The cyclic derivative of 13(S)-hydroperoxolinolenic acid, 12-oxophytodienoic acid, serves as a signal transducer in higher plants, mediating mechanotransductive 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 sn1-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 sn1-O-(12-oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl diglyceride. The novel class of lipids is localized in plastids. Purified monogalactosyl diglyceride was not converted to the sn1-(12-oxophytodienoyl) derivative by the combined action of (soybean) lipoxynase 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) 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), occurs 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 sn1 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 [3H]OPDA and [13C₂]JA (wide 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 MgCl₂, 10 mM Na₂S₂O₄, 0.1% bovine serum albumin using 1 ml of buffer per g of fresh weight. The homoge-

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nate was centrifuged at 10,000 × g, and the supernatant was used to analyze the enzymatic degradation of OPDA. To do this, 1 ml of the protein extract (1.5 mg of protein), 1 μg/ml [2H5]OPDA and [13C2]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 assay of OA 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 Buchner 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 removed by vacuum distillation (25 mbar, 30 °C). The aqueous phase was concentrated acetic acid (4 ml) and centrifuged, and the residue was redissolved in 1 ml of 2-(2-hydroxyethyl)-1-piperazinylethanesulfonic acid (Sigma) and dissolved in 50 ηl of 2-(2H5)OPDA and [13C2]JA (250 ng), and the sample was dried in a stream of nitrogen. A Novel Class of Oxylipins

To analyze the isomeric compositions, optical purity, and quantity of OPDA, the methods described in detail previously, based on GC-MS, were employed (15, 19, 20).

**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).

**Analysis of Jasmonic Acid**—If desired, JA was quantitated by GC-MS concurrently with OPDA using the same procedures and standards and an internal standard of [13C2]jasmonic acid. [13C2]Jasmonic acid was synthesized according to Ref. 21 as follows. [13C2]Malonic acid (49 mg, >99 atom % 13C, Isotec Inc.) was converted to its dimethyl ester using CH3OH and dissolved in 50 μl of 2-2H5 penta-2-lycyclcopentan-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 1.1 ml NaHCO3 (2–3 M) and extracted twice with chloroform. The product (racemic [13C2]methylyjasmonate) 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, 1.0 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. The splitless injection time was 2 min. The ion trap mass spectrometer in chemical ionization mode was set at 7.4 GHz with a 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 ml ammonium acetate (90:10, v/v) as eluent (isocratic flow rate of 50 μl min−1 obtained with an Applied Biosystems 110 B dual-piston pump). The retention time of OPDA-lipid was determined by using anion column (2 × 10−4 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-con- taining lipid (~20 mg) was dissolved in 0.7 ml of CD3OD. 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° mixing pulse; HMQC, phase-sensitive mode using time-proportional phase increment, bilinear rotation decoupling sequence, globally optimized alternating-phase rectangular pulses decoupled; HMBLC, phase-sensitive using time-proportional phase increment, delay tuned to long range couplings, 7Ts.

**Enzymatic Conversion of Monogalactosyldiacylglycerides (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 + Na]+ (m/z 764.6). The purified MGDG (10 mg) was treated with soybean lipase (2 mg of protein) and recombinant allene oxide synthase (2 mg of protein representing total soluble protein from *Erhicheria coli* lysates from cells expressing the A. thaliana enzyme (16, 19)) in 50 ml 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 at 100 °C. The released acyl moieties were analyzed by GC-MS (19). To monitor the enzymatic activity, the reaction mixture contained, in addition to plant MGDG, [2H5]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. arhizus* (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- [2H5]OPDA (250 ng), and the sample was dried in a stream of nitrogen. The sample was then redissolved in 1 ml of 50 ml/ml 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 was added to the 1 ml of 50 ml/ml Tris borate (pH 7.5). Additional lipases tested were those from Candida rugosa (Sigma), Muco rouxianus (Sigma), and wheat germ (Sigma), and phospholipases were the phospholipases A2 (EC 3.1.1.4) from *Apis mellifica* (Sigma) and *Naja mambasica* (Sigma). All lipases were assayed at pH 7.5; the two phospholipases A2 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.
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 metabolite released from the lipid, but was efficiently by phospholipases a2 (Fig. 3). Whereas there was no (or marginal) release of OPDA by phospholipase A2, as well as by the wheat germ lipase, the two lipases that exhibited a preference for the sn1 position of glycerolipids (black bars), but was inefficient by phospholipases with sn2 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. mellifera phospholipase A2; G, N. mossambica phospholipase A2; fw, fresh weight.

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 sn1 position of glycerolipids (black bars), but was inefficient by phospholipases with sn2 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. mellifera phospholipase A2; G, N. mossambica phospholipase A2; fw, fresh weight.

Kinetic analysis of acyl release from purified OPDA-lipid treated with sn1-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).

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 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, (9S,13S)-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 (9S,13R)-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).
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 $\omega$-linolenic acid and OPDA. After a lag phase of $\approx 5$ min, when the rates of OPDA and $\omega$-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 (7Z,10Z,13Z)-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 $\omega$-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. 6A) and positive (Fig. 6B) 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. 6B) to be 760.5 Da. MS/MS of the $[M + Ac - H]^{-}$ ion yielded several diagnostic fragments (cf. Fig. 6A), 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 $\alpha$-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 $\delta$ 4.22 ppm ($J = 7.4$ Hz, which proves the

Fig. 5. MALDI-TOF analysis of the purified OPDA-lipid (30 $\mu$g). 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]$^+$/18:3/16:3).
broad doublet of the H-4 at 174.6 and 175.0 ppm, making it impossible to assign the linkage position at the glycerol skeleton. However, the sn1-linkage of the OPDA has been proven by the enzymatic methods already described above. The chemical shifts of the carbon signals C-1 and C-1' of the unsaturated fatty acids appear at 132.8, 133.8 ppm. The overlapping of the proton resonances, as well as the multiplicity, are in agreement, with the data reported formerly for OPDA (24) and methyl-(7Z,10Z,13Z)-hexadecatrienoate (25). The overlapping of the signals of the olefinic protons 7', 8', 10', 11', 13', 14', 15', and 16' at 5.30–5.50 ppm did not allow the determination of the EZ configuration. However, the configuration of the sn2-hexadecatrienoic acid of MGDG from A. thaliana had earlier been shown to be exclusively the 7Z,10Z,13Z isomer (26), and our GC-MS data (see above) proved that the same isomer was present in the sn2 position of the OPDA lipid. The collective evidence thus firmly established the structure of the isolated metabolite to be sn1-O-(9S,13S-12-oxophytodienoyl)-sn2-O-(7Z,10Z,13Z-hexadecatrienoyl)-monogalactosyl diglyceride (MGDG-O).

Table I

| Position | Chemical shift (δ in ppm) |
|----------|--------------------------|
| 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 (dd, 7.4, 2.4) |
| 2'       | 72.4 3.51 (t, 7.4)        |
| 3'       | 74.9 3.44 (dd, 7.4, 3.2)  |
| 4'       | 70.2 3.82 (dd, 3.2)       |
| 5'       | 76.8 3.50 (m)             |
| 6a       | 62.5 3.74 (m)             |
| 6b       | 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)       |
| 10''     | 28.6, 28.7               |
| 4'', 7'' | 29.8, 30.1 1.25–1.41 (m)  |
| 4'', 5'' | 30.3, 30.4               |
| 10''     | 30.8                     |
| 6''      | 26.6 2.08 (m)             |
| 7'', 8'' | 128.2, 128.3 5.30–5.50 (m) |
| 10'', 11'' | 128.9, 129.2 5.30–5.50 (m) |
| 13'', 14'' | 129.3, 130.9            |
| 15'', 16'' | 132.9, 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''     | 132.8b 6.15 (dd, 6.9, 1.9) |
| 12       | n.d.                     |
| 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)       |
| 16'', 18'' | 14.4, 14.7 0.98 (t, 7.4)  |

a 1H NMR was measured at 500 MHz; 13C NMR was measured at 50 MHz. Chemical shifts are reported relative to residual solvent peaks (CD3OD, δ 3.30, δ 13C 49.0).

Interchangeable.

* Not detectable.

**β-linkage** couples via HMBC with the sn3 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 sn1-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-(7Z,10Z,13Z)-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 EZ configuration. However, the configuration of the sn2-hexadecatrienoic acid of MGDG from A. thaliana had earlier been shown to be exclusively the 7Z,10Z,13Z isomer (26), and our GC-MS data (see above) proved that the same isomer was present in the sn2 position of the OPDA lipid. The collective evidence thus firmly established the structure of the isolated metabolite to be sn1-O-(9S,13S-12-oxophytodienoyl)-sn2-O-(7Z,10Z,13Z-hexadecatrienoyl)-monogalactosyl diglyceride (MGDG-O).

*Fig. 7. Expanded plots of COSY, HMQC, and HMBC spectra of sn1-O-(12-oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl diglyceride (MGDG-O) showing signals of the β-o-galactopyranose moiety.*

*Fig. 8. Structure of the OPDA-lipid identified as sn1-O-(9S,13S-12-oxophytodienoyl)-sn2-O-(7Z,10Z,13Z-hexadecatrienoyl)-sn3-O-(β-D-galactopyranosyl)-glycerol (MGDG-O).*

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).
phytodi-amine to yield, among other products, the 12-oxo-lipoxygenase and allene oxide synthase, act on plant pathogen defense (32). It has recently been shown that identified in plants (31), and these are thought to play a role in Phospholipases that exhibit a preference for phosphatidylcholine (34) and related and MGDG-O in leaf tissue of (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 hydroperoxy 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 phosphatidylethanolamine 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-5-phytodi-enoylaminolates (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 sn1 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 (7Z,10Z,13Z)-hexadecatrienic acid (16:3) in the sn2 position of MGDG-O. MGDG molecular species carrying sn2–16:3 are synthesized in plastids by a prokaryotic pathway (22, 26). A large fraction of sn2–16:3-MGDG carries α-linolenic acid in the sn1 position, the precursor of OPDA. It is thus possible that OPDA is generated in situ from the α-linolenic acid in the sn1 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 sn1-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).

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A Novel Class of Oxylipins
A Novel Class of Oxylypins, sn1-O-(12-Oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl Diglyceride, from Arabidopsis thaliana

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Page 12837, Fig. 9: The labeling is incorrect. The x axis in both A and B is in hours (h), not minutes (min). The correct figure is shown below.

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.

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