THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 16, Issue of April 20, pp. 12832–12838, 2001
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

A Novel Class of Oxylipins, *sn*1-*O*-(12-Oxophytodienoyl)-*sn*2-*O*-(hexadecatrienoyl)-monogalactosyl Diglyceride, from *Arabidopsis thaliana**

Received for publication, November 29, 2000, and in revised form, January 22, 2001
Published, JBC Papers in Press, January 25, 2001, DOI 10.1074/jbc.M010743200

Boguslawa A. Stelmach‡, Axel Müller‡, Peter Hennig‡§, Steffen Gebhardt¶, Manfred Schubert-Zsilavecz¶, and Elmar W. Weiler‡‖

From the ‡Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität, D-44780 Bochum, Germany and the ¶Institut für Pharmazeutische Chemie, Johann Wolfgang Goethe-Universität, D-60439 Frankfurt am Main, Germany

The cyclic derivative of 13(*S*)-hydroperoxolinolenic acid, 12-oxophytodienoic acid, serves as a signal transducer in higher plants, mediating mechanotransductory processes and plant defenses against a variety of pathogens, and also serves as a precursor for the biosynthesis of jasmonic acid, a mediator of plant herbivore defense. Biosynthesis of 12-oxophytodienoic acid from α-linolenic acid occurs in plastids, mainly in chloroplasts, and is thought to start with free linolenic acid liberated from membrane lipids by lipase action. In *Arabidopsis thaliana*, the glycerolipid fraction contains esterified 12-oxophytodienoic acid, which can be released enzymatically by *sn*1-specific, but not by *sn*2-specific, lipases. The 12-oxophytodienoyl glycerolipid fraction was isolated, purified, and characterized. Enzymatic, mass spectrometric, and NMR spectroscopic data allowed us to establish the structure of the novel oxylipin as *sn*1-*O*-(12-oxophytodienoyl)-*sn*2-*O*-(hexadecatrienoyl)-monogalactosyl diglyceride. The novel class of lipids is localized in plastids. Purified monogalactosyl diglyceride was not converted to the *sn*1-(12-oxophytodienoyl) derivative by the combined action of (soybean) lipoxygenase and (*A. thaliana*) allene oxide synthase, an enzyme ensemble that converts free α-linolenic acid to free 12-oxophytodienoic acid. When leaves were wounded, a significant and transient increase in the level of (12-oxophytodienoyl)-monogalactosyl diglyceride was observed. In *A. thaliana*, the major fraction of 12-oxophytodienoic acid occurs esterified at the sn1 position of the plastid-specific glycerolipid, monogalactosyl diglyceride.

Oxylipins are a diverse class of acyclic or cyclic oxidation products of fatty acids, many of which have regulatory functions in the cell (1). Among the plant oxylipins, the cyclic octadecanoids, collectively called jasmonates, have received much recent interest because of their diverse biological functions as mediators of mechanotransduction, herbivore and pathogen defense reactions, senescence, and male fertility (2–4).

Jasmonate biosynthesis is thought to start with induced release of α-linolenic acid from membrane glycerolipids (5), and the pathway from α-linolenic acid to jasmonic acid (JA)[1] has been worked out in detail (6, 7), whereas the processes of α-linolenic acid release are not understood. The oxidation of α-linolenic acid to 13*(S)*-hydroperoxolinolenic acid catalyzed by 13-lipoxygenase and the conversion of this intermediate to the first cyclic metabolite of the pathway, 12-oxophytodienoic acid (OPDA), occur in plastids and thus, in the shoot, predominantly in the chloroplast (8–10). OPDA then leaves the chloroplast and is reduced by the NADPH-dependent flavoprotein oxophytodienoate reductase 3 (11, 12), and the reaction product is converted to JA by β-oxidation (11).

It is known that plant lipoxygenases may act on glycerolipids as well as on free fatty acids (13 and references therein). This prompted us to survey plant membrane lipids for the presence of cyclic octadecanoids. As a result of this study, we report here that the major fraction of OPDA in *Arabidopsis thaliana* occurs esterified in the *sn*1 position of plastid-specific galactolipids. The implications of this novel class of glycerolipids for octadecanoid physiology and chloroplast membrane biology are discussed.

EXPERIMENTAL PROCEDURES

Plant Material—Greenhouse-grown rosettes of 6–8-week-old *A. thaliana* C24 were used for all experiments. Plants were raised individually in standard soil at 18 °C (night) and 24 °C (day) (average temperatures) between 50 and 70% relative humidity and no less than 150 μmol of photons m^{-2} s^{-1} (photosynthetically active radiation; supplementary light, if required, from sodium vapor lamps). Plants were cut above soil and immediately extracted, or they were immersed in liquid nitrogen and stored at −70 °C for minimum amounts of time.

Wounding Experiments—Wounding experiments were carried out as previously described (14). Briefly, all leaves of a rosette were crushed on one half of the leaf blade with a hemostat so that ~10% of the total leaf area of the plant was wounded. After incubation for the times indicated, 1 g of leaf tissue was collected using the whole leaf blades of wounded leaves and analyzed for OPDA and JA content as described in detail in Ref. 15, using [$^2H_5$]OPDA and [$^{13}C_2$]JA (*vide infra*) as internal standards. Extraction (16) and quantification of lipid-bound OPDA were done as described above.

Preparation of Protein Extracts—Leaves of *A. thaliana* were homogenized in a mortar with pestle in 12% sucrose, 1 mM EDTA, 10 mM Tricine, pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 10 mM $Na_2S_2O_4$, 0.1% bovine serum albumin using 1 ml of buffer per g of fresh weight. The homoge-

* This work was supported by the Deutsche Forschungsgemeinschaft, Bonn and by Fonds der Chemischen Industrie, Frankfurt (literature provision). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Merck KGaA ZD-A/ZFA 1, Frankfurter Str. 250, 64271 Darmstadt, Germany.

‖ To whom correspondence should be addressed: Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, Universitätsstraβe 150, D-44801 Bochum, Germany. Tel.: 49 234 3224291; Fax: 49 234 3214187; E-mail: Elmar.Weiler@ruhr-uni-bochum.de.

[1] The abbreviations used are: JA, jasmonic acid; OPDA, 12-oxophytodienoic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GC, gas chromatography; MS, mass spectrometry; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; MGDG, monogalactosyl diglyceride; EI, electron impact ionization.

This paper is available on line at http://www.jbc.org

This is an Open Access article under the CC BY license.

nate was centrifuged at 10,000 × *g*, and the supernatant was used to analyze the enzymatic degradation of OPDA. To do this, to 1 ml of the protein extract (1.5 mg of protein), 1 μg/ml [$^2H_5$]OPDA and [$^{13}C_2$]JA were added, and the assay mixture was then incubated at room temperature. Aliquots were taken at the times indicated and worked up for the analysis of OPDA and JA.

Lipid Extraction and Purification—Initial experiments were carried out using the standard procedure of Bligh and Dyer (16). It was later found that yields and purity of the glycolipid fraction improved when the following preparative procedure was employed. Leaf tissue (500 g) was immersed in 600 ml of boiling methanol containing, as an antioxidant, 200 mg of 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene). After boiling for 15 min, the hot mixture was homogenized with a Polytron mixer, the homogenate was filtered on a Buechner funnel and then over two sequential paper filters, and the methanol was removed by vacuum distillation (25 mbar, 30 °C). The aqueous phase was adjusted to 70% (v/v) methanol (crude lipid extract).

The crude lipid extract was passed over a column of 30 g of LiChroprep RP-18 (Merck). The column was eluted with 150 ml isopropyl alcohol: isohexane (80:55, v/v) and further fractionated on Chromabond NH_2 (Macherey-Nagel; aliquots of one-eighth of the LiChroprep RP-18 eluate passed over 3 g of column material that was eluted with 10 ml of the above solvent). The effluents and eluates of the Chromabond NH_2 column were pooled, the lipid extract was concentrated in a rotary evaporator (25 mbar, 30 °C), and the crude lipid was finally dried in a stream of N_2 (yield, ~11.3 mg from 500 g of leaf tissue). The residue was stored in 4–6 ml of chloroform:methanol (1:1, v/v) at −28 °C under nitrogen, if not used directly.

The dried crude lipid was redissolved in 4–5 ml of *i*-propanol:*i*-hexane (4:3, v/v) and centrifuged to remove particles. Aliquots (1 ml) of the clear solution were injected onto a semipreparative Nucleosil 100 HPLC column (Knauer; 25 mm × 8 mm inner diameter, particle size 15–25 μm) and eluted at a flow rate of 5 ml min^{-1} as follows: 1 min of eluent A, then a linear gradient in 10 min to 50% eluent B, then 27 min of isocratic flow at 50% solvent B, followed by a linear gradient over 10 min to 100% solvent B (solvent A: isopropyl alcohol: isohexane (4:3, v/v); solvent B: isopropyl alcohol: isohexane:H_2O (8:6:1.5, v/v/v)) (17). UV detection was at 205 nm. The lipid composition was checked in each fraction by TLC according to (17). The presence of esterified OPDA was checked by enzymatic treatment of each lipid fraction with *Rhizopus arrhizus* lipase (18). Esterified OPDA-containing fractions ($R_t \approx 15$ min) were purified further. To do so, the OPDA-containing lipid from each run on the silica column was pooled, the solvent was removed in a stream of nitrogen, and the residue was redissolved in 1 ml of acetonitrile:aqueous 50 mM ammonium acetate (70:30, v/v), centrifuged, and applied to a Dynamax C18 reversed phase HPLC column (Rainin; 250 mm × 21.4 mm inner diameter, 8 μm particle size, 60 Å pore size) at a flow rate of 20 ml min^{-1} (isocratic elution for 100 min, UV detection at 221 nm). The OPDA-containing fractions ($R_t \approx 52$–57 min) were pooled and rechromatographed on the same column to yield a homogenous peak ($R_t = 55$ min), which was used for all further characterizations. From 1 kg of plant material, ~1–2 mg of ester-OPDA lipid was obtained.

Analysis of 12-Oxophytodienoic Acid—To analyze the isomeric composition, optical purity, and quantity of OPDA, the methods described in detail previously, based on GC-MS, were employed (15, 19, 20,). Pentadeuterated OPDA (17[$^2H_2$],18[$^2H_3$]-*cis*-OPDA), synthesized according to Ref. 19, was used as an internal standard in all experiments.

Analysis of Jasmonic Acid—If desired, JA was quantitated by GC-MS concurrently with OPDA using the same procedures and samples and an internal standard of [$^{13}C_2$]jasmonic acid. [$^{13}C_2$]Jasmonic acid was synthesized according to Ref. 21 as follows. [$^{13}C_3$]Malonic acid (49 mg, >99 atom % ^{13}C, Isotec Inc.) was converted to its dimethyl ester using CH_2N_2 and dissolved in 50 μl of 2-(2*Z* pentyl)-2-cyclopenten-1-one (Firmenich SA, Geneva, Switzerland), followed by the addition of 170 μl of 0.2 M sodium methanolate. The mixture was stirred for 1.5 h under nitrogen at room temperature. The reaction was terminated with concentrated acetic acid (4 μl), and the sample was dried with nitrogen, redissolved in doubly distilled water (150 μl), and hydrolyzed under nitrogen for 18 h at 225 °C. After cooling, the reaction mixture was diluted into 0.1 M $NaHCO_3$ (2–3 ml) and extracted twice with chloroform, and the reaction product (racemic [$^{13}C_2$]methyljasmonate) recovered from the chloroform phase was treated for about 2 h at 60 °C with 0.5 M KOH to release the free acid, which was purified by HPLC as described in Ref. 15 (yield, ~30%; purity, >99%; isotopic abundance, >99% as analyzed by GC-MS).

Mass Spectrometry of Lipids—Routine analyses of fatty acid and OPDA methyl esters were performed on a Finnigan MAT Magnum ion trap mass spectrometer in chemical ionization (reactant gas, methanol) mode (scan range, 50–398 amu; scan rate, 1 Hz). GC settings were as follows: DB-35 column (J & W Scientific, 30 m × 0.25 mm × 0.25 μm coat), helium carrier gas, splitless injection mode (250 °C), and temperature program of 1 min at 80 °C, 30 °C min^{-1} to 200 °C, 5 °C min^{-1} to 250 °C, and 10 min at 250 °C. Typical retention times (methyl esters) were as follows: *cis*-OPDA, 16.73 min (m/z = 307 $[M + H]^+$); (9*Z*,12*Z*,15*Z*)-octadecatrienoic acid (α-linolenic acid), 12.07 min (m/z = 293 $[M + H]^+$); (7*Z*,10*Z*,13*Z*)-hexadecatrienoic acid, 9.57 min (m/z = 265 $[M + H]^+$).

LC-MS analyses were performed on a triple-quadrupole instrument (TSQ 7000, Finnigan MAT) operated in electrospray (positive or negative ion) mode (4.5 kV; capillary temperature, 200 °C; atmospheric pressure ionization-collision-induced dissociation voltage, 15 V). The compounds to be analyzed were introduced (injection volume, 5 μl) through a Luna C18 HPLC column (Phenomenex; 150 mm × 1 mm inner diameter, 5 μm particle size) using methanol, 20 mM ammonium acetate (90:10, v/v) as eluent (isocratic flow rate of 50 μl min^{-1} obtained with an Applied Biosystems 140 B dual-piston pump). The retention time of the OPDA-lipid was ~30 min. MS/MS measurements were performed using argon collision gas (2×10^{-3} torr) and a collision energy at quadrupole 2 of 10 V. MALDI-TOF-MS was performed on a PE Biosystems Voyager instrument (matrix, dihydroxybenzoic acid) with positive ion detection (25 kV acceleration voltage, calibrated with human angiotensin I (Sigma)).

Nuclear Magnetic Resonance Spectroscopy—The purified OPDA-containing lipid (~20 mg) was dissolved in 0.7 ml of CD_3OD. NMR spectra were recorded with Bruker AC 200 and AMX 500 (5-mm inverse TXI probe head) instruments. NMR conditions were as follows: H-H COSY, 45^0 mixing pulse; HMQC, phase-sensitive mode using time-proportional phase increment, bilinear rotation decoupling sequence, globally optimized alternating-phase rectangular pulses decoupled; HMBC, phase-sensitive using time-proportional phase increment, delay tuned to long range couplings, 71 ms.

Enzymatic Conversion of Monogalactosyl Diglyceride (MGDG)—MGDG was isolated from *Brassica napus* according to Bligh and Dyer (16). After drying, the crude lipid was purified as described above using solid phase extraction and semipreparative HPLC (Nucleosil 100, 12–14-min fraction). The identity of the isolated MGDG was verified by electrospray ionization-MS. The presence of the 18:3/16:3 molecular species was indicated by the molecular ion $[M + NH_4]^+$; m/z = 764.61. The purified MGDG (10 mg) was treated with soybean lipoxygenase (1 mg of protein) and recombinant allene oxide synthase (2 mg of protein representing total soluble protein from *Escherichia coli* lysates from cells expressing the *A. thaliana* enzyme (16, 19)) in 50 mM potassium phosphate, pH 8.0, at room temperature for 6 h. Thereafter, the reaction mixture was reextracted (16), and the lipids were hydrolyzed for 1 h in 0.5 N KOH (100 °C). The released acyl moieties were analyzed by GC-MS (19). To monitor the functionality of the enzymes used, the reaction mixture contained, in addition to plant MGDG, [$^2H_5$]linolenic acid. This allowed us to monitor in parallel by GC-MS the conversion of the esterified α-linolenic acid in the MGDG substrate as well as that of free α-linolenic acid by the two enzymes.

General and Biochemical Procedures—The presence and quantity of lipid-bound (esterified) OPDA was determined by lipase treatment of the corresponding fractions using an adaptation of the procedure described in Ref. 18, followed by GC-MS analysis (see above) of the released metabolites (OPDA, fatty acids) as their methyl esters. OPDA was released by the *sn*1-selective lipase from *R. arrhizus* (triacylglycerol lipase, EC 3.1.1.3, Roche Molecular Biochemicals) as follows. To each fraction to be analyzed was added an internal standard of *cis*-[$^2H_5$]OPDA (250 ng), and the sample was dried in a stream of nitrogen. The residue was then redissolved in 1 ml of 50 mM Tris borate (pH 7.5). To this solution were added 25 units of lipase in 50 μl of 100 mM Tris buffer (pH 7.5); controls received only buffer. The samples were then incubated at 37 °C (for the times indicated or overnight, if reactions were to go to completion), acidified (20 μl of concentrated HCl), and extracted two times with 2 ml of ethyl acetate followed by standard workup (20). In preparative experiments aimed at purification of the OPDA-lipid, no internal standard ([$^2H_5$]OPDA) was used.

Additional lipases tested were those from *Candida rugosa* (Sigma), *Mucor javanicus* (Sigma), and wheat germ (Sigma), and phospholipases tested were the phospholipases A_2 (EC 3.1.1.4) from *Apis mellifica* (Sigma) and *Naja mossambica* (Sigma). All lipases were assayed at pH 7.5; the two phospholipases A_2 were assayed at pH 8.9. Alkaline hydrolysis of lipids was for 1 h at 100 °C in 0.5 M KOH, followed by acidification and further workup as above.

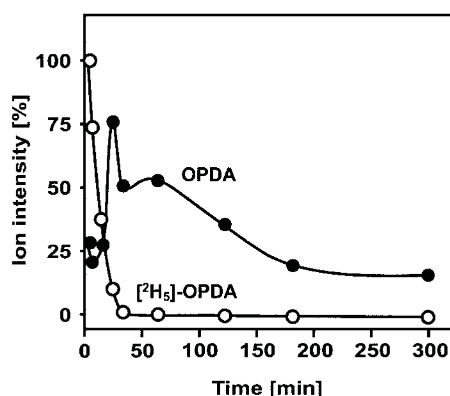

FIG. 1. **Metabolism of OPDA in aqueous crude leaf extracts.** Exogenous, labeled [$^2H_5$]OPDA (*open circles*); endogenous, unlabeled compound (*closed circles*). The ion intensity of 100% represents 3.4 nmol of substance.

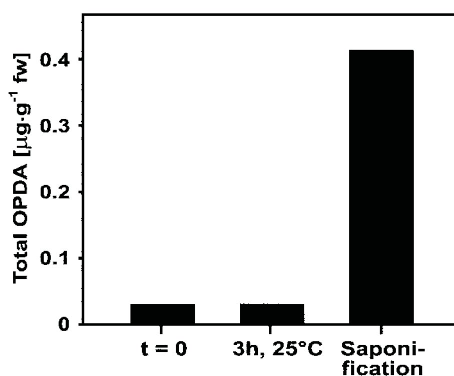

FIG. 2. **Release of OPDA from the crude lipid of *A. thaliana* by alkaline treatment.** OPDA from nonhydrolyzed (*left* and *middle bars*) and hydrolyzed (saponification for 3 h at 25 °C in 1 N KOH, *right bar*) lipid was quantified by GC-MS. *fw*, fresh weight.

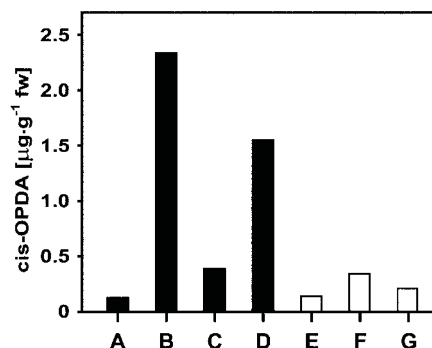

FIG. 3. **Enzymatic release of OPDA from crude lipid of *A. thaliana*, quantified by GC-MS.** Cleavage of OPDA from the lipid was most efficient using lipases, which show a preference for the *sn*1 position of glycerolipids (*black bars*), but was inefficient by phospholipases with *sn*2 preference (*white bars*). *A*, control (pH 7.5); *B*, *R. arrhizus* lipase; *C*, wheat germ lipase; *D*, *M. javanicus* lipase; *E*, control (pH 8.9); *F*, *A. mellifica* phospholipase A_2; *G*, *N. mossambica* phospholipase A_2; *fw*, fresh weight.

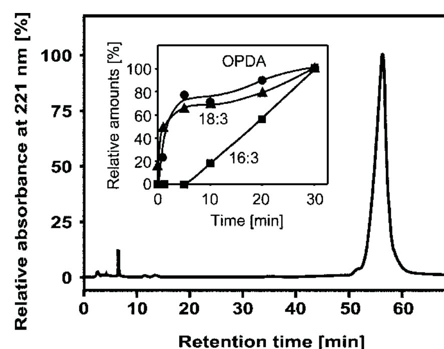

FIG. 4. **Kinetic analysis of acyl release from purified OPDA-lipid treated with *sn*1-specific *R. arrhizus* lipase.** The *inset* shows the normalized kinetics, whereas the elution behavior of the substrate (3 mg injected) used on reversed phase HPLC is shown in the main frame using relative absorbance units (100% = peak maximum).

RESULTS

Discovery of Membrane Lipid-bound 12-Oxophytodienoic Acid—During the course of studies on OPDA metabolism in *A. thaliana*, it was noticed that [$^2H_5$]OPDA added to leaf crude extracts was rapidly metabolized, whereas metabolism of endogenous (*i.e.* unlabeled) free OPDA followed a completely different time course; over at least 1 h, its level actually increased, before it started to decline also (Fig. 1). The pool from which this OPDA was released in crude extracts could be localized to the membrane fraction (100,000 × *g* pellet), whereas the 100,000 × *g* supernatant harbored the OPDA-metabolizing activity. When crude lipid from *A. thaliana* leaves obtained through Bligh and Dyer extraction (16) was subjected to alkaline hydrolysis (saponification conditions), it released OPDA. Control reactions verified that this OPDA fraction was not just dissolved in the lipid phase but covalently bound in an alkali-labile manner, *i.e.* most likely esterified (Fig. 2).

To scrutinize these initial results, the crude lipid was subjected to treatments with various lipases including phospholipases of the A_2 type (Fig. 3). Whereas there was no (or marginal) release of OPDA by phospholipase A_2 as well as by the wheat germ lipase, the two lipases that exhibited a preference for the *sn*1 position of glycerolipids (the fungal enzymes from *R. arrhizus* and *M. javanicus* (18)) efficiently released OPDA from the crude lipid fraction. It was estimated that ~80–90% of the total OPDA content of *A. thaliana* shoots occurred covalently bound in the lipid fraction in plants not subjected to any stimulus inducing the jasmonate signaling system. The endogenous activity that released OPDA from the lipid-bound form was associated with the 5,000 × *g* pellet but was not detected in the 12,000 × *g* supernatant of crude extracts and, thus, most likely represents a membrane-associated system. This activity was not characterized further in the present study.

Analysis by chiral GC-MS of the isomeric status of OPDA released from the lipid revealed that the lipase-released metabolite was the *cis*(+) enantiomer, (9*S*,13*S*)-12-oxophytodienoic acid, the same enantiomer that is also extracted in free form from plants (19, 20). Alkaline hydrolysis, of course, yielded predominantly the trans isomer (9*S*,13*R*)-OPDA), which is produced from the cis form by enolization, particularly under alkaline conditions. The method of analysis of OPDA enantiomers has been described in detail elsewhere (19).

Purification and Structural Characterization of the Lipase-degradable, Membrane-associated OPDA-Lipid—The solubility of the OPDA-lipid in aqueous methanol proved optimum around 80% (v/v), whereas free OPDA is most soluble around 50% (v/v) methanol. Thus, although less polar than the free acid, the OPDA-lipid turned out to be a remarkably polar metabolite that could be extracted from the plant material more selectively with aqueous methanol (70% v/v) than with conventional, less polar, lipid-solvent mixtures (*e.g.* see Ref. 16). Using the procedure detailed under "Experimental Procedures," from 600 mg of crude lipid (corresponding to 26.5 kg of leaf tissue), 20 mg of an apparently homogenous OPDA-lipid fraction was obtained for structure elucidation. During the course of the purification, the compound, in all steps, behaved most similarly to the plastid lipid MGDG. The OPDA-lipid

eluted at $R_t \approx 15$ min from the first HPLC column (Nucleosil 100, coeluting with MGDG) and at $R_t \approx 52$–57 min from the reversed phase column separating the lipid molecular species. The purified material, upon rechromatography, eluted from the same column as a single peak with $R_t = 55$ min (Fig. 4).

Treatment of the purified lipid fraction with the *sn*1-specific *R. arrhizus* lipase (Fig. 4, *inset*) resulted in the immediate release of two acidic metabolites, which were identified by GC-MS as α-linolenic acid and OPDA. After a lag phase of ~5 min, when the rates of OPDA and α-linolenic acid release began to level off and the enzyme began to attack the *sn*2 position, a third acidic metabolite began to accumulate, which was identified as (7*Z*,10*Z*,13*Z*)-hexadecatrienoic acid (16:3). These results indicated that (i) a mixture of two lipids had been obtained by the purification procedures employed (attempts to resolve this further were unsuccessful), and (ii) compound A of that mixture was a diacyl lipid with α-linolenic acid in the *sn*1 position and 16:3 in the *sn*2 position, and compound B was a diacyl lipid with OPDA in the *sn*1 position and 16:3 in the *sn*2 position. The occurrence of 16:3 in the *sn*2 position is typical for membrane lipids synthesized in plastids (22).

MALDI-TOF analysis of the sample (Fig. 5) revealed two masses of *m/z* 769.5 and *m/z* 783.4, which were Na^+ adducts of metabolites having molecular masses of 746.5 and 760.4 Da, respectively. These masses are in agreement with calculated masses of monogalactosyl-(18:3/16:3)-diacylglycerol (compound A) and of monogalactosyl-(OPDA/16:3)-diacylglycerol (compound B).

From the known plastid lipids, no other class could produce these two masses with incorporation of the acyl moieties identified after lipase treatment. The analysis thus confirmed the enzymatic data that the purified lipid was a mixture of two components, differing in the substituent at the *sn*1 position.

LC-MS and LC-MS/MS with electrospray ionization further corroborated the proposed structure of the OPDA-lipid (compound B) in both negative (Fig. 6*A*) and positive (Fig. 6*B*) ion mode. The compound eluted from the LC column at $R_t = 29.5$ min. The mass of the molecular ion was identified from the $[M - H]^-$ ion (*m/z* 759.5), from the acetate adduct $[M + Ac - H]^-$ (*m/z* 819.7), and from the $[M + H]^+$ ion (*m/z* 761.6) as well as from several positively charged solvent adducts (see Fig. 6*B*) to be 760.5 Da. MS/MS of the $[M + Ac - H]^-$ ion yielded several diagnostic fragments (*cf.* Fig. 6*A*), among them the $[OPDA - H]^-$ fragment (*m/z* 291.1) and the $[16:3 - H]^-$ fragment (*m/z* 249.3), most likely representing the carboxylate anions of the two acyl moieties, as has been observed with other monogalactosyl diacylglycerol species (23). No 18:3(linolenic acid)-derived ions were observed in the 29.5-min peak. Likewise, MS/MS of the $[M + H]^+$ ion (*m/z* 761.1) yielded the α-fragmentation derivatives showing loss of the esterified 16:3 fatty acid (*m/z* 349.2) and the corresponding loss of OPDA (*m/z* 307.2). Thus, the mass spectrometric analysis was in agreement with a proposed structure of a MGDG species in which one of the acyl groups is a 16:3 fatty acid, and the other is OPDA.

The structure of *sn*1-*O*-(12-oxophytodienoyl)-*sn*2-*O*-(hexadecatrienoyl)-monogalactosyl diglyceride could be further supported by two-dimensional NMR experiments such as COSY, HMQC, and HMBC (Table I, Fig. 7). The resonances of *sn*1, *sn*2, and *sn*3 were assigned by inspection of chemical shifts and multiplicities (Table I). As seen in Fig. 7, the anomeric proton of the sugar moiety at δ 4.22 ppm ($J = 7.4$ Hz, which proves the

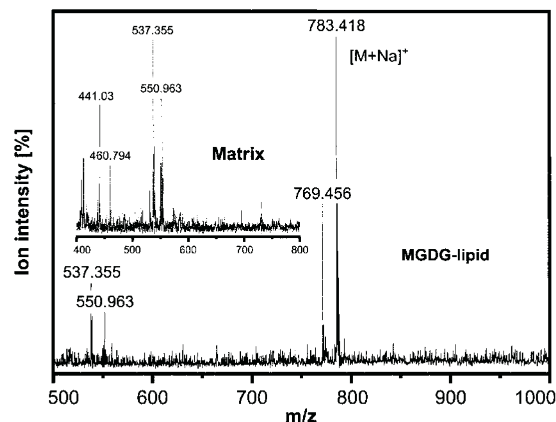

FIG. 5. **MALDI-TOF analysis of the purified OPDA-lipid (30 μg).** Monogalactosyl-(OPDA/hexatrienoyl)-diacylglycerol is indicated by the mass fragment with *m/z* of 783.4 ($[M + Na]^+$). The fragment with *m/z* of 769.4 corresponds to the accompanying (18:3/16:3)-MGDG ($[M + Na]^+$).

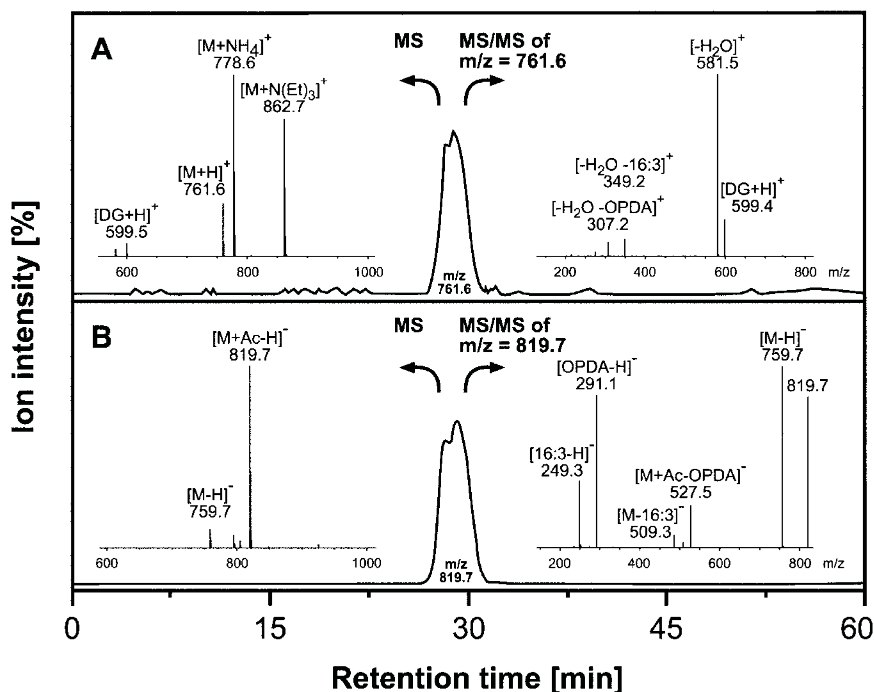

FIG. 6. **Electrospray ionization-LC/MS/MS analysis of the purified OPDA-lipid (100 μg).** Molecular ions of monogalactosyl-(OPDA/hexatrienoyl)-diacylglycerol are represented by *m/z* = 761.6 in positive ion mode (*A*) and *m/z* = 759.7 in negative ion mode (*B*), shown by full scan MS analysis of the eluting lipid compound (*left*). MS/MS analysis of characteristic ions gave specific fragment patterns, which confirmed the presence of the indicated acyl moieties (*right*).

TABLE I
NMR data for sn1-O-(12-oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl diglyceride

See Fig. 8 for numbering of positions.

Position	Chemical shift (δ in ppm)	
	$^{13}C^a$	$^{1}H^a$ (*J* in Hz)
1a	63.9	4.42 (dd, 12.0, 2.8)
1b	63.9	4.20 (dd, 12.0, 5.8)
2	71.8	5.26 (m)
3a	68.7	3.98 (dd, 11.1, 5.5)
3b	68.7	3.72 (dd, 11.1, 5.5)
1′	105.4	4.22 (d, 7.4)
2′	72.4	3.51 (t, 7.4)
3′	74.9	3.44 (dd, 7.4, 3.2)
4′	70.2	3.82 (d, 3.2)
5′	76.8	3.50 (m)
6′a	62.5	3.74 (m)
6′b	62.5	3.71 (m)
1″, 1‴	174.6, 175.0	
2″, 2‴	34.9, 35.1	2.35 (t, 7.0)
3″, 3‴	25.9, 26.0	1.65 (m)
	28.6, 28.7	
4‴–7‴	29.8, 30.1	1.25–1.41 (m)
4″, 5″	30.3, 30.4	
	30.8	
6″	26.6	2.08 (m)
7″, 8″,	128.2, 128.3	
10″, 11″,	128.9, 129.2	5.30–5.50 (m)
13″, 14″,	129.3, 130.9	
15‴, 16‴	132.9[b], 133.8	
8‴a	31.8	1.15 (m)
8‴b	31.8	1.77 (m)
9‴	45.8	3.04 (m)
9″, 12″	26.4, 26.6	2.80 (t, 5.5)
10‴	170.2	7.90 (dd, 5.5, 2.8)
11‴	132.8[b]	6.15 (dd, 6.9, 1.9)
12‴	n.d.[c]	
13‴	51.0	2.49 (m)
14‴a	24.9	2.18 (m)
14‴b	24.9	2.45 (m)
15″, 17‴	21.5, 21.7	2.10 (m)
		0.97 (t, 7.4)
16″, 18‴	14.4, 14.7	0.98 (t, 7.4)

[a] ^{1}H NMR was measured at 500 MHz; ^{13}C NMR was measured at 50 MHz. Chemical shifts are reported relative to residual solvent peaks (CD_3OD, δ ^{1}H 3.30, δ ^{13}C 49.0).
[b] Interchangeable.
[c] Not detectable.

β-linkage) couples via HMBC with the *sn*3 carbon at δ 63.9 ppm. The galactopyranosyl nature of this sugar moiety could be proven by analysis of the spin system, showing *e.g.* the typical broad doublet of the H-4′ at δ 3.82 ppm (*J* = 3.2 Hz). The signals C-1″ and C-1‴ of the unsaturated fatty acids appear at δ 174.6 and 175.0 ppm, making it impossible to assign the linkage position at the glycerol skeleton. However, the *sn*1 linkage of the OPDA has been proven by the enzymatic methods already described above. The chemical shifts of the carbon and proton resonances, as well as the multiplicity, are in agreement with the data reported formerly for OPDA (24) and methyl-(7*Z*,10*Z*,13*Z*)-hexadecatrienoate (25). The overlapping of the signals of the olefinic protons 7″, 8″, 10″, 11″, 13″, 14″, 15‴, and 16‴ at δ 5.30–5.50 ppm did not allow the determination of the E/Z configuration. However, the configuration of the *sn*2-hexadecatrienoic acid of MGDG from *A. thaliana* had earlier been shown to be exclusively the 7*Z*,10*Z*,13*Z* isomer (26), and our GC-MS data (see above) proved that the same isomer was present in the *sn*2 position of the OPDA lipid. The collective evidence thus firmly established the structure of the isolated metabolite to be *sn*1-*O*-(9*S*,13*S*-12-oxophytodienoyl)-*sn*2-*O*-(7*Z*,10*Z*,13*Z*-hexadecatrienoyl)-monogalactosyl diglyceride (MGDG-O, Fig. 8).

Dynamics of MGDG-O Levels in Leaves from Wounded Plants—It is well established that the level of JA, a wound signal produced via OPDA (6), rises rapidly and transiently after wounding of leaves (*e.g.* see Ref. 27). We therefore analyzed the levels of MGDG-O in parallel with those of free OPDA and JA in wounded *A. thaliana* leaves (Fig. 9). The results revealed complex dynamics different for each of the three compounds.

Using a combination of soybean lipoxygenase and recombinant allene oxide synthase, it is possible to convert free α-linolenic acid via 13(*S*)hydroperoxylinolenic acid to the allene oxide 12,13-epoxylinolenic acid, which then rearranges spontaneously, with the formation, among other products, of racemic *cis*-OPDA (19). When purified MGDG (17) was subjected to the same procedures, MGDG-O was not detected, although α-linolenic acid used as a control was readily converted (data not shown).

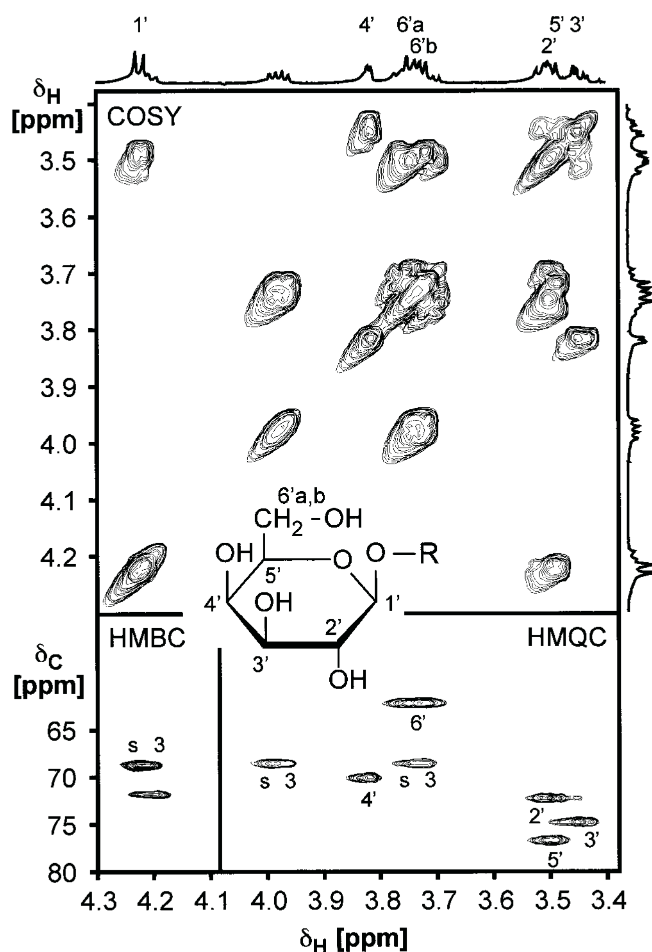

FIG. 7. **Expanded plots of COSY, HMQC, and HMBC spectra of *sn*1-*O*-(12-oxophytodienoyl)-*sn*2-*O*-(hexadecatrienoyl)-monogalactosyl diglyceride (MGDG-O) showing signals of the β-D-galactopyranose moiety.**

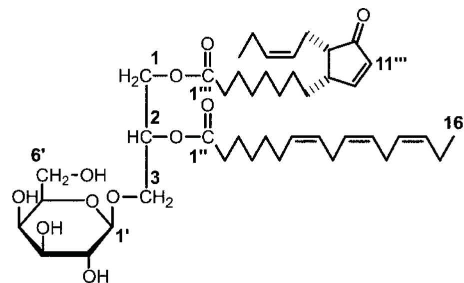

FIG. 8. **Structure of the OPDA-lipid identified as *sn*1-*O*-(9*S*,13*S*-12-oxophytodienoyl)-*sn*2-*O*-(7*Z*,10*Z*,13*Z*-hexadecatrienoyl)-*sn*3-*O*-(β-D-galactopyranosyl)-glycerol (MGDG-O).**

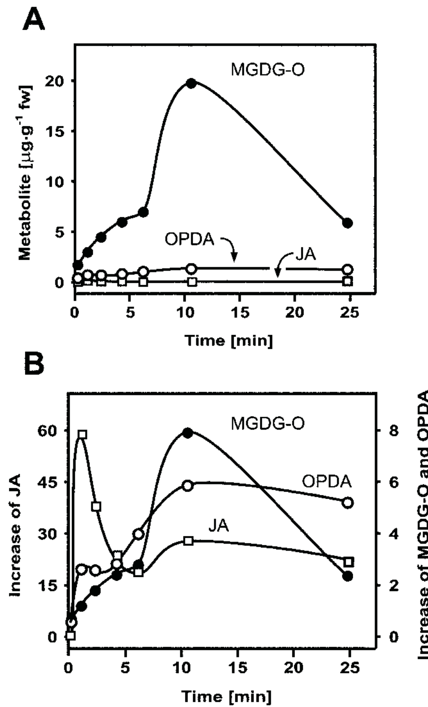

FIG. 9. **Kinetic analysis of the endogenous levels of JA, OPDA, and MGDG-O in leaf tissue of *A. thaliana* after wounding.** Shown are the means of typical experiments plotted in absolute (*A*) and relative (*B*) amounts. *fw*, fresh weight.

DISCUSSION

During recent years, it has become obvious that plants harbor a rich variety of oxidized lipids, collectively known as oxylipins (1), many of them inducible and of physiological importance as signaling compounds in herbivore defense, pathogen defense, and mechanotransduction or involved in the regulation of developmental processes such as senescence and pollen maturation (2–4). The majority of oxylipins occur in free form (1), which is believed to originate from fatty acids released from membrane lipids (5, 9). Recently, however, it has become clear that esterified or amidated fatty acids can be oxidized by enzymes of plant oxylipin biosynthesis. Thus, lipoxygenase attacks unsaturated fatty acids of triglycerides to convert them to hydroperoxo derivatives, which are then released by lipases and enter β-oxidation (28), but lipoxygenase has also been shown to oxidize fatty acids esterified in phospholipids (29). Phospholipases that exhibit a preference for phosphatidylcholine with oxygenated acyl groups have been identified in plant microsomes (30). Moreover, *N*-acyl(ethanol) amines have been identified in plants (31), and these are thought to play a role in plant pathogen defense (32). It has recently been shown that enzymes of the octadecanoid biosynthetic pathway, namely lipoxygenase and allene oxide synthase, act on *N*-acyl(ethanol) amines to yield, among other products, the 12-oxo-*N*-phytodienoylamines (33). Homologs of isoprostanes that are formed nonenzymatically on phospholipids (34) have meanwhile been found in plants, the phytoprostanes (35). Thus, although the biological relevance of these compounds is still not understood, the generation, *in situ*, of oxylipins in plant membranes probably complements their formation from free fatty acids, and it will be important to elucidate the enzymology of their formation and their physiological role(s).

We show, for the first time, that OPDA, a signal in mechanotransduction (36) and the precursor of JA (6), a wound and pathogen defense signal (2–4), occurs in esterified form in the *sn*1 position of the plastid galactolipid MGDG. This strongly suggests a defined route of its formation. It is likely that this route is a plastidic one, *i.e.* in green tissue localized in chloroplast. The first indication is the (7*Z*,10*Z*,13*Z*)-hexadecatrienoic acid (16:3) in the *sn*2 position of MGDG-O. MGDG molecular species carrying *sn*2–16:3 are synthesized in plastids by a prokaryotic pathway (22, 26). A large fraction of *sn*2–16:3-MGDG carries α-linolenic acid in the *sn*1 position, the precursor of OPDA. It is thus possible that OPDA is generated *in situ* from the α-linolenic acid in the *sn*1 position of MGDG. However, we have been unable to show conversion of MGDG to MGDG-O *in vitro* using soybean lipoxygenase and *A. thaliana* allene oxide synthase, an enzyme combination that converts free α-linolenic acid to OPDA. This means that either the enzymes involved in formation of MGDG-O *in vivo* differ from those involved in synthesizing OPDA or the substrate has to be presented to them in a particular environment (*e.g.* a bilayer membrane). Alternatively, it is conceivable that esterified OPDA is not formed from MGDG *in situ* but that free OPDA is synthesized first and then incorporated in the membrane lipid. These issues will be addressed in future studies. It was shown earlier that allene oxide synthase is associated with chloroplast membranes from which it has to be released with detergent (9, 37). The recombinant enzyme is soluble and, as such, acts on free fatty acid 13(*S*)-hydroperoxides (38, 39). The chloroplast membrane docking site of allene oxide synthase is unknown, but the membrane association may reflect the fact that the enzyme, *in vivo*, acts on a membrane substrate rather than on a free fatty acid.

MGDG-O conceivably is a metabolite with a biological significance of its own, and/or it may form a pool from which OPDA could be released immediately when the need arises. Indeed, a membrane-associated enzymatic activity that liberates OPDA from MGDG-O was identified in *A. thaliana* in this study but awaits its full characterization. The data in Fig. 9, on the other hand, do not show decreases in MGDG-O correlated with the initial accumulation of JA (and OPDA) induced by wounding. This may indicate resynthesis of MGDG-O or simply reflect the fact that only a minor fraction of it is being converted to release OPDA. A decisive answer about precursor-product relationships awaits pulse-chase analyses of metabolic fluxes through these metabolites and experiments with isolated chloroplasts. It is clear, though, that wounding induces a complex, biphasic increase in the MGDG-O level. The transient nature of this increase shows clearly that MGDG-O is not simply a metabolic end product but that it has a significant turnover. MGDG-O levels fluctuate quite independently from those of JA. Thus, distinct physiological roles of MGDG-O and JA should be envisaged. It is interesting to note that the two *sn*1-specific lipases that effectively cleaved OPDA from MGDG-O are enzymes of fungal origin and that both species, *R. arrhizus* and *M. javanicus*, are plant pathogenic saprophytes. A potential function of MGDG-O could thus be as a reporter of lipolytic fungal activity indicative of invading phytopathogenic fungi. OPDA released locally upon fungal attack would (directly or through conversion to JA) signal to the plant the presence of an intruder and help to mount immediate local defense responses. Indeed, octadecanoids act as local, and not as systemic, defense signals in *A. thaliana* (40).

Acknowledgment—We thank Rolf Breuckmann, Bioorganic Chemistry, Faculty of Chemistry, Ruhr-Universität Bochum, for the MALDI-TOF analyses.

REFERENCES

1. Hamberg, M. (1993) *J. Lipid Mediators* **6,** 375–384
2. Wasternack, C., and Parthier, B. (1997) *Trends Plant Sci.* **2,** 302–307
3. Creelman, R. A., and Mullet, J. E. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 355–381
4. Weiler, E. W. (1997) *Naturwissenschaften* **84,** 340–349

5. Farmer, E. E., and Ryan, C. A. (1992) *Trends Cell Biol.* **2,** 236–241
6. Vick, B. A., and Zimmerman, D. C. (1984) *Plant Physiol.* (*Bethesda*) **75,** 458–461
7. Vick, B. A., and Zimmerman, D. C. (1987) *Plant Physiol.* (*Bethesda*) **85,** 1073–1078
8. Vick, B. A., and Zimmerman, D. C. (1981) *Plant Physiol.* (*Bethesda*) **67,** 92–97
9. Blée, E., and Joyard, J. (1996) *Plant Physiol.* (*Bethesda*) **110,** 445–454
10. Hamberg, M. (1988) *Biochem. Biophys. Res. Commun.* **156,** 543–550
11. Vick, B. A., and Zimmerman, D. C. (1986) *Plant Physiol.* (*Bethesda*) **80,** 202–205
12. Schaller, F., Biesgen, C., Müssig, C., Altmann, T., and Weiler, E. W. (2000) *Planta* **210,** 979–984
13. Brash, A. R. (1999) *J. Biol. Chem.* **274,** 23679–23682
14. Laudert, D., and Weiler, E. W. (1998) *Plant J.* **15,** 675–684
15. Stelmach, B. A., Müller, A., Hennig, P., Laudert, D., Andert, L., and Weiler, E. W. (1998) *Phytochemistry* **47,** 539–546
16. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **31,** 911–917
17. Demandre, C., Tremolières, A., Justin, A. M., and Mazliak, P. (1985) *Phytochemistry* **24,** 481–485
18. Williams, J. P., Khan, M. U., and Wong, D. (1995) *J. Lipid Res.* **36,** 1407–1412
19. Laudert, D., Hennig, P., Stelmach, B. A., Müller, A., Andert, L., and Weiler, E. W. (1997) *Anal. Biochem.* **246,** 211–217
20. Schaller, F., and Weiler, E. W. (1997) *J. Biol. Chem.* **272,** 28066–28072
21. Knöfel, H. D., and Gross, D. (1988) *Z. Naturforsch.* **43,** 29–31
22. Somerville, C., and Browse, J. (1991) *Science* **252,** 80–87
23. Kim, Y. H., Yoo, J. S., and Kim, M. S. (1997) *J. Mass Spectrom.* **32,** 968–977
24. Bohlmann, F., Jakupovic, J., Ahmed, M., and Schuster, A. (1983) *Phytochemistry* **7,** 1623–1636
25. Morimoto, T., Nagatsu, A., Murakami, N., Sakakibawa, J., Tokuda, H., Nishini, H., and Iwashima, A. (1995) *Phytochemistry* **5,** 1433–1437
26. Browse, J., McCourt, P. J., and Somerville, C. R. (1986) *Anal. Biochem.* **152,** 141–145
27. Albrecht, T., Kehlen, A., Stahl, K., Knöfel, H. D., Sembdner, G., and Weiler, E. W. (1993) *Planta* **191,** 86–94
28. Feussner, I., Wasternack, C., Kindl, H., and Kühn, H. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11849–11853
29. Brash, A., Ingram, C. D., and Harris, T. M. (1987) *Biochemistry* **26,** 5465–5471
30. Banás, A., Johansson, I., and Stysune, S. (1992) *Plant Sci.* **84,** 137–144
31. Schmid, H. H. O., Schmid, P. C., and Natarjan, V. (1990) *Prog. Lipid Res.* **29,** 1–43
32. Tripathy, S., Venables, B. J., and Chapman, K. D. (1999) *Plant Physiol.* (*Bethesda*) **121,** 1299–1308
33. van der Stelt, M., Noordermeer, M. A., Kiss, T., van Zadelhoff, G., Merghart, B., Veldink, G. A., and Vliegenthart, J. F. G. (2000) *Eur. J. Biochem.* **267,** 2000–2007
34. Morrow, J. D., Hill, K. E., Burk, R. F., Nammour, T. N., Bader, K. F., and Roberts, J. F., II (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9383–9387
35. Mueller, M. J. (1998) *Chem. Biol.* **5,** R323–R333
36. Blechert, S., Bockelmann, C., Füβlein, M., von Schrader, T., Stelmach, B. A., Niesel, U., and Weiler, E. W. (1999) *Planta* **207,** 470–479
37. Song, W.-C., and Brash, A. R. (1991) *Science* **253,** 781–784
38. Song, W.-C., Funk, C. D., and Brash, A. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8519–8523
39. Laudert, D., Pfannschmidt, U., Lottspeich, F., Holländer-Czytko, H., and Weiler, E. W. (1996) *Plant Mol. Biol.* **31,** 323–335
40. Kubigsteltig, I., Laudert, D., and Weiler, E. W. (1999) *Planta* **208,** 463–471