

# Traumatin- and Dinortraumatins-containing Galactolipids in *Arabidopsis*

## THEIR FORMATION IN TISSUE-DISRUPTED LEAVES AS COUNTERPARTS OF GREEN LEAF VOLATILES\*

Received for publication, May 23, 2013, and in revised form, July 23, 2013. Published, JBC Papers in Press, July 25, 2013, DOI 10.1074/jbc.M113.487959

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**Background:** The pathway to form green leaf volatiles is important for plant defense against pathogens and herbivores.

**Results:** Galactolipids with truncated oxoacids are formed as counterparts of green leaf volatiles.

**Conclusion:** Galactolipid-hydroperoxides are substrates for hydroperoxide lyase.

**Significance:** The biosynthetic pathway partly proceeds directly with galactolipids, without the involvement of lipases, to form free fatty acids.

Green leaf volatiles (GLVs) consisting of six-carbon aldehydes, alcohols, and their esters, are biosynthesized through the action of fatty acid hydroperoxide lyase (HPL), which uses fatty acid hydroperoxides as substrates. GLVs form immediately after disruption of plant leaf tissues by herbivore attacks and mechanical wounding and play a role in defense against attackers that attempt to invade through the wounds. The fates and the physiological significance of the counterparts of the HPL reaction, the 12/10-carbon oxoacids that are formed from 18/16-carbon fatty acid 13-/11-hydroperoxides, respectively, are largely unknown. In this study, we detected monogalactosyl diacylglycerols (MGDGs) containing the 12/10-carbon HPL products in disrupted leaf tissues of *Arabidopsis*, cabbage, tobacco, tomato, and common bean. They were identified as an MGDG containing 12-oxo-9-hydroxy-(*E*)-10-dodecenoic acid and 10-oxo-7-hydroxy-(*E*)-8-decenoic acid and an MGDG containing two 12-oxo-9-hydroxy-(*E*)-10-dodecenoic acids as their acyl groups. Analyses of *Arabidopsis* mutants lacking HPL indicated that these MGDGs were formed enzymatically through an active HPL reaction. Thus, our results suggested that in disrupted leaf tissues, MGDG-hydroperoxides were cleaved by HPL to form volatile six-carbon aldehydes and non-volatile 12/10-carbon aldehyde-containing galactolipids. Based on these results, we propose a novel oxylipin pathway that does not require the lipase reaction to form GLVs.

leaves of most plants (1). GLVs form rapidly after the disruption of plant tissues (2, 3). Insecticidal, fungicidal, and bactericidal activities have been reported for (*Z*)-3-hexenal and its related aldehydes (4–7). A portion of the C6 aldehydes that form in disrupted tissues diffuses into adjacent intact tissues, where they are reduced to C6 alcohols and further acetylated to C6 acetates (8). They function as airborne infochemicals in specific plant-herbivore, plant-carnivore, and plant-plant relationships (9).

The biosynthetic pathway that produces GLVs (Fig. 1) is widespread in the plant kingdom. Lipoxygenase (LOX) adds dioxygen (O<sub>2</sub>) at position 13 of  $\alpha$ -linolenic acid to produce  $\alpha$ -linolenic acid 13-hydroperoxide (13-HPOT). The hydroperoxide (HPO) is cleaved by fatty acid hydroperoxide lyase (HPL) at the C12–C13 bond to produce two carbonyl compounds; (*Z*)-3-hexenal and 12-oxo-(*Z*)-9-dodecenoic acid ((9*Z*)-traumatins) (10). Hexadecatrienoic acid 11-hydroperoxide (11-HPHT) is also a substrate for HPL, yielding (*Z*)-3-hexenal and 10-oxo-(*Z*)-7-decenoic acid ((7*Z*)-dinortraumatins) as the primary products. If allene oxide synthase (AOS) acts on 13-HPOT, the hydroperoxide is diverted into the jasmonate pathway to form 12-oxophytodienoic acid and jasmonic acid (JA) after several enzymatic reaction steps (11).

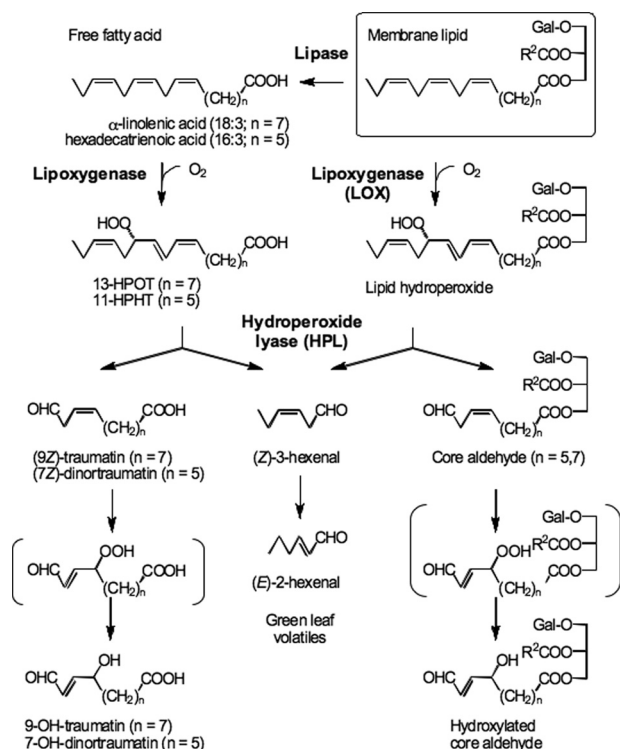
Green leaf volatiles (GLVs),<sup>2</sup> which consist of six-carbon (C6) aldehydes, alcohols, and their esters, are ubiquitous in the

\* This work was supported in part by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant 23580151 and Core-to-Core Project 20004 and by the Yamaguchi University Yobimizu Project.

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<sup>2</sup> The abbreviations used are: GLV, green leaf volatile; HPL, hydroperoxide lyase; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacyl-

glycerol; 13-HPOT,  $\alpha$ -linolenic acid 13-hydroperoxide; 11-HPHT, hexadecatrienoic acid 11-hydroperoxide; HPO, hydroperoxide; (9*Z*)-traumatins, 12-oxo-(*Z*)-9-dodecenoic acid; (10*E*)-traumatins, 12-oxo-(*E*)-10-dodecenoic acid; (7*Z*)-dinortraumatins, 10-oxo-(*Z*)-7-decenoic acid; 9-OH-traumatins, 12-oxo-9-hydroxy-(*E*)-10-dodecenoic acid; 7-OH-dinortraumatins, 10-oxo-7-hydroxy-(*E*)-8-decenoic acid; AOS, allene oxide synthase; JA, jasmonic acid; IS, internal standard; ESI, electrospray ionization; EMS, enhanced mass spectrometry; EPI, enhanced product ion scan; LOX, lipoxygenase; DETAPAC, diethylenetriamine-*N,N,N',N'*-pentaacetic acid; CaHPL, bell pepper hydroperoxide lyase; FW, fresh weight; HR, high resolution; SELCOSY and SELTOCSY, selective COSY and TOCSY, respectively.



**FIGURE 1. Proposed biosynthetic pathway for green leaf volatiles and their counterparts.** Complex membrane lipids are hydrolyzed by lipases to form free fatty acids (18:3 and 16:3). Then the free fatty acid is oxygenated by lipoxygenase, and resulting hydroperoxides are cleaved to form (Z)-3-hexenal and free traumatin or dinotraumatins derivatives. In disrupted leaves of *Arabidopsis* and some other plant species, galactolipids are directly oxygenated by lipoxygenase to form galactolipid-hydroperoxides which are subsequently cleaved by hydroperoxide lyase to form (Z)-3-hexenal and traumatin- and dinotraumatins-containing galactolipids.

In general, GLVs and the corresponding traumatins, as well as JAs, are thought to be formed from free fatty acids that are released from glycerolipids by lipases; however, there are only a few reports on the identification of lipases involved in JA synthesis (11). Mono- and digalactosyldiacylglycerols (MGDGs and DGDGs) that contain 12-oxophytodienoic acid and/or 12-oxodinorphytodienoic acid (collectively known as arabidopsides) have been identified in *Arabidopsis* (*Col-0*) (12, 13). The oxidative modification of MGDG to yield arabidopsides occurs on esterified fatty acids (14). Thus, the first enzyme acting on the lipid may be LOX, especially AtLOX2 in *Arabidopsis*, because this LOX is known to contribute the major portion of arabidopsides formed after leaf wounding (15). The oxylipin galactolipids containing divinyl ether residues (linolipins) that are present in flax were thought to be biosynthesized without free fatty acid intermediates derived from the lipase reaction (16). Therefore, in some plant species, lipases are not essential to form oxylipins.

Nonenzymatic oxidation of lipids can also occur, especially in chloroplasts, because reactive oxygen species such as  $^1\text{O}_2$  are inevitably formed as by-products of photosynthesis (17). The chloroplast-abundant lipids, such as MGDGs and DGDGs, are prone to conversion into their peroxides, a portion of which are modified by radical-catalyzed fragmentation reactions to yield a wide variety of compounds (e.g. malondialdehyde (18),

trioxigenated products, phytoprostanes, or even MGDGs harboring C12 or C9 oxoacids) (19–20).

Studies with purified HPL and 13-HPOT as substrate showed that the C6 and C12 aldehydes formed in an equimolar ratio, as expected from the reaction catalyzed by HPL (10). An isomer of (9Z)-traumatins, 12-oxo-(E)-10-dodecenoic acid ((10E)-traumatins), was found in runner bean (*Phaseolus vulgaris*) (21). Recently, Kallenbach *et al.* (22) quantified the C12 counterparts and their derivatives in wounded *Nicotiana attenuata* leaves. After mechanical wounding, they found only small amounts of (9Z)-traumatins but substantial amounts of its hydroxylated metabolite 12-oxo-9-hydroxy-(E)-10-dodecenoic acid (9-OH-traumatins), which was formed via peroxidation of traumatins either enzymatically by NaLOX2 or nonenzymatically, and its glutathione conjugate. However, they provided no data to precisely compare the amount of GLVs released with the amount of traumatins derivatives retained. Hence, it remains uncertain whether the C12 and C10 counterparts are formed in an equimolar ratio. Inspired by the findings by Kallenbach *et al.* (22), we tried to quantify C12/C10 compounds, including (9Z)-traumatins, (10E)-traumatins, and 9-OH-traumatins, (7Z)-dinotraumatins, 10-oxo-(E)-8-decenoic acid ((10E)-dinotraumatins), and 10-oxo-7-hydroxy-(E)-8-decenoic acid (7-OH-dinotraumatins) in wounded *Arabidopsis* leaves (*No-0*). We generally used the *No-0* ecotype instead of the *Col-0* ecotype, because the latter has a deletion in the *HPL* gene and thus lacks HPL activity (23). We found that the amounts of these C12/C10 compounds were still much lower than those expected based on the amounts of C6 volatiles released. Therefore, we assumed that the C12/C10 compounds were present as esterified galactolipids. Because MGDGs are the most abundant source for octadecatrienoic acid (18:3) and hexadecatrienoic acid (16:3) in *Arabidopsis* leaves (24), we focused on this lipid class to find the corresponding traumatins and dinotraumatins derivatives.

## EXPERIMENTAL PROCEDURES

**Plant Materials**—Seeds of *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.) wild-type ecotypes *Col-0*, *Ler-0*, *No-0*, and *WS* and the transfer DNA-inserted line *aos::Col-6* (*gl1*) (25) were obtained from ABRC (Columbus, OH). *aos::Col-6* (*gl1*) was crossed with *Ler-0*, and a line showing the *aos* (male-sterile) and *HPL* (ability to form GLVs) phenotypes was selected from the resultant F2 progenies. The line was denoted as *aos::Ler-0*. *hpl1* has a *Col-0*-derived deletion of the *HPL* gene in the *Ler-0* background; thus, it has no HPL activity (26). The seeds were germinated in soil (Metro-Mix, Sun Gro Horticulture Distribution Inc., Bellevue, WA) in plastic pots (6-cm inner diameter). Plants were cultivated in a growth chamber at 22 °C under fluorescent lights ( $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with a 14-h light/10-h dark photoperiod for 30 days until they reached the stage just before bolting. Seeds of *Brassica oleracea*, *Nicotiana tabacum*, *Solanum lycopersicum*, and *Phaseolus vulgaris* were purchased from a local market and were cultivated in a growth chamber at 25 °C under fluorescent lights ( $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with a 14-h light/10-h dark photoperiod for 30 days. Clover (*Trifolium repens* L.)

## Formation of Galactolipids Containing Traumatin Derivatives

leaves were harvested from the experimental farm at the Faculty of Agriculture, Yamaguchi University, in June 2009. Spinach (*Spinacia oleracea* L.) was purchased from a local market.

**Chemicals**—(9Z)-Traumatin was purchased from Larodan (Malmö, Sweden). 9-OH-traumatin was generated by nonenzymatic oxidation of (9Z)-traumatin at 60 °C for 80 min (22, 27). (7Z,10Z,13Z)-7,10,13-Hexadecatrienoic acid (16:3) was purified from MGDGs isolated from spinach leaves. Crude lipids were extracted from 1 kg of spinach leaves using the Bligh-Dyer method (28) and fractionated by column chromatography on silica gel (Wakogel C-300, Wako Pure Chemicals, Osaka, Japan) with a solvent system consisting of chloroform and acetone. The fractions eluted with chloroform/acetone (3:2, v/v) were collected. A portion (100 mg) of the purified MGDGs was submitted to alkaline hydrolysis with 50% ethanolic 3.5 M KOH in boiling water for 2 h. Then, after acidification of the reaction mixture, the fatty acids were extracted with diethyl ether. The fatty acid mixture was fractionated by preparative HPLC using a Mightysil RP-18 column (250 mm × 4.6-mm inner diameter, Kanto Chemicals, Tokyo, Japan) with acetonitrile as the elution solvent at a flow rate of 1 ml min<sup>-1</sup>. Compounds were detected by monitoring absorbance at 220 nm. Purified 16:3 eluting at ~4 min was collected, and its identity was confirmed by GC-MS analysis after methyl esterification with 2.0 M trimethylsilyldiazomethane in hexane (Sigma-Aldrich).

Hexadecatrienoic acid was oxygenated by the activity of partially purified LOX-1 from soybean seeds. The reaction proceeded in 0.1 M sodium borate buffer at pH 9.5. LOX-1 was obtained from soybean seeds (*Glycine max* L., cv. Yumeyutaka) that contained only the LOX-1 isoform instead of the three isozymes found in normal soybean seeds (29). The resulting 11-HPHT was recovered with a 64% yield and was subjected to a cleavage reaction in 50 mM MES-KOH (pH 5.5) containing 0.1 mM diethylenetriamine-*N,N,N',N',N''*-pentaacetic acid (DETAPAC) using recombinant bell pepper hydroperoxide lyase (CaHPL) expressed in *E. coli* (30). The products were applied to a Sep-pak C18 Plus cartridge (Waters, Milford, MA) and eluted with methanol. The yield of (7Z)-dinortraumatin from 11-HPHT was 11%. 7-OH-Dinortraumatin was generated nonenzymatically by treating (7Z)-dinortraumatin at 60 °C for 80 min. The structure was assigned by LC-MS/MS analysis.

MGDG bis18:3 and MGDG 18:3/16:3 were purified from MGDG isolated from clover leaves and spinach leaves, respectively, by HPLC using a Mightysil RP-18 column (250 mm × 4.6-mm inner diameter) with methanol/water/acetonitrile (90.5:7.0:2.5, v/v/v; flow rate of 1 ml min<sup>-1</sup>). Compounds were detected by monitoring absorbance at 210 nm. Structures of the acyl groups were confirmed by GC-MS analysis after transesterification with 5% HCl/methanol at 80 °C for 2 h. According to the literature (24, 31), MGDG 18:3/16:3 was defined as *sn*1-*O*-C18:3-*sn*2-*O*-C16:3 MGDG.

MGDG-HPOs were prepared by treating purified MGDG bis18:3 and MGDG 18:3/16:3 with soybean LOX-1 (isolated as described above). Hydroperoxidation of both of the acyl groups, at the 13-position of 18:3 and the 11-position of 16:3, was confirmed by HPLC analysis as described previously (29). Bis-*O*-(13-hydroperoxy-(9Z,11E,15Z)-9,11,15-octadecatrienoyl) monogalactosyl diglyceride (MGDG bis13-HPOT) or *O*-(13-

hydroperoxy-(9Z,11E,15Z)-9,11,15-octadecatrienoyl)-*O*-(11-hydroperoxy-(7Z,9E,13Z)-7,9,13-hexadecatrienoyl) monogalactosyl diglyceride (MGDG 13-HPOT/11-HPHT) (1 mg) was dissolved in 500 μl of methanol and diluted with 50 ml of 0.1 M MES-KOH (pH 5.5) containing 0.1 mM DETAPAC. After sonication to suspend the lipid-HPOs in the buffer, 50 μl of purified recombinant CaHPL (see above) was added to the mixture. The reaction was monitored by measuring absorbance at 234 nm after diluting a 50-μl aliquot of the reaction mixture with 950 μl of acetonitrile/water (1/1, v/v). After confirming consumption of the HPOs, the products were loaded onto a Sep-pak C18 (500 mg) cartridge that had been washed with methanol and subsequently equilibrated with 0.1 M MES-KOH (pH 5.5). The products were eluted from the cartridge with methanol. Their hydroxylated products were generated by spontaneous oxygenation at 60 °C for 80 min.

**Extraction and Analysis of Free Traumatin Derivatives**—*Arabidopsis* (*No-0*) leaves (2.5 g FW) were homogenized in 5 ml of 20 mM sodium phosphate buffer (pH 6.3) containing 0.1 mM DETAPAC. The homogenate was incubated for 5 min at 25 °C. Then the homogenate was acidified to pH 4.0 by adding HClO<sub>4</sub>, and 10 nmol each of 15-hydroxy-(11Z,13E)-11,13-icosadecadienoic acid (prepared from (11Z,14Z)-11,14-icosadienoic acid (Sigma-Aldrich) with soybean LOX-1) and 10-hydroxy-(*E*)-2-decenoic acid (Wako Pure Chemicals) were added as internal standards. Then 25 ml of methanol (containing 0.0025% butylated hydroxytoluene), 12.5 ml of chloroform, and 12.5 ml of 1% KCl solution were added to the mixture, and the aqueous and organic phases were allowed to separate. The aqueous phase was extracted again with chloroform (12.5 ml), and the combined organic phase was washed with 1% KCl solution. The solvent was removed under vacuum, and the remaining residue was dissolved in 0.7 ml of chloroform. To isolate lipids from intact leaves, the leaves (2.5 g FW) were homogenized with 10 ml of methanol containing 0.0025% butylated hydroxytoluene to avoid any enzymatic modifications of the lipid composition, and then extraction was conducted as described above.

A portion (~1 mg) of the crude lipids was dried in a stream of N<sub>2</sub> gas and suspended in 1 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.01% sodium deoxycholate with the aid of an ultrasonic bath (US Cleaner, AS ONE Co., Osaka, Japan) under an N<sub>2</sub> atmosphere. To the suspension 0.1 ml of 2.2% CaCl<sub>2</sub> solution and 0.5 ml of 20 mg ml<sup>-1</sup> pancreatin (Wako Pure Chemicals) were added. The mixture was incubated at 37 °C for 30 min. After hydrolysis, the mixture was acidified to pH 4.0 with HClO<sub>4</sub> and applied to a Bond Elut C18 cartridge (200 mg, Agilent Technologies Inc., Santa Clara, CA) equilibrated with water. The hydrolyzed compounds were eluted with methanol.

Free forms of traumatin derivatives were analyzed by LC-MS/MS (3200 Q-TRAP LC/MS/MS System (AB Sciex, Framingham, MA) equipped with a Prominence UFLC (Shimadzu, Kyoto, Japan)). Products were separated on a Mightysil RP18 column (150 mm × 2-mm inner diameter) with a binary gradient consisting of water/formic acid (100:0.1, v/v, solvent A) and acetonitrile/formic acid (100:0.1, v/v, solvent B). The run consisted of a linear increase from 20% B to 90% B over 25 min (flow rate, 0.2 ml min<sup>-1</sup>). Compounds were detected with a photodiode array

TABLE 1

## Parameters used for detection of traumatin and its derivatives

DP, declustering potential; EP, energy potential; CEP, collision energy potential; CE, collision energy.

Compound	Q1 mass	Q3 mass	DP	EP	CEP	CE	Response factor
	<i>Da</i>	<i>Da</i>	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>	
10-Hydroxy-(2 <i>E</i> )-decanoic acid (IS)	184.86	139.0	-30	-9.0	-12.00	-22.0	NA <sup>b</sup>
(7 <i>Z</i> )-Dinortraumatin	182.79	155.0	-50	-10.5	-18.42	-10.0	0.605
7-OH-dinortraumatin	198.72	180.8	-30	-10.0	-19.01	-6.0	0.321
(9 <i>Z</i> )-Traumatin	210.78	183.0	-45	-10.5	-19.45	-12.0	6.503
9-OH-traumatin	226.79	208.8	-45	-10.5	-20.05	-12.0	3.161
Formononetin (IS)	266.96	252.0	-50	-8.5	-16.00	-16.0	NA
MGDG-9-OH-traumatin-7-OH-dinortraumatin	645.20	209.1	-40	-12.0	-35.53	-46.0	0.060
MGDG-bis-9-OH-traumatin	673.35	209.1	-40	-12.0	-36.57	-35.0	0.060
Arabidopsin A	773.45	291.2	-40	-12.0	-40.27	-35.0	ND <sup>c</sup>
Arabidopsin B	801.48	291.2	-40	-12.0	-41.31	-50.0	ND

<sup>a</sup> The values for free carboxylic acids were determined with 10-hydroxy-(2*E*)-decanoic acid, and those for MGDG-derivatives were determined with formononetin.<sup>b</sup> NA, not applicable.<sup>c</sup> ND, not determined.

detector (SPD-M20A, Shimadzu) and by MS/MS using ESI in the negative ion mode (ion spray voltage, -4500 V; nitrogen as both the curtain gas (set to 40 arbitrary units) and collision gas (set to "high"); collision energy, -10 V; scan range,  $m/z$  100–1200; scan speed, 4,000 Da s<sup>-1</sup>; declustering potential, -10 V) and multiple reaction monitoring (Table 1). The response curves of the traumatin derivatives *versus* the IS were determined and used to calculate the response factors (Table 1). To quantify traumatin derivatives, the areas of corresponding peaks in the chromatogram were integrated and divided by the peak area of the IS, and the values were corrected by the respective response factor. The peak with a signal/noise ratio of more than 10 (0.6 pmol for traumatin and dinortraumatin and 0.4 pmol for their hydroxylated derivatives; roughly corresponding to 0.15 and 0.10 nmol g FW<sup>-1</sup>, respectively) was used for calculation.

Six-carbon aldehydes formed after complete disruption of *Arabidopsis* leaves were quantified by HPLC after derivatization of the aldehydes with 2,4-dinitrophenylhydrazine, as described previously (32). Under these experimental conditions, (Z)-3-hexenal was the main product, accounting for ~85% of the GLVs formed (8).

**Extraction and Analysis of Galactolipid Oxylipins**—Plant leaves were thoroughly ground using a mortar and pestle, and a portion (0.1 g FW) of the slurry was mixed with 1 ml of acetone containing 0.1% butylated hydroxytoluene and 10 ng ml<sup>-1</sup> formononetin (IS). The suspension was mixed vigorously for 10 min and then centrifuged at 17,000 × *g* for 10 min. The resultant green supernatant was analyzed by LC-MS/MS (see below). To determine the amounts in intact leaves, the leaves were frozen in liquid nitrogen immediately after harvest. The extraction was conducted as described above after powdering the frozen tissues.

MGDGs containing traumatin derivatives were qualitatively analyzed using the LC-PDA-MS/MS system described above. Multiple reaction monitoring analysis was conducted to quantify the compounds (Table 1). When the partially purified MGDG-9-OH-traumatin/7-OH-dinortraumatin and MGDG-bis-9-OH-traumatin were analyzed by LC-PDA-MS/MS, we found that there was no other compound with absorbance around 220 nm. Therefore, these compounds were tentatively quantified using the molar absorption coefficient ( $\epsilon$ ) reported

for 4-hydroxy-(*E*)-2-nonenal (13,750) (33), which has the same chromophore. The response curves of MGDG 7-OH-dinortraumatin/9-OH-traumatin and MGDG bis-9-OH-traumatin *versus* those of IS (formononetin) were determined and used to calculate the response factors (Table 1). For quantification, the corresponding peak areas obtained by integration of the ion chromatograms were divided by that of the IS, and the values were corrected by the respective response factor. The detection limit for MGDG 7-OH-dinortraumatin/9-OH-traumatin and MGDG bis-9-OH-traumatin was 0.2 nmol g FW<sup>-1</sup> with a signal/noise ratio on the multiple reaction monitoring chromatogram of more than 10. Arabidopsins A and B were identified from their MS and MS/MS spectra according to parameters reported by Buseman *et al.* (13). Because standard arabidopsins were unavailable, the ratios of their areas to that of the IS were calculated.

**Purification of MGDG Bis-9-OH-traumatin and MGDG 9-OH-traumatin/7-OH-dinortraumatin—Arabidopsis (No-0) leaves (437 g) were completely disrupted with a Polytron homogenizer (PT-20sk, Kinematica AG, Luzern, Switzerland), and the homogenate was stirred vigorously for 5 min to facilitate the enzyme reaction. Crude lipids were extracted with the Bligh-Dyer method as described above. Total lipids were subjected to column chromatography on silica gel (Wakogel C-300, 220 × 20 mm inner diameter). The column was washed with chloroform/acetone (4:6, v/v), and the glycolipids were eluted with acetone/methanol (9:1, v/v). For further purification, the fraction was concentrated and subjected to preparative TLC (20 × 20 cm, silica gel 60 F<sub>254</sub>, Merck) using ethyl acetate/acetone/acetic acid (10:10:1, v/v/v) as the developing solvent. The zones containing the galactolipids of traumatin derivatives were visible under UV light and were scraped off the plates. The lipids were eluted in ethyl acetate. The fraction containing MGDGs with traumatin and its derivatives was further separated by RP-HPLC with a Mightysil RP18 column (250 × 4.6 mm) using a binary gradient consisting of acetonitrile/water/formic acid (20:80:0.1, v/v/v, solvent A) and acetonitrile/water/formic acid (50:50:0.1, v/v/v, solvent B). The run consisted of a linear increase from 100% A to 100% B over 40 min at a flow rate of 1 ml min<sup>-1</sup>. The MGDGs containing oxygenated traumatin derivatives were detected by monitoring UV absorbance at 220 nm and collected.**

## Formation of Galactolipids Containing Traumatin Derivatives

**NMR Analysis of MGDG 9-OH-traumatin/7-OH-dinor-traumatins**—Fractions enriched in MGDG 9-OH-traumatins/7-OH-dinortraumatins were concentrated under vacuum. The residue was dissolved in 70  $\mu\text{l}$  of  $\text{CD}_3\text{OD}$  and transferred into a 2-mm NMR tube. NMR spectra were recorded using a Bruker Avance 500 NMR instrument (Bruker Biospin, Karlsruhe, Germany) operating at 500.13 MHz and equipped with a 5-mm TCI cryoprobe. The spectra were acquired at 300 K and a total of 5,000–7,000 transient signals were recorded as 32,000 data points with a spectral width of 20 ppm using a pulse sequence with water suppression (zgpgur). Selective COSY (SELCOSE) (selcogp) and selective TOCSY (SELTOCSY) (selmlgp.2) spectra were recorded using standard Bruker pulse sequences with a 10-ms excitation range and 200-ms mixing time. Data analysis was conducted using Topspin version 3.1 software (Bruker Biospin, Karlsruhe, Germany).

**HPLC-HR-ESI-MS/MS Analysis of MGDG 9-OH-traumatins/7-OH-dinortraumatins**—High resolution-ESI-MS analysis was performed using an Ultimate 3000 series RSLC (Dionex, Sunnyvale, CA) system and an Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with an ESI source. For HPLC, compounds were separated on an Acclaim C18 column (150  $\times$  2.1-mm inner diameter, 2.2  $\mu\text{m}$ ; Dionex) at a constant flow rate of 300  $\mu\text{l min}^{-1}$  with a linear gradient of 0.1% (v/v) formic acid in water to 0.1% formic acid in acetonitrile.

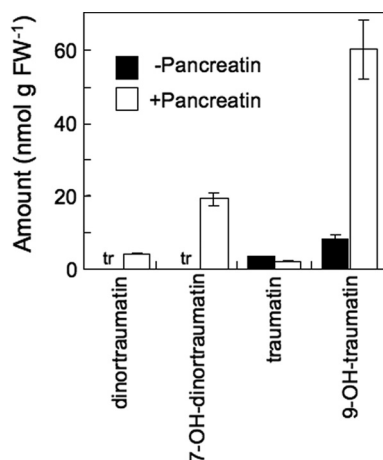
HR-ESI-MS spectra were measured in the negative ionization mode on the Orbitrap mass analyzer using 30,000  $\text{m}/\Delta\text{m}$  resolving power. MS/MS spectra of the  $[\text{M} - \text{H}]^-$  ion were acquired at a collision gas energy of 35 arbitrary units. Data analysis was conducted using XCALIBUR software (Thermo Fisher Scientific).

## RESULTS

**Quantification of Free and Esterified Traumatins Derivatives**—In extracts from intact leaf tissues of *Arabidopsis* (No-0), free C12/C10 oxoacids were undetectable. After complete disruption of the leaves, small amounts of traumatin and 9-OH-traumatins ( $\sim 5$  and 10  $\text{nmol g FW}^{-1}$ , respectively) were detected (Fig. 2). Under these experimental conditions,  $279 \pm 47 \text{ nmol g FW}^{-1}$  (Z)-3-hexenal was formed after disruption of the leaves. The markedly lower yield of free C12/C10 oxoacids prompted us to examine esterified C12/C10 oxoacid derivatives.

To quantify the esterified forms of oxoacids, the crude lipid extract prepared from homogenized *Arabidopsis* (No-0) leaves was hydrolyzed with pancreatin, which has strong galactolipase activity and triacylglycerol lipase activity (34). The pancreatin treatment increased the amounts of 9-OH-traumatins, 7-OH-dinortraumatins, and (7Z)-dinortraumatins (Fig. 2). The amount of (9Z)-traumatins hardly increased after hydrolysis. This result indicated that the oxoacids were present as their esterified forms, probably as the acyl groups of lipids.

**Detection of MGDGs Containing Traumatins Derivatives**—Esterified oxylipins have been found predominantly in galactolipids (12, 13). The most abundant C6 compounds produced after wounding of *Arabidopsis* leaves are formed from octadecatrienoic acid (18:3) and hexadecatrienoic acid (16:3). Most 18:3 ( $\sim 65\%$ ) and 16:3 ( $\sim 96\%$ ) are found in MGDGs (24). We found that MGDGs were substrates for LOX to yield MGDG

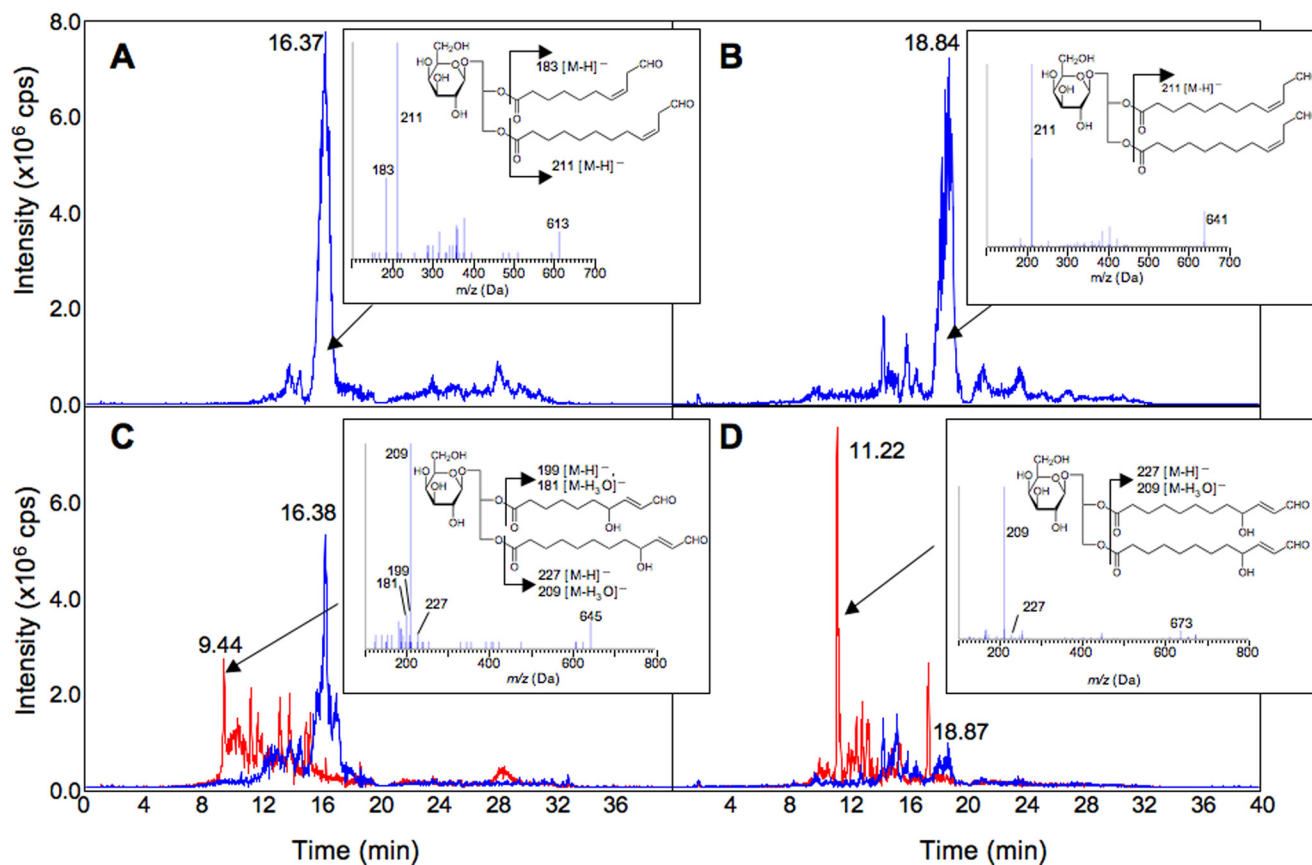


**FIGURE 2. Amounts of free and esterified traumatins and its derivatives in leaves of *Arabidopsis* (No-0).** Crude lipids prepared from disrupted *Arabidopsis* (No-0) leaves were treated with pancreatin or left untreated and then analyzed by LC-MS/MS. In intact leaves, the amounts of free and esterified traumatins and its derivatives were below detection limits. *tr*, trace. Mean values  $\pm$  S.E. (error bars) are shown ( $n = 3$ ).

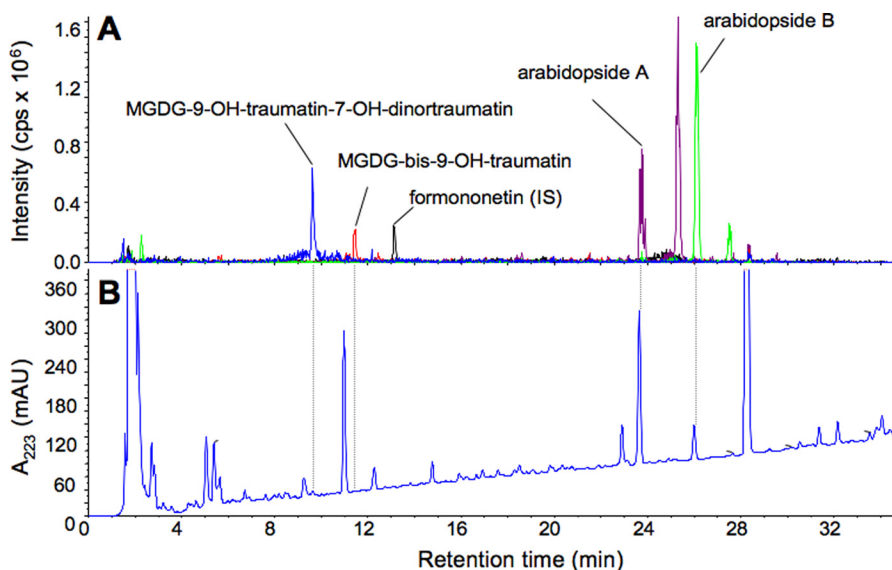
HPOs, which could be the substrates for HPL (29). Together, these findings led us to assume that a portion of esterified traumatins and its derivatives was present as the acyl moieties of MGDG. To explore this idea, we attempted to develop a method to analyze MGDGs containing oxoacids.

The recombinant CaHPL (30) showed significant activity toward either MGDG bis-13-HPOT or MGDG 13-HPOT/11-HPHT in the presence of deoxycholic acid. EMS scanning with LC-MS/MS showed the appearance of peaks with  $[\text{M} - \text{H}]^-$  values corresponding to MGDGs containing oxoacids (Fig. 3). The products formed from MGDG 13-HPOT/11-HPHT and MGDG bis-13-HPOT were analyzed by enhanced product ion (EPI) analysis, which facilitates the detection of fragment ions corresponding to each acyl group of glycerolipids (35). This analysis suggested that the reaction products were MGDGs containing (9Z)-traumatins and/or (7Z)-dinortraumatins as their acyl groups. Incubation of MGDGs containing (9Z)-traumatins and/or (7Z)-dinortraumatins at 60  $^{\circ}\text{C}$  for 80 min resulted in decomposition of the reactants concomitant with the formation of compounds with MS profiles corresponding to MGDGs containing 9-OH-traumatins and/or 7-OH-dinortraumatins (Fig. 3). Although conclusive structural assignment required NMR analysis (as follows), we tentatively assigned the cleavage products of CaHPL with MGDG bis-13-HPOT or MGDG 13-HPOT/11-HPHT as MGDG harboring 9-OH-traumatins and 7-OH-dinortraumatins and MGDG harboring two 9-OH-traumatins, based on their MS/MS-EPI profiles. They were used as standards to detect those MGDGs formed endogenously *in situ*.

A crude lipid extract prepared from intact *Arabidopsis* (No-0) leaves was analyzed by LC-MS/MS to detect MGDGs containing C12 or C10 oxoacids. In intact leaf extracts, peaks corresponding to MGDGs containing C12/C10 acyl moieties were not detected. However, when the leaves were disrupted and incubated for 5 min to allow formation of GLVs, peaks corresponding to MGDG-9-OH-traumatins/7-OH-dinortraumatins and MGDG-bis-9-OH-traumatins were detected (Fig. 4).



**FIGURE 3. Identification of HPL products formed from MGDG-bis-13-HPOT (A and C) or MGDG 13-HPOT/11-HPHT (B and D) by LC-MS/MS analysis.** A portion of the reaction products was autooxidized (60 °C for 80 min) (C and D). LC-MS/MS analyses were carried out with EMS mode. The figure shows selected ion chromatograms with  $m/z = 613.3 \pm 0.5$  (blue chromatogram in A and C) (corresponding to  $[M - H]^-$  of MGDG-(9Z)-traumatatin-(7Z)-dinortraumatatin), with  $m/z = 641.4 \pm 0.5$  (blue chromatogram in B and D) (corresponding to  $[M - H]^-$  of MGDG-bis-(9Z)-traumatatin), with  $m/z = 645.3 \pm 0.5$  (red chromatogram in C) (corresponding to  $[M - H]^-$  of MGDG-9-OH-traumatatin-7-OH-dinortraumatatin), and with  $m/z = 673.4 \pm 0.5$  (red chromatogram in D) (corresponding to  $[M - H]^-$  of MGDG-bis-9-OH-traumatatin). The MS/MS profile (EPI mode) for each prominent peak marked with an arrow is shown in the inset. cps, counts/s.



**FIGURE 4. Representative chromatogram of acetone extract prepared from disrupted *Arabidopsis* (No-0) leaves.** LC-MS/MS analyses were carried out with EMS mode. A, selected ion chromatograms with  $m/z = 645.3 \pm 0.5$  (blue) (corresponding to  $[M - H]^-$  of MGDG-9-OH-traumatatin-7-OH-dinortraumatatin),  $m/z = 673.4 \pm 0.5$  (orange) (corresponding to  $[M - H]^-$  of MGDG-bis-9-OH-traumatatin),  $m/z = 773.5 \pm 0.5$  (purple) (corresponding to  $[M - H]^-$  of arabisidopside A), and  $m/z = 801.5 \pm 0.5$  (green) (corresponding to  $[M - H]^-$  of arabisidopside B). The peak at a retention time of 13.11 min corresponds to formononetin (internal standard;  $m/z = 267.0 \pm 0.5$ ; chromatogram in black). A trace with absorption at 223 nm is also shown at the bottom (B). cps, counts/s; mAU, milliabsorbance units.

## Formation of Galactolipids Containing Traumatin Derivatives

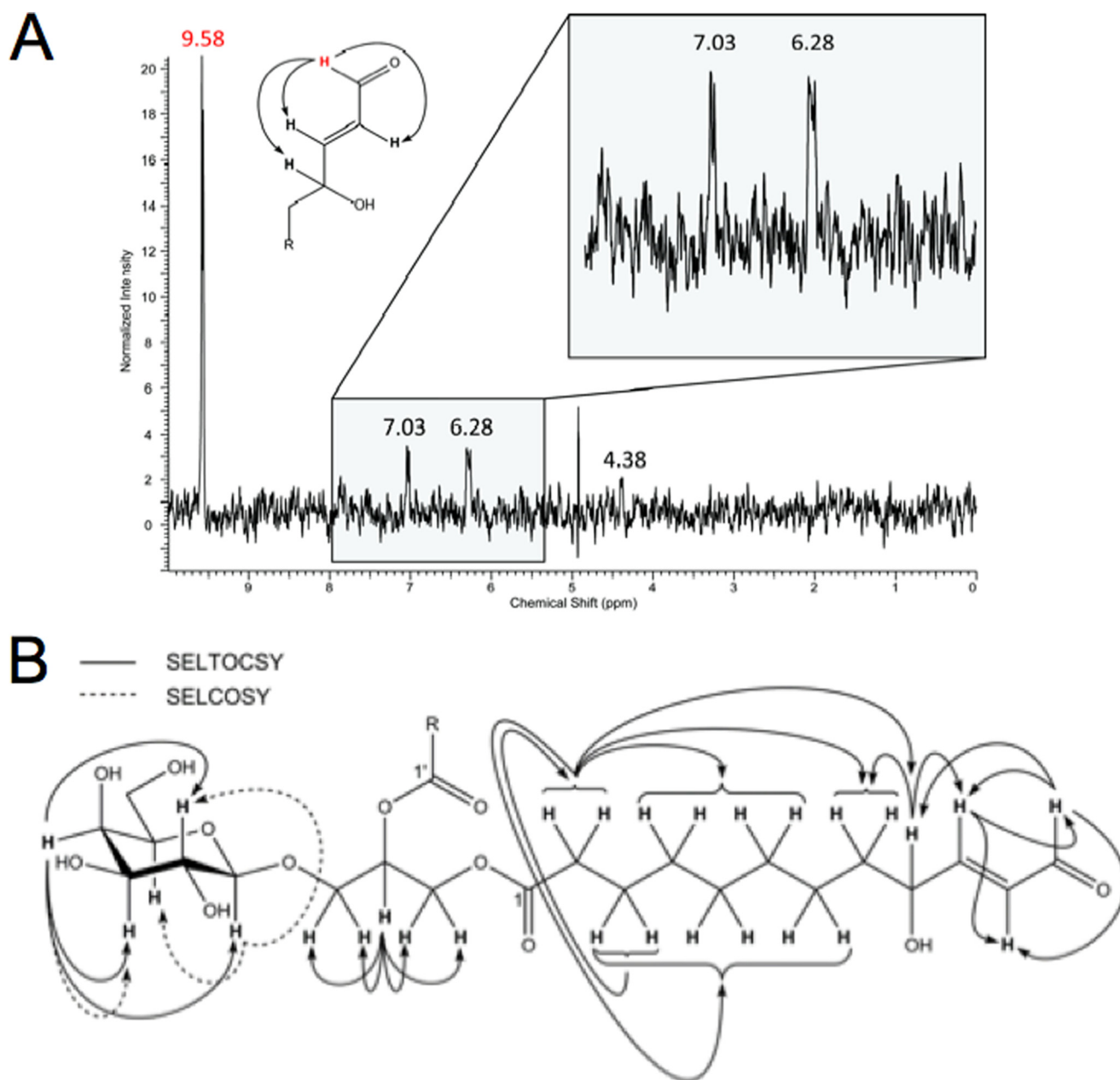


FIGURE 5. **Structure determination with NMR.** A, a representative spectrum used for structure determination. Shown is the SELTOCSY spectrum (500 MHz,  $\text{CD}_3\text{OD}$ , irradiated at  $\delta = 9.58$  ppm, NS 5120) of enriched MGDG-9-OH-traumatins-7-OH-dinortraumatins, showing H,H-correlations between the aldehyde proton ( $\delta = 9.58$  ppm) and the adjacent double bond ( $\delta = 7.03$  and  $6.28$  ppm) as well as the hydroxymethine group ( $\delta = 4.38$  ppm). Further SELTOCSY and SELCOSY experiments were carried out with the other protons to confirm the structure. B, NMR correlations observed in MGDG-9-OH-traumatins-7-OH-dinortraumatins using SELTOCSY and SELCOSY experiments.

Neither MGDGs containing C12/C10 oxoacids without hydroxylation nor those with only one hydroxylation were detected under these experimental conditions. Arabidopsides A and B were present in very low quantities in intact leaves, but their quantities increased markedly after disruption of the leaves as reported previously (13) (Fig. 4).

To confirm the tentative structural assignment of MGDG-9-OH-traumatins/7-OH-dinortraumatins, the compound was purified from crude lipids extracted from disrupted *Arabidopsis* (*No-0*) leaves and submitted to NMR analysis. Because of its instability, complete purification could not be accomplished. The small amount of enriched material rendered the sample unsuitable for two-dimensional NMR analysis. However, using

a combination of one-dimensional SELTOCSY and SELCOSY experiments, all three structural units ( $\beta$ -galactose, glycerol, and fatty acid with a terminal  $\gamma$ -hydroxy- $\alpha,\beta$ -unsaturated aldehyde moiety) were confirmed (Fig. 5). Comparison of the  $^1\text{H}$  NMR data derived from SELTOCSY and SELCOSY spectra with those of a MGDG-derivative (36) and (*E*)-4-hydroxynon-2-enal (37) also confirmed the structural assignment. The molecular formula of  $\text{C}_{31}\text{H}_{50}\text{O}_{14}$  for MGDG-9-OH-traumatins/7-OH-dinortraumatins was unambiguously established by HR-ESI-MS. HR-MS/MS experiments further confirmed the loss of hydroxytraumatins or hydroxydinortraumatins fragments and revealed product ions corresponding to the glyceryl-galactoside moiety as well as dehydrated OH-traumatins and

TABLE 2

HPLC-HR-ESI-MS (negative ion mode) and MS/MS data of MGDG-9-OH-traumatin-7-OH-dinortraumatins

Molecular formula	$m/z$ calc. [M - H]	$m/z$ obs. [M - H]	$\Delta$	MS/MS @cid35	$\Delta$	Assignment
$C_{31}H_{49}O_{14}$	645.3122	645.3141	2.9			[M - H]
$C_{31}H_{47}O_{13}$	627.3017	627.3034	2.7	627.3008	1.4	[M - H <sub>3</sub> O]
$C_{31}H_{45}O_{12}$	609.2911	609.2928	2.8	609.2906	0.8	[M - H <sub>3</sub> O - H <sub>2</sub> O]
$C_{21}H_{35}O_{11}$	463.2179	463.2192	2.8			[M - H - 7-HO dinortraumatins]
$C_{21}H_{33}O_{10}$	445.2074	445.2084	2.2	445.2081	1.6	[M - H <sub>3</sub> O - 7-HO dinortraumatins]
$C_{19}H_{29}O_{10}$	417.1761	417.1763	0.5	417.1765	1.0	[M - H <sub>3</sub> O - 9-HO traumatins]
$C_9H_{17}O_8$	253.0923			253.0928	2.0	Glyceryl-galactoside
$C_{12}H_{17}O_3$	209.1178	209.1184	2.9	209.1182	1.9	[9-OH traumatins - H <sub>3</sub> O]
$C_{10}H_{15}O_4$	199.0970	199.0976	3.0			[7-OH dinortraumatins - H]
$C_{10}H_{13}O_3$	181.0865	181.0871	3.3	181.0873	4.4	[7-OH dinortraumatins - H <sub>3</sub> O]

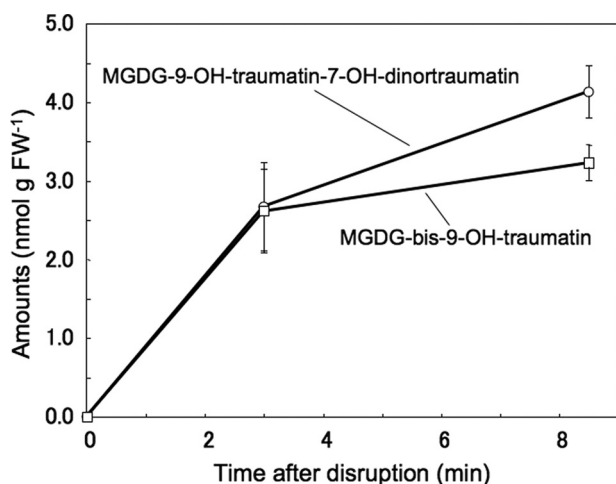


FIGURE 6. Formation of MGDG-9-OH-traumatins-7-OH-dinortraumatins (circle) and MGDG-bis-9-OH-traumatins (square) in disrupted leaves of *Arabidopsis* (*No-0*). *Arabidopsis* (*No-0*) leaves were completely disrupted with a mortar and pestle, and then a portion of the disrupted tissues was used to isolate and quantify MGDGs containing traumatin derivatives. In intact leaves at 0 min (leaves instantly frozen in liquid nitrogen), both compounds were undetectable. Mean values  $\pm$  S.E. (error bars) are shown ( $n = 4$ ).

OH-dinortraumatins (Table 2). Accordingly, the structure was assigned as *O*-(9-hydroxy-12-oxo-(*E*)-10-dodecenyl)-*O*-(7-hydroxy-10-oxo-(*E*)-8-decenyl)-*O*- $\beta$ -D-galactopyranosylglycerol (MGDG-9-OH-traumatins/7-OH-dinortraumatins). Based on the expected analogy in terms of reaction and structure, the MGDG with two 9-OH-traumatins was tentatively assigned as bis-*O*-(9-hydroxy-12-oxo-(*E*)-10-dodecenyl)-*O*- $\beta$ -D-galactopyranosylglycerol (MGDG-bis-9-OH-traumatins).

In intact *No-0* leaves, MGDG-9-OH-traumatins/7-OH-dinortraumatins and MGDG-bis-9-OH-traumatins were undetectable (Fig. 6). Upon disruption, they rapidly formed within 3 min, and their quantities gradually increased by 8.5 min. The amounts of MGDG-9-OH-traumatins/7-OH-dinortraumatins and MGDG-bis-9-OH-traumatins were almost equivalent. Detailed analysis of the extracts with EMS mode revealed that they were the sole HPL products formed after disruption, and MGDGs with non- or monohydroxylated C10 or C12 compounds were not detected.

*Arabidopsis* ecotype *Col-0* has no HPL activity (23). As expected, formation of MGDG-9-OH-traumatins/7-OH-dinortraumatins and MGDG-bis-9-OH-traumatins was hardly observed after disruption of *Col-0* leaves (Fig. 7). This result indicated that HPL activity was essential for the formation of MGDG-9-OH-traumatins/7-OH-dinortraumatins and MGDG-

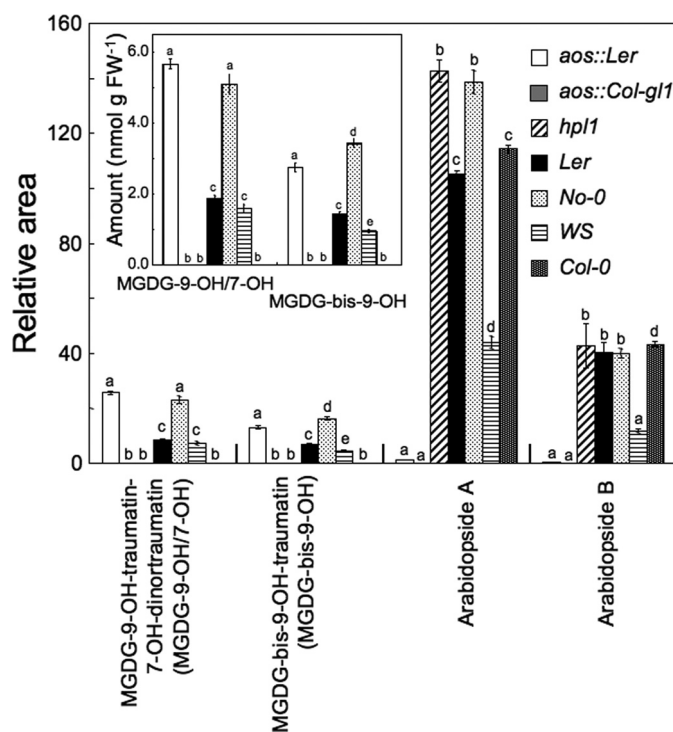


FIGURE 7. Formation of MGDG-9-OH-traumatins-7-OH-dinortraumatins, MGDG-bis-9-OH-traumatins, Arabidopsides A, and Arabidopsides B in *Arabidopsis* ecotypes and mutants. Leaves of each *Arabidopsis* line were completely disrupted, and then the oxylipin-containing MGDGs were extracted after 3 min. For arbitrary quantification, the ratio of each peak area to that of formononetin (IS) is shown. For MGDG-9-OH-traumatins-7-OH-dinortraumatins (MGDG-9-OH/7-OH) and MGDG-bis-9-OH-traumatins (MGDG-bis-9-OH), the amounts calculated based on response factors are shown in the inset. Mean values  $\pm$  S.E. (error bars) are shown ( $n = 4$ ). Different letters indicate significant difference (analysis of variance, Tukey's test,  $p < 0.01$ ).

bis-9-OH-traumatins. This conclusion was confirmed by comparing their formation between *hpl1* (a backcross progeny containing deleted *HPL* gene derived from *Col-0* in *Ler* background) (26) and *Ler* lines. Again, formation of MGDG-9-OH-traumatins/7-OH-dinortraumatins and MGDG-bis-9-OH-traumatins was undetectable in disrupted *hpl1* leaves, whereas significant amounts formed after disruption of *Ler* leaves.

Arabidopsides are MGDGs containing 12-oxophytodienoic acid and/or 12-oxodihydroxyphytyldienoic acid formed via the AOS reaction (12, 13). It is assumed that Arabidopsides are formed from HPOs of MGDGs; therefore, AOS and HPL may compete for the same substrates in disrupted leaf tissues. Massive formation of Arabidopsides A and B was detected in the various *Arabidopsis* ecotypes/genotypes, whereas they were undetectable in the

## Formation of Galactolipids Containing Traumatin Derivatives

**TABLE 3**

Amounts of MGDG form and free form of HPL products in cabbage, tobacco, tomato, and common bean

	MGDG form		Free form			
	(OH)C12/(OH)C10	Bis(OH)C12	9-OH traumatin	Traumatin	7-OH dinortraumatin	Dinortraumatin
	<i>nmol g FW<sup>-1</sup></i>					
<i>B. oleracea</i>	15.1 ± 0.65	8.42 ± 0.94	3.01 ± 0.39	0.17 ± 0.02	1.72 ± 0.20	ND <sup>a</sup>
<i>N. tabacum</i>	0.43 ± 0.07	2.28 ± 0.50	0.67 ± 0.02	ND	ND	ND
<i>S. lycopersicum</i>	3.82 ± 0.52	10.6 ± 1.30	5.84 ± 0.26	0.40 ± 0.03	1.62 ± 0.12	ND
<i>P. vulgaris</i>	ND	53.8 ± 2.21	37.9 ± 1.56	28.61 ± 1.08	ND	ND

<sup>a</sup> ND, not detected. The values are means ± S.E. (*n* = 4).

transfer DNA knock-out mutants, *aos::Ler* and *aos::Col-gl1*. The amounts of MGDG-9-OH-traumatin/7-OH-dinortraumatin and MGDG-bis-9-OH-traumatin formed after disruption of leaves were markedly lower in *Ler* leaves than in *aos::Ler* leaves (Fig. 7). This suggested that HPL and AOS compete at least in part for the same substrate (MGDG HPOs) to form oxylipin-containing MGDGs.

**MGDG-oxoacids in Other Plant Species**—It has been reported that arabinosides occur only in *A. thaliana* and *Arabidopsis arenosa* but not in any other plant species examined so far (38). We examined whether MGDG-oxoacids were present in cabbage (*B. oleracea*, Brassicaceae), tobacco (*N. tabacum*, Solanaceae), tomato (*S. lycopersicum*, Solanaceae), and common bean (*P. vulgaris*, Fabaceae) (Table 3). As expected, arabinosides were not detected in the disrupted leaf tissues of these four plant species. However, significant amounts of MGDG-9-OH-traumatins/7-OH-dinortraumatins and/or MGDG-bis-9-OH-traumatins were detected in all four species. The amounts varied, with lower amounts detected in tobacco than in *Arabidopsis* (*No-0*). Higher quantities formed in cabbage, tomato, and common bean leaves. The highest concentration detected was that of MGDG-bis-9-OH-traumatins (51 nmol g FW<sup>-1</sup>) in common bean leaves. Plants belonging to the Fabaceae have no 16:3, and accordingly, MGDG-9-OH-traumatins/7-OH-dinortraumatins was not detected.

### DISCUSSION

It has been believed that HPLs show higher affinities for free fatty acid-HPOs as substrates; thus, the C12/C10 counterparts of HPL products were assumed to be free oxoacids. In this study, we detected less than 10 nmol g FW<sup>-1</sup> of (9*Z*)-traumatins and 9-OH-traumatins in their free forms, compared with ~300 nmol g FW<sup>-1</sup> of (3*Z*)-hexenal in completely disrupted *Arabidopsis* (*No-0*) leaves. From this result, we propose that the C12/C10 counterparts are formed via a different mechanism from the canonical HPL pathway, in which free fatty acid HPOs are the substrates. It was reported that esterified (10*E*)-traumatins is present in runner bean (21); thus, we tried to identify esterified C12/C10 oxoacid derivatives.

Accordingly, we analyzed MGDGs containing 9-OH-traumatins and/or 7-OH-dinortraumatins not only in disrupted leaves of *Arabidopsis* but also those of cabbage, tobacco, tomato, and common bean. This is the first report of endogenous MGDGs containing traumatins and its derivatives in disrupted plant leaves. Previous reports have described MGDGs with 12-oxodihydrophytyldienoic acid and divinylether, which were thought to be formed by AOS and divinylether synthase, both of which are CYP74 enzymes closely related to HPL. How-

ever, these compounds were detected only in *Arabidopsis* sp. and flax plants (*Linum usitatissimum*), respectively (12–13, 16).

In *Arabidopsis* (*No-0*) leaves, MGDG-9-OH-traumatins/7-OH-dinortraumatins and/or MGDG-bis-9-OH-traumatins were formed quickly after disruption. This rapid formation coincided with the release of C6 aldehydes. The structures of their acyl moieties are those expected for the counterparts of canonical products of HPL activity toward free 13-HPOT and 11-HPHT, possibly followed by enzymatic or nonenzymatic peroxidation and subsequent reduction to yield hydroxylated oxo compounds (22, 27). The requirement for active HPL was confirmed by comparing formation of MGDGs containing traumatins derivatives between *Arabidopsis* ecotypes and mutants with/without an active HPL. We also found that recombinant CaHPL showed catalytic activity toward pure MGDG HPOs. These lines of evidence indicated that MGDG HPOs are substrates of HPL and that volatile C6 aldehydes and nonvolatile MGDG containing C12/C10 oxoacids are the products of HPL in disrupted *Arabidopsis* leaf tissues.

It has been widely believed that the most suitable substrates for HPL are free fatty acid HPOs. However, it was reported that the *Arabidopsis* HPL showed significant activity (80% of that toward the free fatty acid derivative) toward the methyl ester of 13-HPOT (39). Hughes *et al.* (40) reported that detergent micelles massively affect HPL and AOS activities. In leaf tissues disrupted by mechanical wounding or herbivore attack, the enzymes in the oxylipin pathway show catalytic activity toward substrates that co-exist with a wide array of biological molecules, including lipids. Thus, the reaction conditions of these enzymes *in situ* differ greatly from those *in vitro*. This study demonstrated that at least the HPLs examined here were able to utilize lipid HPOs as substrates. The substrate preferences of HPLs and related enzymes involved in oxylipin formation in disrupted leaf tissues should be reevaluated, taking these results into account.

The total amounts of C10/C12 compounds (the sum of their free and MGDG forms) detected in disrupted *Arabidopsis* (*No-0*) leaves were still substantially lower than those expected from the amounts of C6 aldehydes. It is possible that traumatins and its derivatives were esterified to other lipids. Greater amounts of traumatins derivatives than MGDG-containing traumatins derivatives were detected after pancreatin treatment of crude lipids extracted from disrupted *Arabidopsis* (*No-0*) leaves. Zoeller *et al.* (20) reported that in *Arabidopsis* (*Col-0*), in addition to MGDGs, also DGDGs, phosphatidylglycerols, and triacylglycerols contained significant amounts of 18:3 and 16:3 that were partially oxygenated or even fragmented under biotic

stress conditions. In addition, it is likely that MGDG-hydroxylated oxoacids are further converted because 9-OH-traumatolipin and 7-OH-traumatolipin should be very reactive with various kinds of nucleophiles present in cells, such as glutathione, because such compounds contain a highly electrophilic  $\gamma$ -hydroxy- $\alpha,\beta$ -unsaturated aldehyde moiety (41–42). In the present study, the amounts of analytes detected may have been underestimated because of the spontaneous degradation of these reactive compounds, especially the hydroperoxides that may have formed as intermediates, during analysis. To match the reactions that occur in disrupted leaf tissues with the predicted stoichiometry between C6 and C12 products of HPL, an accurate system to quantify the possible metabolites must be established.

A comparison of the amounts of arabidopsides and MGDG with traumatolipin derivatives between *aos::Ler* and *Ler* suggested that AOS and HPL competed for the same substrates, MGDG-HPOs, in disrupted *Arabidopsis* leaf tissues. AtLOX2 is involved in supplying the substrate, at least for AOS (15). Because significant amounts of these lipid oxylipins formed rapidly, it was assumed that substantial amounts of both AOS and HPL were present in intact leaf cells in their latent forms or were located in different cellular compartments, separated from their substrates. Arabidopsides were not formed in leaves of cabbage, tobacco, tomato, and common bean, although these species formed significant amounts of MGDG containing traumatolipin derivatives as well as GLVs after disruption of leaf tissues. These findings suggested that the nature of the *Arabidopsis* AOS differed from that of AOSs in these plants. AOSs in leaves of cabbage, tobacco, tomato, and common bean may not be able to utilize MGDG-HPOs, or the amount of AOS in intact leaf cells might be too low to compete with HPL.

These findings do not exclude the possibility that GLVs can be formed via the lipase reaction with lipids to form 18:3 and 16:3. It is still possible that the free C12/C10 compounds are re-esterified; however, this possibility is unlikely because arabidopsides form on lipids without hydrolysis, as shown in a series of excellent experiments using isotopes (18). The formation of esterified and free oxylipins is regulated differently between natural and stress-induced senescence (43). The biosynthesis of free oxylipins definitely requires lipases, such as AtDAD1 (44) or NaGPA1 (45). GLV formation occurs even in intact plant tissues distant from the site of herbivore damage (46), after pathogen infection (47), under heat stress, after sudden darkness (48), or even after treating the tissues with pathogen-induced plant volatiles (49). In an analogous situation to the formation of free JAs, hydrolysis of free fatty acids may be required for the formation of GLVs in intact tissues under stress. This idea is partly supported by the fact that free JA production was enhanced in transgenic rice suppressing HPL (50).

$\gamma$ -Hydroxy- $\alpha,\beta$ -unsaturated aldehydic esters of phosphatidylcholine, such as 1-palmitoyl-2-(5-hydroxy-8-oxooct-6-enyl)-sn-glycero-3-phosphocholine, have been identified in human atherosclerotic lesions (51). The truncated, terminally oxidized phospholipids, which were formed through autooxidation and subsequent cleavage of phospholipids with polyunsaturated fatty acids, were proposed to be ligands for the macrophage scavenger receptor CD36 (52). Because of the high reactivity of  $\gamma$ -hydroxy- $\alpha,\beta$ -unsaturated aldehydes with bio-

logical molecules through the Michael addition reaction (42), MGDGs with  $\gamma$ -hydroxy- $\alpha,\beta$ -unsaturated aldehyde moieties might also have potent bioactivity. To gain further insights into the physiological and ecological significance of the formation of MGDGs with  $\gamma$ -hydroxy- $\alpha,\beta$ -unsaturated aldehydes, the fate of these lipid oxylipins must be identified.

## REFERENCES

- Matsui, K. (2006) Green leaf volatiles. Hydroperoxide lyase pathway of oxylipin metabolism. *Curr. Opin. Plant Biol.* **9**, 274–280
- D'Auria, J. C., Pichersky, E., Schaub, A., Hansel, A., and Gershenzon, J. (2007) Characterization of BAHD acyltransferase responsible for producing the green leaf volatile (*Z*)-3-hexen-1-yl acetate in *Arabidopsis thaliana*. *Plant J.* **49**, 194–207
- Allmann, S., and Baldwin, I. T. (2010) Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. *Science* **329**, 1075–1078
- Hammond, D. G., Rangel, S., and Kubo, I. (2000) Volatile aldehydes are promising broad-spectrum postharvest insecticides. *J. Agric. Food Chem.* **48**, 4410–4417
- Nakamura, S., and Hatanaka, A. (2002) Green-leaf-derived C6 aroma compounds with potent antibacterial action that act on both gram-negative and gram-positive bacteria. *J. Agric. Food Chem.* **50**, 7639–7644
- Kishimoto, K., Matsui, K., Ozawa, R., and Takabayashi, J. (2008) Direct fungicidal activities of C6-aldehydes are important constituents for defense responses in *Arabidopsis* against *Botrytis cinerea*. *Phytochemistry* **69**, 2127–2132
- Hubert, J., Münzbergová, Z., and Santino, A. (2008) Plant volatile aldehydes as natural insecticides against stored-product beetles. *Pest Manag. Sci.* **64**, 57–64
- Matsui, K., Sugimoto, K., Mano, J., Ozawa, R., and Takabayashi, J. (2012) Differential metabolisms of green leaf volatiles in injured and intact parts of a wounded leaf meet distinct ecophysiological requirements. *PLoS One* **7**, e36433
- Arimura, G., Matsui, K., and Takabayashi, J. (2009) Chemical and molecular ecology of herbivore-induced plant volatiles. Proximate factors and their ultimate functions. *Plant Cell Physiol.* **50**, 911–923
- Grechkin, A. N., and Hamberg, M. (2004) The “heterolytic hydroperoxide lyase” is an isomerase producing a short-lived fatty acid hemiacetal. *Biochim. Biophys. Acta* **1636**, 47–58
- Mosblech, A., Feussner, I., and Heilmann, I. (2009) Oxylipins. Structurally diverse metabolites from fatty acid oxidation. *Plant Physiol. Biochem.* **47**, 511–517
- Stelmach, B. A., Müller, A., Hennig, P., Gebhardt, S., Schubert-Zsilavecz, M., and Weiler, E. W. (2001) A novel class of oxylipins, sn1-O-(12-oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl diglyceride, from *Arabidopsis thaliana*. *J. Biol. Chem.* **276**, 12832–12838
- Buseman, C. M., Tamura, P., Sparks, A. A., Baughman, E. J., Maatta, S., Zhao, J., Roth, M. R., Esch, S. W., Shah, J., Williams, T. D., and Welti, R. (2006) Wounding stimulates the accumulation of glycerolipids containing oxophytodienoic acid and dinor-oxophytodienoic acid in *Arabidopsis* leaves. *Plant Physiol.* **142**, 28–39
- Nilsson, A. K., Fahlberg, P., Ellerström, M., and Andersson, M. X. (2012) Oxo-phytodienoic acid (OPDA) is formed on fatty acids esterified to galactolipids after tissue disruption in *Arabidopsis thaliana*. *FEBS Lett.* **586**, 2483–2487
- Glauser, G., Dubugnon, L., Mousavi, S. A., Rudaz, S., Wolfender, J.-L., Farmer, E. E. (2009) Velocity estimates for signal propagation leading to systemic jasmonic acid accumulation in wounded *Arabidopsis*. *J. Biol. Chem.* **284**, 34506–34513
- Chechetkin, I. R., Mukhitova, F. K., Blufard, A. S., Yarin, A. Y., Antsygina, L. L., and Grechkin, A. N. (2009) Unprecedented pathogen-inducible complex oxylipins from flax. Linolipins A and B. *FEBS J.* **276**, 4463–4472
- Farmer, E. E., and Mueller, M. J. (2013) ROS-mediated lipid peroxidation and RES-activated signaling. *Annu. Rev. Plant Biol.* **64**, 429–450
- Mène-Saffrané, L., Dubugnon, L., Chételat, A., Stolz, S., Gouhier-Darimont, C., and Farmer, E. E. (2009) Nonenzymatic oxidation of trienoic

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- fatty acids contributes to reactive oxygen species management in *Arabidopsis*. *J. Biol. Chem.* **284**, 1702–1708
19. Ibrahim, A., Schültz, A.-L., Galano, J.-M., Herrfurth, C., Feussner, K., Durand, T., Brodhun, F., and Feussner, I. (2011) The alphabet of galactolipids in *Arabidopsis thaliana*. *Front. Plant Sci.* **2**, 95
  20. Zoeller, M., Stingl, N., Krischke, M., Fekete, A., Waller, F., Berger, S., and Mueller, M. J. (2012) Lipid profiling of the *Arabidopsis* hypersensitive response reveals specific lipid peroxidation and fragmentation processes. Biogenesis of pimelic and azelaic acid. *Plant Physiol.* **160**, 365–378
  21. Zimmermann D. C., and Coudron, C. A. (1979) Identification of traumatin, a wound hormone, as 12-oxo-*trans*-10-dodecenoic acid. *Plant Physiol.* **63**, 536–541
  22. Kallenbach, M., Gilardoni, P. A., Allmann, S., Baldwin, I. T., and Bonaventure, G. (2011) C12 derivatives of the hydroperoxide lyase pathway are produced by product recycling through lipoxygenase-2 in *Nicotiana attenuata* leaves. *New Phytol.* **191**, 1054–1068
  23. Duan, H., Huang, M. Y., Palacio, K., and Schuler, M. A. (2005) Variations in CYP74B2 (hydroperoxide lyase) gene expression differentially affect hexenal signaling in the *Columbia* and *Landsberg erecta* ecotypes of *Arabidopsis*. *Plant Physiol.* **139**, 1529–1544
  24. Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M. X., Arondel, V., Bates, P. D., Baud, S., Bird, D., Debono, A., Durrett, T. P., Franke, R. B., Graham, I. A., Katayama, K., Kelly, A. A., Larson, T., Markham, J. E., Miquel, M., Molina, I., Nishida, I., Rowland, O., Samuels, L., Schmid, K. M., Wada, H., Welti, R., Xu, C., Zallot, R., and Ohlrogge, J. (2010) Acyl-lipid Metabolism. *Arabidopsis Book* **8**, e0133
  25. Park, J. H., Halitschke, R., Kim, H. B., Baldwin, I. T., Feldmann, K. A., and Feyereisen, R. (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. *Plant J.* **31**, 1–12
  26. Shiojiri, K., Ozawa, R., Matsui, K., Sabelis, M. W., and Takabayashi, J. (2012) Intermittent exposure to traces of green leaf volatiles triggers a plant response. *Sci. Rep.* **2**, 378
  27. Noordermeer, M. A., Feussner, I., Kolbe, A., Veldink, G. A., and Vliegenhart, J. F. G. (2000) Oxygenation of (3Z)-alkenals to 4-hydroxy-(2E)-alkenals in plant extracts. A nonenzymatic process. *Biochem. Biophys. Res. Commun.* **277**, 112–116
  28. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917
  29. Nakashima, A., Iijima, Y., Aoki, K., Shibata, D., Sugimoto, K., Takabayashi, J., and Matsui, K. (2011) Monogalactosyl diacylglycerol is a substrate for lipoxygenase: Its implications for oxylipin formation directly from lipids. *J. Plant Interact.* **6**, 93–97
  30. Matsui, K., Shibutani, M., Hase, T., Kajiwara, T. (1996) Bell pepper fruit fatty acid hydroperoxide lyase is a cytochrome P450 (CYP74B). *FEBS Lett.* **394**, 21–24
  31. Sakaki, T., Saito, K., Kawaguchi, A., Kondo, N., and Yamada, M. (1990) Conversion of monogalactosyldiacylglycerols to triacylglycerols in ozone-fumigated spinach leaves. *Plant Physiol.* **94**, 766–772
  32. Matsui, K., Sugimoto, K., Kakumyan, P., Khorobrykh, S. A., and Mano, J. (2009) Volatile oxylipins and related compounds formed under stress in plants. *Methods Mol. Biol.* **580**, 17–28
  33. Esterbauer, H., and Weger, W. (1967) Über die Wirkungen von Aldehyden auf gesunde und maligne Zellen. 3. Mitt.: Synthese von homologen 4-Hydroxy-2-alkenalen, II. *Monatsh. Chem.* **98**, 1994–2000
  34. Sias, B., Ferrato, F., Grandval, P., Lafont, D., Boullanger, P., De Caro, A., Leboeuf, B., Verger, R., and Carrière, F. (2004) Human pancreatic lipase-related protein 2 is a galactolipase. *Biochemistry* **43**, 10138–10148
  35. Nakanishi, H., Iida, Y., Shimizu, T., and Taguchi, R. (2010) Separation and quantification of *sn*-1 and *sn*-2 fatty acid positional isomers in phosphatidylcholine by RPLC-ESIMS/MS. *J. Biochem.* **147**, 245–256
  36. Hisamatsu Y., Goto, N., Hasegawa, K., and Shigemori, H. (2006) A glycolipid involved in flower bud formation of *Arabidopsis thaliana*. *Bot. Stud.* **47**, 45–50
  37. Komisarski, M., Kaczmarek, Z., and Kuśmierk, J. T. (2009) Practical highly enantioselective synthesis of (R)- and (S)-(E)-4-hydroxynon-2-enal. *Acta Biochim. Pol.* **56**, 189–193
  38. Böttcher, C., and Weiler, E. W. (2007) Cyclo-oxylipin-galactolipids in plants. occurrence and dynamics. *Planta* **226**, 629–637
  39. Kandzia, R., Stumpe, M., Berndt, E., Szalata, M., Matsui, K., and Feussner, I. (2003) On the specificity of lipid hydroperoxide fragmentation by fatty acid hydroperoxide lyase from *Arabidopsis thaliana*. *J. Plant Physiol.* **160**, 803–809
  40. Hughes, R. K., Belfield, E. J., and Casey, R. (2006) CYP74C3 and CYP74A1, plant cytochrome P450 enzymes whose activity is regulated by detergent micelle association, and proposed new rules for the classification of CYP74 enzymes. *Biochem. Soc. Trans.* **34**, 1223–1227
  41. Bochkov, V. N., Oskolkova, O. V., Birukov, K. G., Levonen, A.-L., Binder, C. J., and Stöckl, J. (2010) Generation and biological activities of oxidized phospholipids. *Antioxid. Redox Signal.* **12**, 1009–1059
  42. Farmer, E. E., and Davoine, C. (2007) Reactive electrophile species. *Curr. Opin. Plant Biol.* **10**, 380–386
  43. Seltmann, M. A., Stingl, N. E., Lautenschlaeger, J. K., Krischke, M., Mueller, M. J., and Berger, S. (2010) Differential impact of lipoxygenase 2 and jasmonates on natural and stress-induced senescence in *Arabidopsis*. *Plant Physiol.* **152**, 1940–1950
  44. Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., and Okada, K. (2001) The DEFECTIVE IN ANther DEHISCENCE1 gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. *Plant Cell* **13**, 2191–2209
  45. Bonaventure, G., Schuck, S., and Baldwin, I. T. (2011) Revealing complexity and specificity in the activation of lipase-mediated oxylipin biosynthesis. A specific role of the *Nicotiana attenuata* GLA1 lipase in the activation of jasmonic acid biosynthesis in leaves and roots. *Plant Cell Environ.* **34**, 1507–1520
  46. Rose, U., Manukian, A., Heath, R. R., and Tumlinson, J. H. (1996) Volatile semiochemicals released from undamaged cotton leaves. A systemic response of living plants to caterpillar damage. *Plant Physiol.* **111**, 487–495
  47. Piesik, D., Lemńczyk, G., Skoczek, A., Lamparski, R., Bocianowski, J., Kotwica, K., and Delaney, K. J. (2011) *Fusarium* infection in maize. Volatile induction of infected and neighboring uninfected plants has the potential to attract a pest cereal leaf beetle, *Oulema melanopus*. *J. Plant Physiol.* **168**, 1534–1542
  48. Loreto, F., Barta, C., Brillì, F., and Nogues, I. (2006) On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. *Plant Cell Environ.* **29**, 1820–1828
  49. Engelberth, J., Alborn, H. T., Schmelz, E. A., and Tumlinson, J. H. (2004) Airborne signals prime plants against insect herbivore attack. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 1781–1785
  50. Liu, X., Li, F., Tang, J., Wang, W., Zhang, F., Wang, G., Chu, J., Yan, C., Wang, T., Chu, C., and Li, C. (2012) Activation of the jasmonic acid pathway by depletion of the hydroperoxide lyase OsHPL3 reveals crosstalk between the HPL and AOS branches of the oxylipin pathway in rice. *PLoS One* **7**, e50089
  51. Deng, Y., Salomon, R. G. (1998) Total synthesis of  $\gamma$ -hydroxy- $\alpha$ ,  $\beta$ -unsaturated aldehydic esters of cholesterol and 2-lysophosphatidylcholine. *J. Org. Chem.* **63**, 7779–7794
  52. Podrez, E. A., Poliakov, E., Shen, Z., Zhang, R., Deng, Y., Sun, M., Finton, P. J., Shan, L., Febbraio, M., Hajjar, D. P., Silverstein, R. L., Hoff, H. F., Salomon, R. G., and Hazen, S. L. (2002) A novel family of atherogenic oxidized phospholipids promotes macrophage foam cell formation via the scavenger receptor CD36 and is enriched in atherosclerotic lesions. *J. Biol. Chem.* **277**, 38517–38523