A Feedback Regulatory Loop between G3P and Lipid Transfer Proteins DIR1 and AZI1 Mediates Azelaic-Acid-Induced Systemic Immunity

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A Feedback Regulatory Loop between G3P and Lipid Transfer Proteins DIR1 and AZI1 Mediates Azelaic-Acid-Induced Systemic Immunity

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SUMMARY

Systemic acquired resistance (SAR), a highly desirable form of plant defense, provides broad-spectrum immunity against diverse pathogens. The recent identification of seemingly unrelated chemical inducers of SAR warrants an investigation of their mutual interrelationships. We show that SAR induced by the dicarboxylic acid azelaic acid (AA) requires the phosphorylated sugar derivative glycerol-3-phosphate (G3P). Pathogen inoculation induced the release of free unsaturated fatty acids (FAs) and thereby triggered AA accumulation, because these FAs serve as precursors for AA. AA accumulation in turn increased the levels of G3P, which is required for AA-conferred SAR. The lipid transfer proteins DIR1 and AZI1, both of which are required for G3P- and AA-induced SAR, were essential for G3P accumulation. Conversely, reduced G3P resulted in decreased AZI1 and DIR1 transcription. Our results demonstrate that an intricate feedback regulatory loop among G3P, DIR1, and AZI1 regulates SAR and that AA functions upstream of G3P in this pathway.

INTRODUCTION

In plants, recognition of a pathogen by specific resistance (R) protein(s) often results in the generation of one or more signals that upon translocation to distal tissues activate broad-spectrum resistance throughout the plant. This form of resistance is commonly called systemic acquired resistance (SAR; Dempsey and Klessig, 2012; Spoel and Dong, 2012). Many chemical signals that contribute to SAR have been discovered, including the phytohormone salicylic acid (SA; Gaffney et al., 1993; reviewed in Vlot et al., 2009), its methylated derivative MeSA (Park et al., 2007), the diterpenoid dehydroabietinal (DA; Chaturvedi et al., 2012), the dicarboxylic acid azelaic acid (AA or AzA; Jung et al., 2009), auxin (Truman et al., 2010), the nonprotein amino acid pipecolic acid (Navarova et al., 2012), and the phosphorylated sugar derivative glycerol-3-phosphate (G3P; Chanda et al., 2011; Mandal et al., 2011). Jasmonic acid has also been suggested to participate in SAR (Truman et al., 2007), although its precise role remains contentious (Attaran et al., 2009). SAR is also dependent on components of the SA signaling pathway (reviewed in Dempsey and Klessig, 2012; Spoel and Dong, 2012), the lipid transfer protein (LTP) DIR1 (Defective in Induced Resistance; Maldonado et al., 2002; Champigny et al., 2011; Chanda et al., 2011), and the LTP-like protein AZI1 (AA insensitive; Jung et al., 2009). In addition, SAR is modulated by physical factors such as the plant cuticle (Xia et al., 2009, 2010, 2012) as well as environmental factors such as light (Griebel and Zeier, 2008; Liu et al., 2011a). The diverse chemical natures of the various SAR signals and the lack of studies targeted at examining their relationships have led to the growing belief that SAR involves the interplay of many diverse and possibly independent signals.

The fact that DIR1 is required for G3P-, AA-, and DA-mediated SAR suggests that these diverse chemical signals might converge into a common pathway. This is further supported by the fact that G3P-, AA-, and DA-mediated SAR is dependent on SA. A link among DIR1, G3P, and MeSA was proposed by Liu et al. (2011b) and also supported by our own observations, including the requirement of DIR1 for systemic movement of the G3P-derived signal and the systemic repression of MeSA biosynthetic genes in response to localized G3P application (Chanda et al., 2011). Although these results point toward the convergence of signaling derived from the various SAR inducers, the relationship among these seemingly unrelated chemicals, the precise point(s) of convergence, and the biochemical basis of the underlying signaling remain largely unclear. Here, we characterized the relationship between AA and G3P in inducing SAR. AA is thought to induce SAR by priming SA biosynthesis, but only when applied in advance of primary infection (Jung et al., 2009). In contrast, exogenous G3P confers SAR without inducing SA biosynthesis (Chanda et al., 2011). We show that AA induces G3P biosynthesis in the absence of pathogen infection and provides genetic, biochemical, and molecular evidence supporting...
an interconnection between AA- and G3P-induced SAR. We also demonstrate that 18 carbon (C) unsaturated fatty acids (FAs) containing a double bond on C9 can serve as the in planta precursors for AA, and provide a mechanistic insight into how AA, G3P, and two LTPs might function together during the onset of SAR.

RESULTS

Unsaturated FAs Serve as Precursors for AA
To define the relationship between AA and G3P, we first considered their biosynthetic pathways. Although G3P biosynthesis in plants has been well investigated (Chanda et al., 2011), the AA biosynthetic pathway(s) is unknown. The C9 dicarboxylic acid structure of AA suggested that it could be derived from the hydrolysis of C18 FAs carrying a double bond at C9 positions, such as oleic acid (18:1) and/or its desaturated derivatives, linoleic acid (18:2) and linolenic acid (18:3; Figure 1A). We tested this by infiltrating 1 mM 18:1 into leaves of wild-type (WT) plants (ecotype Col-0) and analyzing the AA levels in the local (injected) tissues. A significant increase in AA levels was seen in the 18:1-infiltrated leaves but not in control leaves (Figure 1B); AA levels increased at 12 and 24 hr posttreatment with 18:1 and returned to near-basal levels at 48 hr. Similarly, exogenous application of 18:2 or 18:3 also resulted in increased accumulation of AA (Figure S1A), suggesting 18:1, 18:2, and/or 18:3 can serve as precursors for AA. We confirmed this by infiltrating 14C-labeled 18:1 (14C at the C-1 position) into leaves of WT plants and analyzed the methylated leaf extracts by thin-layer chromatography (TLC). The TLC analysis showed a band corresponding to 14C-AA di-methyl ester (ME; Figure 1C) or cold AA-ME standards (Figure S1B). The band corresponding to 14C-AA-ME was further extracted from the TLC plates, demethylated to obtain the free acid form, and rerun on a new TLC plate (Figures 1D and S1C). Only the band corresponding to AA control (Figure S1C, denoted by asterisk) in the first TLC run comigrated with the AA standard in the second TLC run (Figures 1D and S1C). Similar results were obtained when leaves were infiltrated with 14C-18:2 (Figure 1D), thus confirming that unsaturated C18 FAs serve as precursors for AA. This, and the fact that exogenous AA induced SAR in Col-0 plants (Jung et al., 2009), suggested that exogenous application of 18:1 or 18:2 might mimic AA-induced SAR. We tested this by comparing resistance to Pseudomonas syringae pv tomato (Pst) in the distal tissues of WT (Col-0 ecotype) plants preinfiltrated with 18:1, 18:2, or Pst expressing avrRpt2. Localized pretreatment with either 18:1 or 18:2 was sufficient to confer immunity in the distal tissues and was comparable to the SAR induced in response to preinfection with avrRpt2 Pst (Figure 1E). Furthermore, SAR induced in response to 18:1 related well with FA-triggered AA accumulation; 18:1 conferred SAR only during the time frame of AA accumulation (within 24 hr of 18:1 application), and not at 48 hr postapplication, the time at which AA levels were down to basal levels (Figures 1B and 1E). The AA- or 18:1-infiltrated leaves showed WT-like ion leakage (Figure S1D) and PR-1 expression (Figure S1E), suggesting that neither AA nor 18:1 induces SAR by promoting cell death. Together, these data suggested that pathogen-induced AA likely depends on the availability of free C18 unsaturated FAs. To test this, we quantified free FA levels in mock- and avrRpt2 Pst-inoculated leaves of Col-0 plants. Indeed, pathogen-inoculation resulted in an increase in free 18:1, 18:2, and 18:3 (Figure 1F). We next assayed AA levels in fatty acid desaturase (fad) mutants that are unable to accumulate normal levels of 18:1 (ssz2), 18:2 (fad2), or 18:3 (fad3 and fad7 fad8) FAs (Kachroo and Kachroo, 2009). All of these mutants accumulated WT-like levels of AA in response to pathogen infection (Figure S1F), suggesting that 18:1, 18:2, or 18:3 can nonpreferentially serve as substrates for AA. These results are consistent with recent studies showing WT-like levels of AA in the fad3 fad7 fad8 triple mutant (Zoeller et al., 2012), and normal SAR seen in various fad single and double mutants (Xia et al., 2010).

Arabidopsis Mutants Impaired in G3P Biosynthesis Are Insensitive to AA
The observed 18:1 and AA connection was particularly interesting since 18:1 is intricately associated with another SAR inducer, G3P. G3P enters glycerolipid biosynthesis upon acylation with 18:1 in the plant chloroplast (Kunst et al., 1988; Kachroo et al., 2004). We considered the possibility that AA- and G3P-induced signaling was connected somehow in the SAR pathway. We tested this by examining G3P levels in 18:1- and 18:2-treated plants. Interestingly, exogenous application of 18:1 and 18:2 resulted in G3P accumulation (Figures 2A and S2A). This in turn was consistent with our result that 18:1 conferred SAR in WT but not in plants defective in the G3P biosynthesizing enzymes glycerol kinase (GLY1), GLY1/NHO1; Kang et al., 2003; Chanda et al., 2011) or G3P dehydrogenase (G3Pdh; GLY1; Miquel et al., 1998; Nandi et al., 2004; Kachroo et al., 2004; Chanda et al., 2011; Figure 2B). Furthermore, exogenous AA also significantly increased G3P levels in WT plants (Figure 2C) at 24 hr posttreatment. This associated nicely with the time point at which AA induced the most robust SAR in WT plants (Figure S2B). Importantly, AA was unable to induce a significant increase in G3P levels in gly1 and gli1 plants. AA-treated gli1 and gli1 plants showed no or a nominal increase in G3P levels, respectively (Figure 2C). The AA-induced increase in G3P levels was consistent with the transcriptional upregulation of GLI1 and GLY1 in AA-treated plants (Figure 2D). Together, these results suggested that AA likely conferred SAR in WT plants by inducing G3P biosynthesis. To test this, we evaluated AA-mediated SAR in gli1 and gli1 mutant plants. AA was applied locally and the response to virulent bacteria was monitored in distal tissues. As expected, AA-induced SAR in the systemic tissues of WT plants (Figure 2E). In contrast, AA was unable to induce SAR in the G3P biosynthetic mutants gly1 or gli1 (Figure 2E), indicating that AA-induced SAR required the G3P biosynthetic genes GLY1 and GLI1.

gli1 and gli1 Plants Are Not Impaired in Uptake or Transport of AA
A second possibility was that the compromised SAR in gli1 gli1 plants was due to a defect in the uptake and/or transport of AA. We tested this by first determining the time point at which AA was metabolized in infiltrated leaves and transported into distal leaves in WT (Col-0) plants. Leaves were infiltrated with 14C- AA, and methylated leaf extracts that were prepared 24–72 hr
Figure 1. Unsaturated C18 FAs Serve as AA Precursors

(A) Chemical structures (obtained from http://chemistry.about.com) of 18:1, 18:2, 18:3, and AA. Arrow indicates the position of the double bond (carbon 9), which is shared among 18:1, 18:2, and 18:3 FAs.

(B) AA levels (per gram FW) in Col-0 leaves 12, 24, and 48 hr after infiltration with 0.01% ethanol or 18:1. The values are presented as the average of four replicates. Asterisks denote significant differences compared with ethanol-treated (0.01%) plants (t test, p < 0.05) at the respective time points. Error bars indicate SD. Results are representative of three independent experiments.

(C and D) Autoradiographs of TLC plates, samples analyzed on silica TLC using hexane/MTBE/acetic acid (80:20:1, by vol for C; 65:35:1, by vol for D) solvent systems. Vertical arrows indicate the direction of the runs. Results are representative of three independent experiments.

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after AA infiltration were analyzed using TLC. Even though a large portion of the radiolabel was detected as AA, four other derivatives were also detected within 24–72 hr of infiltration (Figure 3A, bands 1–4). Analysis of distal tissues showed that only a small fraction of 14C-AA was transported (7%) and maximum transport took place within 24 hr of 14C-AA infiltration (Figures 3B and S2D). Interestingly, two prominent AA derivatives detected in the infiltrated leaves (marked 1 and 4) were not detected in the distal tissues (Figures 3A, 3B, 3D, 3E, and S2E). The bands corresponding to 1 and 4 were extracted, treated with ethanolic NaOH to remove modifications, and rerun on a fresh TLC plate. The ethanolic NaOH treatment of band 1 yielded a band that comigrated with free AA (Figure 3C), suggesting that band 1 represented a modified form of AA. The ethanolic NaOH treatment of band 4 did not yield a band corresponding to AA, suggesting that it was likely a derivative of AA (Figure 3C). Together, these analyses suggested that only a nominal fraction of AA was converted into various derivatives in planta, and a majority of the 14C-AA was retained as free AA (band marked by arrowhead, Figures 3A, 3B, 3D, and 3E). However, it was possible that AA released from membrane lipids was detected as free AA in the H2SO4-methanol extraction procedure used. Therefore, we extracted intact lipids from 14C-AA-infiltrated leaves and analyzed the presence of AA in these extracts. We found that only a small fraction of total AA was present in its free form. Most of the AA was likely present as a polar derivative (C) MEs of extracts from leaves infiltrated with 14C-18:1 were analyzed together with 14C-AA ME as the standard. 14C-containing products in leaves at 24 hr posttreatment are shown. (D) Comigration of 14C-18:1- and 14C-18:2-derived AA with 14C-AA standard. Products corresponding to 14C-AA in (C) were extracted, hydrolyzed, and analyzed together with 14C-AA standard. The 14C-18:2 used in this experiment had lower specific activity, which accounted for the reduced intensity of the 14C-18:2-derived AA signal. (E) SAR response in distal leaves of Col-0 plants treated locally with MES buffer or AA for 24 hr prior to inoculation of distal leaves with DC3000. The error bars represent SD. Asterisk denotes significant difference (t test, p < 0.001). Results are representative of three independent experiments. See also Figures S2A and S2B.
conjugate(s), since most of the \(^{14}\)C label was retained at the origin of the TLC run (Figure 3F, indicated by an arrow). Furthermore, we were unable to detect any free AA in the distal tissues even though much of the radiolabel in the distal leaf was also retained at the origin in the TLC run. To determine whether the radiolabel that was retained at the origin consisted of polar derivatives, we extracted this region and reran the constituents on a fresh TLC plate using a solvent system that allowed us to resolve polar lipids. The radiolabel that was retained at the origin in Figure 3F resolved into one major and several minor polar derivatives, one of which comigrated with phosphatidylcholine (Figure 3G). Together, these results suggested that AA is transported as one or more polar derivatives and/or is rapidly converted into these derivatives upon transport into distal tissues.

The positive association between AA and G3P also prompted us to test the in planta derivatization and movement of AA in plants treated with exogenous G3P. However, addition of G3P did not alter AA derivatization (Figures 3D and S2C) or its transport to distal leaves (Figures 3E and S2D). Likewise, inoculation with \textit{avrRpt2 Pst} did not alter transport of AA to distal leaves (Figure S2D, right panel). Next, we infiltrated \(^{14}\)C-AA in \textit{gly1} and \textit{gli1} leaves and analyzed the profile of AA in local and distal leaves. We profiled methylated esters because this procedure allowed us to analyze AA as a single band on TLC. The levels of \(^{14}\)C-AA and its derivatives in \textit{gly1} and \textit{gli1} plants were similar to that in Col-0 plants, suggesting that \textit{gly1} and \textit{gli1} plants are able to metabolize exogenous AA similarly to Col-0 plants (Figures 3D and S2C). Moreover, in most experiments, \textit{gly1} and \textit{gli1} plants also showed WT-like levels of \(^{14}\)C-AA in their distal...
tissues (Figures 3E, S2D, and S2E), suggesting that these plants were not affected in the transport of exogenously provided AA. These results, together with our earlier observation that exogenous G3P can restore SAR in gly1 and gli1 plants (Chanda et al., 2011), suggest that the compromised AA-inducible SAR in gly1 and gli1 plants is due to their inability to synthesize G3P.

**AA- and G3P-Mediated SAR Is Dependent on DIR1 and AZI1**

Previously it was shown that AA is unable to induce SAR in mutant plants lacking the LTPs AZI1 or DIR1 (Jung et al., 2009). Notably, G3P-induced SAR also requires DIR1 (Chanda et al., 2011). Therefore, it was reasonable to assume that G3P-mediated SAR might require AZI1 as well, particularly since AA activated SAR by inducing G3P accumulation. As shown earlier (Chanda et al., 2011), G3P alone induced marginal SAR in WT plants but triggered a more robust SAR response when infiltrated together with petiole exudates (Figures 4A and 4B). Additionally, WT plants infiltrated with G3P+avrRpt2Pst showed much stronger SAR compared with plants treated with avrRpt2Pst alone (Figure 4A). However, exogenous G3P induced only partial SAR on azi1 plants when infiltrated together with avrRpt2Pst (Figure 4A) or petiole exudates (Figure 4B). This suggested that, similarly to AA, G3P-mediated SAR was dependent on the AZI1 and DIR1 proteins, which likely functioned downstream of AA and G3P. To clarify this further, we quantified AA and G3P levels in azi1 and dir1 plants. The azi1 and dir1 plants accumulated WT levels of AA (Figure S3A) and showed WT patterns of AA and its derivatives in leaves infiltrated with 14C-AA (Figures 3D and S2C), as well as normal transport of 14C-AA into the distal tissues (Figures 3E, S2D, and S2E). Intriguingly, the azi1 and dir1 plants were compromised in pathogen-induced G3P levels (Figures 4C, S3B, and S3C). One possible explanation for this was that DIR1 and AZI1, directly or indirectly, regulate G3P biosynthesis. To test this, we evaluated GLY1 and GLI1 transcript levels in dir1 and azi1 plants. Interestingly, both basal and AA-responsive expression of GLY1 and GLI1 was significantly reduced in the dir1 and azi1 mutant plants compared with WT plants (Figure S3D). Although it is not uncommon (Jeong et al., 2010), pathogen inoculation did not mimic an elicitor-induced response; unlike AA, inoculation with avrRpt2Pst did not induce GLY1 or GLI1 expression (Table S1). This suggests that pathogen-induced G3P biosynthesis involves transient induction of GLY1 and GLI1 transcripts or may involve posttranscriptional regulation of their enzymatic activities. Nonetheless, these results and the dependence of G3P-mediated SAR on AZI1 and DIR1 suggested that AZI1 and DIR1 might function as a unit, alterations in which would be sufficient to disrupt G3P- and AA-mediated SAR, as well as AA-/avr-induced accumulation of G3P. We tested this by examining the interaction between AZI1 and DIR1 through bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation (co-IP) assays. Interestingly, DIR1 and AZI1 interacted with self and with each other (Figures 4D–4F and S4A), but not with GST or GFP (Figures S4A–S4C). Consistent with the interaction studies, a significant portion of the AZI1-GFP and DIR1-RFP proteins colocalized (Figures 4G and S5A). Furthermore, similarly to DIR1, AZI1 protein localized to the endoplasmic reticulum (Figure 4H) and plasmodesmata (Figures 4I and S5B; Chanda et al., 2011, Champigny et al., 2011). We determined the plasmodesmata localization by analyzing the colocalization of AZI1-GFP with the plasmodesmata-localizing movement protein (MP) 30 from tobacco mosaic virus (Figure 4I) as well as by staining plasmodesmata callose with aniline blue in AZI1-GFP expressing plants (Figure S5B). As expected, GFP alone was distributed uniformly throughout the cell (Figure S5C).

**G3P Regulates the Transcriptional Stability of AZI1 and DIR1 Genes**

The interaction between AZI1 and DIR1 proteins prompted us to investigate whether these proteins could compensate for each other when overexpressed. The 35S-AZI1-GFP and 35S-DIR1-GFP transgenic lines were generated in the Col-0 background and then analyzed for transgene expression (Figures S6A and S6B) and SAR phenotypes (Figure S6C). Two independent lines (lines 1 and 2) were analyzed per transgene and each showed WT-like SAR. Interestingly, overexpression of DIR1, but not AZI1, conferred a small but significant increase in basal resistance (Figure S6C). These results with DIR1 were consistent with earlier results from Maldonado et al. (2002), although these authors did not observe enhanced resistance in all DIR1-overexpressing lines. The DIR1-GFP- and AZI1-GFP-overexpressing lines were each crossed with azi1 and dir1 plants, and the F2 and F3 progeny were assayed for SAR response. Overexpression of AZI1-GFP and DIR1-GFP transgenes was able to complement the respective azi1 and dir1 mutations, suggesting that these fusion proteins were biologically functional (Figure 5A). Notably, overexpression of AZI1 and DIR1 genes also complemented the reciprocal mutation, i.e., overexpression of DIR1 complemented the azi1 mutant and overexpression of AZI1 complemented the dir1 mutation (Figure 5A). Furthermore, the DIR1:azi1 and AZI1:dir1 plants accumulated elevated levels of basal and pathogen-induced G3P as compared with the azi1 and dir1 mutant plants (Figure 5B). In contrast, overexpression of DIR1 or AZI1 did not alter the basal levels of SA or its stored form, SA glucoside (SAg), suggesting that the SAR in these plants was likely not due to changes in SA levels (Figures S6D and S6E). Together, these results suggested that overexpression of either DIR1 or AZI1 genes complemented the reciprocal mutation and, when overexpressed, DIR1 and AZI1 were functionally redundant for G3P accumulation.

These results also raised the possibility that the partial SAR induced in G3P-treated azi1 plants might be due to G3P-mediated increase in DIR1 levels (Figures 4A and 4B). To test whether G3P induced an increase in DIR1 levels, we generated transgenic Col-0 plants stably expressing Dir1-GFP under the Dir1 native promoter. Two independent lines were analyzed and the mock-treated pDir1-Dir1-GFP plants from both lines showed very low expression of Dir1-GFP protein, which was induced to high levels upon treatment with G3P (Figures 5C and S7A). Exogenous G3P did not increase Dir1 transcript levels (Figure S7B; Table S2), suggesting that G3P-induced accumulation of Dir1 involved posttranscriptional regulation. More importantly, local application of G3P induced Dir1 in distal leaves as well (Figures 5C and S7A). Intriguingly, in contrast to our results (Figures S7A–S7C), Champigny et al. (2011) showed that Ws
Figure 4. DIR1 and AZI1 Interact with Each Other and Regulate G3P Biosynthesis

(A and B) SAR response in Col-0 and azi1 plants. Asterisks denote significant difference from MgCl2-infiltrated plants for the respective genotypes (t test, p < 0.05) and the error bars represent SD. Results are representative of three independent experiments.

(A) Primary leaves were inoculated with MgCl2, avrRpt2, G3P (100 μM), or avrRpt2 + G3P, and the distal leaves were inoculated 24 hr later with a virulent strain of P. syringae (DC3000).

(B) Primary leaves were inoculated with MgCl2, avrRpt2, or petiole exudate (Ex) with or without G3P. The Ex was collected from mock (MgCl2)-inoculated Col-0 plants as described in Experimental Procedures. The distal leaves were inoculated 24 hr later with DC3000.

(C) G3P levels in petiole exudates of Col-0, dir1, and azi1 plants at 24 hr postinoculation with avrRpt2. Asterisk denotes significant difference (t test, p < 0.001). The error bars represent SD. The petiole exudates of dir1 and azi1 plants also showed basal levels of G3P at 6 hr postinoculation with avrRpt2.

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plants expressing DIR1-GUS under the DIR1 native promoter showed a higher level of basal GUS staining/activity, which was suppressed in pathogen-inoculated plants. The same study also showed that DIR1 transcript increased in avrRpt2 Pst-inoculated plants, and a more pronounced increase was seen in plants inoculated with Pst hrpS mutants that are impaired in...
were the reduced protein levels in the gly1 or azi1 backgrounds as detected by reduced fluorescence and protein levels in western analysis (Figures 5D versus the 14 hr photoperiod used in our study) might contribute to the discrepancies in DIR1 expression. Nonetheless, our results are consistent with overexpression studies and support the possibility that the G3P-triggered partial SAR in azi1 plants might be due to increased accumulation of the DIR1 protein.

To test whether SAR in DIR1- or AZI1-overexpressing plants was G3P dependent, we crossed gly1 and gil1 plants with the same DIR1-GFP- or AZI1-GFP-overexpressing plants that were used in crosses with dir1 and azi1. Interestingly, we noticed that the DIR1-GFP or AZI1-GFP proteins were significantly reduced in the gly1 or gil1 backgrounds as suggested by reduced fluorescence and protein levels in western analysis (Figures 5D and 5E). This was not the case in the dir1 or azi1 plants expressing AZI1-GFP or DIR1-GFP (Figure S7E). To determine whether the reduced protein levels in the gly1 and gil1 backgrounds were due to transcript instability, we analyzed DIR1-GFP and AZI1-GFP transcript levels in gly1 and gil1 backgrounds. Interestingly, RNA gel-blot and qRT-PCR analyses revealed that AZI1-GFP and DIR1-GFP transgenes were expressed poorly in the gly1 and gil1 backgrounds (Figures 5F and S7F), which was associated with the reduced fluorescence and protein levels observed in these transgenic plants (Figures 5D and 5E).

Together, these results suggest that G3P regulates the stability of the AZI1 and DIR1 transcripts.

**Effect of AA and G3P on Pathogenic Responsive SA Accumulation**

Earlier work suggested that AA confers SAR by priming for SA biosynthesis (Jung et al., 2009). This raised two possibilities: either AA application induced both SA and G3P accumulation or AA induced G3P accumulation, which in turn primed for SA biosynthesis. To test this, we assayed SA levels in WT plants that were pretreated with buffer, AA, or G3P, followed by inoculation with virulent pathogen (Figures S8A–S8C). Samples were collected 0, 6, 12, and 24 hr after pathogen inoculation and assayed for free SA levels as described previously (Jung et al., 2009). As expected, pathogen inoculation induced SA accumulation in 2-(N-morpholino)ethanesulfonic acid (MES)-, AA-, and G3P-pretreated plants (Figure S8A). Interestingly, and in contrast to the Jung et al. (2009) report, the pathogen-induced levels of SA in AA-pretreated plants were comparable to those of MES-pretreated plants. This was also the case for the G3P-pretreated plants. However, compared with the 0 time point, the percentage increase in SA levels at 6 or 12 hr after pathogen inoculation was noticeably higher in AA- and G3P-pretreated plants than in the MES-pretreated plants (Figure S8A). This was consistent with a small but significant increase in PR-1 expression in the AA- or G3P-pretreated plants at 12 hr after pathogen inoculation (Figure S8B). Intriguingly, this increase in PR-1 expression was detectable by qRT-PCR analysis but not by RNA gel-blot analysis, regardless of whether AA was suspended in MES buffer (Figure S8C, upper panel) or water (Figure S8C, lower panel). Together, these results suggest that AA and G3P might promote a slightly more rapid accumulation of SA in response to pathogen infection, although the biological relevance of this early induction needs further investigation.

**DISCUSSION**

Based on the above results, we conclude that AA application confers SAR by inducing G3P biosynthesis, and demonstrate a mechanistic link among unsaturated C18 FAs, AA, and G3P. Pathogen-induced release of free C18 FAs likely serves as the precursor for AA biosynthesis, which involves oxidative cleavage at the double bond on carbon 9. Recent results suggest that a single reaction rather than enzymatic reactions (Zoeller et al., 2012). However, WT-like AA levels in ssi2, cpr5, and ssi4 mutants that accumulate increased ROS levels (Jing et al., 2008; Zhou et al., 2008; Mandal et al., 2012; Figure S8D) suggest that other chemical and/or enzymatic mechanisms might also participate in AA biosynthesis.

The fact that only ~7% of AA is transported to the distal tissues suggests that either AA transport is not critical or transport of low levels of AA is sufficient for the induction/establishment of SAR. Notably, like G3P, AA is either transported as a derivative(s) or rapidly derivatized upon transport into distal tissues. However, unlike G3P, transport of AA does not appear to be associated with its de novo synthesis in the distal tissues. It is possible...
that transport of ~7% AA is responsible and sufficient for the induction of G3P in the distal tissues. Since pathogen inoculation does not increase AA levels in the distal leaves, the induction/establishment of SAR in distal leaves may not be associated with the release of free FAs.

We show that AA induces SAR by increasing G3P levels, which in turn is required for the transcriptional stability of DIR1 and AZI1 genes (Figure 6). This, together with the fact that DIR1 and AZI1 are required for pathogen-induced biosynthesis of G3P, suggests that G3P operates in a feedback loop with DIR1 and AZI1. Notably, a mutation in either the GLY1 or GLI1 gene is sufficient to compromise G3P biosynthesis and thus AA-induced SAR. This suggests that the activities of the plastidal enzyme GLY1 and the cytosolic enzyme GLI1 may be under feedback regulation from a common substrate and/or cofactor. Thus, a mutation in either enzyme (GLY1 or GLI1) would also impair the reaction catalyzed by the reciprocal protein. This notion is further supported by the fact that a mutation in the plastidal enzyme GLY1 results in reduced accumulation of the cytosolic proteins DIR1 and AZI1. Altogether, these results show that a feedback regulatory loop among G3P, DIR1, and AZI1 orchestrates the precise induction of SAR, and that the C18 FA-derived AA functions upstream of this loop.

**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 hr photoperiod. These chambers were equipped with cool white fluorescent bulbs (F096/841/XP/ECO; Sylvania, Beverly, MA, USA). The photon flux density (PFD) of the day period was 106.9 μmol m⁻² s⁻¹ (measured with a digital light meter; Phytotronics, Earth City, MO, USA). The gly1, gli1, and dir1 plants have been described elsewhere (Miquel et al., 1998; Maldonado et al., 2002; Kachroo et al., 2004; Chanda et al., 2008, 2011). The azi1-2 plants were isolated from the SALK_085727 line as described previously (Jung et al., 2009). The gly1, gli1, and dir1 plants to generate lines containing the transgenes and the respective mutations. For the pDIR1-DIR1-GFP construct, the DIR1 promoter (1 kb upstream of ATG that does not contain the 3'-UTR/terminator from the adjacent gene; Table S4) and open reading frame were amplified from the Col-0 genome and fused upstream of GFP or RFP in pSITE-2NA vector. The pSITE-DIR1-GFP/RFP and pSITE-AZI1-GFP/RFP constructs were introduced into Agrobacterium tumefaciens strain LBA4404. Agrobacterium strains carrying pSITE-DIR1-GFP/RFP, pSITE-AZI1-GFP/RFP, or 35S-GFP were infiltrated into Nicotiana benthamiana plants and 48 hr later, water-mounted sections of leaf tissue were examined by confocal microscopy. For colocalization studies, Agrobacterium strains carrying pSITE-30-GFP and AZI1-RFP constructs or pSITE-DIR1-GFP/RFP and pSITE-AZI1-GFP/RFP were infiltrated together. Transgenic Arabidopsis plants expressing 3SS-DIR1-GFP, 3SS-AZI1-GFP or pDIR1-DIR1-GFP were generated in the Col-0 background and at least two independent lines were analyzed in the T2 generation. These T2 plants expressing 3SS-DIR1-GFP, 3SS-AZI1-GFP were also crossed with dir1, azi1, gly1, and gli1 plants to generate lines containing the transgenes and the respective mutations. For the pDIR1-DIR1-GFP construct, the DIR1 promoter (1 kb upstream of ATG that does not contain the 3'-UTR/terminator from the adjacent gene; Table S4) and open reading frame were amplified from the Col-0 genome.
and cloned into a pGWB4 vector. Transgenic pDIR1-DIR1-GFP plants were generated in the Col-0 background.

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, Carlsbad, CA, USA) according to 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 qRT-PCR was carried out as described previously (Zhang et al., 2009; Table S4).

G3P, SA, FA, AA, and Free FA Quantifications
G3P quantifications were carried out as described previously (Chanda et al., 2008, 2011). SA and SAG were extracted and measured from ~0.3 g of fresh weight (FW) leaf tissue as described previously (Chandra-Shekara et al., 2006). FA and AA extraction was carried out as described previously (Xia et al., 2009; Chanda et al., 2011). Free FA analysis was based on previously published procedures (Hamilton and Comai, 1988; Norman et al., 2001).

Synthesis of Radiolabeled AA
14C-AA was synthesized as described previously (Best et al., 2012).

AA Precursor Analysis and TLC
Cold unsaturated FAs 18:1, 18:2 and 18:3 (1 mM each) were infiltrated into Col-0 leaves, sampled 12, 24, or 48 hr posttreatment, and processed for AA analysis as described above. For AA precursor analysis, 13C-18:1 (20 μM) was infiltrated into Col-0 leaves, sampled 12 and 24 hr postinfiltration, methylated with 3% H2SO4 in methanol, and run on silica TLC plates using hexane/methyl tertiary butyl ether (MTBE)/acetonic acid (80:20:1, by vol). For detection of AA dimethyl ester, TLC plates were sprayed with 50% H2SO4 and heated on a hotplate at 230°C for ~20 min. The TLC plates containing radiolabeled samples were autoradiographed using a Typhoon PhosphorImager (GE Healthcare, Piscataway, NJ, USA). The bands corresponding to AA dimethyl ester were scraped individually, hydrolyzed with ethanolic 2N NaOH (1:1 by vol), refluxed at 80°C for ~30 min, neutralized with 0.3 vol of 1M 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 AA. The lanes containing radiolabeled samples were autoradiographed and the lane containing AA was stained with 50% H2SO4 on a hotplate as described above.

1[14C]AA (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 AA. The labeled AA with or without 100 μM G3P was injected into the abaxial surface of 4-week-old Arabidopsis leaves. Three leaves per plant were infiltrated with ~0.2 ml of 13C-AF solution. The untreated leaves were individually covered with Saran wrap to avoid any splillow. The plants were then kept in a growth chamber set at 14 hr light and 10 hr dark photoperiods. The leaf samples were extracted using a lipid extraction (Bligh and Dyer, 1959) or an acid methylation method. For acid methylation, the leaf samples were added to 3 ml of 3% H2SO4 in methanol and heated at 80°C for 15 min followed by two extractions with hexane (2 ml each). The samples were quantified using a liquid scintillation counter and extracts containing same amount of 14C radioactivity were loaded onto silica gel 60. The TLC plates were exposed in a storage PhosphorImager screen (GE) and the bands were visualized with a Typhoon 9400 Variable Mode Imager (GE).

Bands from methylated samples were scraped off the TLC plates, eluted with MTBE, evaporated with a stream of nitrogen gas, and hydrolyzed with 1 ml of ethanol/2 N NaOH (9:1, by vol) at 80°C for 30 min in a 15 ml screw-capped test tube. The polar lipids from the chloroform extract were scraped off the TLC plate and eluted with 3 ml of methanol.

AA, FA, and G3P Treatments
AA, FA (18:1, 18:2, and 18:3), and G3P treatments were carried out by spraying (AA) or infiltrating (FA and G3P) 1 mM (AA and FA) or 100 μM (G3P) solutions. G3P was dissolved in water. FAs 18:1, 18:2, and 18:3 were dissolved in ethanol and diluted to 1 mM solution with water. AA was dissolved in MES buffer or methanol and the methanol stock of AA was diluted in water. AA prepared in methanol induced better SAR compared to AA dissolved in MES.

Conductivity Assays
Electrolyte leakage was measured in 4-week-old plants. Leaves were infiltrated with Pst avrRpt2 (107 colony-forming units [cfu]/ml), 18:1, or AA. At 0, 6, 12, and 24 hr posttreatment, five leaf discs per plant (7 mm) were removed with a cork borer, floated in distilled water for 50 min, and subsequently transferred to tubes containing 5 ml of distilled water. The conductivity of the solution was determined with a NiST traceable digital Conductivity Meter (Fisher Scientific, Waltham, MA, USA) at the indicated time points. The SD was calculated from four replicate measurements per genotype per experiment.

Pathogen Infection and Collection of Phloem Exudate
Inoculations with Pseudomonas syringae DC 3000 were conducted as described previously (Xia et al., 2009; Chanda et al., 2011). The bacterial cultures were grown overnight in King’s B medium containing rifampicin and/or kanamycin. For analysis of SAR, the primary leaves were inoculated with MgCl2 or avr bacteria (105 cfu/ml) and 24 hr later the systemic leaves were inoculated with vir bacteria (107 cfu/ml). Unless noted otherwise, samples from the systemic leaves were harvested at 3 dpi. Petiole exudates were collected as described earlier (Maldonado et al., 2002; Chanda et al., 2011).

Confocal Microscopy and BiFC Assays
For confocal imaging, samples were scanned on an Olympus FV1000 microscope (Olympus America, Melville, NY, USA) as described previously (Jeong et al., 2010; Chanda et al., 2011). Constructs were made using pSITE (Martin et al., 2009), pEarlyGate, or pGWB (Nakagawa et al., 2007) binary vectors using Gateway technology and introduced in A. tumefaciens strain LBA4404 or MP90 for agroinfiltration into N. benthamiana or Arabidopsis, respectively. BiFC assays were carried out as described previously (Jeong et al., 2010).

Protein Extraction and Immunoblot Analysis
Protein extraction and coimmunoprecipitations were carried out as described earlier (Jeong et al., 2010). Detailed experimental procedures are included in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, eight figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.03.030.

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