

The *glabra1* Mutation Affects Cuticle Formation and Plant Responses to Microbes^{1[C][W][OA]}

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Systemic acquired resistance (SAR) is a form of defense that provides resistance against a broad spectrum of pathogens in plants. Previous work indicates a role for plastidial glycerolipid biosynthesis in SAR. Specifically, mutations in *FATTY ACID DESATURASE7* (*FAD7*), which lead to reduced trienoic fatty acid levels and compromised plastidial lipid biosynthesis, have been associated with defective SAR. We show that the defective SAR in *Arabidopsis* (*Arabidopsis thaliana*) *fad7-1* plants is not associated with a mutation in *FAD7* but rather with a second-site mutation in *GLABRA1* (*GL1*), a gene well known for its role in trichome formation. The compromised SAR in *gl1* plants is associated with impairment in their cuticles. Furthermore, mutations in two other components of trichome development, *GL3* and *TRANSPARENT TESTA GLABRA1*, also impaired cuticle development and SAR. This suggests an overlap in the biochemical pathways leading to cuticle and trichome development. Interestingly, exogenous application of gibberellic acid (GA) not only enhanced SAR in wild-type plants but also restored SAR in *gl1* plants. In contrast to GA, the defense phytohormones salicylic acid and jasmonic acid were unable to restore SAR in *gl1* plants. GA application increased levels of cuticular components but not trichome formation on *gl1* plants, thus implicating cuticle, but not trichomes, as an important component of SAR. Our findings question the prudence of using mutant backgrounds for genetic screens and underscore a need to reevaluate phenotypes previously studied in the *gl1* background.

Plants have evolved a large array of defense mechanisms to resist infection by pathogens. Upon recognition, the host plant initiates one or more signal transduction pathways that activate various plant defenses and thereby prevent pathogen colonization. In many cases, resistance is associated with increased expression of defense genes, including the pathogenesis-related (*PR*) genes and the accumulation of salicylic acid (SA) in the inoculated leaf. Induction of these responses is accompanied by localized cell death at the site of pathogen entry, which can often restrict the spread of pathogen to cells within and immediately surrounding the lesions. This phenomenon, known as the hypersensitive response, is one of the earliest visible manifestations of induced defense responses and resembles programmed cell death in animals (Dangl et al., 1996; Gray, 2002; Glazebrook, 2005; Kachroo and Kachroo, 2006). Concurrent with hyper-

sensitive response development, defense reactions are triggered in sites both local and distal from the primary infection. This phenomenon, known as systemic acquired resistance (SAR), is accompanied by a local and systemic increase in SA and jasmonic acid (JA) and a concomitant up-regulation of a large set of defense genes (Durrant and Dong, 2004; Truman et al., 2007; Vlot et al., 2009).

SAR involves the generation of a mobile signal in the primary leaves that, upon translocation to the distal tissues, activates defense responses resulting in broad-spectrum resistance. The production of the mobile signal takes place within 3 to 6 h of avirulent pathogen inoculation in the primary leaves (Smith-Becker et al., 1998), and the inoculated leaf must remain attached for at least 4 h after inoculation for immunity to be induced in the systemic tissues (Rasmussen et al., 1991). Mutations compromising SA synthesis or impairing SA, JA, or auxin signaling abolish SAR (Durrant and Dong, 2004; Truman et al., 2007, 2010). SAR is also dependent on the SALICYLIC ACID-BINDING PROTEIN2 (*SABP2*)-catalyzed conversion of methyl SA to SA in the distal tissues (Kumar and Klessig, 2003). Recent studies have suggested that methyl SA is the mobile signal required to initiate SAR in distal tissues in tobacco (*Nicotiana tabacum*; Park et al., 2007) and *Arabidopsis* (*Arabidopsis thaliana*; Liu et al., 2010), although another group reported a disparity in their findings related to the role of methyl SA in *Arabidopsis* (Attaran et al., 2009). Notably, the time point of requirement of *SABP2* activity (between 48 and 72 h post inoculation; Park et al., 2009) does not coincide with the early

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generation and/or translocation of the mobile signal into distal tissues (within 6 h post inoculation).

The mutations *acyl carrier protein4* (*acp4*), *long-chain acyl-CoA synthetase2* (*lacs2*), and *lacs9*, which are impaired in fatty acid (FA)/lipid flux (Schnurr et al., 2004; Xia et al., 2009), also compromise SAR (Xia et al., 2009). Detailed characterization has shown that the SAR defect in *acp4*, *lacs2*, and *lacs9* mutants correlates with their defective cuticles. Analysis of the SAR response in *acp4* plants has shown that these plants can generate the mobile signal required for inducing SAR but are unable to respond to it. It is likely that the defective cuticle in these plants impairs their ability to perceive the SAR signal, because mechanical abrasion of cuticles disrupts SAR in wild-type plants (Xia et al., 2009). This SAR-disruptive effect of cuticle abrasion is highly specific, because it does not alter local defenses and hinders SAR only during the time frame during which the mobile signal is translocated to distal tissues.

SAR is also compromised in plants that contain a mutation in glycerol-3-phosphate dehydrogenase (Nandi et al., 2004). The glycerol-3-phosphate dehydrogenase (*GLY1*) reduces dihydroxyacetone phosphate to generate glycerol-3-phosphate, an obligatory component and precursor for the biosynthesis of all plant glycerolipids. Consequently, a mutation in *GLY1* results in reduced carbon flux through the prokaryotic pathway of lipid biosynthesis, which leads to a reduction in the hexadecatrienoic (16:3) FAs (Miquel et al., 1998; Kachroo et al., 2004). Carbon flux and SAR are also impaired in plants containing mutations in *FATTY ACID DESATURASE7* (*FAD7*; Chaturvedi et al., 2008). The *FAD7* enzyme desaturates 16:2 and 18:2 FA species present on plastidial lipids to 16:3 and 18:3, respectively. Consequently, the *fad7* mutant plants accumulate significantly reduced levels of trienoic FAs (16:3 and 18:3). Compromised SAR in mutants affected in certain plastidial FA/lipid pathways has prompted the suggestion that plastidial FA/lipids participate in SAR (Chaturvedi et al., 2008). Such a tempting conclusion is also favored by the fact that SAR requires the *DIR1*-encoded nonspecific lipid transfer protein, which is required for the generation

and/or translocation of the mobile signal (Maldonado et al., 2002). In addition, azelaic acid, a dicarboxylic acid, was recently shown to prime SA biosynthesis and thereby SAR (Jung et al., 2009). The fact that azelaic acid is derived from oleic acid, a FA well known for its role in defense (Kachroo et al., 2003, 2004, 2005, 2007, 2008; Chandra-Shekhara et al., 2007; Jiang et al., 2009; Venugopal et al., 2009; Xia et al., 2009), further suggests that FA/lipids might participate in SAR.

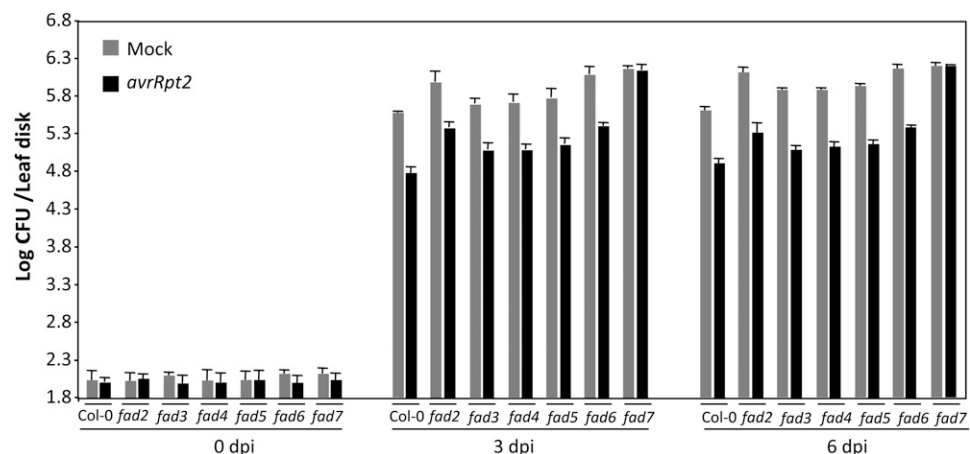
This study was undertaken to reexamine the role of the FA/lipid pathways in SAR and to determine the nature of the FA/lipid species mediating SAR in *fad7-1* plants. Our results show that impaired FA/lipid flux is not associated with compromised SAR in *fad7-1* plants but, rather, with an abnormal cuticle, which is the result of a nonallelic mutation in the *GLABRA1* (*GL1*) gene. Besides *GL1*, other mutations affecting trichome formation also compromised cuticle and thereby SAR. A compensatory effect of exogenous GA on *gl1* plants suggests that GA might participate in resistance to bacterial pathogens by restoring cuticle formation.

RESULTS

SAR Is Compromised in the *fad7-1* Plants But Not in Other *fad* Mutants

To determine which, if any, of the desaturated FA species contributed to SAR, we first evaluated SAR in all *fad* mutants that are defective in the desaturation of various FA species present on plastidial or extraplastidial membrane lipids (for the respective FAD-catalyzed activities, see Supplemental Fig. S1; for review, see Kachroo and Kachroo, 2009). The *fad2-1*, *fad3-2*, *fad4-1*, *fad5-1*, *fad6-1*, and *fad7-1* mutants were first inoculated with $MgCl_2$ or avirulent bacteria (*avrRpt2*) followed by a second inoculation with virulent bacteria on distal tissues at 48 h post primary inoculation. The growth of the virulent bacteria was monitored at 0, 3, and 6 d post inoculation (dpi; Fig. 1). As expected, $MgCl_2$ -infiltrated leaves of wild-type (ecotype Columbia [Col-0]) plants supported more growth of the secondary

Figure 1. SAR response in *fad* mutants. Primary leaves were inoculated with $MgCl_2$ (gray bars) or *P. syringae* expressing *avrRpt2* (black bars), and the systemic leaves were inoculated 48 h later with a virulent strain of *P. syringae*.



virulent pathogen than the plants that were pre-inoculated with the *avrRpt2* strain, indicating appropriate induction of SAR (Fig. 1). Similar to wild-type plants, the *fad2-1*, *fad3-2*, *fad4-1*, *fad5-1*, and *fad6-1* mutants also showed proper induction of SAR. In agreement with a previous report (Chaturvedi et al., 2008), the *fad7-1* mutant plants showed compromised SAR; plants infiltrated with $MgCl_2$ or *avrRpt2* bacteria were equally susceptible to the secondary virulent bacteria in the distal tissues. Together, these data suggested that the FAD7-catalyzed desaturation of 18:2 to 18:3 on plastidial membrane lipids might be important for the proper induction of SAR.

fad7-1 Plants Are Compromised in the Pathogen-Induced Accumulation of SA

Since SA plays a critical role in SAR, we first tested if the *fad7-1* mutant plants were competent in pathogen-

responsive accumulation of SA. SA levels in wild-type and *fad7-1* plants were determined before and after inoculation of *Pseudomonas syringae* containing *avrRpt2*. As expected, wild-type plants inoculated with avirulent pathogen showed a significant increase in both free SA and salicylic acid glucoside (SAG) in their primary as well as systemic tissues. Although the *fad7-1* plants also showed an increase in SA and SAG levels in the primary tissues, levels of SA/SAG were significantly lower in their distal leaves in comparison with wild-type plants (Fig. 2A). Thus, impaired SAR in *fad7-1* plants correlated with their inability to accumulate SA in the distal tissues. To confirm this, we tested the effect of exogenously supplied SA on SAR in *fad7-1* plants. The wild-type and *fad7-1* leaves were infiltrated with $MgCl_2$ (mock) or *avrRpt2* bacteria. Vascular exudates collected from these infiltrated leaves were mixed with water or the SA analog benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) and

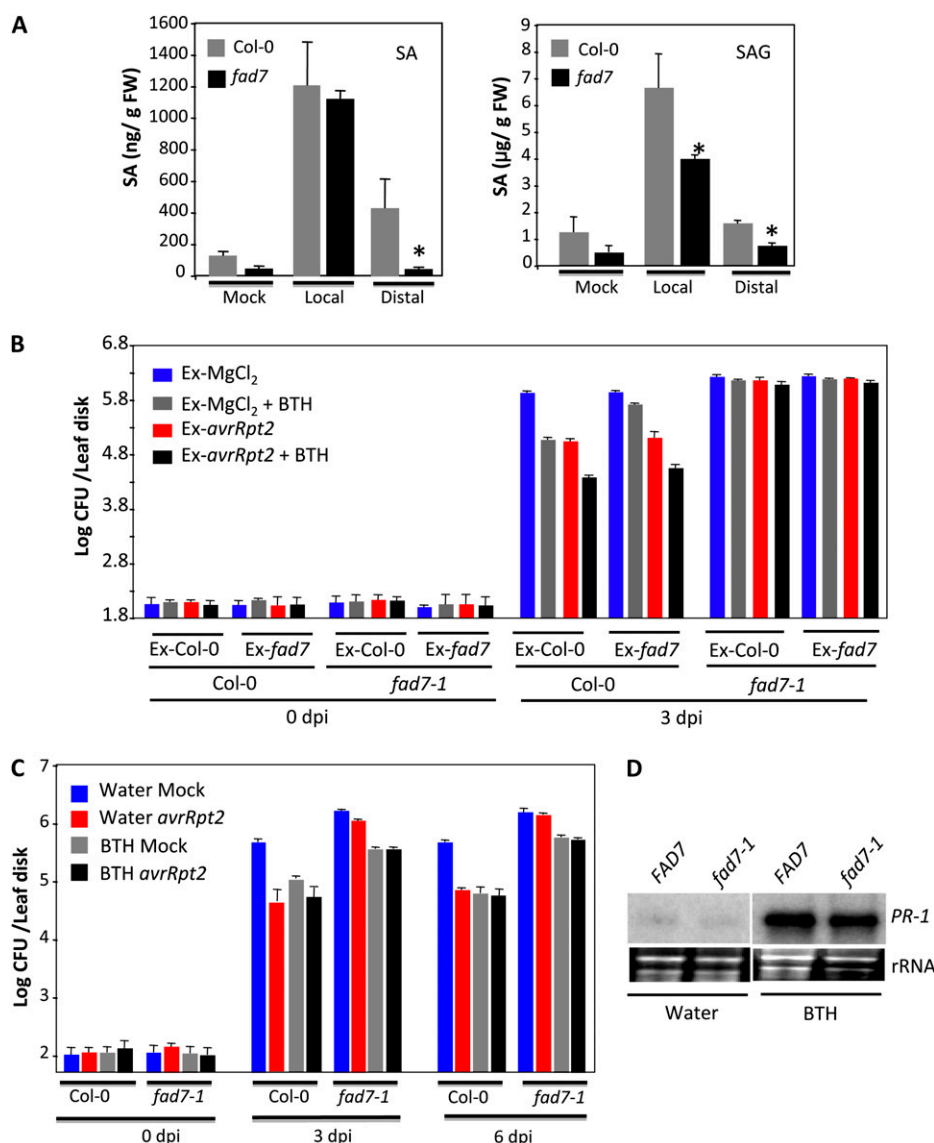


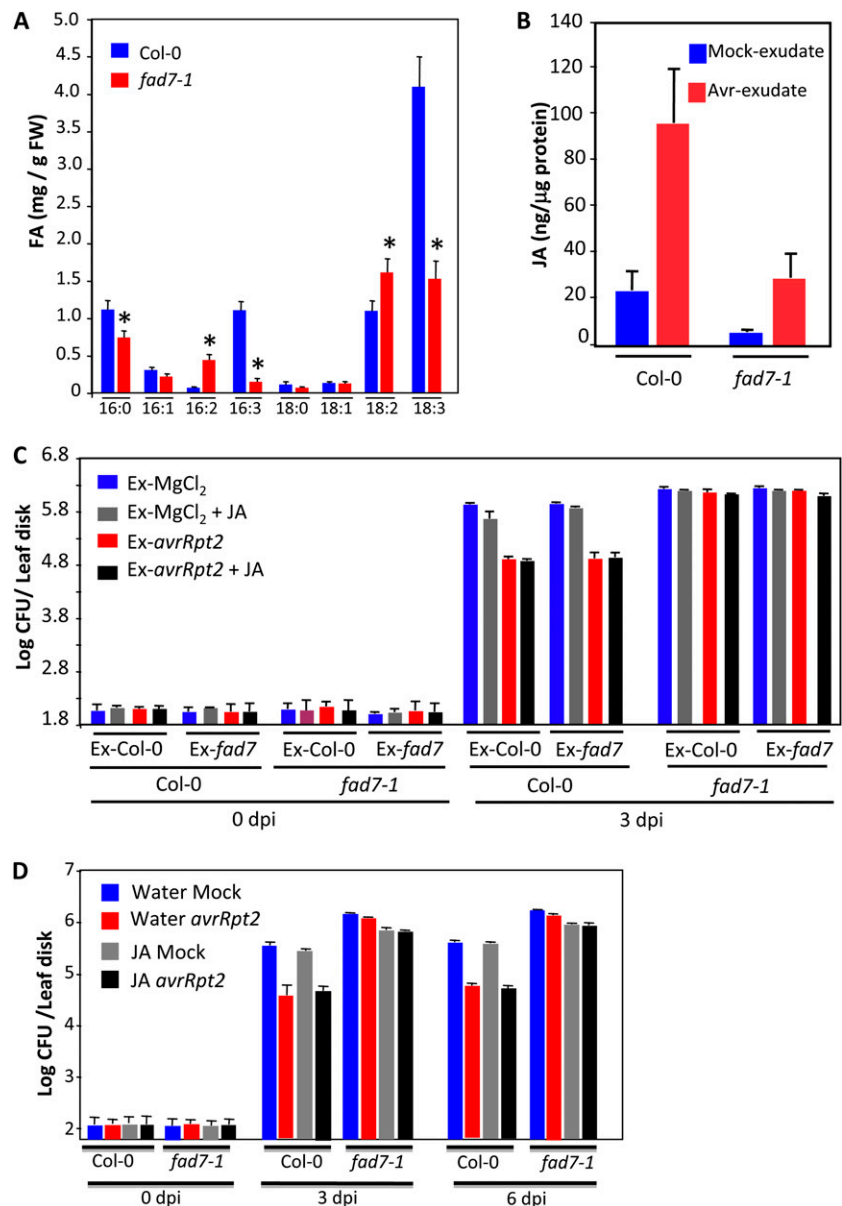
Figure 2. SA levels and pathogen response of Col-0 and *fad7-1* plants after exogenous application of BTH. **A**, SA and SAG levels in Col-0 and *fad7-1* plants inoculated with $MgCl_2$ or *P. syringae* expressing *avrRpt2*. FW, Fresh weight. **B**, SAR response in Col-0 and *fad7-1* plants infiltrated with exudates collected from wild-type or *fad7-1* plants that were treated either with $MgCl_2$ or *P. syringae* expressing *avrRpt2*. Exudates (Ex) were mixed with water or 100 μM BTH prior to infiltration into a fresh set of plants. **C**, SAR in Col-0 or *fad7-1* plants treated with water or BTH for 48 h prior to inoculation. **D**, RNA gel blot showing transcript levels of *PR-1* gene in plants treated with water or BTH for 48 h. [See online article for color version of this figure.]

injected into the leaves of a fresh set of wild-type and *fad7-1* plants. The distal leaves of this second set of plants were then inoculated with virulent bacteria, and proliferation of the virulent bacteria was monitored at 0 and 3 dpi (Fig. 2B). Notably, BTH-containing exudate from MgCl₂-infiltrated Col-0 plants conferred SAR on Col-0 plants but not on *fad7-1* plants. In comparison, the BTH-containing exudate from MgCl₂-infiltrated *fad7-1* was unable to confer SAR on either Col-0 or *fad7-1* plants. Interestingly, the exudate from *avrRpt2*-infiltrated *fad7-1* plants conferred SAR on Col-0 plants but not on *fad7-1* plants. The BTH-containing exudate from *avrRpt2*-infiltrated Col-0 or *fad7-1* plants produced better SAR on Col-0 but not on *fad7-1*, suggesting that exogenous BTH had an additive effect on SAR in Col-0. Together, these results suggested that exog-

enously supplied BTH was unable to restore the defective SAR in *fad7-1* plants.

To determine if pretreatment of the whole plant with BTH restored SAR in *fad7-1* plants, we treated Col-0 and *fad7-1* plants with BTH for two consecutive days followed by inoculation of primary leaves with MgCl₂ (mock) or *avrRpt2* bacteria. The virulent bacteria were then infiltrated into the distal leaves 48 h after primary infiltration (MgCl₂/*avrRpt2*), and growth of virulent bacteria was monitored at 3 and 6 dpi (Fig. 2C). Whole plant application of BTH conferred enhanced resistance to virulent pathogen in both wild-type and *fad7-1* plants. However, the BTH-treated *fad7-1* plants continued to support higher growth of virulent bacteria compared with BTH-treated Col-0, suggesting that BTH application on *fad7-1* enhanced resistance but not

Figure 3. FA and JA levels and pathogen response of Col-0 and *fad7-1* plants after exogenous application of JA. A, Levels of FAs in 4-week-old Col-0 and *fad7-1* leaves. The error bars represent sd. Asterisks denote a significant difference with Col-0 (*t* test, *P* < 0.05). FW, Fresh weight. B, JA levels in Col-0 and *fad7-1* plants inoculated with MgCl₂ or *P. syringae* expressing *avrRpt2*. C, SAR response in Col-0 and *fad7-1* plants infiltrated with exudates collected from wild-type or *fad7-1* plants that were treated either with MgCl₂ or *P. syringae* expressing *avrRpt2*. Exudates (Ex) were mixed with water or 50 μM JA prior to infiltration into a fresh set of plants. D, SAR in Col-0 or *fad7-1* plants treated with water or JA for 48 h prior to inoculation. [See online article for color version of this figure.]



to wild-type-like levels. To determine if this was due to a partial insensitivity to SA, we compared the levels of the SA-inducible marker, *PR-1*, in *fad7-1* plants. Similar levels of *PR-1* transcript were induced in response to BTH in both Col-0 and *fad7* plants (Fig. 2D), suggesting that *fad7-1* plants are able to induce SA-derived marker gene expression.

fad7-1 Plants Are Compromised in Pathogen-Induced Accumulation of JA

The FAD7 enzyme catalyzes the desaturation of 18:2 to 18:3 FA on membranous lipids, and a mutation in *FAD7* reduces trienoic FA levels in the plant (Fig. 3A).

Notably, the *fad7-1* plants accumulated reduced levels of basal and pathogen-induced JA (Fig. 3B), which correlated well with reduced levels of the JA precursor 16:3 and 18:3 FAs. To determine if reduced accumulation of JA was responsible for compromised SAR, we tested if exogenously supplied JA restored SAR in *fad7* plants. The wild-type and *fad7-1* leaves were infiltrated with MgCl₂ (mock) or *avrRpt2* bacteria. Vascular exudates collected from the MgCl₂- or *avrRpt2*-infiltrated leaves were mixed with water or JA and injected into the leaves of a fresh set of wild-type and *fad7-1* plants. The distal leaves of this second set of plants were then inoculated with virulent bacteria, and their proliferation was monitored at 0 and 3 dpi (Fig. 3C). Unlike

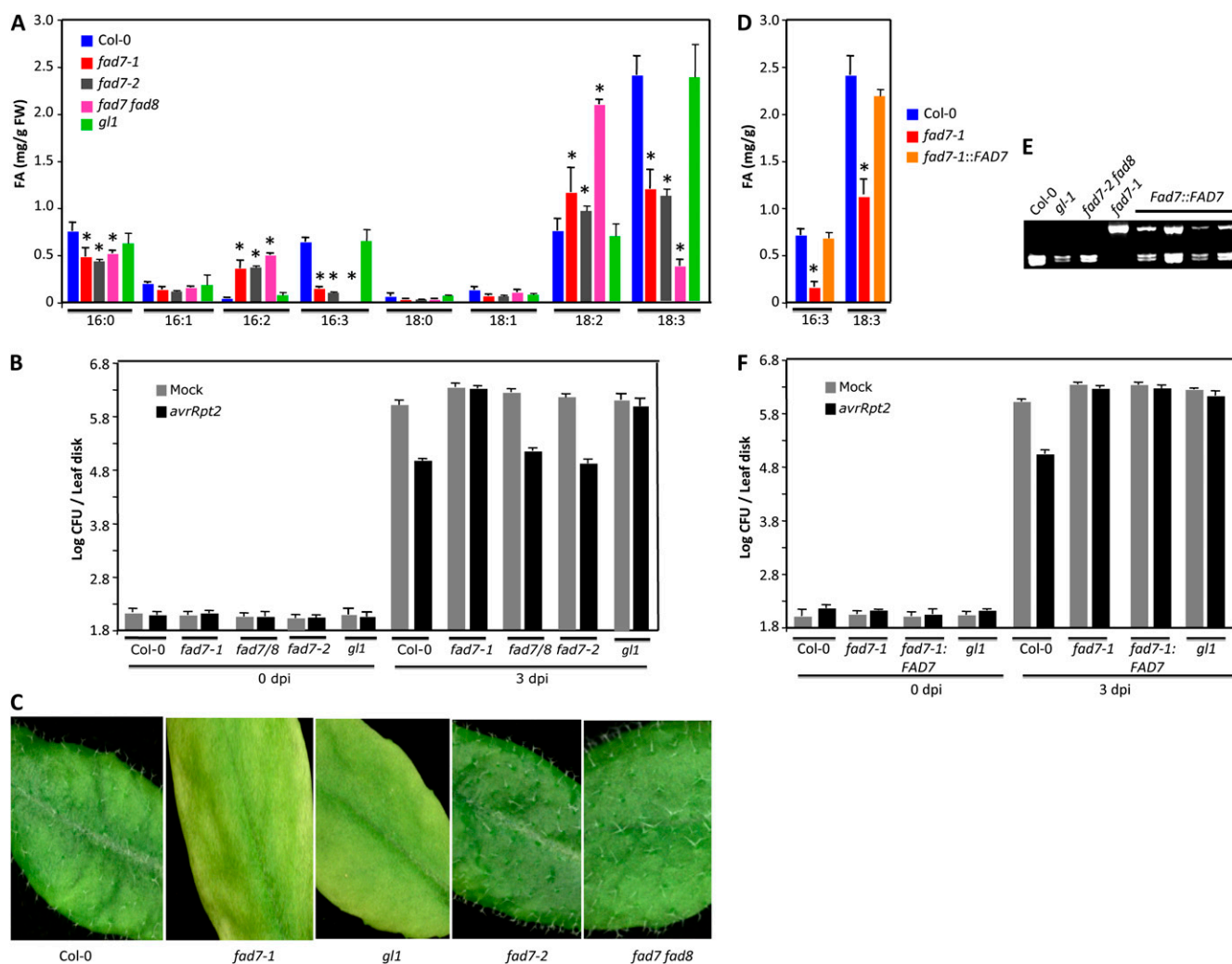


Figure 4. FA levels, SAR response, and trichome phenotypes in *fad7* and *fad7 fad8* plants. A, Levels of FAs in 4-week-old leaves. The error bars represent sd. Asterisks denote a significant difference with Col-0 (*t* test, $P < 0.05$). FW, Fresh weight. B, SAR response in the indicated genotypes. C, Leaves from the indicated genotypes showing the presence or absence of trichomes. D, Levels of FAs in the indicated genotypes. At least 10 different T2 transgenic plants expressing a genomic copy of *FAD7* in the *fad7-1* background were analyzed, and all showed a similar profile. The error bars represent sd. Asterisks denote a significant difference with Col-0 (*t* test, $P < 0.05$). E, Cleaved amplified polymorphic sequence analysis of the indicated genotypes for *fad7-1* mutation. F, SAR response in the indicated genotypes. Two independent transgenic lines were analyzed, and both showed compromised SAR.

BTH, addition of JA to exudates from MgCl_2 -infiltrated Col-0 or *fad7-1* plants did not reduce the growth of virulent bacteria. Furthermore, addition of JA to exudate from *avrRpt2*-infiltrated Col-0 plants also did not increase the potency of SAR in Col-0. Application of exudate on the *fad7-1* plants did not induce SAR, regardless of the source of the exudate (Col-0 or *fad7-1* plants). Together, these data suggested that the inability to accumulate JA likely did not contribute to the compromised SAR in *fad7-1* plants. This was further confirmed by whole plant application of JA (Fig. 3D). The Col-0 and *fad7-1* plants were treated with JA for 2 d followed by inoculation of primary leaves with MgCl_2 (mock) or *avrRpt2* bacteria. The virulent bacteria were inoculated on distal leaves 48 h after primary infiltrations, and their growth was monitored at 3 and 6 dpi. JA-treated *fad7-1* plants showed a marginal reduction in the growth of the virulent pathogen but they did not show SAR. Additionally, similar to results obtained in the exudate-related experiment (Fig. 3C), whole plant pretreatment with JA did not improve SAR in either mock- or *avrRpt2*-inoculated wild-type plants. Together, these results argue against reduced JA as the cause of defective SAR in *fad7-1* plants.

A *gl1* Mutation in the *fad7-1* Plants Is Responsible for Their Defective SAR

In addition to FAD7, the Arabidopsis FAD8 enzyme also catalyzes the desaturation of 18:2 to 18:3 on plastidial lipids. This is evident in the *fad7-2 fad8-1* double mutant plants, which are more severely reduced in their trienoic FA levels. To test if trienoic FAs contribute to SAR, we assayed SAR in the *fad7-2 fad8-1* double mutant plants. As expected, the *fad7-2 fad8-1* plants showed negligible levels of 16:3 and a significant reduction in 18:3 levels (Fig. 4A). Surprisingly, the *fad7-2 fad8-1* plants showed normal SAR (Fig. 4B). One possibility was that the *fad8* mutation restored SAR in the *fad7-2 fad8* double mutant. However, this was rather unlikely, given the fact that both FAD7 and FAD8 catalyze the same desaturation event (18:2–18:3). It was also not easily testable, since the *fad8* single mutation has no detectable effect on overall FA composition (McConn et al., 1994). Therefore, we retested the requirement of FAD7 in SAR induction by evaluating SAR in the *fad7-1* allelic mutant, *fad7-2*. Both *fad7-1* and *fad7-2* showed similar profile of FAs and resulted in a similar decrease in the levels of trienoic FAs (Fig. 4A). However, unlike *fad7-1*, the *fad7-2* plants showed normal SAR (Fig. 4B). Together, these data suggested that compromised SAR in *fad7-1* plants was not associated with their reduced trienoic FA levels or their altered FA/lipid profile.

A comparison of the genetic backgrounds used for isolating *fad7-1* and *fad7-2* mutants revealed that *fad7-1* was isolated in the *gl1* background whereas *fad7-2* was isolated in the wild-type background. The *GL1* gene encodes the R2R2-MYB transcription factor, which is required for trichome differentiation in Arabidopsis

(Oppenheimer et al., 1991). A mutation in the *GL1* gene results in a glabrous phenotype due to a lack of trichomes on leaves. Indeed, similar to leaves from *gl1* plants, *fad7-1 gl1* leaves were devoid of any trichomes (Fig. 4C). On the other hand, wild-type, *fad7-2*, and *fad7-2 fad8-1* leaves showed normal trichomes (Fig. 4C). Quantitative FA profiling showed a wild-type-like profile of FAs in *gl1* plants (Fig. 4A), suggesting that, unlike *fad7-1*, the *gl1* mutation was not associated with an altered FA profile. However, similar to *fad7-1*, the *gl1* plants were compromised in SAR (Fig. 4B). Together, these data suggested that the *gl1* mutation was responsible for compromised SAR in *fad7-1* plants.

To confirm this further, we expressed a genomic copy of the *FAD7* gene in *fad7-1 gl1* plants. All the T1 plants showed wild-type-like levels of trienoic acid but were still devoid of trichomes, suggesting that these transgenic plants were complemented for *fad7-1* but not for the *gl1* mutation. Three independent T1 lines were analyzed in the T2 generation, and these segregated for the *fad7-1* mutation in an approximately 3:1 wild-type-like:*fad7*-like manner; the *fad7-1* plants were scored based on their genotype as well as FA profile (Fig. 4, D and E). However, all the T2 plants lacked trichomes (Supplemental Fig. S2), further confirming that the *FAD7* transgene complemented only the *fad7-1* and not the *gl1* mutation. The *fad7:FAD7* transgenic plants showed compromised SAR, like *fad7-1* and *gl1* plants (Fig. 4F), further reiterating the fact that the *fad7-1* mutation was not responsible for compromised SAR.

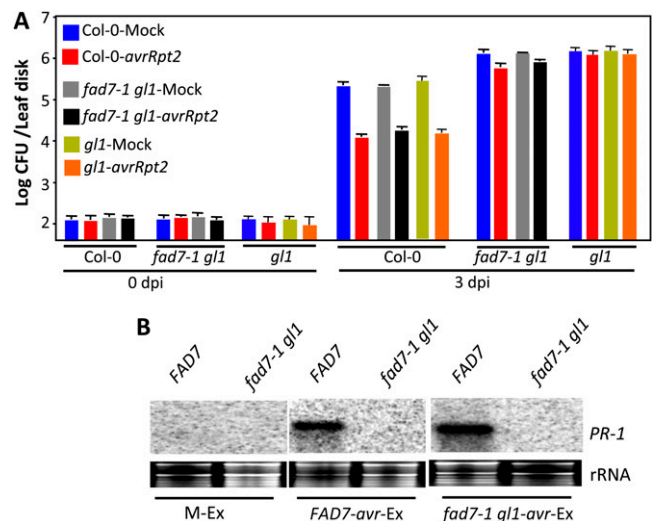


Figure 5. Responsiveness of *gl1* plants to petiole exudates collected from pathogen-inoculated plants. A, SAR response in Col-0, *fad7-1 gl1*, and *gl1* plants infiltrated with exudates collected from wild-type or *acp4* plants that were treated either with MgCl_2 or *avrRpt2*. B, RNA gel blot showing transcript levels of *PR-1* in Col-0 and *fad7-1 gl1* leaves infiltrated with petiole exudates (Ex). *PR-1* transcript levels were analyzed 48 h after treatments. M and *avr* indicate petiole exudates collected from leaves infiltrated with MgCl_2 or *P. syringae* containing *avrRpt2*. [See online article for color version of this figure.]

gl1 Plants Are Impaired in the Perception, But Not the Generation, of the Mobile Signal

We next assessed whether GL1 contributed to mobile signal production or perception in the SAR response. We evaluated the response of wild-type, *gl1*, and *fad7-1 gl1* plants to vascular exudates collected from pathogen-inoculated wild-type, *gl1*, or *fad7-1 gl1* leaves. The wild-type, *gl1*, or *fad7-1 gl1* leaves were infiltrated with $MgCl_2$ or *avrRpt2* bacteria, and vascular exudates collected from the inoculated leaves were injected into the leaves of a fresh set of wild-type, *gl1*, or *fad7-1 gl1* plants. Distal leaves of the exudate-infiltrated plants were then inoculated with virulent bacteria, and proliferation of virulent bacteria was monitored at 0 and 3 dpi (Fig. 5A). As expected, exudates from *avrRpt2*-infected wild-type plants conferred protection against virulent pathogen in wild-type plants. Similar to the wild type, exudates from *avrRpt2*-infected *gl1* or *fad7-1 gl1* plants also conferred resistance against virulent pathogen in wild-type plants. In comparison, the same exudates (wild-type, *gl1*, or *fad7-1 gl1* plants) conferred only marginal SAR

on *gl1* or *fad7-1 gl1* plants. These data also correlated with *PR-1* gene expression; the *fad7-1 gl1* plants showed basal levels of *PR-1* transcript in response to exudate application compared with wild-type plants (Fig. 5B). Together, these results suggested that *gl1* plants are competent in generating the mobile SAR signal(s) but are defective in its perception.

The *gl1* Plants Are Defective in Their Cuticle

The inability of the *fad7-1 gl1* plants to perceive SAR signal(s) was reminiscent of phenotypes associated with *acp4* plants (Fig. 5A; Xia et al., 2009), which are defective in cuticle formation. These similarities prompted us to investigate whether the *fad7-1 gl1* and *gl1* plants also contained defective cuticles, similar to *acp4* plants. The *fad2-1*, *fad3-2*, *fad4-1*, *fad5-1*, *fad6-1*, *fad7-2*, and *fad7-2 fad8-1* mutants were also included in this analysis. The known cuticle-impaired mutant *gpat4 gpat8* was included as a positive control (Fig. 6A; Li et al., 2007). Toluidine blue, a hydrophilic dye that only penetrates leaves with permeable cuticles

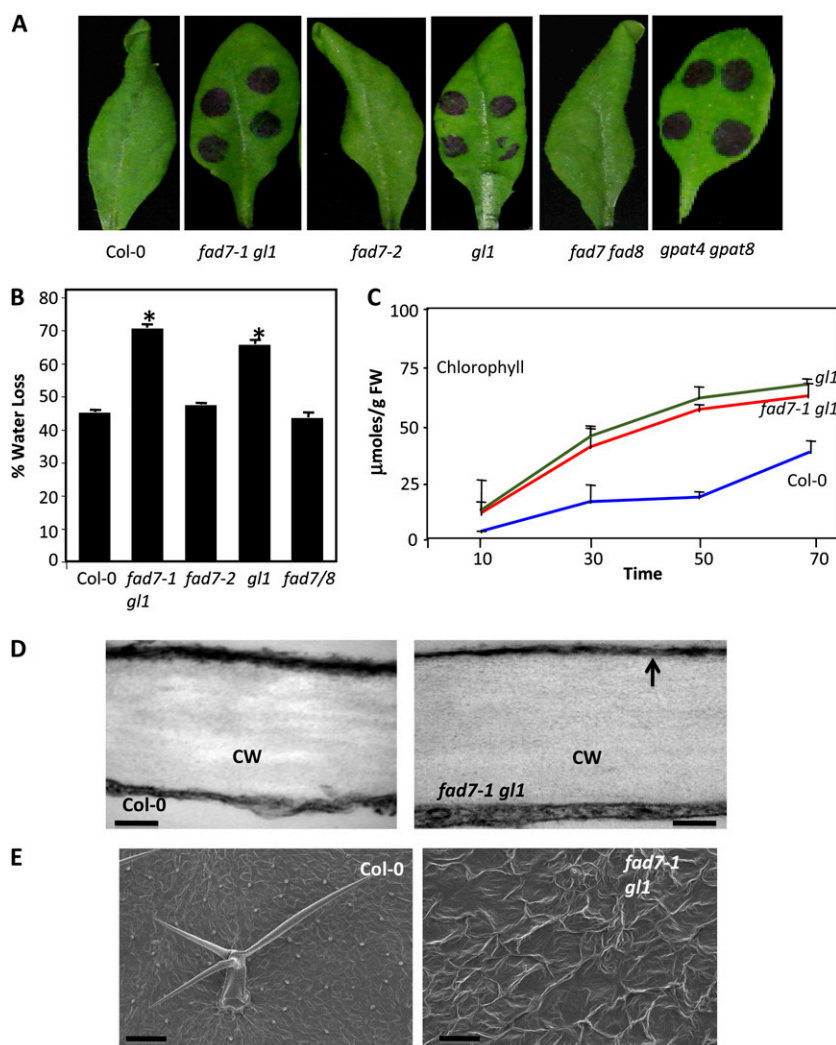


Figure 6. Evaluation of cuticle-associated phenotypes in *fad7-1 gl1* plants. A, Toluidine blue-stained leaves from 4-week-old plants of the indicated genotypes. B, Measurement of water lost from leaves subjected to drought conditions for 4 d. C, A time-course measurement of chlorophyll leaching in the indicated genotypes. FW, Fresh weight. D, Transmission electron micrographs showing the cuticle layer on the adaxial surface of leaves from the indicated genotypes. The arrow indicates electron-opaque regions. CW, Cell wall. Bars = 50 nm. E, Scanning electron micrographs showing adaxial surface of leaves from the indicated genotypes. Bars = 200 μm . [See online article for color version of this figure.]

(Tanaka et al., 2004), stained leaves in *fad7-1 gl1*, *gl1*, and *gpat4 gpat8* mutants. Furthermore, *fad7-1 gl1* and *gl1* also showed increased water loss (Fig. 6B) and chlorophyll leaching (Fig. 6C), further supporting their defective cuticle phenotype.

We also analyzed the outermost cell wall of the epidermis of *fad7-1 gl1* and *gl1* leaves by transmission electron microscopy (TEM). As expected, the cuticle of the wild-type leaf appeared as a continuous and regular electron-dense osmiophilic layer outside the cell wall (Fig. 6D). In comparison, *fad7-1 gl1* mutants showed electron-opaque cuticles (Fig. 6D, arrow). Comparison of the scanning electron micrograph (SEM) results of wild-type, *fad7-1 gl1*, and *gl1* leaf surfaces showed that the mutants each had an uneven surface, which was greatly folded compared with that of wild-type leaves (Fig. 6E; data not shown for *gl1*).

To determine if this defect in cuticle structure was associated with alterations in the content and/or composition of cuticular waxes or cutin polyester monomers, we compared levels of waxes and cutin monomers between wild-type, *fad7-1 gl1*, and *gl1* leaves (Supplemental Fig. S3). The *fad7-1 gl1* and *gl1* leaves showed reduction in several FAs, alkane and primary alcohol species, compared with wild-type plants (Supplemental Fig. S3A). The *gl1* leaves showed an approximately 32% overall decrease in cutin aliphatic monomer content. In the case of *gl1*, the decrease was more pronounced in two major monomers (16:0-, 18:1-, and 18:2-dicarboxylic acid and 16-OH-16:0 [Supplemental Fig. S3B]). Taken together, these results show that *GL1* is required for the biosynthesis of the cuticular wax and cutin polymers in leaves.

The *gl1* Plants Show Compromised Resistance to Fungal Pathogens

Since cuticle plays an important role in defense against fungal pathogens (Kurdyukov et al., 2006; Bessire et al., 2007; Chassot et al., 2007; Li et al., 2007; Mang et al., 2009), we next evaluated the response of *fad7-1 gl1* and *gl1* plants to the necrotrophic pathogen *Botrytis cinerea* and the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*. Interestingly, both *fad7-1 gl1* and *gl1* plants showed enhanced susceptibility to *B. cinerea* and *C. higginsianum*; spray and spot inoculations showed significantly larger lesions on *fad7-1 gl1* and *gl1* leaves (Fig. 7; Supplemental Fig. S4). Unlike *fad7-1 gl1* and *gl1* plants, the *fad7-2* and *fad7-2 fad8* plants showed wild-type-like response to *B. cinerea* and *C. higginsianum*, arguing against a role for trienoic FAs in resistance to necrotrophic or hemibiotrophic fungal pathogens.

Mutations in *GL3* and *TTG1* Impair the Cuticle and Compromise SAR

Since the *GL1* gene is involved in trichome formation, we next tested if trichome formation was generally associated with a defective cuticle and thereby

impaired SAR. The cuticular phenotypes were assessed in two known mutants, *gl3* and *transparent testa glabra1 (ttg1)*, which are affected in the differentiation of trichomes (Supplemental Fig. S5). The *GL3* and *TTG1* genes encode a basic helix-loop-helix protein and WD40 repeat protein, respectively. *GL3* and *TTG1* along with *GL1* are thought to form a combinatorial regulatory complex during trichome differentiation (Walker et al., 1999; Payne et al., 2000; Zhang et al., 2003; Morohashi et al., 2007). Indeed, similar to *gl1*, both *gl3-1* and *ttg1* mutants do not form any trichomes on their leaves (Supplemental Fig. S5). Interestingly, the *gl3* and *ttg1* leaves rapidly stained with toluidine blue (Fig. 8A) and also showed increased chlorophyll leaching (Fig. 8B). These results suggest that *gl3* and *ttg1* mutants contain a defective cuticle. Consistent with the proposed role for cuticle in SAR, both *gl3* and *ttg1* plants were unable to induce proper SAR (Fig. 8C).

GA application is known to stimulate trichome formation (Chien and Sussex, 1996; Perazza et al., 1998). Since our results indicated that trichome differ-

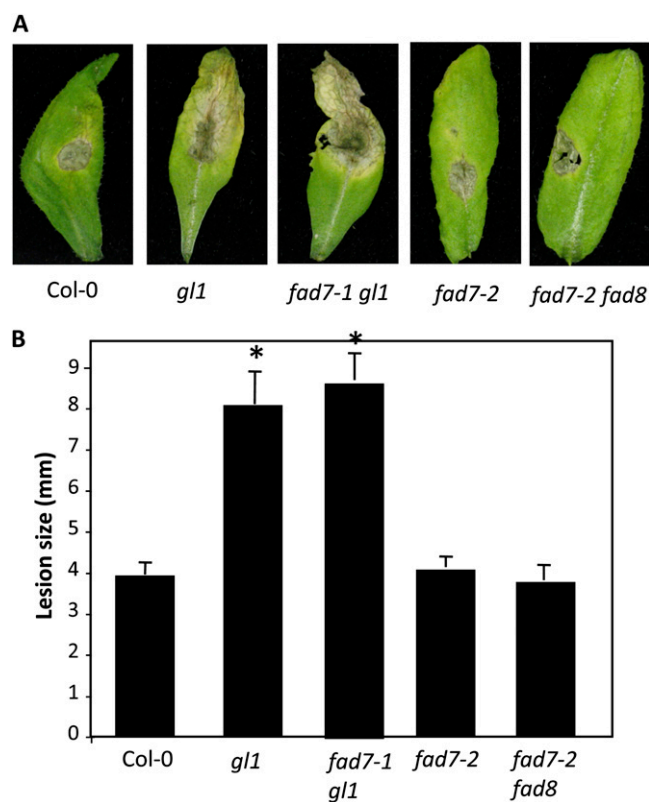


Figure 7. Response of *gl1* to the hemibiotrophic pathogen *C. higginsianum*. A, Disease symptoms on the indicated genotypes spot inoculated with 10^6 spores mL^{-1} *C. higginsianum*. B, Lesion size in spot-inoculated genotypes. The plants were spot inoculated with 10^6 spores mL^{-1} *C. higginsianum*, and the lesion size was measured from 20 to 30 independent leaves at 6 dpi. Statistical significance was determined using Student's *t* test. Asterisks indicate data significantly different from those of the control (Col-0; $P < 0.05$). Error bars indicate sd. [See online article for color version of this figure.]

entiation was associated with cuticle formation, we next tested whether GA can restore trichome and/or cuticle formation on *gl1* leaves. Exogenous application of GA to wild-type and *gl1* plants caused a significant enhancement in their growth (Supplemental Fig. S6A). Although GA application was unable to induce trichome formation on *gl1* leaves (Supplemental Fig. S6A), the GA-treated *gl1* plants showed a significant reduction in the toluidine blue-stained areas (Fig. 9A). Furthermore, GA-treated wild-type and *gl1* plants showed a significant reduction in chlorophyll leaching from these leaves (Supplemental Fig. 6B). These data suggested that GA application led to enhancement of the cuticle in both wild-type and *gl1* plants. Consistent with toluidine blue-staining and chlorophyll-leaching phenotypes, the GA-treated wild-type and *gl1* plants showed a pronounced increase in the levels of cuticular components (Fig. 9B). Interestingly, GA-treated wild-type plants showed significantly better SAR compared with water-treated plants (Fig. 9C). Exogenous application of GA also improved SAR on *gl1* plants, but the effect was less pronounced compared with wild-type plants, likely because the cuticle might not be completely restored. Nonetheless, unlike SA or JA, GA was able to partially restore defective SAR in *gl1* plants.

DISCUSSION

The *gl1* mutant was first isolated based on its glabrous phenotype (Koornneef et al., 1982), and this phenotype has served as a useful marker for numerous mutant isolations and/or screenings. A cursory database search identified over 200 articles in which the *gl1* background has been used to study molecular, genetic, and biochemical aspects of diverse aspects of plant physiology (Liu and Zhu, 1997; Aarts et al., 1998; Bender and Fink, 1998; Cecchini et al., 1998; Xie et al.,

1998; Yi and Jack, 1998; Bieza and Lois, 2001; Ellis and Turner, 2001; Laby et al., 2001; Devoto et al., 2002; He et al., 2002; Collins et al., 2003; Quiel and Bender, 2003; Barkan et al., 2006; Zhu et al., 2007; Speth et al., 2009; Jung and Niyogi, 2010; Rowe et al., 2010). Notably, many of the mutants that were isolated or crossed into the *gl1* background have been studied for the defense physiologies. Interestingly, most studies were conducted with the assumption that the *gl1* mutant itself does not affect any phenotype but the loss of trichomes. Our work shows that this is clearly not the case and that *gl1* plants are compromised in defense to both bacterial and fungal pathogens. Additionally, the *gl1* mutation affects cuticular development. Our findings question the prudence of using mutant backgrounds to design genetic screens and underscore the need to reevaluate phenotypes that were previously studied in the *gl1* background.

This is clearly the case for the *fad7-1* mutant, which was originally isolated in the *gl1* background (Browse et al., 1986). Subsequent studies suggested a role for glycerolipid synthesis in SAR due to the inability of the *fad7-1* (*fad7-1 gl1*) plants to induce SAR (Chaturvedi et al., 2008). However, normal SAR phenotype in allelic *fad7-2* and the *fad7-2 fad8* double mutant argue against a role for trienoic FA/lipid metabolism in SAR. This is further reiterated by the fact that trienoic FA levels but not the ability to induce SAR were restored in *fad7-1* transgenic lines expressing a wild-type copy of *FAD7*. Characterization of the *fad7-1* mutant showed that its impaired SAR is associated with a second-site mutation in the *GL1* gene. Notably, a mutation in the *GL1* gene impaired cuticle development, thereby compromising the plant's ability to induce SAR. Interestingly, besides *GL1*, two other mutations affecting trichome formation (*gl3* and *ttg1*) also contained deformed cuticles and were defective in inducing SAR. These data suggest that trichome differentiation and

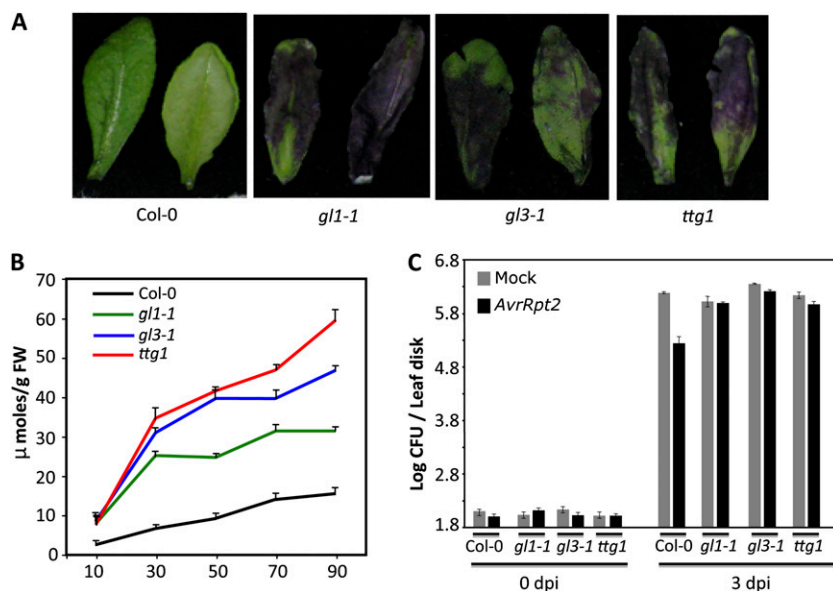
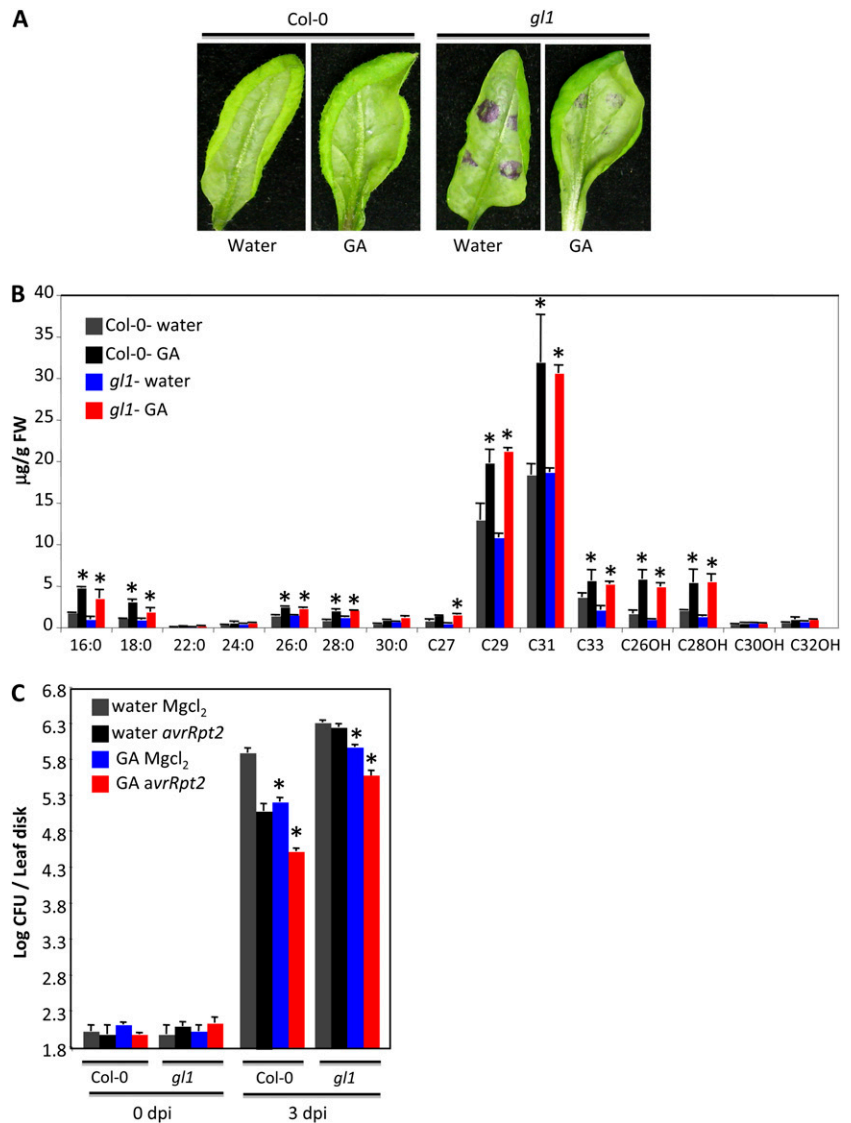


Figure 8. Evaluation of cuticle-associated phenotypes and SAR response in *gl3* and *ttg1* plants. A, Toluidine blue-stained leaves from 4-week-old plants of the indicated genotypes. B, A time-course measurement of chlorophyll leaching in the indicated genotypes. FW, Fresh weight. C, SAR response in the indicated genotypes. [See online article for color version of this figure.]

Figure 9. Evaluation of cuticle-associated phenotypes and SAR response in plants treated with GA. A, Toluidine blue-stained leaves from 4-week-old plants of the indicated genotypes. B, Analysis of wax components from leaves of 4-week old Col-0 and *gl1* plants treated with water or GA. 16:0 to 30:0 are FAs, C27 to C33 are alkanes, and C26OH to C32OH are primary alcohols. FW, Fresh weight. C, SAR response in Col-0 and *gl1* plants pretreated with water or GA. Asterisks denote significant differences with respect to water-treated and MgCl₂-infiltrated plants (*t* test, *P* < 0.05). [See online article for color version of this figure.]



cuticle development may involve an overlapping biochemical basis. However, a correlation between the lack of trichomes and the presence of a defective cuticle was not observed in transgenic plants expressing the *FATTY ACID ELONGATION1* gene specifically in the epidermis; these plants do not contain trichomes but have a normal cuticle (Reina-Pinto et al., 2009).

Is there a connection between trichome formation and cuticle development? To begin with, epidermal cells are involved in both of these processes. Trichomes differentiate from the pluripotent epidermal cells in a patterned manner, which is determined by genetic interactions between trichome-promoting and trichome-repressing factors (for review, see Schellmann and Hülskamp, 2005; Ishida et al., 2008). Proteins encoded by *GL1*, *GL3*, and *TTG1* act as positive regulators, and mutations in these genes affect trichome development. Both *GL1* and *GL3* act as transcriptional regulators and are known to regulate genes involved in various other processes besides trichome develop-

ment (Morohashi and Grotewold, 2009). Interestingly, one of the direct targets of *GL3/GL1* includes *FIDDLEHEAD (FDH)*, which encodes a protein related to β -ketoacyl-CoA synthase involved in cuticle development (Pruitt et al., 2000; Voisin et al., 2009). Notably, the *fdh* mutant shows a significant reduction in the number of trichomes (Yephremov et al., 1999). Similarly, several other mutants, including *lcr* (for *LACERATA*), *bdg* (for *BODYGUARD*), *yre* (for *YORE-YORE*, allelic to *WAX2/CER3*), and *dso* (for *DESPERADO/AtWBC11*), which are defective in cuticle formation, exhibit abnormal differentiation of trichomes and reduced trichome numbers (Wellesen et al., 2001; Kurata et al., 2003; Kurdyukov et al., 2006; Panikashvili et al., 2007). Suppression of ketoacyl reductase activity, which catalyzes the first reduction step leading to the synthesis of very-long-chain FAs, also results in abnormal trichomes and correlates with reduced cuticular wax levels (Beaudoin et al., 2009). These findings, together with our results, support the notion that overlapping pathways involv-

ing GL1, GL3, and TTG1 might regulate cuticle development as well as trichome formation. This is further supported by the fact the defective trichome phenotypes of the *yre gl2* and *yre ttg1* double mutant plants are more severe than the *yre*, *ttg1*, and *gl2* single mutants (Kurata et al., 2003).

The ability of exogenous GA to complement the SAR defect in *gl1* plants but not restore trichome formation suggests that trichomes might not play a role in SAR. This is further supported by the fact that compromised SAR in *acp4* plants is not associated with trichome formation; *acp4* plants show wild-type-like trichomes on their leaves (Supplemental Fig. S7). It appears that GA might participate in systemic immunity by affecting cuticle development. This is based on the fact that GA application reduced chlorophyll leaching by increasing the levels of cuticular components. Increased incorporation of palmitic acid and oleic acid into cutin monomers was also seen in GA-treated rice (*Oryza sativa*) and pea (*Pisum sativum*) plants (Bowen and Walton, 1988; Hoffmann-Benning and Kende, 1994), suggesting that GA-mediated increased carbon flux into cuticle might be a common phenomenon in plants. GA application also induced stronger SAR in wild-type plants. GA application is known to derepress its signaling pathway by inducing the degradation of DELLA proteins (Achard and Genschik, 2009), which negatively regulates the accumulation of reactive oxygen species (ROS; Achard et al., 2008). Notably, GA treatment, in the absence of pathogen inoculation, did not alter ROS levels; ROS levels in uninoculated, GA-treated wild-type or *gl1* plants were similar to those in water-treated plants. This suggests that the GA-triggered increased carbon flux into cuticle is not mediated via ROS. However, it does not rule out a role for ROS in GA-triggered SAR. The fact that exogenous application of hydrogen peroxide is unable to improve the resistance response of wild-type plants to *Pseudomonas* discounts a role for ROS in GA-triggered *gl1* plants. This is further supported by the result that exogenous application of SA, which potentiated a defense response leading to increased accumulation of ROS (Shirasu et al., 1997), was unable to trigger SAR in *gl1* plants. Similarly, SA and JA treatments were also unable to complement defective SAR in another cuticle-deficient mutant, *acp4* (Xia et al., 2009). Together, these observations support the notion that GA-mediated restoration of cuticle might be responsible for the restored SAR in *gl1* plants. More importantly, these and our earlier results (Xia et al., 2009) reconfirm the fact that cuticle-deficient mutants are impaired in their ability to perceive mobile signal.

MATERIALS AND METHODS

Plant Growth Conditions and Complementation Analysis

Arabidopsis (*Arabidopsis thaliana*) plants were grown in MTPS 144 Conviron walk-in chambers at 22°C, 65% relative humidity, and a 14-h photoper-

iod. For complementation, an approximately 3.5-kb region encompassing the *FAD7* coding region was amplified from Col-0 genomic DNA using *Bam*HI and *Xba*I linker primers, 5'-AAATTCATGCGGAATCAGAGAACC-3' and 5'-TCTATGTTCCGATACTGAAGC-3', respectively, and cloned into the pBARI binary vector. After confirmation of the DNA sequence, the binary vector was transformed into *fad7-1* plants using the floral dip method. The transgenic plants were selected on soil sprayed with the herbicide Basta. The complementation was confirmed by FA and genotype analysis of the T1 plants and by analyzing the segregation of FA phenotypes in the T2 generation. The *fad7-1* mutation was identified as a C-to-T transition that converted the amino acid at position 253 from Pro to Leu. The coding region of *FAD7* was amplified using 5'-ATGGCGAACTTGGTCTTATCAGAA-3' and 5'-GAGGTCAAAGTAAGAGCAGATTGA-3' primers. Genomic cleaved-amplified polymorphic sequence analysis for the *fad7-1* was performed by amplifying a 481-bp fragment using primers 5'-GAGGAGTCTCCATTGGAGAA-3' and 5'-CATGTTGCTAGTAGCAACCC-3', which was digested with *Msp*I.

RNA Extraction and Northern Analyses

Small-scale extraction of RNA from one or two leaves was performed in the TRIzol reagent (Invitrogen) following the manufacturer's instructions. Northern analyses and synthesis of random primed probes was carried out as described before (Kachroo et al., 2005).

SA, FA, and JA Quantifications

SA and SAG were extracted and measured from approximately 0.3 g fresh weight of leaf tissue as described before (Chandra-Shekara et al., 2006). Measurements were performed in triplicate, and the experiment was repeated twice.

FA extraction was carried out by placing leaf tissue ($n = 6$) in 2 mL of 3% H_2SO_4 in methanol. After 30 min of incubation at 80°C, 1 mL of hexane with 0.001% butylated hydroxytoluene 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 \times 50-m column and quantified with flame ionization detection. For quantification of FAs, leaves (50 mg) were extracted together with an internal standard, 19:0, 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. FA quantifications were repeated three times.

For JA estimations, leaves (1 g) were ground in liquid nitrogen and extracted in 100% methanol using dihydro-JA as an internal standard. The extract was acidified to pH ≤ 4 with 1 M HCl and passed through C-18 Sep-Pak columns (Waters; 500 mg, 3 mL), which were preequilibrated with 75% methanol containing 0.2% acetic acid. The column-purified extract was saturated with sodium chloride and reextracted with diethyl ether. The ether extract was completely dried under a stream of nitrogen gas and methylated with diazomethane. The oxylipins were solubilized in 0.5 mL of hexane and dried to approximately 10 μ L under a gentle stream of nitrogen gas, and 1 μ L was injected for GC-mass spectrometry (MS) as described above. The JA peaks were identified using mass spectrometry. For quantitative analysis of JA, the mass spectrometer was run in selective ion monitoring mode for mass-to-charge ratio (m/z) 224 and 226. The ratio of m/z 224 to 226 in the peak area (retention time between 9.5 and 11.0 min) was used to calculate the JA levels based on the abundance of m/z 224 and 226 generated by standard JA, dihydro-JA, and their equal-level mixture. Measurements were performed in triplicate, and the experiment was repeated twice.

For JA levels in vascular exudates, samples were extracted using a solution containing glacial acetic acid, methanol, chloroform, and potassium chloride (0.9%; 1:4:8:8, v/v) and 17:0 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 methyl *tert*-butyl ether, transferred to a glass insert, dried again under a stream of nitrogen gas, and reconstituted in a minimum volume of acetonitrile. Samples (1 μ L) were analyzed with GC-MS as described above. Measurements were performed in triplicate, and the experiment was repeated twice.

SA, JA, and GA Treatments

SA, JA, and GA (GA_4 and GA_7) treatments were carried out by spraying 500, 50, or 100 μ M solutions, respectively. JA-treated plants were covered with a transparent plastic dome to maximize exposure to JA.

Pathogen Infections

Inoculations with the bacterial pathogen *Pseudomonas syringae* were conducted as described before (Kachroo et al., 2005). The bacterial cultures were grown overnight in King's B medium containing rifampicin and/or kanamycin. The cells were washed and suspended in 10 mM MgCl₂. The bacterial suspension was injected into the abaxial surface of the leaf using a needleless 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 avirulent bacteria (10⁷ colony-forming units [CFU] mL⁻¹), and 48 h later the systemic leaves were inoculated with virulent bacteria (10⁵ CFU mL⁻¹). Unless noted otherwise, samples from the systemic leaves were harvested at 3 dpi. All SAR experiments were repeated at least three times.

Colletotrichum higginsianum (IMI 349063) and *Botrytis cinerea* were maintained on potato dextrose agar and V8 medium, respectively. Four-week-old *Arabidopsis* plants were used for both spray and spot inoculations. Spore suspensions at concentrations of 10⁴ to 10⁶ spores mL⁻¹ were used for various experiments. For spot inoculations, 10 μL of spore suspension was used to inoculate *Arabidopsis* leaves. After inoculations, the plants were transferred to a PGV36 Conviron walk-in chamber and covered with a plastic dome to maintain high humidity. Disease symptoms were scored between 4 and 11 dpi. A digital Vernier caliper was used to measure lesion size in spot-inoculated leaves. Each experiment was repeated at least twice, and each included 30 to 50 individual plants. Statistical significance was determined using Student's *t* test.

Collection of Phloem Exudate

Leaf exudate was collected as described earlier (Maldonado et al., 2002). In brief, plants were induced for SAR by inoculation with *P. syringae* containing *avrRpt2* (10⁶ CFU mL⁻¹). Twelve to 24 h later, petioles were excised, surface sterilized in 50% ethanol and 0.0006% bleach, rinsed in sterile 1 mM EDTA, and submerged in approximately 1.9 mL of 1 mM EDTA and 100 μg mL⁻¹ ampicillin. Exudates were collected over 48 h and infiltrated into healthy plants. Infiltrated leaves were harvested after 2 d for *PR-1* gene expression studies. For SAR studies, virulent pathogen was inoculated in the distal leaves 2 d after infiltration of exudate.

Microscopy, Chlorophyll Leaching, and Water Loss

For SEM analysis, both abaxial and adaxial surfaces of the leaf samples were mounted on sample holders with 12-mm conductive carbon tabs (Ted Pella, Inc.), sputter coated with gold-palladium, and observed on a Hitachi S-3200 SEM device with and without a backscatter detector at 5 and 20 kV.

For TEM analysis, leaves were fixed in paraformaldehyde and embedded in Epon-Araldite. Leaves were sectioned on a Reichert-Jung Ultracut E microtome with a Diatome diamond knife and observed with a Philips Tecnai Biotwin 12 TEM device.

For chlorophyll leaching assays, 100 mg of leaves was weighed and gently agitated, in the dark at room temperature, in tubes containing 80% ethanol. Absorbance of each sample was measured at 664 and 647 nm, and micromolar concentrations of total chlorophyll per gram fresh weight were calculated using the following formula: total micromoles of chlorophyll = 7.93 (*A*₆₆₄) + 19.3 (*A*₆₄₇).

For water loss measurements, 4-week-old plants were either subjected to drought or kept moist. The leaf weight was measured from approximately 50 leaves.

Analysis of Wax and Cutin Components

For analysis of the wax component, 500 mg of 4-week-old leaves was immersed in 10 mL of chloroform for 10 s. The leaves were rinsed once more with 10 mL of chloroform. An internal standard (100 μg of *n*-tetracosane) was added, and the sample volume was evaporated under a gentle stream of nitrogen. The samples were dried under a stream of nitrogen gas and methylated with diazomethane, dried again, and derivatized with 100 μL of acetic anhydride in 100 μL of pyridine, and the sealed tubes were incubated for 60 min at 60°C. The samples were again dried under a stream of nitrogen and dissolved in 1 mL of acetonitrile. Samples (1 μL) were injected into an HP-5 column of the GC device equipped with a flame ionization detector. The same samples were also run on an HP-5 column (30-m × 0.25-mm × 0.25-mm film thickness) on a GC device equipped with a mass spectrometer. Various

components were identified based on their retention times as compared with standards and by MS analysis. Quantification was based on flame ionization detector peak areas as compared with the peak areas of the internal standard *n*-tetracosane added prior to derivatization. Measurements were performed in triplicate, and the experiment was repeated twice.

Cutin monomer composition and content were determined using the sodium methoxide-catalyzed transmethylation method followed by acetylation of the hydroxyl groups with acetic anhydride and GC-MS slightly modified from a previous description (Bonaventure and Ohlrogge, 2002; Molina et al., 2006). After methanolysis, the methylene dichloride extract of cutin monomers was washed with 0.9% potassium chloride instead of 0.5 M sodium chloride. For GC-MS analysis, the Varian FAME capillary column used was as described in wax analysis with helium carrier gas at 1 mL min⁻¹. The mass spectrometer was run in scan mode over 35 to 450 atomic mass units (electron impact ionization). Measurements were performed in triplicate, and the experiment was repeated twice.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. An abbreviated scheme for fatty acid and lipid biosynthesis.

Supplemental Figure S2. Leaves from the indicated genotypes showing the presence or absence of trichomes.

Supplemental Figure S3. Cuticular wax and cutin monomer profiles.

Supplemental Figure S4. Response of *gl1* to the necrotrophic pathogen *B. cinerea*.

Supplemental Figure S5. Leaves of the indicated genotypes showing the absence of trichomes on *gl3* and *ttg1* plants.

Supplemental Figure S6. Effects of GA treatment on trichome formation, leaf size, and chlorophyll leaching.

Supplemental Figure S7. Scanning electron micrographs of leaves from *Nö* and *acp4* plants.

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