Mono- and Digalactosyldiacylglycerol Lipids Function Nonredundantly to Regulate Systemic Acquired Resistance in Plants

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INVENTORY- Supplementary Items

Extended Experimental Procedures

Plant growth conditions and genetic analysis

Plants were grown in MTPS 144 Conviron (Winnipeg, MB, Canada) walk-in chambers at 22 °C, 65% relative humidity and 14 h photoperiod. These chambers were equipped with cool white fluorescent bulbs (Sylvania, FO96/841/XP/ECO). The photon flux density (PFD) of the day period was 106.9 µmoles m⁻² s⁻¹ (measured using a digital light meter, Phytotronic Inc, MO). Plants were grown on autoclaved Pro-Mix soil (Premier Horticulture Inc., PA, USA). Soil was fertilized once using Scotts Peter’s 20:10:20 peat lite special general fertilizer that contained 8.1% ammoniacal nitrogen and 11.9% nitrate nitrogen (Scottspro.com). The mgd1-1, dgd1-1 and transgenic plants expressing DGD1 or the bacterial enzyme glycosyltransferase are described before (Jarvis et al., 2000; Hölzl et al. 2006; Aronsson et al., 2008). The mgd1 dgd1 double mutant plants were obtained by pollinating flowers of the mgd1 plant with pollen from dgd1 plants. The double mutants were identified based on FA profile as well as genotype analysis.

Each experiment was performed in triplicate sets and repeated at least twice with similar results. Due to the natural fluctuations only data from representative experiments are shown.

RNA extraction, RNA gel-blot analyses and RT-PCR

Small-scale extraction of RNA from two or three leaves (per sample) was performed with the TRIZol reagent (Invitrogen, CA) following the manufacturer’s instructions. RNA gel blot analysis and synthesis of random-primed probes were carried out as described previously (Kachroo et al., 2004). Real-time quantitative RT-PCR was carried out as described before (Zhang et al., 2009; Yu et al., 2013).
SA, G3P, AzA, FA and JA quantifications

SA and SA glucoside (SAG) were extracted and measured from ~ 0.3 g of fresh weight leaf tissue as described before (Chandra-Shekara et al., 2006). G3P and AzA quantifications were carried out as described earlier (Chanda et al., 2008; 2011).

FA extraction was carried out by placing leaf tissue in 2 ml of 3% H₂SO₄ in methanol. After 30 min incubation at 80 °C, 1 ml of hexane with 0.001% butylated hydroxytoluene (BHT) was added. The hexane phase was then transferred to vials for gas chromatography (GC) analysis. One-microliter samples were analyzed by GC on a Varian FAME 0.25 mm x 50 m column and quantified with flame ionization detection. For quantification of FAs, leaves (50 mg) were extracted together with an internal standard 17:0 and the FA levels were calculated based on the detected peak areas corresponding to the FA retention time relative to the areas of the internal standard. Free FA analysis was based on previously published procedures (Hamilton et al., 1988; Norman et al., 2001; Yu et al., 2013).

For JA levels, samples were extracted using a solution containing glacial acetic acid, methanol, chloroform and potassium chloride (0.9%) (1:4:8:8, vol/vol) and 17:0 was used as an internal standard. The lower phase was removed and dried under a stream of nitrogen gas and samples were derivatized with diazomethane, dried and reconstituted in MTBE, transferred to a glass insert and dried again under a stream of nitrogen gas and reconstituted in a minimum volume of acetonitrile. Samples (1μl) were analyzed with GC as described above. The JA peaks were identified using mass spectrometry (MS).

SA, AzA and G3P treatments

SA, AzA and G3P treatments were carried out by spraying or infiltrating 500 μM, 1 mM or 100 μM solutions, respectively. SA and G3P were dissolved in water. AzA was prepared in methanol and diluted in water (Yu et al., 2013).

NO staining and confocal microscopy

For NO staining, adaxial side of leaves were infiltrated with 4 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) and, after 5 min incubation in dark, leaves were observed under Olympus FV1000 laser-scanning confocal microscope.
For imaging, water-mounted sections of leaf tissue were examined by confocal microscopy using a water immersion PLAPO60XWLSM 2 (NA 1.0) objective on a FV1000 point-scanning/point-detection laser scanning confocal microscope (Olympus America, Melville, NY) equipped with lasers spanning the spectral range of 405–633 nm. GFP (488 nm) and Ds-RED2 (543 nm) channels were used to analyze DAF-FM DA stained leaves and overlay images (40X magnification) were acquired at a scan rate of 10 ms/pixel. Olympus FLUOVIE 1.5 was used to control the microscope, image acquisition and the export of TIFF files.

**ROS quantification**

For ESR spectra, 0.1 g leaves were homogenized in 500 μl of 50 mM HEPS buffer (pH6.9) containing 50 mM POBN and 10 μl of this homogenate was loaded onto graduated capillary tube in a flat cell. EPR spectra were measured at room temperature using a Bruker ESP 300 X-band spectrometer set at 5 mW microwave power, 100 kHz modulation frequency, 1 G modulation amplitude, and 9.687 GHz microwave frequency. Values of ESR signals were calculated from the maximum-signal/noise ratio of recorder traces and corrected, if necessary, by subtracting reagent blanks determined in parallel. Signal intensity was evaluated as the peak height in ESR spectra. Standard spectra for carbon centered radicals were created by incubating POBN with xanthine (1 mM) and xanthine oxidase (0.05 units/ml) reaction mixture.

**Protein extraction and immunoblot analysis**

Proteins were extracted in buffer containing 50 mM Tris-HCl (pH7.5), 10% glycerol, 150 mM NaCl, 10 mM MgCl₂, 5 mM EDTA, 5 mM DTT, and 1 X protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentration was measured by the Bio-RAD protein assay (Bio-Rad, CA). For Ponceau-S staining, PVDF membranes were incubated in Ponceau-S solution (40% methanol (v/v), 15% acetic acid (v/v), 0.25% Ponceau-S). The membranes were destained using deionized water. Proteins (30-100 μg) were fractionated on a 7-10% SDS-PAGE gel and subjected to immunoblot analysis using α-NOA1 antibody. Immunoblots were developed using ECL detection kit (Roche) or alkaline phosphatase-
Pathogen infections and collection of phloem exudate

Inoculations with bacterial pathogen *Pseudomonas syringae* were conducted as described before (Kachroo et al., 2005). The bacterial cultures were grown overnight in King’s B medium containing rifampicin and/or kanamycin. The cells were washed and suspended in 10 mM MgCl₂. The bacterial suspension was injected into the abaxial surface of the leaf using needle-less syringe. Three discs from the inoculated leaves were collected and homogenized in 10 mM MgCl₂. The extract was diluted and appropriate dilutions were plated on King’s B medium. For analysis of SAR, the primary leaves were inoculated with MgCl₂ or the *avr* bacteria (10⁷ CFU ml⁻¹) and 48 h later the systemic leaves were inoculated with *vir* bacteria (10⁵ CFU ml⁻¹). Unless noted otherwise, samples from the systemic leaves were harvested at 3 dpi.

Leaf exudate was collected as described earlier (Maldonado et al., 2002). In brief, plants were induced for SAR by inoculation with *P. syringae* containing *avrRpt2* (10⁶ CFU ml⁻¹). Twelve hours later, petioles were excised, surface sterilized in 50% ethanol, 0.0006% bleach, rinsed in sterile 1 mM EDTA and submerged in ~1.9 ml of 1 mM EDTA and 100 μg ml⁻¹ ampicillin. Exudates were collected over 48 h and infiltrated into healthy plants. Infiltrated leaves were harvested after two days for PR-1 gene expression studies. For SAR studies, *vir* pathogen was inoculated in the distal leaves two days after infiltration of exudate.

Thin layer chromatography

For conversion of 18:1 to AzA, [¹⁴C]-18:1 (20 μM) was infiltrated into Col-0, *mgd1* or *dgd1* leaves, sampled 24 h post-infiltration, methylated with 3% H₂SO₄ in methanol and ran on silica TLC plates using hexane: MTBE: acetic acid (80: 20: 1, by vol). The TLC plates were autoradiographed using Typhoon 9400 Variable Mode Imager (GE). The bands corresponding to AzA dimethyl ester were scraped individually, hydrolyzed with ethanolic 2N NaOH (9:1 by vol), refluxed at 80 °C for ~30 min, neutralized with 0.3 volumes of 1 M HCl and extracted with hexane:MTBE (96:4 by vol). These fractions were dried completely.
under a stream of nitrogen gas, resuspended in a minimal volume of hexane:MTBE (96:4 by vol) and rerun on a fresh silica plate along with [1-\(^{14}\)C] AzA.

For AzA transport, [1-\(^{14}\)C] AzA (1 µCi/ml, specific activity 16 mCi/mmol) was dissolved in 50 µl of ethanol and diluted to 1 ml with water. The resulting solution contained 62.5 µM of AzA and was injected into abaxial surface of four-week-old Arabidopsis leaves. Three leaves per plant were infiltrated with ~0.1 ml of \(^{14}\)C-AzA solution. The untreated leaves were individually covered with Saran wrap to avoid any spillover. The plants were then kept in a growth chamber set at 14 h light and 10 h dark photoperiods. The leaf samples were extracted using an acid methylation method as described below. The leaf samples were added to 3 ml of 3% H\(_2\)SO\(_4\) in methanol and heated at 80 °C for 15 min followed by two extraction with hexane (2 ml each). The samples were quantified using a liquid scintillation counter and extracts containing same amount of \(^{14}\)C radioactivity were loaded onto silica gel 60. The TLC plates were exposed in a storage phosphorimage screen (GE) and the bands were visualized by Typhoon 9400 Variable Mode Imager (GE).

TLC analysis of galactolipids of MGDG and DGDG was carried out as described before (Wang and Benning, 2011). For MGDG and DGDG recovery from TLC plates, 0.005% primulin in 80% acetone in water was sprayed on the TLC and bands were visualized under long wave UV light. The MGDG and DGDG bands were scraped off the TLC plates and eluted with approximately 10 ml of the TLC developing solvent mixture acetone:toluene:water (90:30:7.5, by vol). A small portion of the eluted galactolipids was used for quantification and the remaining portion was dried under a stream of nitrogen gas and re-constituted in 1 ml of acetone. For MGDG and DGDG quantification, ~300 mg of Arabidopsis leaf tissue was suspended in 600 µl of chloroform:methanol:formic acid (20:10:1, by vol), vortexed vigorously for 5 min followed by addition of 300 µl of 0.2 M H\(_3\)PO\(_4\) and the samples were revortexed for additional 1 min. After a brief centrifugation for 1 min at 12,000 rpm, the lower phase was transferred to a glass test tube and the upper phase was re-extracted with 300 µl of chloroform. The extract was combined and dried under a stream of nitrogen gas. The samples were reconstituted in 1 ml of chloroform and 100 µl was loaded on a TLC plate prepared as described earlier (Wang and Benning, 2011). The MGDG and DGDG bands were scraped and added to a glass test tube containing 20 µg
of triheptadecanoin in 100 µl chloroform:methanol (2:1, by vol). To this 500 µl of 4.8% sodium methoxide was added and the samples were shaken for 40 min at 150 rpm. The samples were mixed with 1 ml of hexane:MTBE (96:4, v/v) and 600 µl of 0.9% KCl, centrifuged at 500 rpm for 1 min followed by the transfer of the upper layer to a GC vial. The samples were dried, resuspended in 400 µl of hexane and analyzed by GC equipped with a Varian VF-17ms (0.25mm x 50 m) column.

In vitro FA oxidation assay

FAs (18:2) and MGDG or DGDG (10 µg each) each with 2 µg of triheptadecanoin were incubated with 100 µl of methanol and 300 µl of 1 mM methylene blue under dark or strong light for 14 hours. FAs were extracted twice with 1 ml of ethyl acetate. The upper phase was transferred to a 13 x 100 mm test tube, dried under a stream of nitrogen gas and methylated with 10 drops of ethereal diazomethane. The ethereal diazomethane was vortexed and dried under a stream of nitrogen gas. Galactolipids were extracted with 1 ml of methanol:chloroform:formic acid (20:10:1, by vol) and 350 µl of 0.2 M phosphoric acid. The lower phase was transferred to a 13 x 100 mm test tube. The samples were re-extracted with 500 µl of chloroform and combined. The extract was dried under a stream of nitrogen gas. The dried samples from FA and galactolipids as described above were methylated with 500 µl of sodium methoxide (4.8% in methanol), extracted with hexane, and analyzed by GC-MS as described earlier.

References:


SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Data used in this study is related to Figure 1. SAR model depicting the proposed NO- and SA-mediated signaling pathways and analysis of DGDG levels. (A) Infection by avirulent pathogen triggers signaling events that result in the accumulation of salicylic acid (SA) and nitric oxide (NO). NO acts in a feedback loop with reactive oxygen species (ROS), with the different ROS species functioning in an additive manner to catalyze the oxidation of C18 unsaturated fatty acids (FAs). Hydrolysis of C18 FAs at the Δ9 double bond (indicated by an arrowhead) generates azelaic acid (AzA), which triggers the biosynthesis of glycerol-3-phosphate (G3P) by upregulating genes encoding the G3P biosynthetic enzymes, glycerol kinase (GK) and G3P dehydrogenase (G3Pdh). G3P and the lipid transfer-like proteins DIR1 (defective in induced resistance) and AZI1 (AzA insensitive) operate in a feedback loop and are interdependent on each other. The SA and NO/ROS pathways cross-talk at several levels, including the S-nitrosylation of a key positive regulator of SA signaling, NPR1. (B) TLC showing MGDG and DGDG profile in indicated genotypes. Lipids were stained by iodine vapor. Vertical arrow indicates direction of the TLC run. (C) Relative levels of MGDG and DGDG in wild-type, mgd1 and mgd1 dgd1 leaves. MGDG and DGDG lipids were separated by TLC and quantified as described in methods. The experiment was repeated two times with similar results. (D) RNA gel blot showing transcript levels of DGD1 in Col-0, dgd1, and transgenic dgd1:DGD1 plants. Ethidium bromide staining of rRNA was used as loading control. The experiment was repeated two times with similar results.

Figure S2. Data used in this study is related to Figures 2 and 3. Exogenous treatment with SA does not restore SAR in dgd1 plants. (A) RNA gel blot showing transcript levels of PR-1 in Col-0 and dgd1 leaves infiltrated with petiole exudates from Col-0 plants treated either with MgCl₂ (EX_MgCl₂) or avrRpt2 (EX_avrRpt2). PR-1 transcript levels were analyzed 48 h after treatments. Ethidium bromide staining of rRNA was used as loading control. The experiment was repeated three times with similar results. (B) SAR response in Col-0 and dgd1 plants infiltrated with EX_MgCl₂ or EX_avrRpt2 from Col-0 or dgd1 plants. Exudates were mixed with water or 500 μM SA (EX_MgCl₂/avrRpt2 +SA) prior to infiltration. Error bars
represent SD (n=4). Asterisks denote a significant difference with Col-0 (t test, P<0.01). The experiment was repeated two times with similar results. (C) RNA gel blot showing transcript levels of PR-1 gene in Col-0 and dgd1 plants treated with water or SA (500 µm) for 48 h. Ethidium bromide staining of rRNA was used as loading control. The experiment was repeated two times with similar results. (D) Levels of FAs in four-week-old indicated genotypes. Error bars represent SD (n=6). Asterisks denote significant differences from Col-0 (t test, P<0.05). FW indicates fresh weight. The experiment was repeated three times with similar results. (E) Quantification of radioactivity in local (infiltrated) and distal tissues (untreated) of mgd1 (left panel) and dgd1 (right panel) leaves infiltrated with 14C-AzA. The Col-0 plants were used as control. Leaves were infiltrated with 1 µCi/ml solution of 14C-AzA and sampled 24 h post treatments. The error bars indicate SD (n=3). Per t-test analysis, values for mgd1 are not statistically significant. The dgd1 plants showed more AzA levels in local and distal leaves in two of four experiments. DPM and FW indicate disintegrations per minute (quantified using scintillation counts) and fresh weight, respectively. NS indicates not significant.

Figure S3. Data used in this study is related to Figures 4 and 5. The mgd1 and dgd1 plants show increase tolerance to paraquat. (A) Morphological phenotypes of mgd1 dgd1 double mutant plants. The photograph shows four-week-old soil grown plants. The mgd1 dgd1 plants show a pale phenotype. (B) Confocal micrograph of DAF-FM DA stained leaves showing induction of NO in wild-type (Col-0) or dgd1 plants treated with 0.1 mM NO donor SNP or DETA-NONOate. Control Col-0 and dgd1 plants (data not shown for dgd1) were treated with DMSO or SULFO-NONOate and plants were analyzed 24 h post treatment. Scale bar, 10 µm. Chloroplasts were visualized using DS-Red2. At least four independent leaves were analyzed in two experiments with similar results. (C) Typical morphological phenotype seen in leaves spot inoculated with 20 µM paraquat. The leaves were photographed 48 h post treatment. Scale bar, 8.6 mm. (D) Lesion size in Col-0 and mgd1 leaves treated with 20 µM paraquat. Lesion size was not quantified for dgd1 since 20 µM paraquat killed the entire dgd1 leaf. Asterisks denote a significant difference with Col-0 (t test, P<0.001). Results are representative of two independent experiments. (E) Real-time quantitative RT-PCR analysis showing relative expression of indicated genes in Col-0, mgd1
or dgd1 plants. The error bars indicate SD (n=3). Asterisk denotes significant differences from Col-0 plants (t test, P<0.05). The experiment was repeated two times with similar results. (F) Lesion size in Col-0, dgd1 and mgd1 leaves treated with 150 µM H₂O₂. Results are representative of two independent experiments. (G) Quantitative RT-PCR analysis showing relative expression levels of PR-1 in mock- and avrRpt2 inoculated Col-0 (wt), dgd1::DGD1 and dgd1::GT plants. The error bars indicate SD (n=3). Asterisks denote significant differences from mock-inoculated plants. The experiment was repeated twice with similar results.

**Figure S4. Data used in this study is related to all the Figures. A simplified model showing galactolipid and chemical signaling during SAR.** Inoculation of avirulent pathogen triggers activation of as yet unknown signal(s) (indicated by X) that is dependent on DGDG to induce accumulation of salicylic acid (SA) and nitric oxide (NO). NO operates in a feedback loop with reactive oxygen species (ROS), which act in an additive manner to catalyze oxidation of free C18 unsaturated fatty acids (FA) present on MGDG and DGDG lipids. Oxidation of C18 FAs at C9 carbon (indicated by arrow and arrowahead) generates AzA, which triggers biosynthesis of G3P via upregulation of genes encoding G3P biosynthetic enzymes. Normal levels of MGDG and DGDG are also required for pathogen induced ROS accumulation. R1 and R2 indicate FA species.

**Figure S5. Data used in this study is related to Figures 2 and 4. The dgd1 plants show compromised chloroplast-nuclear signaling.** (A) A simplified model showing retrograde signaling between chloroplast and nucleus. Infection by avirulent pathogen triggers signaling events that result in transcriptional upregulation of nuclear genes SID2 and EDS5, protein products of which localize to chloroplast and initiate biosynthesis or transport of salicylic acid (SA), respectively. A mutation in DGD1 leads to reduction in DGDG pool of galactolipids and dgd1 plants are unable to increase SID2 or EDS5 transcript levels in response to pathogen infection. Consequently, pathogen challenged dgd1 plants are unable to accumulate wt-like SA levels. (B) Relative levels of AzA and ONA (9-oxononanoic acid) generated in in vitro reactions where 18:2 FA or galactolipids (MGDG or DGDG) purified
from Arabidopsis leaves (10 µg each) were incubated under light with methylene blue, which generates superoxide radical. The control reactions containing FA/galactolipid and methylene blue were incubated in dark. The *in vitro* assays were repeated two times with similar results.
S. FIGURE 1

B

Col-0  mgd1  dgd1  mgd1 dgd1  dgd1::DGD1  dgd1::GT  dgd2

DGDG →

MGDG →

C

Col-0  mgd1  mgd1 dgd1

μmoles/g FW

MGDG  DGDG

D

Col-0  dgd1  dgd1::DGD1

DGD1

rRNA
**S. Figure 2**

**A**

![Image of gel electrophoresis with bands labeled](image1)

**B**

Bar chart showing Log (CFU/leaf disk) for different treatments:
- Ex-MgCl₂
- Ex-MgCl₂ + SA
- Ex-avrRpt2
- Ex-avrRpt2 + SA

**C**

![Image of gel electrophoresis with bands labeled](image2)

**D**

Line graph showing μmol/g FW for different fatty acids:
- 16:0
- 16:1 Δ7
- 16:2
- 16:3
- 18:0
- 18:1 Δ9
- 18:2
- 18:3

**E**

![Images of DPM/g FW](image3)
S. FIGURE 3

C

Col-0  mgd1  dgd1

D

Lesion size (mm)

Col-0  mgd1

* 0.001

E

Relative Expression

Col-0  mgd1  dgd1

SOD2  GSTU6  GSTU24

F

Lesion size (mm)

Col-0  dgd1  mgd1

G

Relative Expression (PR-1)

Col-0  dgd1::GT  dgd1::DGD1

Mock  avrRpt2

*
S. FIGURE 4

HOST

SA

HO

HO

AzA

O

O

G3P

HO

OH

OH

OH

P

O

ROS

Membrane

SAR

DGDG

DGDG

MGDG

AzA

G3P

SAR