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

Graphical Abstract

Highlights
Galactolipids (MGDG and DGDG) act nonredundantly in systemic acquired resistance (SAR)

DGDG is required for pathogen-responsive nitric oxide and salicylic acid accumulation

MGDG and DGDG contribute to the biosynthesis of the SAR signal azelaic acid

The α-galactose-β-galactose head group of DGDG is essential for SAR

Authors
Qing-ming Gao, Keshun Yu, ..., Aardra Kachroo, Pradeep Kachroo

Correspondence
apkach2@uky.edu (A.K.), pk62@uky.edu (P.K.)

In Brief
The galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) constitute ~80% of total membrane lipids in plants. Gao et al. now show that these galactolipids function nonredundantly to regulate systemic acquired resistance (SAR). Furthermore, they show that the terminal galactose on the α-galactose-β-galactose head group of DGDG is critical for SAR.
Mono- and Digalactosyldiacylglycerol Lipids Function Nonredundantly to Regulate Systemic Acquired Resistance in Plants

Qing-ming Gao,1 Keshun Yu,1 Ye Xia,1,4 M.B. Shine,1 Caixia Wang,1,2 DuRoy Navarre,3 Aardra Kachroo,1,* and Pradeep Kachroo1,4

1Department of Plant Pathology, University of Kentucky, Lexington, KY 40546, USA
2Qingdao Agricultural University, Number 700, Changcheng Road, Chengyang District, Qingdao City 266109, PRC
3Agricultural Research Service, United States Department of Agriculture, Washington State University, Prosser, WA 99350, USA
4Present address: Department of Horticulture, University of Kentucky, Lexington, KY 40546, USA

*Correspondence: apkach2@uky.edu (A.K.), pk62@uky.edu (P.K.)

http://dx.doi.org/10.1016/j.celrep.2014.10.069
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

The plant galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) have been linked to the anti-inflammatory and cancer benefits of a green leafy vegetable diet in humans due to their ability to regulate the levels of free radicals like nitric oxide (NO). Here, we show that DGDG contributes to plant NO as well as salicylic acid biosynthesis and is required for the induction of systemic acquired resistance (SAR). In contrast, MGDG regulates the biosynthesis of the SAR signals azelaic acid (AzA) and glycerol-3-phosphate (G3P) that function downstream of NO. Interestingly, DGDG is also required for AzA-induced SAR, but MGDG is not. Notably, transgenic expression of a bacterial glucosyltransferase is unable to restore SAR in dgd1 plants even though it does rescue their morphological and fatty acid phenotypes. These results suggest that MGDG and DGDG are required at distinct steps and function exclusively in their individual roles during the induction of SAR.

INTRODUCTION

Systemic acquired resistance (SAR) is a form of defense response that protects plants against a broad spectrum of secondary infections by related or unrelated pathogens (reviewed in Shah and Zeier, 2013; Kachroo and Robin, 2013; Gao et al., 2014; Wendehenne et al., 2014). Components of SAR include glycerol-3-phosphate (G3P; Chanda et al., 2011; Yu et al., 2013; Wang et al., 2014), the dicarboxylic acid azelaic acid (AzA; Jung et al., 2009), and the free radicals nitric oxide (NO) and reactive oxygen species (ROS) (Wang et al., 2014). Recent in vitro and in planta studies have shown that AzA is derived from the oxidation of C18 unsaturated fatty acids (FAs) that contain a double bond on C9 and that this process is facilitated by ROS (Zoeller et al., 2012; Yu et al., 2013; Wang et al., 2014). Consequently, AzA is unable to confer SAR on mutants impaired in GLY1-encoded G3P dehydrogenase (G3Pdh) or GL1-encoded glycerol kinase (GK) activities (Yu et al., 2013). Notably, the NO-ROS-AzA-G3P branch of SAR operates in parallel with salicylic acid (SA), a well-known regulator of SAR. Both branches are essential for the activation of SAR (Figure S1A). Consistent with this scheme of function, neither AzA nor G3P induces SA accumulation when applied alone or together with pathogen (Yu et al., 2013; Wang et al., 2014).

Galactolipids and cuticle have also been associated with SAR (Chaturvedi et al., 2008; Xia et al., 2010, 2012), although the underlying mechanism remains unknown. The plant galactolipids constitute ~80% of total membrane lipids, and of these, monogalactosyldiacylglycerol (MGDG) is considered to be the most abundant, accounting for ~50% of the total thylakoid lipids (Kelly and Dörmann, 2004). MGDG is synthesized by the monogalactosyl synthase 1 (MGD1)-mediated transfer of a galactose residue to diacylglycerol (Awai et al., 2001). A partial or complete defect in MGD1 reduces the MGDG level by ~42% (mgd1-1; Janvis et al., 2000) or ~98% (mgd1-2; Kobayashi et al., 2007), respectively. Consistent with their MGDG level, mgd1-1 plants show wild-type-like morphology, but the mgd1-2 mutants germinate as albino (Kobayashi et al., 2007). MGDG is subsequently converted to digalactosyldiacylglycerol (DGDG) via digalactosyl...
functions downstream of NO. Our study highlights the importance of the axial hydroxyl group at C4 of DGDG galactose in NO biosynthesis and SAR. This study also highlights the similar function of lipids in the regulation of NO in plants and animals.

RESULTS

The dgd1 Mutant Is Compromised in SAR

Consistent with an earlier report (Chaturvedi et al., 2008), we found that mgd1 plants were compromised in SAR (Figure 1A). To examine the biochemical basis of this defect in the context of galactolipid pools, we analyzed SAR in dgd plants that accumulate reduced levels of DGDG. In Arabidopsis, two genes (DGD1 and DGD2) are involved in the synthesis of DGDG, of which DGD1 is the major isomerase impacting DGDG biosynthesis (Kelly et al., 2003; Figures S1B and S1C). Wild-type (WT) and dgd1 plants were first inoculated with MgCl2 or avirulent bacteria (Pseudomonas syringae pv. tomato [Pst] expressing avrRpt2) followed by a second inoculation with virulent bacteria on distal tissues at 48 hr after primary inoculation. Growth of the virulent bacteria was monitored at 0 and 3 days postinoculation (dpi) (Figure 1A). As expected, MgCl2-infiltrated leaves of WT (Col-0 ecotype) plants supported more growth of the secondary virulent pathogen than the plants that were preinoculated with avrRpt2, indicating appropriate induction of SAR (Figure 1A). In comparison, the dgd1 mutant plants showed compromised SAR; plants infiltrated with MgCl2 or avrRpt2 bacteria were equally susceptible to the secondary virulent bacteria in the distal tissues. Unlike dgd1, the dgd2 plants showed normal SAR, and this correlated with the detectable levels of DGDG in these plants (Figure S1B). Together, these data suggested that DGD1 is required for the proper induction of SAR and the compromised SAR in dgd1 plants correlated with the reduced DGDG levels (Kelly et al., 2003; Figures S1B and S1C). Transgenic dgd1 plants expressing a wild-type copy of DGD1 (dgd1::DGD1) showed normal SAR (Figures 1B and S1D), suggesting that compromised SAR in dgd1 plants was specifically associated with the dgd1 mutation.

Next, we assessed whether DGDG was required for production or perception of the SAR mobile signal. We evaluated the response of WT and dgd1 plants to vascular exudates collected from pathogen-inoculated WT and dgd1 leaves. The WT or dgd1 leaves were infiltrated with MgCl2 or avrRpt2 bacteria, and vascular exudates (ExMgCl2/avrRpt2) collected from the inoculated leaves were injected into the leaves of a fresh set of WT or dgd1 plants. Distal leaves of plants infiltrated with ExMgCl2/avrRpt2 were then inoculated with virulent bacteria and proliferation of virulent bacteria monitored at 0 and 3 dpi (Figure 1C). As expected, ExavrRpt2 from WT plants conferred protection against virulent pathogen in WT plants, as did ExavrRpt2 from dgd1 plants, suggesting that dgd1 mutant plants were able to generate the SAR signals. In comparison, neither WT ExavrRpt2 nor dgd1 ExavrRpt2 induced SAR in dgd1 plants, suggesting that dgd1 plants were unable to sense the SAR signals. We tested this further by assaying ExavrRpt2-induced SAR in mgd1 plants, since they accumulate WT-like levels of DGDG (Figures S1B and S1C; Kelly et al., 2003). Interestingly, as in dgd1, mgd1 ExavrRpt2 was able to confer SAR on WT plants, but not on mgd1 plants (Figure 1D). Conversely, WT ExavrRpt2 was unable to confer SAR on mgd1 plants. Together, these data
suggested that both mgd1 and dgd1 plants were able to make the SAR signal(s) but unable to respond to them.

**The dgd1 Mutant Is Impaired in the SA Branch of SAR**

Recently, we showed that induction of SAR is dependent on two parallel pathways, one of which is regulated by SA (Figure S1A; Wang et al., 2014). To test the effect of mgd1 and dgd1 mutations on the SA pathway, we first evaluated the expression of the SA marker gene, PR-1, in mock- and avrRpt2-infected plants. Interestingly, compared to WT (Col-0) and mgd1 plants, which showed comparable induction of PR-1 expression, the dgd1 plants showed significantly lower levels of PR-1 in their inoculated leaves (Figure 2A). Similar results were obtained when we assayed PR-1 levels in Col-0 and dgd1 plants infiltrated with ExMgCl2/avrRpt2 from Col-0 plants (Figure S2A). These results suggested that dgd1 plants were impaired in either pathogen-responsive SA accumulation or signaling. Therefore, we tested the basal and pathogen-responsive SA levels in dgd1 plants. Although the dgd1 plants accumulated WT-like levels of basal free SA and the SA glucoside (SAG), they contained significantly reduced free SA and SAG after pathogen infection (Figure 2B). In contrast and as expected, WT leaves infected with avrRpt2 showed a significant increase in free SA and SAG (Figure 2B).

To determine if the reduced accumulation of SA in dgd1 plants was responsible for their defective SAR, we assayed SAR after inoculation of SA to ExavrRpt2, but not dgd1 was responsible for their defective SAR, we assayed SAR after inoculation of SA to ExavrRpt2-infected plants. Interestingly, compared to WT (Col-0) and mgd1 plants, which showed comparable induction of PR-1 expression, the dgd1 plants showed significantly lower levels of PR-1 in their inoculated leaves (Figure 2A). Similar results were obtained when we assayed PR-1 levels in Col-0 and dgd1 plants infiltrated with ExMgCl2/avrRpt2 from Col-0 plants (Figure S2A). These results suggested that dgd1 plants were impaired in either pathogen-responsive SA accumulation or signaling. Therefore, we tested the basal and pathogen-responsive SA levels in dgd1 plants. Although the dgd1 plants accumulated WT-like levels of basal free SA and the SA glucoside (SAG), they contained significantly reduced free SA and SAG after pathogen infection (Figure 2B). In contrast and as expected, WT leaves infected with avrRpt2 showed a significant increase in free SA and SAG (Figure 2B).

Although the distal tissues of dgd1 plants also showed a marked reduction in free SA level, they accumulated slightly higher levels of SAG compared to WT plants (Figure 2B), the biological significance of which remains unclear at this point. Congruent with their inability to accumulate SA after avrRpt2 inoculation, the dgd1 plants showed compromised local resistance to avrRpt2-infected Pst but displayed WT-like response to virulent bacteria (Figure 2C). Thus, the reduced pathogen-responsive PR-1 expression of dgd1 plants associated with their reduced SA levels and local R-mediated resistance.

To determine if the reduced accumulation of SA in dgd1 plants was responsible for their defective SAR, we assayed SAR after SA treatment. Localized application of SA induced SAR in WT, but not dgd1, plants (Figure 2D). Next, we tested whether addition of SA to ExavrRpt2 improved the SA response of dgd1 plants. ExMgCl2/avrRpt2 collected from WT or dgd1 plants was mixed with water or SA and injected into the leaves of a fresh set of WT and dgd1 plants. Col-0 ExMgCl2 + SA induced SAR on Col-0 plants, but not on dgd1 plants (Figure S2B). In comparison, dgd1 ExMgCl2 + SA was unable to confer SAR on either Col-0 or dgd1 plants. As seen above (Figure 1C), dgd1 ExavrRpt2 conferred SAR on Col-0 plants, but not on dgd1 plants. Both Col-0 and dgd1 ExavrRpt2 + SA induced SAR on Col-0 plants than ExavrRpt2 alone (p < 0.05), but not on dgd1. This suggested that although addition of SA to WT/dgd1 ExavrRpt2 improved SAR in Col-0 plants, SA was unable to restore the SAR response to ExavrRpt2 in dgd1 plants. To determine if this was due to reduced sensitivity to SA, we compared the expression of the SA-inducible marker PR-1 in dgd1 and WT plants. Similar levels of PR-1 transcript were induced in response to SA in both Col-0 and dgd1 plants (Figure S2C), suggesting that dgd1 plants are as responsive to SA as WT plants. Together, these results suggested that although DGDG is required for pathogen-triggered SA accumulation, the SAR defect in dgd1 could not be entirely attributed to their inability to accumulate SA.

**The dgd1 Mutant Accumulates Normal JA Levels**

Since dgd1 plants are also impaired in photosynthesis (Klaus et al., 2002), it was possible that the defective SA accumulation was the consequence of a generalized defect in chloroplast (site of SA synthesis) biogenesis. We assessed this by monitoring pathogen-inducible JA accumulation in the dgd1 plants, since JA synthesis also begins in the chloroplasts. The dgd1 mutant plants accumulated JA in response to pathogen infection at levels comparable to those of WT plants (Figure 2E). We next compared total and free FA levels in WT and dgd1 plants, because FAs not only are synthesized de novo in the chloroplast but also serve as a precursor for JA synthesis. The dgd1 plants accumulated reduced levels of 16:3 FA (Figure S2D), which is consistent with their reduced DGDG pool (Figures S1B and S1C). In comparison, both dgd2 and dgd::DGDG1 showed WT-like 16:3 levels. Normal JA levels in dgd1 plants were consistent with their total as well as free 18:3 FA levels; like wild-type plants, the dgd1 plants showed increased accumulation of free 18:3 in response to pathogen infection (Figure 2F). Together, these results suggested that the defect in pathogen-induced SA accumulation of dgd1 plants was unlikely to be associated with a generalized defect in chloroplast function.

Since dgd1 showed impaired import of nuclear proteins into plastids (Chen and Li, 1998), it was possible that the reduced SA accumulation in these plants was associated with altered import of proteins involved in SA biosynthesis. We first assayed the transcript levels of nuclear-encoded genes that were induced in response to pathogen infection and contributed to SA or JA levels. Notably, unlike WT and mgd1 plants, dgd1 mutant plants showed significantly reduced induction of EDS5 and SId2, which are involved in transport or biosynthesis of SA, respectively (Figure 2G). Likewise, Pst avrRpt2-infected dgd1 plants showed no induction of EDS1 and PAD4 genes, which indirectly contribute to SA biosynthesis (Figure 2H). In comparison, the induced levels of JA pathway genes LOX3 and OPR3 in dgd1 plants were comparable to the WT plants (Figure 2G). Unlike dgd1, the mgd1 plants showed WT-like induction of EDS5 and SId2 genes and higher-than-WT induction of LOX3 and OPR3 (Figure 2G). Together, these results suggest that reduced SA levels in dgd1 plants are likely due to their inability to induce the transcription of genes involved in SA biosynthesis.

**The dgd1 and mgd1 Plants Are Compromised in Pathogen-Induced G3P and A2A Levels**

We next assayed if the SAR defect in dgd1 was associated with deficiencies in other chemical signals of the NO-derived branch of SAR (see Figure S1A). We first assayed G3P levels, since it acts downstream of NO, ROS, and A2A (Figure S1A; Chanda et al., 2011; Yu et al., 2013, Wang et al., 2014). As shown earlier (Chanda et al., 2011; Yu et al., 2013; Wang et al., 2014), avrRpt2-infected WT plants accumulated ~3- to 4-fold higher levels of G3P in their vascular exudates (Figure 3A). In comparison, pathogen-infected dgd1 plants did not accumulate higher-than-basal levels of G3P. This was also the case for mgd1 plants (Figure 3A). Together, these results suggested that the compromised SAR in mgd1 and dgd1 plants might be associated with
Figure 2. The dgd1 Mutant Is Impaired in Pathogen-Induced SA Accumulation, but Not JA

(A) Quantitative RT-PCR analysis showing relative expression levels of PR-1 in mock (MgCl₂-) and avrRpt2-inoculated local leaves from Col-0 (WT), mgd1, and dgd1 plants.

(B) SA and SAG levels in Col-0 and dgd1 plants inoculated with MgCl₂ or P. syringae expressing avrRpt2. FW indicates fresh weight.

(C) Local resistance response of Col-0 (WT), mgd1 and dgd1 plants to virulent (DC3000) and avirulent (avrRpt2) Pst bacteria (10⁴ colony-forming units ml⁻¹). NS indicates not significant.

(D) SAR response in Col-0 and dgd1 plants locally infiltrated with MgCl₂, Pst avrRpt2, or SA (500 μM). The virulent Pst DC3000 was inoculated 24 hr after local treatments.

(E) JA levels in petiole exudates collected from Col-0 and dgd1 plants infiltrated with MgCl₂ (EXMgCl₂) or Pst expressing avrRpt2 (EXavrRpt2).

(F) Free 18:3 levels in indicated genotypes 24 hr postinfiltration of MgCl₂ or avrRpt2.

(legend continued on next page)
their inability to accumulate G3P in response to pathogen infection. We tested this further by assaying SAR in G3P-treated mgd1 and dgd1 plants. Interestingly, exogenous G3P restored SAR in both mgd1 and dgd1 plants, suggesting that their compromised SAR was indeed associated with the inability to accumulate G3P and that these plants were defective in a step upstream of G3P (Figure 3B).

Previously, we showed that Aza acts upstream of G3P and confers SAR by increasing G3P levels (Yu et al., 2013). Therefore, we analyzed pathogen-responsive Aza levels in mgd1 and dgd1 plants. The avrRpt2-infected mgd1 and dgd1 plants accumulated significantly lower levels of Aza as compared to WT plants (Figure 3C). Given that Aza is derived from C18 unsaturated FAs containing a double bond at C9 (Zoeller et al., 2012; Yu et al., 2013).

(G) Quantitative RT-PCR analysis showing relative induction of indicated genes in avrRpt2 infected Col-0, mgd1, and dgd1 plants compared to MgCl2 (mock)-inoculated respective genotypes. Leaves were sampled 24 hr postinoculation.

(H) Quantitative RT-PCR analysis showing relative induction of EDS1 and PAD4 genes in avrRpt2 infected Col-0 and dgd1 plants compared to MgCl2 (mock)-inoculated respective genotypes. Leaves were sampled 24 hr postinoculation. All experiments were repeated at least two times with similar results. Error bars represent SD (n = 4). Asterisk denotes a significant difference (Student’s t test).
Figure 4. The dgd1 Mutant Is Impaired in Pathogen-Induced NO Accumulation

(A) Confocal micrographs showing pathogen-induced NO accumulation in indicated genotypes at 12 hr postinoculation. The leaves were infiltrated with MgCl₂ (mock) or avrRpt2 Pst, with or without c-PTIO (500 μM). Scale bar, 10 μm. 

(B) Confocal micrographs showing NO accumulation in indicated genotypes postinoculation with petiole exudates collected from MgCl₂ (EXMgCl₂) or avrRpt2 (EXavr)-infected Col-0, mgd1, or dgd1 plants. Scale bar, 10 μm. "R" indicates genotype that received the EX.

(C) Protein immunoblot showing NOA1 levels in total protein extracts from indicated genotypes. Ponceau-S staining of the immunoblot was used as the loading control. Equal amount of total proteins were also loaded for noa1, which accumulates reduced levels of RUBISCO (Mandal et al., 2012).

(legend continued on next page)
and because mgd1 and dgd1 plants accumulate normal levels of free 18:3 FA (Figure 2F), it was possible that these mutants are unable to hydrolyze the C9 double bond that is required for the conversion of FA to Aza. To test this, we monitored the in planta conversion of 14C-18:1 to Aza in mgd1 and dgd1 plants. We infiltrated 14C-labeled 18:1 (14C at C-1 position) into leaves of WT, mgd1, and dgd1 plants and analyzed the methylated leaf extracts by thin layer chromatography (TLC). The TLC analysis showed a band corresponding to 14C-Aza dimethyl ester (ME) (Figure 3D). This band was extracted from the TLC plates, demethylated to obtain the free acid form, and rerun on a new TLC plate (Figure 3E). The band corresponding to Aza control in the first TLC run comigrated with the Aza standard in the second TLC run (Figure 3E), thus arguing that mgd1 and dgd1 plants were not affected in conversion of C18 unsaturated FAs to Aza.

This did not, however, rule out a possible defect in the uptake and/or transport of Aza in mgd1 and dgd1 plants. To test this, we infiltrated WT, mgd1, and dgd1 leaves with 14C-Aza, prepared methylated leaf extracts at 24 hr postinfiltration, and analyzed them on TLC. As shown earlier (Yu et al., 2013), a large portion of the radiolabel was present as Aza in addition to several other minor derivatives in WT plants (Figure 3F). The pattern and relative levels of Aza and its derivatives in mgd1 and dgd1 plants were similar to WT plants (Figures 3F and S2E), suggesting that mutations in MGD1 or DGD1 did not alter the uptake or derivatization of Aza in the infiltrated leaves. Likewise, analysis of distal tissues of mgd1 and dgd1 mutants showed WT-like profile of Aza derivatives (Figure 3G) and WT-like levels of total 14C-Aza (Figure S2E). Together, these results suggest that mgd1 and dgd1 plants are not altered in the uptake or transport of Aza or conversion of C18 FA to Aza. Thus, reduced levels of Aza in mgd1 and dgd1 plants were likely associated with their reduced MGDG and DGDG levels, respectively. Notably, this was not associated with a reduced free FA pool; both mgd1 and dgd1 plants accumulated WT-like levels of free 18:3 after pathogen infection (Figure 2F).

Based on the above results, we expected that exogenous treatment with Aza would compensate for the reduced Aza and confer SAR in mgd1 and dgd1 plants. Aza was applied locally, and the response to virulent bacteria was monitored in distal tissues. As expected, Aza-induced SAR in the systemic tissues of WT plants (Figure 3H). Intriguingly, although Aza also induced SAR in mgd1 plants, it was unable to do so in dgd1 plants (Figure 3H). This, together with the fact that both mgd1 and dgd1 plants accumulated reduced levels of G3P and that G3P was able to confer SAR in both these mutants, suggested that DGD1-derived DGDG galactolipids were likely involved in Aza-induced accumulation of G3P. To address this, we first assayed Aza-mediated SAR in dgd1::DGD1 plants and found these to respond normally to Aza; the dgd1::DGD1 plants showed WT-like SAR to exogenous Aza (Figure 3H). Next, we evaluated Aza-mediated transcriptional upregulation of the G3P biosynthesis genes GLY1 and GLU1, which is associated with an Aza-mediated increase in G3P levels (Yu et al., 2013). Exogenous Aza application induced GLY1 and GLU1 expression in WT and mgd1 plants, though GLU1 transcript levels in mgd1 plants were consistently lower compared to those in WT plants (Figures 3I and 3J). Notably, exogenous Aza was unable to induce expression of GLY1 or GLU1 genes in dgd1 plants (Figures 3I and 3J), which correlated with the inability of Aza to confer SAR in dgd1 plants. To determine if dgd1 plants were able to respond to Aza, we analyzed the expression of AzaA-responsive gene AZI1 in these plants (Jung et al., 2009). AzaA-treated dgd1 plants showed high expression of AZI1 (Figure 3K), suggesting that dgd1 plants were able to sense Aza but unable to induce SAR in response to Aza. Together, these results suggest that the inability of Aza to induce GLY1/GLU1 expression and confer SAR on dgd1 mutant was likely associated with the reduced pool of DGDG galactolipids in dgd1 plants.

**DGDG Is Required for NO Biosynthesis and Accumulation**

We recently showed that NO and ROS act upstream of Aza to confer SAR (Wang et al., 2014). This and reduced accumulation of Aza in dgd1 and dgd1 mutants prompted us to analyze NO and ROS levels in these plants. Analysis of NO levels was carried out using the NO-sensitive dye 4-amino-5-methylamino-2,7-di-fluorofluorescein diacetate (DAF-FM DA). Confocal microscopy of Pst avrRpt2-infected leaves detected increased DAF-FM DA staining 24 hr postinoculation (detected as green fluorescence) of Col-0 leaves compared to mock-inoculated or NO scavenger carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide)-treated plants (Figure 4A). Unlike in WT plants, NO could not be detected in pathogen-infected dgd1 plants (Figure 4A). However, NO accumulation was restored in the dgd1::DGD1 complemented lines (Figure 4A), which were also SAR competent (Figures 1B and 3H). Interestingly, unlike dgd1, the pathogen infected mgd1 plants did accumulate NO (Figure 4A). To test whether NO accumulation was primarily associated with DGDG lipids, we evaluated NO levels in mgd1 dgd1 double-mutant plants. The double mutant, which shows dgd1-like morphology (Figure S3A), did not show detectable NO accumulation after pathogen infection like the dgd1 single mutant (Figure 4A). This suggested that dgd1 was epistatic to mgd1 and that DGDG was perhaps required for normal biosynthesis and/or accumulation of NO. To determine if the defect was associated with biosynthesis or accumulation, we assayed NO accumulation in dgd1 plants treated with the NO donors sodium nitroprusside (SNP) or 2-(N,N-diethylamino)-diazenolate-2-oxide (DETA-NONOate) or the nitrous oxide donor SULFO-NONOate (negative control) (Figure S3B). Notably, dgd1 plants showed WT-like NO staining after SNP or DETA-NONOate treatments, suggesting that dgd1 plants were likely affected in biosynthesis of NO, but not its accumulation.

(D) Electron spin resonance (ESR) spectrometry assay showing relative levels of 4-POBN adduct in local tissues of mock and avrRpt2 inoculated Col-0, mgd1, dgd1, and dgd1::DGD1 plants. The leaves were sampled at 12 hr postinoculation.

(E) ESR spectrometry spectra of carbon centered radical levels in mock- and avrRpt2-inoculated leaves of Col-0, mgd1, and dgd1 plants. For (A) and (B), at least six independent leaves were stained with DAF-FM DA and analyzed in three independent experiments with similar results. Experiments shown in (C)–(E) were repeated at least two times with similar results. Error bars in (D) represent SD (n = 3). Asterisk denotes a significant difference (Student’s t test).
Intriguingly, even though dgd1 plants were impaired in the biosynthesis of multiple SAR signals like NO, AzA, and G3P, the ExavrRpt2 from dgd1 plants were fully capable of inducing SAR on WT plants (Figure 1C). One possibility was that dgd1 ExavrRpt2 contained signals that act upstream of NO and can induce NO biosynthesis in WT plants. To test this, we evaluated NO levels in WT and dgd1 plants treated with ExmgD12avrRpt2 from WT and dgd1 plants. As an additional control, we also included mgd1 plants in this analysis. As predicted, WT ExavrRpt2 was able to induce NO biosynthesis in WT and mgd1 plants, but not dgd1 plants (Figure 4B). Likewise, mgd1 ExavrRpt2 induced NO accumulation in mgd1 and WT plants. Notably, dgd1 ExavrRpt2 induced NO biosynthesis in WT, but not dgd1 plants. Together, these results suggested that DGD1, and thereby DGDG lipids, are essential for NO synthesis, but not for generating the SAR signal(s). These results further suggest that such a signal(s) from dgd1 is able to activate NO synthesis, and thereby SAR, in a plant that contains normal DGDG levels. One plausible reason for impaired NO accumulation in dgd1 plants could be that it is affected in the plastidal import and/or stability of NOA1, which is an indirect major contributor of pathogen-responsive NO accumulation. However, comparison of NOA1 levels in WT, mgd1, dgd1, and dgd1::DGD1 plants did not detect significant differences in NOA1 levels in these plants (Figure 4C).

Since ROS act downstream of NO, we monitored ROS levels in the infected tissues of dgd1 and mgd1 mutants using electron spin resonance spectrometry (ESRS). ESRS using z-(4-pyridyl N-oxide)-N-tert-butylamine (POBN), which detects hydroxyl and carbon-centered radicals, detected increased accumulation of free radicals in the Pst avrRpt2-infected tissues of WT plants (Figures 4D and 4E). Quantification of POBN trapped free radicals in local tissues of dgd1 and mgd1 plants showed significantly higher basal levels, which did not increase further after Pst avrRpt2 inoculation (Figures 4D and 4E). In comparison, dgd1::DGD1 plants showed WT-like basal and pathogen-induced POBN trapped free radicals (Figure 4D). Higher basal ROS levels in dgd1 plants were unexpected, since they are unable to accumulate NO that acts upstream of ROS (Wang et al., 2014). To test if higher basal ROS in mgd1 and dgd1 plants were due to their impaired photosynthesis, we assayed their sensitivity to paraquat (methyl viologen), an agent that promotes the formation of ROS by inhibiting electron transport during photosynthesis. The plants were treated by placing a 10 μl droplet of 10 to 20 μM paraquat on individual leaves, and the lesion size was monitored 48 hr posttreatment. The dgd1 and mgd1 plants developed larger lesions, suggesting that they are defective in the regulation/sequestration of ROS derived from photosynthetic electron transport (Figures S3C and S3D). Consistent with this result, both mgd1 and dgd1 plants showed increased expression of antioxidant genes (Figure S3E). This result further suggests that increased expression of antioxidants genes in mgd1 and dgd1 plants could be responsible for scavenging the excess ROS induced upon pathogen infection. In contrast to paraquat, dgd1 and mgd1 plants showed WT-like sensitivity to exogenous H2O2 (Figures S3F), Thus, the impaired photosynthesis in mgd1 and dgd1 plants was likely responsible for their higher basal ROS levels.

Transgenic Expression of Bacterial Glucosyltransferase Rescues Morphological and FA Phenotype, but Not SAR Phenotype, of dgd1 Plants

To determine the importance of the galactose sugar moiety in DGDG for SAR, we evaluated the response of transgenic dgd1 plants expressing a glucosyltransferase (GT) gene from the photosynthetic bacteria Chloroflexus aurantiacus. The transgenic expression of GT introduces glucose instead of galactose on the MGDG, resulting in formation of α-glucose-β-galactose diacylglycerol (Hölzl et al., 2006; Figures 5A and S1B). Notably, WT-like morphology could be restored in transgenic dgd1 plants expressing either the bacterial GT (dgd1::GT) or native DGD1 (dgd1::DGD1) (Figure 5B; Hölzl et al., 2006). Furthermore, transgenic expression of either GT or DGD1 was able to normalize 16:3 levels in dgd1 plants (Figure S2D). Interestingly, unlike native DGD1, the bacterial GT was unable to restore SAR in dgd1 plants (Figure 5C). Pathogen-infected dgd1::GT plants accumulated nominal NO at levels significantly lower than those in pathogen-infected WT or dgd1::DGD1 plants (Figure 5D). Similarly, compared to WT or dgd1::DGD1 plants, pathogen-induced PR-1 expression was only slightly higher in dgd1::GT plants (Figure S3G). The small increase of NO in dgd1::GT plants was insufficient to increase their AzA levels; dgd1::DGD1 plants accumulated WT-like levels of AzA in response to pathogen infection, whereas both dgd1 and dgd1::GT plants contained much lower AzA levels (Figure 5E). Furthermore, reduced accumulation of AzA did not associate with free FA pool, since dgd1::GT plants accumulated WT-like levels of free 18:3 (Figure 2F). Thus, even though dgd1::GT plants did exhibit a nominal increase in NO levels and PR-1 expression in response to pathogen infection, this slight increase was clearly insufficient to confer SAR. To reconfirm this, we assayed SA-mediated SAR in dgd1::GT plants, since exogenous SA would require functional NO-ROS-AzA-G3P branch to confer SAR (Wang et al., 2014). As predicted, exogenous SA was unable to confer SAR on dgd1::GT plants (Figure 5F). Together, these results suggest that the α-galactose-β-galactose head group is essential for normal induction of the NO-ROS-AzA-G3P branch of the SAR pathway as well as AzA-conferred SAR.

DISCUSSION

Galactolipids are one of the most abundant classes of lipids in plants, constituting ~80% of total membrane lipids in Arabidopsis. Although Arabidopsis leaves contain a higher proportion of MGDG compared to DGDG, the total and relative levels of these two galactolipids can vary in other plants (Christensen, 2009). Notably, in addition to serving as important constituents of chloroplastic membranes, galactolipids have also been shown to have other unique functions and are often associated with the medicinal and nutritional properties of vegetable plants (Christensen, 2009). For example, MGDG containing 18:3/18:3 or 16:3/18:3 FAs isolated from spinach leaves has antitumor activity (Wang et al., 2002). In this regard, it is interesting to note that MGDG and DGDG play a very specific role in plant defense leading to the induction of SAR (Figure S4). This is based on the specific phenotypes of mgd1 and dgd1 plants; mgd1

1688 Cell Reports 9, 1681–1691, December 11, 2014 ©2014 The Authors
plants, which accumulate WT-like levels of DGDG, are impaired in pathogen-induced ROS and AzA accumulation, but not NO levels or induction of the SA signaling marker, PR-1. In comparison, dgd1 plants, which are drastically reduced in DGDG and contain WT levels of MGDG, are unable to accumulate NO or SA after pathogen infection. Thus, DGDG is required at an early step during SAR signaling and appears to link the NO- and SA-derived branches of this pathway.

The inability of dgd1 plants to accumulate SA after pathogen infection is associated with reduced induction of the nuclear genes SID2 and EDS5, which regulate SA biosynthesis or its export from the chloroplast, respectively. Notably, DGDG is also required for AzA-mediated induction of GLY1 and GLI1 genes. Consequently, exogenous AzA, which induced high expression of GLY1 and GLI1 genes and conferred SAR in mgd1 plants, was unable to do so in dgd1 plants. Thus, the SA pathway is regulated by chloroplastic DGDG levels (Figure S5A).

Figure 5. Transgenic Expression of Bacterial Glucosyltransferase Restores Morphology in dgd1 Plants but Not SAR Phenotype

(A) Chemical structures and enzymatic steps catalyzed by the bacterial glucosyltransferase (GT) and DGD1 enzymes. Glu and gal indicate glucose and galactose sugars, respectively.

(B) Typical morphological phenotype of 4-week-old plants.

(C) SAR response in indicated genotypes treated locally with MgCl2 (gray bars) or avrRpt2 Pst (black bars).

(D) Confocal micrographs showing pathogen-induced NO accumulation in indicated genotypes at 12 hr postinoculation. The leaves were infiltrated with MgCl2 (mock) or avrRpt2 Pst, and at least six independent leaves were stained with DAF-FM DA and analyzed in three independent experiments with similar results. Scale bar, 10 μm.

(E) AzA levels in mock- (MgCl2) or avrRpt2 P. syringae (Avr)-inoculated leaves of Col-0, mgd1, or dgd1 plants at 24 hr postinoculation.

(F) SAR response in Col-0 or dgd1 plants treated locally with water or 500 μM SA for 24 hr prior to inoculation of distal leaves with virulent Pst. All experiments were repeated at least two times with similar results. Error bars in (C), (E), and (F) represent SD (n = 3 or 4). Asterisk denotes a significant difference (Student’s t test).

DGDG, which is synthesized in the outer envelope of plastids (Froehlich et al., 2001), might be important for retrograde signaling between the chloroplast and nucleus, since it regulates the expression of nuclear genes. Conversely, it is possible that impaired SA biosynthesis in chloroplast might result in the feedback suppression of nuclear genes directly or indirectly responsible for maintaining the pathogen-induced SA pool. Regardless, these results show that pathogen-activated signaling resulting in induction of
possible that interaction involving this axial hydroxyl group of galactose confers a structural feature that allows DGDG to associate with membrane proteins, cofactors, or other FA/lipid molecules.

Interestingly, even though mgd1 and dgd1 plants were impaired in the biosynthesis of multiple SAR signals, the petiole exudates obtained from these plants were fully capable of inducing SAR on WT plants. This was especially true for dgd1 plants, which are impaired in the biosynthesis of all major signals associated with SAR. These results suggest that mgd1 and dgd1 are able to make the SAR signal(s) but unable to respond to them. Thus, MGDG and DGDG lipids could play a role in the perception of the SAR signals. An alternate possibility is that mgd1 and dgd1 plants are able to make SAR signal(s) that act upstream of the SA–NO branch point, which in turn are sufficient to initiate SAR in the presence of WT-like levels of MGDG and DGDG (Figure S4).

Clearly, both MGDG and DGDG are required to generate sufficient AzA and in turn induce SAR, since mgd1 (containing WT levels of DGDG) as well as dgd1 (containing WT levels of MGDG) plants are defective for SAR. It is possible that a total threshold level of these galactolipids is important to generate AzA sufficient to induce SAR. This is supported by the fact that both MGDG and DGDG lipids are able to generate AzA and its precursor, 9-oxononanoic acid (ONA), when exposed to superoxide radical under in vitro conditions (Figure S5B). Notably, MGDG contained higher basal levels of ONA and its derivative might facilitate the induction of AzA synthesis in vitro conditions (Figure S5B; Yu et al., 2013). These results suggest that pathogen-inducible AzA synthesis might occur via the oxidation of C18 FAs on MGDG and DGDG lipids rather than free FAs and that an AzA-DGDG derivative might facilitate the induction of GLY1 and GL11 genes. Indeed, AzA functions upstream of G3P, and both mgd1 and dgd1 plants are compromised in pathogen-responsive G3P accumulation. However, at this point, we are unable to rule out a role for free FA in AzA biosynthesis, since both mgd1 and dgd1 are impaired in pathogen-induced ROS accumulation, which can generate AzA from FAs under in vitro conditions (Figure S5B; Yu et al., 2013).

Consistent with the notion that SAR requires the concomitant activation of both the SA and NO/ROS branches, exogenous application of SA did not restore the defective SAR in dgd1 plants. In contrast, G3P application did confer SAR in dgd1 plants, likely because G3P-conferred SAR only requires basal levels of SA and dgd1 plants are not affected in basal SA levels. Notably, petiole exudates from both mgd1 and dgd1 plants were able to confer SAR on WT, though not on themselves, suggesting that these mutants are able to generate signal(s) upstream of galactolipid-mediated NO production (Figure S4). Identification of this signal(s) would help unravel the role that DGDG plays in pathogen-induced SA and NO accumulation.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions and Genotypes Used

Plants were grown in MTPS 144 Conviron walk-in chambers at 22°C, 65% relative humidity, and 14 hr photoperiod. These chambers were equipped with cool white fluorescent bulbs (Sylvania, F096/841/XP/ECO). The mgd1-1, dgd1-1, and transgenic plants expressing DGD1 or the bacterial enzyme glycosyltransferase have been described before (Jarvis et al., 2000; Hözl 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.

Detailed experimental procedures are included in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.069.

AUTHOR CONTRIBUTIONS

The bulk of the SAR experiments, expression analysis, G3P and AzA quantifications, and NO analysis were carried out by Q.-m.G. with help from Y.Y. and C.W. Lipid and fatty acid analyses were carried out by K.Y. with help from Q.-m.G. AzA transport assay was carried out by K.Y. EPR experiments were carried out by M.B.S. with help from Q.-m.G. N.D. estimated SA. P.K. and A.K. supervised the project and wrote the manuscript with help from all the authors.

ACKNOWLEDGMENTS

We thank Georg Hözl and Peter Dörmann for dgd1 complemented lines and dgd2 seeds, Henrik Aronsson for mgd1 seeds, John Johnson for help with gas chromatography, Andrew Gifford and Joanna Fowler for 14C-AzA, Joanne Holden for help with SA analysis, and Ludmila Lapchyk for technical help. We thank Christoph Benning for useful advice on galactolipid profiling and Peter Dörmann, David Wendehenne, Maelor Davies, Wolf-Dieter Reiter, and Meenakshi Upreti for useful suggestions. This work was supported by grants from National Science Foundation (IOS#0749731, MCB#0421914) and Kentucky Science and Engineering Foundation (2390-RDE-016).

Received: June 2, 2014
Revised: August 7, 2014
Accepted: October 30, 2014
Published: November 26, 2014

REFERENCES


Christensen, L.P. (2009). Galactolipids as potential health promoting compounds in vegetable foods. Recent Pat Food Nutr Agric 1, 50–58.


Mono- and Digalactosyl Diacyl Glycerol Lipids Function Nonredundantly to Regulate Systemic Acquired Resistance in Plants

Qing-ming Gao, Keshun Yu, Ye Xia, M.B. Shine, Caixia Wang, DuRoy Navarre, Aardra Kachroo, and Pradeep Kachroo
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 TRIlzol 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% H$_2$SO$_4$ 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 (1 µl) 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 FLUOVIEW 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 \textit{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 \textit{avr} bacteria \((10^7 \text{ CFU ml}^{-1})\) and 48 h later the systemic leaves were inoculated with \textit{vir} bacteria \((10^5 \text{ CFU ml}^{-1})\). 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 \textit{P. syringae} containing \textit{avrRpt2} \((10^6 \text{ CFU ml}^{-1})\). 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 \(\mu\text{g ml}^{-1}\) ampicillin. Exudates were collected over 48 h and infiltrated into healthy plants. Infiltrated leaves were harvested after two days for \textit{PR-1} gene expression studies. For SAR studies, \textit{vir} pathogen was inoculated in the distal leaves two days after infiltration of exudate.

Thin layer chromatography

For conversion of 18:1 to AzA, \(^{14}\text{C}-18:1\) \((20 \mu\text{M})\) was infiltrated into Col-0, \textit{mgd1} or \textit{dgd1} leaves, sampled 24 h post-infiltration, methylated with 3% \(\text{H}_2\text{SO}_4\) in methanol and ran on silica TLC plates using hexane: MTBE: acetic acid \((80: 20: 1\text{, by vol})\). 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 \(2\text{N NaOH (9:1 by vol)}\), refluxed at 80 °C for ~30 min, neutralized with 0.3 volumes of 1 M HCl and extracted with hexane:MTBE \((96:4\text{ by vol})\). 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 \(\mu\)Ci/ml, specific activity 16 mCi/mmol) was dissolved in 50 \(\mu\)l of ethanol and diluted to 1 ml with water. The resulting solution contained 62.5 \(\mu\)M 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% \(\text{H}_2\text{SO}_4\) in methanol and heated at 80 \(^\circ\)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 \(\mu\)l of chloroform:methanol:formic acid (20:10:1, by vol), vortexed vigorously for 5 min followed by addition of 300 \(\mu\)l of 0.2 M \(\text{H}_3\text{PO}_4\) 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 \(\mu\)l 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 \(\mu\)l 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 \(\mu\)g
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 H$_2$O$_2$. 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 arrowhead) 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.
**FIGURE 2**

**A**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Col-0</th>
<th>dgd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EX(_{MgCl2})</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>EX(_{avrRpt2})</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Col-0</th>
<th>dgd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex-Col-0</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>Ex-dgd1</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

**D**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Col-0</th>
<th>dgd1</th>
<th>dgd2</th>
<th>dgd1::DGD1</th>
<th>dgd1::GT</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>16:1(\Delta7)</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>16:2</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>16:3</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>18:0</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>18:1(\Delta9)</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>18:2</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>18:3</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

**E**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Col-0</th>
<th>mgd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>Distal</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Col-0</th>
<th>dgd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td>Distal</td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

**S. FIGURE 2**

Log (CFU/leaf disk)

Ex-MgCl2

Ex-MgCl2 + SA

Ex-avrRpt2

Ex-avrRpt2 + SA

DPM/g FW

NS

NS

*
**S. FIGURE 3**

**A**

<table>
<thead>
<tr>
<th>Col-0 DMSO</th>
<th>dgd1 SNP</th>
<th>mgd1 dgd1</th>
</tr>
</thead>
</table>

**B**

<table>
<thead>
<tr>
<th></th>
<th>DAF-FM DA</th>
<th>DS-RED2</th>
<th>Transmission</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 DMSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col-0 SNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dgd1 SNP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col-0 SULFO NONOate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col-0 DETA NONOate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dgd1 SULFO NONOate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dgd1 DETA NONOate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
S. FIGURE 3

C

Col-0  mgd1  dgd1

D

Lesion size (mm)

Col-0  mgd1

E

Relative Expression

SOD2  GSTU6  GSTU24

* 0.001

F

Lesion size (mm)

Col-0  dgd1  mgd1

G

Relative Expression (PR-1)

Mock  avrRpt2

Col-0  dgd1::GT  dgd1::DGD1

*
S. FIGURE 5

A

Pathogen

SID2

EDS5

SID2

EDS5

B

<table>
<thead>
<tr>
<th>Fatty acid (mol%)</th>
<th>9-ONA</th>
<th>AzA</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGDG</td>
<td>4.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Light</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>DGDG</td>
<td>3.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

FaYy acid (mol%)