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Supporting Online Material

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Priming in Systemic Plant Immunity

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Plants possess inducible systemic defense responses when locally infected by pathogens. Bacterial infection results in the increased accumulation of the mobile metabolite azelaic acid, a nine-carbon dicarboxylic acid, in the vascular sap of *Arabidopsis* that confers local and systemic resistance against the pathogen *Pseudomonas syringae*. Azelaic acid primes plants to accumulate salicylic acid (SA), a known defense signal, upon infection. Mutation of the *AZELAIC ACID INDUCED 1 (AZI1)* gene, which is induced by azelaic acid, results in the specific loss of systemic immunity triggered by pathogen or azelaic acid and of the priming of SA induction in plants. Furthermore, the predicted secreted protein AZI1 is also important for generating vascular sap that confers disease resistance. Thus, azelaic acid and AZI1 are components of plant systemic immunity involved in priming defenses.

Whole plant immunity, called systemic acquired resistance (SAR), often develops after localized foliar infections by diverse pathogens. In this process, leaves distal to the localized infection become primed to activate a stronger defense response upon secondary infection (*1*). Leaves infected with SAR-inducing bacteria produce vascular sap, called petiole exudate, which confers disease resistance to previously unexposed (naïve) plants (*2*, *3*). This indicates that a mobile systemic signal(s) is involved in SAR (*4*). Although the hormone jasmonic acid (JA) accumulates to a high level in petiole exudates from leaves infected with SAR-inducing bacteria, JA does not seem to be the critical signal for SAR (*5*, *6*). Instead, SAR and the production of active exudates require the DIR1 protein, a predicted secreted protein and putative signal carrier in the lipid transfer protein family, and other proteins involved in glycerolipid biosynthesis (*2*, *3*, *7*). Additionally, SAR and exudate-induced resistance appears to require the phenolic metabolite salicylic acid (SA) (*3*, *8*) and possibly methylsalicylate (MeSA) and its methyl

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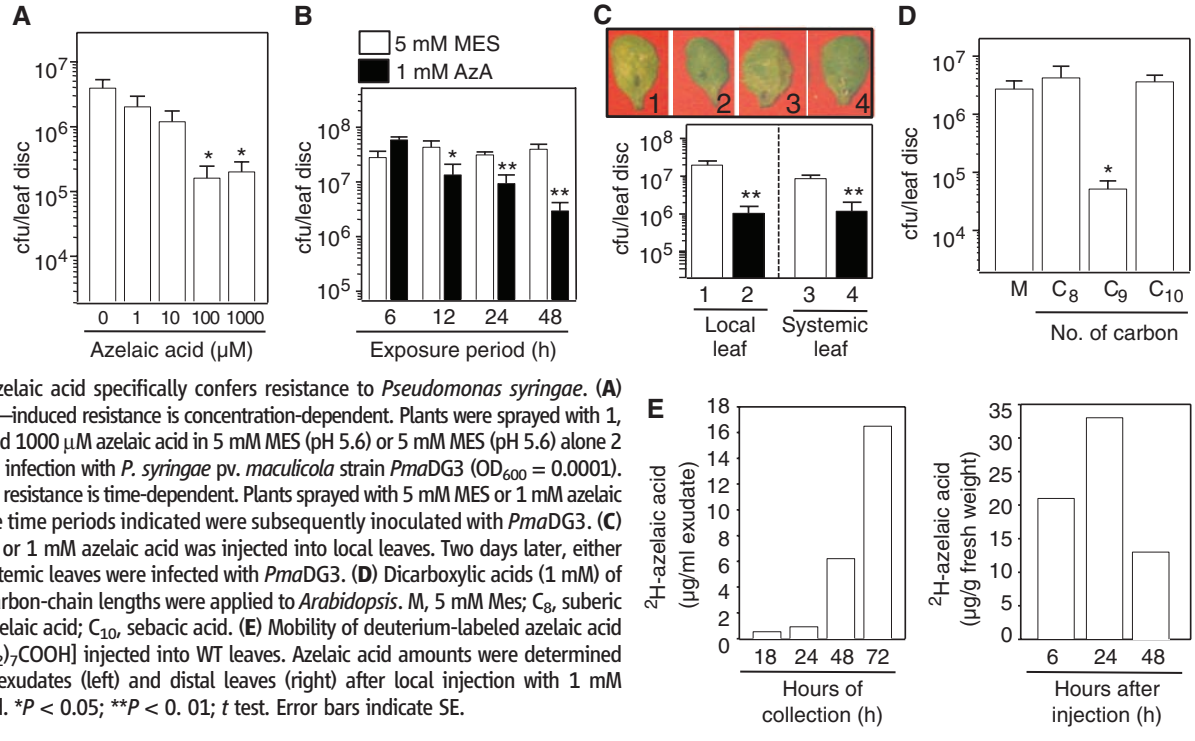

Fig. 1. Azelaic acid specifically confers resistance to *Pseudomonas syringae*. (**A**) Azelaic acid–induced resistance is concentration-dependent. Plants were sprayed with 1, 10, 100, and 1000 μM azelaic acid in 5 mM MES (pH 5.6) or 5 mM MES (pH 5.6) alone 2 days before infection with *P. syringae* pv. *maculicola* strain *Pma*DG3 (OD_{600} = 0.0001). (**B**) Induced resistance is time-dependent. Plants sprayed with 5 mM MES or 1 mM azelaic acid for the time periods indicated were subsequently inoculated with *Pma*DG3. (**C**) 5 mM MES or 1 mM azelaic acid was injected into local leaves. Two days later, either local or systemic leaves were infected with *Pma*DG3. (**D**) Dicarboxylic acids (1 mM) of different carbon-chain lengths were applied to *Arabidopsis*. M, 5 mM Mes; C_8, suberic acid; C_9, azelaic acid; C_{10}, sebacic acid. (**E**) Mobility of deuterium-labeled azelaic acid [$HOOC(CD_2)_7COOH$] injected into WT leaves. Azelaic acid amounts were determined in petiole exudates (left) and distal leaves (right) after local injection with 1 mM azelaic acid. *$P < 0.05$; **$P < 0.01$; *t* test. Error bars indicate SE.

esterase activity (which converts MeSA to SA) in distal leaves (*9*, *10*).

Effective establishment of SAR is not always correlated with elevated systemic SA accumulation, despite its necessary presence, before secondary infections (*11*). This finding implicates that there are additional signal(s) important for priming during SAR. We hypothesize that any such signal should: (i) show elevated levels in petiole exudates of tissue treated with a SAR-inducing pathogen, (ii) confer local and systemic disease resistance through a priming event, (iii) be mobile in plants, and (iv) act in a manner that depends on SA.

To identify this hypothetical signal, we obtained petiole exudates from SAR-induced plants that, unlike exudates from mock-treated plants, conferred disease resistance. We found that pathogen-induced exudates (Col-Pex), but not mock-induced exudates (Col-Mex), conferred resistance to a virulent *Pseudomonas syringae* strain (*Pma*DG3) (fig. S1). Importantly, the SAR-induced exudates did not induce disease resistance when applied to many SAR-defective mutants (fig. S2). Thus, these exudates were biologically active and induced disease resistance in a manner that requires many of the same genes important for SAR.

Because small molecules are often involved in plant signaling, we used gas chromatography coupled with mass spectrometry with electron impact ionization to scan for small molecules (70 to 550 dalton) enriched in the active (SAR-induced) versus inactive (mock-induced) exudates. In four independent experiments, the active exudates consistently had an average of 6.2-fold higher levels of the dicarboxylic acid azelaic acid ($C_9H_{16}O_4$) as compared with inactive exudates [5.1 μM (± 2.3 SEM) in mock-induced exudates versus 31.6 μM (± 10.0 SEM) in active exudates, $P = 0.042$, *t* test]. In vitro, azelaic acid showed weak antimicrobial activity against phytopathogenic bacteria but no activity against fungi (table S1).

To examine if azelaic acid induces disease resistance, we measured *Pma*DG3 growth after spraying plants with different concentrations of azelaic acid and found that more than 10 μM azelaic acid was required to confer disease resistance (Fig. 1A). Additionally, plants needed to be exposed to azelaic acid for at least 12 hours before infection, suggesting that azelaic acid does not directly inhibit *Pma*DG3 during infection (Fig. 1B). Local application of azelaic acid (1 mM) conferred both local and systemic resistance to *Pma*DG3 (Fig. 1C). The immunity-inducing properties of azelaic acid appeared to be specific, as the related C_8 and C_{10} dicarboxylic acids did not induce disease resistance (Fig. 1D). Furthermore, azelaic acid accumulated in distal systemic tissue as well as petiole exudates when it was locally applied to leaves, showing that it was mobile in plants when labeled with deuterium [$HOOC(CD_2)_7COOH$] (Fig. 1E and fig. S3).

To test which genes are necessary for azelaic acid–induced resistance, we monitored *Pma*DG3 growth in leaves of various mutants affecting SAR.

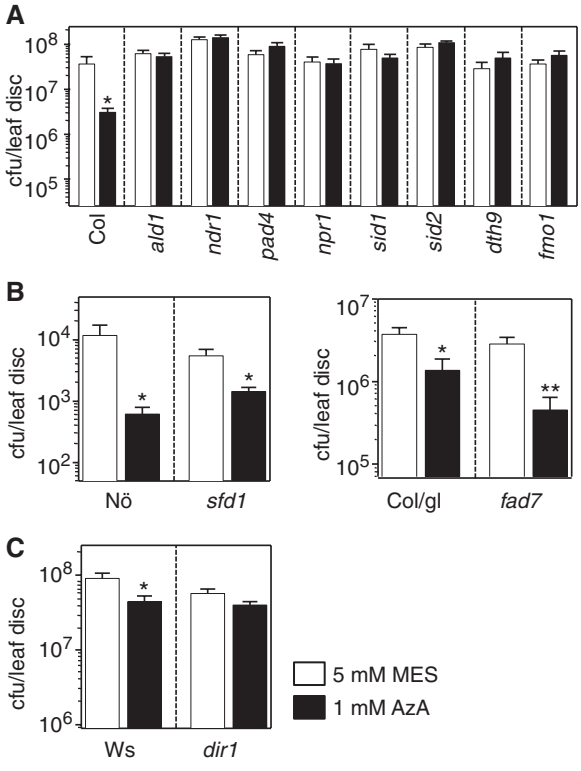

Fig. 2. Genes involved in azelaic acid–induced resistance. 1 mM azelaic acid in 5 mM MES and 5 mM MES alone were sprayed on to WT *Arabidopsis* accessions and the indicated SAR-defective SA pathway mutants (**A**) and glycerolipid mutants (**B** and **C**) 2 days before challenge inoculation with *Pma*DG3 (A and B) or *P. syringae* pv. *tomato* DC3000 (C). Bacterial growth in azelaic acid–treated plants should only be compared with those of the same genotype pre-treated with 5 mM MES, because different genotypes were grown separately. $*P < 0.05$; $**P < 0.01$; *t* test. Error bars indicate SE.

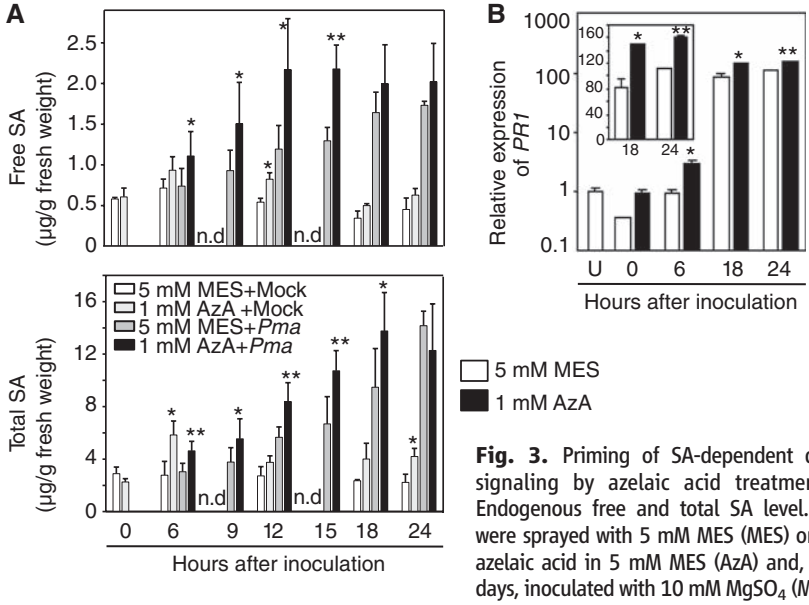

Fig. 3. Priming of SA-dependent defense signaling by azelaic acid treatment. (**A**) Endogenous free and total SA level. Plants were sprayed with 5 mM MES (MES) or 1 mM azelaic acid in 5 mM MES (AzA) and, after 2 days, inoculated with 10 mM $MgSO_4$ (Mock) or *Pma*DG3 (*Pma*). (**B**) Relative *PR1* expression in leaves treated as in (A). Expression of *PR1* is plotted on a log scale; *PR1* levels at 0 hours were not statistically different ($P = 0.065$). (Inset) Increased *PR1* levels in azelaic acid–treated plants at 18 and 24 hours after subsequent *Pma*DG3 infection. $*P < 0.05$; $**P < 0.001$; *t* test. Error bars indicate SD [number of samples analyzed per treatment (*n*) = 5 (A) or 3 (B)]. U, untreated; n.d., not determined.

We found that azelaic acid did not induce resistance in known SAR-defective SA pathway mutants (Fig. 2A) but did confer resistance to *sfd1* and *fad7* mutants (Fig. 2B), which lack an unidentified glycerolipid-requiring SAR signal (*3*, *7*). In contrast, the SAR-defective *dir1-1* mutant was insensitive to azelaic acid, suggesting that a DIR1-mediated signal is required for azelaic acid–induced resistance (Fig. 2C). The hormone mutants, *jar1* and *etr1*, that are not SAR-defective were responsive to azelaic acid (fig. S4).

Azelaic acid's effects may be to directly induce SA production. Although intact SA synthesis and signaling was required for azelaic acid–induced resistance, free and total SA levels were not significantly elevated up to 48 hours after treatment, compared with those of mock-treated plants (fig. S5). Alternatively, it may be that azelaic acid primes

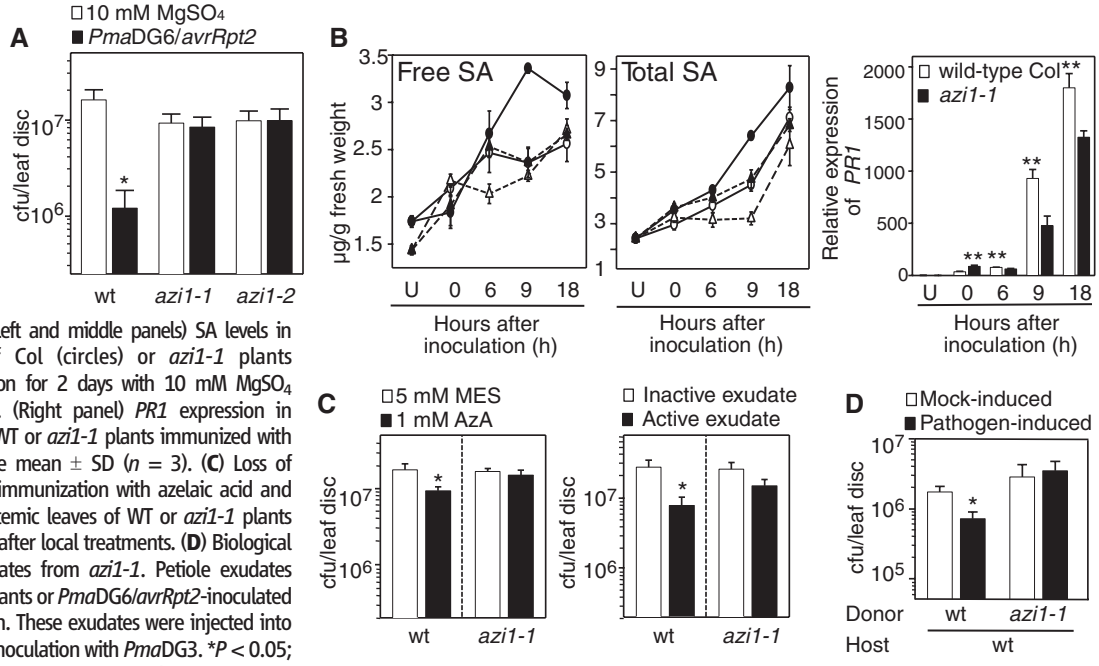

Fig. 4. Signaling defects of *azi1* mutants. (**A**) Loss of SAR as measured by *Pma*DG3 growth in immunized *azi1* plants. WT Col or *azi1* plants were immunized with 10 mM MgSO4 or *Pma*DG6/*avrRpt2* 2 days before secondary infection of distal systemic leaves with *Pma*DG3 and differences identified with a *t* test. (**B**) Reduced defense priming in distal systemic leaves of *Pma*DG6/*avrRpt2*-inoculated *azi1*. (Left and middle panels) SA levels in *Pma*DG3–infected distal leaves of Col (circles) or *azi1-1* plants (triangles) after local immunization for 2 days with 10 mM $MgSO_4$ (white) or *Pma*DG6/*avrRpt2* (black). (Right panel) *PR1* expression in *Pma*DG3–infected distal leaves of WT or *azi1-1* plants immunized with *Pma*DG6/*avrRpt2*. Data present the mean ± SD (n = 3). (**C**) Loss of systemic response of *azi1* to local immunization with azelaic acid and Col-Pex (active exudate). Distal systemic leaves of WT or *azi1-1* plants were infected with *Pma*DG3 2 days after local treatments. (**D**) Biological activity of pathogen-induced exudates from *azi1-1*. Petiole exudates were collected from mock-treated plants or *Pma*DG6/*avrRpt2*-inoculated plants for 72 hours after inoculation. These exudates were injected into WT plants 2 days before challenge inoculation with *Pma*DG3. *P < 0.05; **P < 0.001; *t* test. Error bars indicate SE. U, untreated.

plant cells to mount a faster and/or stronger defense response, including SA production, upon infection. Azelaic acid–treated plants that were subsequently infected had higher levels of both SA (Fig. 3A) and transcripts of the SA-associated signaling marker *PR1* compared with mock-treated plants (Fig. 3B). Thus, azelaic acid primes SA production upon infection upstream of both the SA-dependent SAR signaling pathway and the DIR1-dependent signal and downstream or independent of SFD1 and FAD7.

We examined possible effectors of azelaic acid with microarray analysis (*12*) but observed no statistically significant altered expression above twofold (table S2) of 464 defense-related genes. Thus, azelaic acid does not cause dramatic reprogramming of the plant transcriptome. However, *AZI1* (*AZELAIC ACID INDUCED 1*, At4g12470), which showed modest induction at 24 hours (1.8-fold, P = 0.13), was transiently and significantly induced at 3 to 6 hours by azelaic acid and active exudates (fig. S6). AZI1 encodes a predicted secreted protease inhibitor/seed storage/lipid transfer protein family protein, has no significant similarity to DIR1 (*2*), and confers disease resistance when overexpressed (*13*).

Two independent *azi1* mutants (SALK_017709 and SALK_085727, fig. S7) were found to be defective in SAR (Fig. 4A). However, *azi1* plants showed normal susceptibility to local infection with several strains of *P. syringae* (fig. S8). We observed that after local immunization with SAR-inducing bacteria, wild-type (WT) plants appeared to be primed to accumulate SA and *PR1* transcripts in distal leaves upon secondary infection (Fig. 4B). In contrast, *azi1* plants showed reduced priming during SAR (Fig. 4B), although they showed normal SA and *PR1* accumulation during local immunization (fig. S9).

SAR can be impaired because of a failure to recognize a defense/priming signal, generate the signal(s) in local infected-leaves, or translocate the signal(s) from local infected-leaves. To test if *azi1* plants fail to recognize a defense/priming signal(s), we infiltrated azelaic acid or active petiole exudate into leaves of *azi1* mutants. Two days later, we inoculated the same leaves with *Pma*DG3. *azi1* plants were resistant to subsequent local infection (fig. S10), indicating that *azi1* mutants still recognize defense/priming signal(s). To test whether AZI1 functions in long-distance signaling for systemic immunity, we examined the growth of *Pma*DG3 in systemic leaves of *azi1* plants in which azelaic acid or active petiole exudate (Col-Pex) was locally infiltrated. Azelaic acid and petioles exudates failed to induce systemic immunity in *azi1* plants, although these treatments protected WT plants against subsequent infection (Fig. 4C). Additionally, pathogen-induced exudates from *azi1* were inactive when applied to WT plants (Fig. 4D). Thus, AZI1 modulates production and/or translocation of a mobile signal(s) during SAR.

In summary, azelaic acid has the expected properties of a SAR component in that the molecule is mobile in plants, shows increased accumulation in biologically active exudates, confers pathogen resistance to local and systemic tissues, requires genes important for SA production and action to confer disease resistance, and primes SA accumulation and SA-associated gene expression. AZI1 appears to be induced by azelaic acid and regulates and/or directly translocates a SAR signal(s) from local infected tissues. The identification of previously unknown SAR components may be useful for plant protection and provides insight into how some interactions trigger systemic plant immunity.

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Supporting Online Material

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