characterized by ions of high intensity at *m/z* 55 [$CH_3CH_2(CH_2)_2$], 149, and 185 (α cleavage on either side of the keto group), 237 (M-69, loss of C_5H_9), and 306 (M). The mass spectrum of the second peak presented major fragment ions at *m/z* 55 [$CH_3CH_2(CH_2)_2$], 97 [$CH_3(CH_2)_2(CH)_2CO$], 149 [M-157, loss of $(CH_2)_7COOCH_3$], 237 (M-69, loss of C_5H_9), 275 (M-31), and 306 (M), indicating that this latter compound was 13-keto-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid. These keto derivatives were formed by metabolism of [1-^{14}C]13-HPOT by spinach chloroplast envelope preparations and eluted in one single peak (Fig. 4A, peak 6; time of elution 37 min) on radio HPLC.

The formation of such oxoenes has been described as resulting from the hydroperoxidase activity of lipoxygenase under anaerobic and aerobic conditions. Generally, this activity occurs during oxidation of linoleic acid by plant lipoxygenases, e.g. pea, soybean, and wheat (Verhagen et al., 1977; Jolivet and Bergeron, 1988; Kühn et al., 1991), but also by recombinant arachidonate 15-lipoxygenase from human reticulocytes (Sigal et al., 1990) and by platelet 12-lipoxygenase (Fruteau de Laclos and Borgeat, 1987). To determine whether our membrane preparation contained

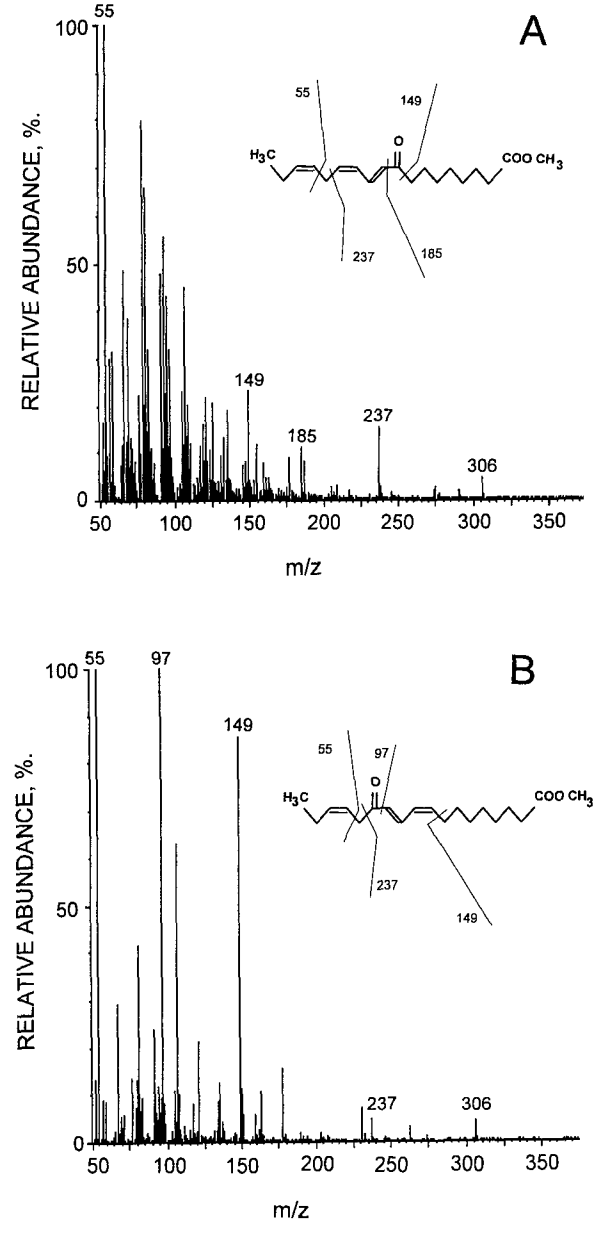

Figure 7. GC-MS analysis of methyl esters from 9-keto-10(*E*),12(*Z*),15(*Z*)-octadecenoic acid (A) and its 13-keto-regioisomer (B) formed during the metabolism of [1-^{14}C]13-HPOT by spinach chloroplast envelope membranes. One single peak (peak 6 in Fig. 4A; time of elution 37 min) was obtained by RP-HPLC and was further analyzed by GC-MS. The gas chromatogram showed two major peaks, containing the compounds shown as an inset in A and B.

lipoxygenase activity, we incubated [1-^{14}C]13-HPOD in the presence of envelope and nordihydroguaiaretic acid, a classical inhibitor of lipoxygenase. Nordihydroguaiaretic acid completely abolished the formation of labeled compounds eluting as peak 6 on radio HPLC (Fig. 4C), suggesting strongly the involvement of a lipoxygenase in the formation of ketotrienoic fatty acids. However, the mechanism of

their formation remains unclear. Since the keto-compounds found were labeled, they must be derived from the secondary decomposition of hydroperoxy lipids. However, hydroperoxidase activity was always observed during dioxygenation of fatty acids by lipoxygenase, and it was suggested that ketodienoic fatty acids can be regarded as dehydration products of the hydroperoxy compounds that may be generated via a radical mechanism (Kühn et al., 1991). These radicals are typically produced during the catalytic cycle of the lipoxygenase. The formation of an equimolar ratio of the two regioisomers keto derivatives of the 13-hydroperoxides would be in favor of the involvement of a radical mechanism, but since no exogenous fatty acid was added to the reaction mixture, this may imply that some endogenous polyenoic fatty acid from the envelope was oxygenated by the envelope lipoxygenase.

To provide further evidence for a lipoxygenase to exist in envelope membranes, we added a fresh preparation of spinach chloroplast envelope membranes (3–5 μg protein/mL) to an acetate buffer (pH 4.5) containing linoleic acid and followed by spectrophotometry the rapid increase of A_{234}. In contrast, boiled envelope showed no activity. All of the substrate (70 nmol in 1 mL) was consumed in less than 2 min, thus providing further evidence for the occurrence of a lipoxygenase activity in envelope membranes. Specific activities of 3 to 5 μmol linoleic acid consumed min^{-1} mg^{-1} protein were observed in such experimental conditions. The optimum pH value for linoleic acid metabolism was around 4.5, but the activity was still detectable above pH 7.0. Above 6 to 7 μg protein/mL, although all linoleic acid was readily consumed in envelope membranes, no increase of A_{234} could be followed because of the very rapid metabolism of hydroperoxides in envelope membranes, thus making further characterization of the envelope lipoxygenase difficult. Such experiments are in progress at present. Finally, we observed that the envelope lipoxygenase was labile and did not withstand freezing. A fresh envelope preparation should be used to measure this activity, since after freezing the envelope fraction to −80°C, we observed that (a) formation of the ketone derivatives of fatty acid hydroperoxides and (b) linoleic acid metabolism were highly reduced (not shown).

DISCUSSION

To date, chloroplast envelope membranes have been demonstrated to play a major role in the regulation of plant cell metabolism and membrane biogenesis (Douce et al., 1973; Douce and Joyard, 1980, 1982, 1990; Joyard et al., 1991). The results presented in this article demonstrate a new type of function for the plastid envelope as a site of synthesis of oxylipins, a polyunsaturated fatty acid-derived family of plant growth regulators and defense compounds. Enzymes metabolizing fatty acids already have been measured in chloroplast preparations (Götz-Schmidt et al., 1986; Vick and Zimmerman, 1987; Bowsher et al., 1992; Hatanaka, 1993). Further indirect evidence for such a localization has been obtained: although hydroperoxide dehydratase is usually described as a microsomal enzyme, the cDNA of flaxseed hydroperoxide dehydratase encodes a transit peptide sequence characteristic of a plastidial location (Song et al., 1993). A similar transit peptide sequence was also found in a gene expressed after rice infection by pathogens and encoding a lipoxygenase, suggesting that this latter enzyme is also located in plastids. However, little information on the location of these enzymes within chloroplasts was available. For instance, hydroperoxide lyase was found to be bound to thylakoids (Hatanaka, 1993), and a lipoxygenase associated with thylakoid membranes was recently purified from tomato fruit (Bowsher et al., 1992). In contrast, the results presented in this article unambiguously demonstrate that envelope membranes contain enzymes such as hydroperoxide lyase, alcohol dehydrogenase, hydroperoxide dehydratase, and lipoxygenase. Therefore, in chloroplasts, hydroperoxide metabolism is associated with envelope membranes, and not with thylakoids.

During the last few years, oxylipins have been proposed to play an important role in the reactions of higher plants to pathogen attack or wounding. For instance, it was shown that volatile C_6-aldehydes (Croft et al., 1993), jasmonic acid (Neto et al., 1991), and epoxy-hydroxy- and trihydroxy-octadecanoids (Kato et al., 1984) derived from hydroperoxide lyase, dehydratase, or peroxygenase pathways, respectively, possess antifungal properties. In addition, there is increasing evidence that wound induction of plant defense reactions is mediated by endogenous jasmonates. In addition, some of these compounds are known to influence embryo development and seed maturation (Mason and Mullet, 1990; Wilen et al., 1991). In general, the questions that are addressed concern the identification of endogenous substances that control plant development, gene expression and response to the environment, and the characterization of their mode of action. However, unlike most hormones that have been characterized in plant signal transduction pathways, oxilipins are not stored but rather are synthesized and released rapidly (some of them are volatile) in response to extracellular stimuli. In addition, the hydrophobic nature of most of these molecules, together with their rapid metabolization, complicates efforts to correlate their biosynthesis with putative physiological roles. Thus, a crucial question is where and how such a chemical signal is generated and further mobilized to distant sites. Very few studies have addressed such questions in plants. The occurrence of enzymes catalyzing the formation of lipoxygenase-derived products within envelope membranes clearly indicates that plastids, although absent from most signal transduction pathways described so far, are an essential component of these pathways because they generate potentially important signaling molecules.

The data presented above demonstrate that envelope membranes are a complex membrane system in which all the enzymes involved in hydroperoxide metabolism share identical precursors and therefore could compete together, in vitro as well as in vivo. In vitro, we observed that the activity of each enzymatic pathway and therefore the relative proportion of the various products

formed are likely to vary from one envelope preparation to another. In vivo, the possible competition of the different enzymes for the same substrate is a crucial aspect of regulation. Timing during plant defense processes appears fundamental. Oxilipin (e.g. C_6-aldehydes, jasmonic acid, etc.) production is detectable very soon (30 min) after wounding (Albrecht et al., 1993), but a more or less long lag phase (in the range of hours) was observed after elicitation (Mueller et al., 1993) or pathogen attack (Croft et al., 1993). These observations first suggest that plants discriminate between wound and pathogen signals (Choi et al., 1994). They also suggest that enzymes required for jasmonate production are expressed constitutively in plants (Farmer and Ryan, 1992b; Albrecht et al., 1993). Such observations are supported by our data, since hydroperoxide metabolism by isolated envelope membranes occurs immediately upon addition of the substrate; therefore, the enzymes involved were already present. In addition, the lipoxygenase activity we found to be associated with the chloroplast envelope (a) would more readily attack membrane lipids than a soluble lipoxygenase and (b) could very rapidly deliver fatty acid hydroperoxides to the lyase and dehydratase, since they are located in the same membrane.

In conclusion, the presence of various enzymes involved in hydroperoxide formation and metabolism in chloroplast envelope membranes provides a new insight in the regulation of signaling processes in plants and opens a new field of research on this membrane system.

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