The Arabidopsis *RESURRECTION1* Gene Regulates a Novel Antagonistic Interaction in Plant Defense to Biotrophs and Necrotrophs[1][W][OA]

Hyung Gon Mang, Kristin A. Laluk, Eugene P. Parsons, Dylan K. Kosma, Bruce R. Cooper, Hyeong Cheol Park, Synan AbuQamar, Claudia Boccongelli, Saori Miyazaki, Federica Consiglio, Gabriele Chilosi, Hans J. Bohnert, Ray A. Bressan, Tesfaye Mengiste, and Matthew A. Jenks*

Department of Horticulture and Landscape Architecture (H.G.M., E.P.P., D.K.K., B.R.C., H.C.P., R.A.B., M.A.J.) and Department of Botany and Plant Pathology (K.A.L., S.A., T.M.), Purdue University, West Lafayette, Indiana 47907–2054; Department of Plant Protection, Università degli Studi Della Tuscia, 01100 Viterbo, Italy (C.B., G.C.); Department of Plant Biology and Crop Science, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 (S.M., H.J.B.); Consiglio Nazionale delle Ricerche-Istituto di Genetica Vegetale, Institute of Plant Genetics, 80055 Portici, Italy (F.C.); and Division of Applied Life Science, Gyeongsang National University, Jinju 660–701, Korea (R.A.B.)

We report a role for the Arabidopsis (*Arabidopsis thaliana*) *RESURRECTION1* (*RST1*) gene in plant defense. The *rst1* mutant exhibits enhanced susceptibility to the biotrophic fungal pathogen *Erysiphe cichoracearum* but enhanced resistance to the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola*. *RST1* encodes a novel protein that localizes to the plasma membrane and is predicted to contain 11 transmembrane domains. Disease responses in *rst1* correlate with higher levels of jasmonic acid (JA) and increased basal and *B. cinerea*-induced expression of the plant defensin *PDF1.2* gene but reduced *E. cichoracearum*-inducible salicylic acid levels and expression of pathogenesis-related genes *PR1* and *PR2*. These results are consistent with *rst1*'s varied resistance and susceptibility to pathogens of different life styles. Cuticular lipids, both cutin monomers and cuticular waxes, on *rst1* leaves were significantly elevated, indicating a role for *RST1* in the suppression of leaf cuticle lipid synthesis. The *rst1* cuticle exhibits normal permeability, however, indicating that the disease responses of *rst1* are not due to changes in this cuticle property. Double mutant analysis revealed that the *coi1* mutation (causing defective JA signaling) is completely epistatic to *rst1*, whereas the *ein2* mutation (causing defective ethylene signaling) is partially epistatic to *rst1*, for resistance to *B. cinerea*. The *rst1* mutation thus defines a unique combination of disease responses to biotrophic and necrotrophic fungi in that it antagonizes salicylic acid-dependent defense and enhances JA-mediated defense through a mechanism that also controls cuticle synthesis.

Plants are constantly exposed to a variety of pathogenic microbes that often suppress plant growth and decrease crop yield. Plant resistance to these diverse pathogens is controlled by multiple plant defense pathways, which include both constitutive and inducible factors. Salicylic acid (SA) is a primary signal against biotrophic pathogens, whereas jasmonic acid (JA), ethylene (ET), and oleic acid (OA; 18:1 fatty acid) are utilized as primary signaling compounds activated in response to necrotrophic infections (Kachroo et al., 2003a, 2003b; Loake and Grant, 2007).

For biotrophs, gene-for-gene resistance is one of the strongest forms of plant defense, wherein the product of a plant *R* gene recognizes, either directly or indirectly, race-specific elicitors produced by the pathogen. This type of resistance is often coupled to the hypersensitive response (Dangl and Jones, 2001). Pathogen-induced hypersensitive response is often associated with activation of SA-dependent defense mechanisms, which leads to systemic acquired resistance (SAR) characterized by an increase in endogenous SA, transcriptional activation of *PATHOGENESIS-RELATED* (*PR*) genes *PR1*, *PR2*, *PR5*, and *GLUTATHIONE-S-TRANSFERASE1*, and protection against biotrophic pathogens (Cao et al., 1997; Reuber et al., 1998; Dewdney et al., 2000). SA is thought to be necessary for SAR, since the removal of SA blocks the onset of SAR (Gaffney et al., 1993). SA accumulation in plants, either by genetic modification of SA metabolism or exogenous SA application, induces SAR (Malamy et al., 1990, 1992; Métraux et al., 1990; Rasmussen et al., 1991;

[1] This work was supported by the National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service (grant no. 2006–35304–17323).

* Corresponding author; e-mail jenksm@purdue.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Matthew A. Jenks (jenksm@purdue.edu).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.109.142158

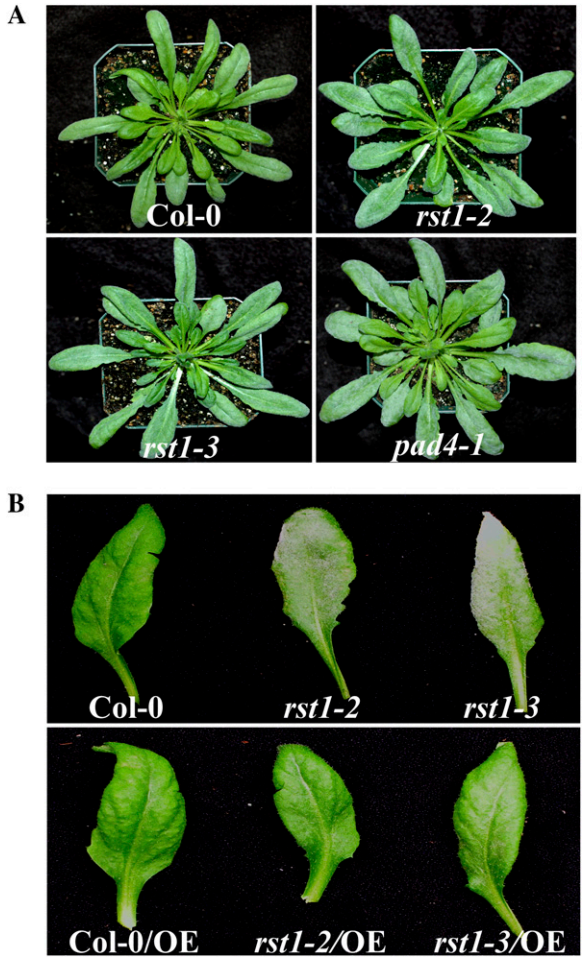

Figure 1. Loss of *RST1* function increases Arabidopsis susceptibility to *E. cichoracearum*. A, The *rst1-2* and *rst1-3* plants exhibit enhanced susceptibility to *E. cichoracearum*. The *pad4-1* mutant shows susceptibility to powdery mildew and was used as a control for the disease assay. B, Constitutive expression of *RST1* rescues the *E. cichoracearum* susceptibility of *rst1-2* and *rst1-3* to wild-type (Col-0) levels. Plants were grown on soil for 30 d and infected with *E. cichoracearum*. At least 10 plants were tested per genotype. Photographs were taken 10 to 14 dpi. The experiment was repeated at least three times, and representative results are shown. OE (overexpression) indicates plants expressing the *RST1* genomic region including the introns from the CaMV 35S promoter (*CaMV 35S$_{pro}$:RST1*).

Yalpani et al., 1991; Enyedi et al., 1992). On the other hand, JA, ET, and OA are signal molecules required for defense responses to necrotrophic pathogens such as *Botrytis cinerea* (Thomma et al., 1999; Kachroo et al., 2001; Diaz et al., 2002; Ferrari et al., 2003). Arabidopsis (*Arabidopsis thaliana*) mutants with altered JA and/or ET signaling or biosynthesis, or the synthesis of OA, show increased susceptibility to the necrotrophs *B. cinerea* and *Alternaria brassicicola* (Thomma et al., 1999; Kachroo et al., 2001; Nandi et al., 2005). Transcription of the plant defense genes *PDF1.2* and *Thi2.1* is enhanced in response to *B. cinerea* and *A. brassicicola* infection and is dependent on ET, JA, and OA signals (Epple et al., 1995; Penninckx et al., 1996, 1998; Kachroo et al., 2003a, 2003b). Recent studies demonstrate cross talk between these signaling networks, revealing antagonistic relationships between the SA and JA/ET signaling pathways and an associated role for OA (Doares et al., 1995; Kunkel and Brooks, 2002; Kachroo et al., 2003a, 2003b). As a case in point, *eds4* and *pad4* mutants that are deficient in SA accumulation have impaired responses to SA and display enhanced JA-regulated gene expression (Gupta et al., 2000)

The cuticle, composed primarily of free epicuticular and intracuticular waxes and an insoluble polymer composed primarily of cutin, covers the aerial epidermal cell walls of plants and serves as the outermost boundary between the plant and its environment (Nawrath, 2002, 2006; Goodwin and Jenks, 2005). Besides their role in abiotic stress tolerance, chemical components of the cuticle are thought to play an important role in plant defense against fungal (Jenks et al., 1994) and bacterial (Xiao et al., 2004) pathogens, potentially through direct influence on innate immunity (Reina-Pinto and Yephremov, 2009) and/or perception (or generation) of signals required for SAR (Xia et al., 2009). Saturated, desaturated, and hydroxylated fatty acids are major substrates for cuticle lipid synthesis and have been implicated in barley (*Hordeum vulgare*) and rice (*Oryza sativa*) resistance to *Erysiphe graminis* and *Magnaporthe grisea*, respectively (Schweizer et al., 1994, 1996). Cutin monomers have been shown to induce developmental processes in pathogenic fungi such as the germination and formation of appressoria in the rice blast fungus *M. grisea* and appressorial tube formation in *E. graminis* (Francis et al., 1996; Gilbert et al., 1996). Additionally, both cutin monomers and cuticular waxes serve as general elicitors of plant defense response pathways (Fauth et al., 1998). Mutations that cause increased cuticle permeability, such as occurs in the *long-chain acyl-CoA synthetase2* (*lacs2*) mutant, provide full immunity to

Table I. *E. cichoracearum development on leaves of Arabidopsis wild-type Col-0, rst1-2, and rst1-3 plants*

Detached leaves were inoculated and incubated on petri plates as described in "Materials and Methods." Leaves were fixed and stained with trypan blue.

Genotype	Germination[a]	Hyphal Length[b]	Conidiophores per Colony[c]
	%	*mm*	
Col-0	62.1 (100)[d]	0.887 ± 0.074 (8)	8.9 ± 4.0 (7)
rst1-2	59.3 (100)	1.053 ± 0.056 (7)	19.3 ± 6.1 (9)
rst1-3	68.6 (100)	1.188 ± 0.069 (9)	46.8 ± 5.5 (9)

[a]Asexual spore germination measured at 1 dpi. [b]The length of secondary hyphae per germling measured at 4 dpi. Values are means ± SD. [c]Conidiophores were counted on five to six randomly selected single fungal colonies per leaf on three to five leaves at 5 dpi. Values are means ± SD. [d]Numbers within parentheses indicate the number of replicates.

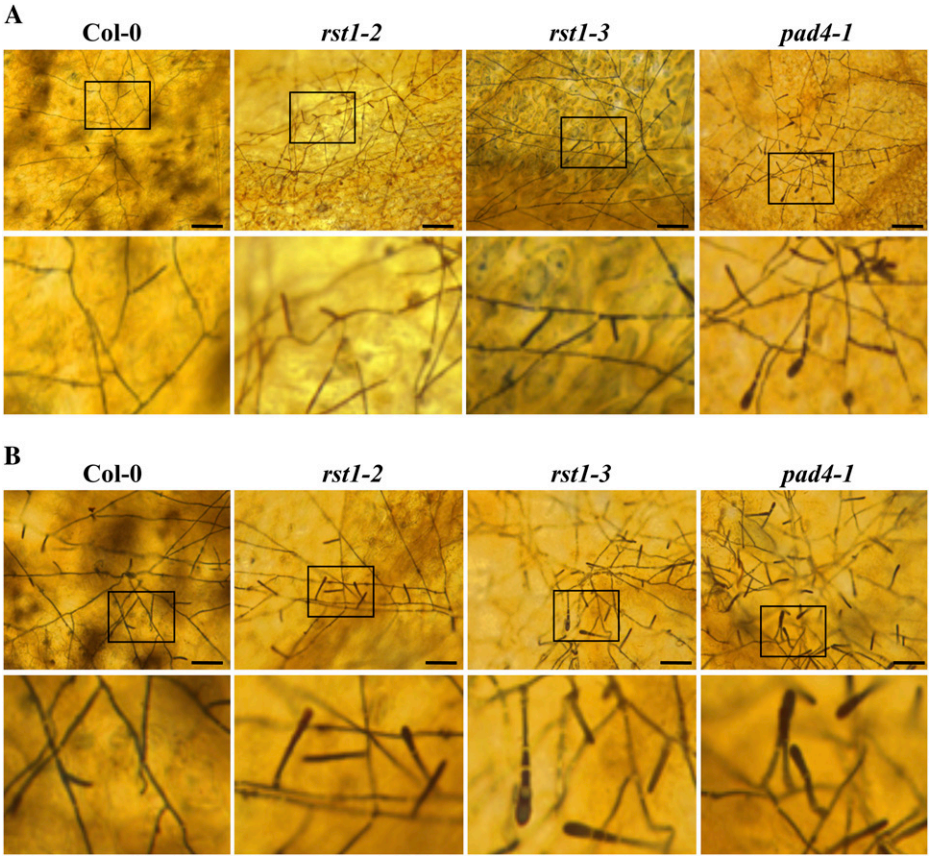

Figure 2. Microscopic analyses showing the growth of *E. cichoracearum* in inoculated plants. A, *E. cichoracearum* growth at 4 dpi showing hyphal branching on leaves of wild-type Col-0, *rst1-2*, *rst1-3*, and *pad4-1* at 4 dpi. B, *E. cichoracearum* growth at 5 dpi showing sporulation in *rst1-2* and *rst1-3* leaves. Bottom panels of both A and B are magnified views of the boxes on the respective top panels. Representative samples are shown. The experiment was repeated at least three times. Bars = 100 μm.

B. cinerea and *Sclerotiorum sclerotinia* (Chassot et al., 2007). Mutation in the α/β-hydrolase-encoding *BODYGUARD* (*BDG*) gene and ectopic expression of a fungal cutinase in Arabidopsis (CUTE) likewise increase cuticle permeability and confer enhanced resistance to *B. cinerea* (Sieber et al., 2000; Kurdyukov et al., 2006; Chassot et al., 2007). It is still unclear, however, whether this resistance is due to changes in plant defense response signaling resulting from altered cuticle properties, from enhanced secretion of antifungal or effector compounds, or from some other undiscovered mechanism.

We recently described the Arabidopsis *resurrection1* (*rst1*) mutant as having altered cuticular waxes (Chen et al., 2005). In this report, biochemical analyses were expanded to reveal that the amount of cutin monomers was significantly elevated on *rst1* leaves, just as previously reported for the waxes on *rst1* leaves. Further analysis revealed that *rst1* mutants were more susceptible to the obligate biotrophic fungus *Erysiphe cichoracearum* but more resistant to the necrotrophic fungi *B. cinerea* and *A. brassicicola*. Analysis of defense gene expression and SA and JA levels in *rst1* suggests that SA-dependent defense responses are attenuated, whereas JA-dependent defense is enhanced. A novel role for *RST1* and cuticle lipids in an antagonistic interaction between the SA- and JA/ET-mediated pathogen response pathways is described.

RESULTS

Loss-of-Function Mutation in *RST1* Results in Enhanced Susceptibility to *E. cichoracearum*

The original *rst1-1* mutant was identified from a T-DNA-mutagenized population of Arabidopsis in the C24 genetic background using visual screening for altered glaucousness of the inflorescence stem. The *rst1-2* and *rst1-3* allelic mutants were isolated from the SALK T-DNA insertion collection in the Columbia ecotype (Col-0) obtained from the Arabidopsis Biological Resource Center (Chen et al., 2005). Transcript analysis by reverse transcription (RT)-PCR detected truncated *RST1* transcripts in *rst1-1* and *rst1-3* (Supplemental Fig. S1A). Sequence analyses suggest that RST1 is a membrane-bound protein with 11 transmembrane domains (Supplemental Fig. S1B; Hofmann and Stoffel, 1993).

The *rst1-1* mutant exhibited elevated susceptibility to *E. cichoracearum* under naturally occurring powdery mildew infections in the greenhouse (Supplemental Fig. S2). Although the C24 ecotype is completely immune to *E. cichoracearum* infection, the *rst1-1* mutant showed extreme susceptibility to the pathogen. Subsequent studies of the *rst1-2* and *rst1-3* allelic mutants, as well as a *pad4-1* positive control, clearly showed enhanced growth of *E. cichoracearum* on leaves relative to the normally susceptible Col-0 wild type (Fig. 1).

Thus, *RST1* contributes to resistance in both ecotypes. To clearly establish the role of *RST1* in resistance to *E. cichoracearum*, inoculated leaves of Col-0, *rst1-2*, *rst1-3*, and *pad4-1* were examined for fungal growth and development. Detached leaves from 4-week-old plants inoculated with a low density of conidia were assessed for the percentage of germinating conidia, hyphal length, and the number of conidiophores per colony at 1, 4, and 5 d post inoculation (dpi; Table I). At 1 dpi, no significant difference in the asexual spore germination and development of appressorial germ tubes for the wild type and the mutants was observed; however, from 2 to 4 dpi, *E. cichoracearum* hyphal growth became highly branched and produced more conidiophores on *rst1-2* and *rst1-3* plants compared with the wild type (Fig. 2A; Table I). At 5 dpi, *E. cichoracearum* produced two to four times more conidiophores on the *rst1* mutants than on wild-type plants (Fig. 2B; Table I). Although *rst1-2* and *rst1-3* are allelic mutants, *rst1-3* displayed more susceptibility to powdery mildew infection than *rst1-2* (Table I). By comparison, the development of *E. cichoracearum* was marginally faster on the leaves of *pad4-1* than on the *rst1* mutants, with all mutants displaying more rapid pathogen development than wild-type plants (Fig. 2A). These observations indicate that *E. cichoracearum* colonizes *rst1* mutant leaves more rapidly than wild-type leaves once the penetration peg growth phase is reached.

To confirm genetic complementation, the *RST1* gene including approximately 200 bp of both upstream and downstream untranslated regions was expressed in wild-type and *rst1* plants. The overexpression of *RST1* in the wild type does not affect responses to *E. cichoracearum*, whereas in the mutant, the *RST1* gene rescued the *E. cichoracearum* susceptibility back to wild-type levels (Fig. 1B). Complementation tests using reciprocal crosses of *rst1-2* with *rst1-3* further confirmed that the observed phenotypes in the mutants are due to defects in *RST1*. All F1 plants resulting from crosses between the two mutant alleles exhibited a clear *rst1* mutant glossy stem phenotype and enhanced disease susceptibility comparable to the parental mutant plants (data not shown). These results confirm that the phenotypes of *rst1-2* and *rst1-3* mutants are solely caused by defects in the *RST1* gene.

The *rst1* Mutant Has Elevated Levels of Leaf Cuticular Lipids But Displays Normal Cuticle Permeability

Previously, we showed that mutation in *RST1* caused a 43% elevation in cuticular wax amounts on *rst1* leaves (Chen et al., 2005). To provide a more complete analysis of leaf cuticle lipids, we examined the amount and composition of the leaf cutin monomers on wild-type Col-0 and the isogenic allelic mutants *rst1-2* and *rst1-3*. Overall, the total amount of cutin monomers was significantly higher in both *rst1-2* (16.4%) and *rst1-3* (32.1%) compared with wild-type plants (Table II). Just as in their powdery mildew response, the *rst1-3* mutation showed a stronger effect than *rst1-2* on cutin monomers. In both allelic mutants, the C18:2 dicarboxylic acids were significantly higher than wild-type levels, rising from 47.5% of the total cutin monomers in the wild type to 56.2% and 66.3% of total cutin monomers for *rst1-2* and *rst1-3*, respectively (Table II). Other cutin monomers changed very little in these allelic mutants (Table II). As such, both cuticular wax (Chen et al., 2005) and cutin amount per leaf area are significantly elevated in the *rst1* mutants. Since the leaf areas of *rst1-2* and *rst1-3* are unchanged from their isogenic wild-type parent (data not shown), both cutin and wax synthetic pathways appear to have been activated by the mutation in *RST1*.

Previous studies have implicated leaf permeability due to altered cuticle composition as a factor in pathogen response, so we examined leaf permeability of *rst1* using measures of transpiration rate, stomatal index, toluidine blue staining, and sensitivity to xenobiotics as criteria. Transpiration rates of detached leaves of soilless medium-grown *rst1* plants did not differ from those of the wild type. Moreover, sensitivity to herbicide (BASTA) was not significantly different between the wild type and *rst1*, nor did the leaves of *rst1* and the wild type show differences in the rate of uptake of toluidine blue stain (Supplemental Figs. S3–S5). Additionally, no difference was observed in stomatal index or trichome number of the adaxial and abaxial leaf surfaces on *rst1* compared with the wild type (Chen et al., 2005). These results provide strong evidence that, although *rst1* has an increased amount

Table II. *Effects of the rst1 mutation on Arabidopsis leaf cutin monomers*

The amount (μg dm^{-2}) of total cutin monomers was higher in rosette leaves of the two isogenic allelic mutants *rst1-2* and *rst1-3* than in wild-type Col-0, due primarily to an increase in C18:2 dicarboxylic acids. Values represent means ± SD (n = 5). Asterisks indicate significant differences from the wild-type Col-0 amount as determined by Student's t test ($P < 0.05$).

Cutin Monomers[a]	Col-0	*rst1-2*	*rst1-3*
16-OH C16:0	1.3 ± 0.4	1.8 ± 0.1	1.3 ± 0.2
10(9),16-OH C16:0	1.9 ± 0.8	2.3 ± 0.2	2.1 ± 1.2
C16:0 dioic acid	6.6 ± 0.6	7.2 ± 0.8	8.1 ± 0.7
18-OH C18:2	5.2 ± 0.9	6.0 ± 1.0	7.1 ± 1.7
18-OH C18:1	2.7 ± 0.6	2.8 ± 0.7	2.9 ± 0.2
18-OH C18:0	1.2 ± 0.3	1.3 ± 0.0	1.0 ± 0.1
C18:2 dioic acid	47.5 ± 4.5	56.2 ± 0.2*	66.3 ± 7.7*
C18:1 dioic acid	5.2 ± 0.6	5.6 ± 1.3	5.9 ± 0.3
C18:0 dioic acid	1.4 ± 0.2	1.6 ± 0.1	1.7 ± 0.2
Total	73.0 ± 6.0	84.8 ± 3.8*	96.4 ± 8.0*

[a]16-OH C16:0, 16-Hydroxy hexadecanoic acid; 10(9),16-OH C16:0, 10(9),16-dihydroxy hexadecanoic acid; C16:0 dioic acid, hexadecane-1,16-dioic acid; 18-OH C18:0, 18-hydroxy octadecanoic acid; 18-OH C18:1, 18-hydroxy octadec-9-enoic acid; 18-OH C18:2, 18-hydroxy octadeca-9,12-dienoic acid; C18:0 dioic acid, octadecane-1,18-dioic acid; C18:1 dioic acid, octadecene-1,18-dioic acid; C18:2 dioic acid, octadecadien-1,18-dioic acid. OH denotes a hydroxyl functional group.

of leaf cutin monomers and cuticular waxes, these cuticular modifications cause no significant changes in general leaf cuticle membrane permeability, although it is possible that permeability to specific chemical(s) has been altered.

The *rst1* Mutant Has Attenuated SA-Dependent Defense to *E. cichoracearum*

To determine whether enhanced susceptibility to *E. cichoracearum* in *rst1* is associated with altered defense responses, we determined the expression of defense genes *PR1*, *PR2*, and *PDF1.2* following *E. cichoracearum* or *B. cinerea* inoculation. The expression of both *PR1* and *PR2* was slightly elevated in noninoculated *rst1-2* and *rst1-3* compared with the wild type (Supplemental Fig. S6). After *E. cichoracearum* infection, both the wild type and mutants showed a dramatic induction of *PR1* and *PR2* transcripts (Fig. 3). However, induction of *PR1* and *PR2* in *rst1-2* and *rst1-3* was much less than in Col-0, averaging 21% to 26% and 11% to 14%, respectively, of wild-type induction levels. The lower expression of these *PR* genes in *rst1-3* than in *rst1-2* corresponds well with the relatively greater increase in susceptibility of *rst1-3* over *rst1-2* to *E. cichoracearum* (Table I). Both noninoculated and inoculated *pad4* mutants show very low levels of *PR1* and *PR2* expression (Fig. 3). Expression of the *PDF1.2* gene, a marker for JA/ET-dependent defense responses, was increased in *rst1-2*, *rst1-3*, and *pad4-1* compared with the wild type following *E. cichoracearum* infection (Fig. 3). The *RST1* gene is also responsive to pathogen infection, with expression in the wild type being slightly induced in response to *E. cichoracearum* (Fig. 3). The expression of SA signaling genes *NPR1* and *PAD4* is significantly reduced in the *rst1* mutants relative to the wild type. We next examined the accumulation of SA in wild-type, *rst1*, and *pad4* plants with and without powdery mildew inoculation. The amount of SA was not altered in *rst1-2* and *rst1-3* compared with the wild type in noninoculated plants (Fig. 4). However, in the *rst1-2* and *rst1-3* mutants, accumulation of SA after inoculation with *E. cichoracearum* was severely reduced relative to wild-type plants (Fig. 4), providing further evidence for an association between SA synthesis or accumulation and *RST1* function. Thus, *RST1* is required for normal pathogen-induced SA accumulation and downstream responses.

The *rst1* Mutant Displays Enhanced Resistance to Necrotrophic Pathogens *A. brassicicola* and *B. cinerea*

To determine the effects of impaired *RST1* function on plant responses to necrotrophic pathogens, we examined the response of *rst1* to *A. brassicicola* and *B. cinerea*. The *rst1-2* and *rst1-3* mutants displayed elevated resistance to both necrotrophs relative to the wild type based on disease symptoms and pathogen growth (Figs. 5 and 6A). The size of disease lesions on inoculated leaves of *B. cinerea* was approximately 3-fold smaller than on the wild type (Fig. 6B). The inoculated *rst1* mutant alleles supported significantly lower pathogen sporulation and showed confined disease lesions, suggesting that *rst1* suppresses pathogen growth and disease symptoms. Consistent with

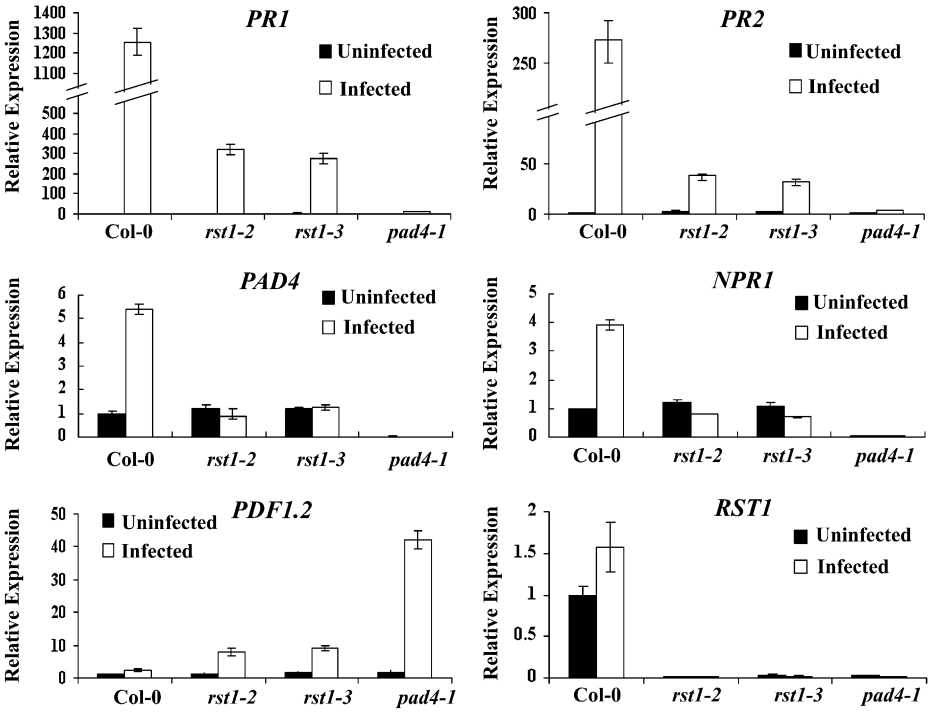

Figure 3. Basal and *E. cichoracearum*-induced expression of defense response and *RST1* genes. Quantitative RT-PCR data showing the expression of the SA pathway defense genes *PR1*, *PR2*, *PAD4*, and *NPR1* and the ET/JA-pathway defense genes *PDF1.2* and *RST1*. Total RNA was extracted from leaves of plants at 7 dpi. Arabidopsis *TUBULIN* was used as an internal control. Control samples were normalized to 1. Values represent means ± SD ($n = 4$).

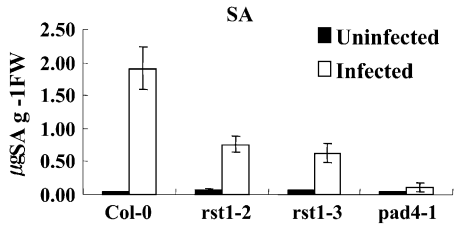

Figure 4. The *rst1* mutants accumulate less SA than the wild type after *E. cichoracearum* inoculation. The amount of free SA in leaves from 4-week-old plants was analyzed using HPLC. Samples were collected with or without *E. cichoracearum* infection at 7 dpi. Values represent means ± SD ($n = 3$). FW, Fresh weight.

restoration of *E. cichoracearum* resistance in *rst1* plants expressing the wild-type *RST1* gene (Fig. 1B), *B. cinerea* susceptibility was restored to wild-type levels in *rst1* mutants expressing the *35Spro*:*RST1* construct (as was the wild-type wax phenotype), further confirming that expression of the wild-type *RST1* gene promotes infection by the necrotrophic fungi tested (Fig. 6B).

Expression of the plant defensin gene *PDF1.2* positively correlates with activation of JA/ET-dependent defenses and resistance to necrotrophic pathogens (Penninckx et al., 1996, 1998). In an effort to further examine the role of *RST1* in JA/ET-mediated defenses, we determined the expression levels of *PDF1.2* in wild-type and *rst1* plants at various time points after inoculation with *B. cinerea*, as described previously (Veronese et al., 2006). The expression of *PDF1.2* in uninoculated *rst1* plants was significantly higher than in wild-type plants. After inoculation, the expression continued to increase in both the wild-type and *rst1* plants at comparable levels up to 24 h post inoculation (hpi). However, in the *rst1* mutants, the expression of *PDF1.2* surpassed that of the wild type by 48 hpi (Fig. 7A). After 48 h, *PDF1.2* expression began to decline in wild-type plants but continued to increase in the *rst1* mutants (Fig. 7A). The induced expression of the *PDF1.2* gene was sustained in a manner consistent with the sustained resistance response to the pathogen. The highly induced expression of *RST1* in wild-type plants at 48 hpi preceded the observed increase in *PDF1.2* expression (Fig. 7B). Consistent with their observed disease responses, the *rst1-2* and *rst1-3* mutants had 2.6- to 3.5-fold higher basal levels of JA than the wild-type plants (Fig. 8). Together, these results demonstrate that *RST1* regulates plant responses to *A. brassicicola* and *B. cinerea*, likely through the modulation of JA-dependent plant defenses.

The Mutation of *RST1* Appears to Induce Several JA-Regulated Genes and Elevates JA Levels

To determine the genome-wide effects of loss of *RST1* function on gene expression, we performed transcriptome analysis of the *rst1* mutant to obtain preliminary indications of genes that have altered transcript levels in the *rst1* genetic background. Hundreds of genes were significantly up- and down-regulated in *rst1* compared with the wild type (data not shown). The full set of the raw intensity microarray data are deposited at http://www.ncbi.nlm.nih.gov/, with GEO accession numbers GSE16875, GSM422925, GSM422926, and GSM422927. As a means to verify microarray data, we used RT-PCR to examine the expression of numerous genes revealed as highly expressed in the mutant array, including *PR1*, *PR2*, *PDF1.2*, *BG1*, *GLYCOSYL HYDROLASE FAMILY19* (*CHITINASE*), and *ATHILA RETROELEMENT*, to find similar high expression (Figs. 3 and 7A; Supplemental Figs. S6 and S7). Of the genes that were up-regulated in *rst1*, six were associated with JA synthesis, two with JA signaling, and 18 were JA responsive, indicating a strong impact of the *RST1* gene mutation on JA synthesis and signaling, results consistent with the elevated JA levels in the mutant (Table III). By comparison, ET synthesis-related genes were not altered in *rst1*, and only three ET-specific signaling or stimulus genes had elevated expression,

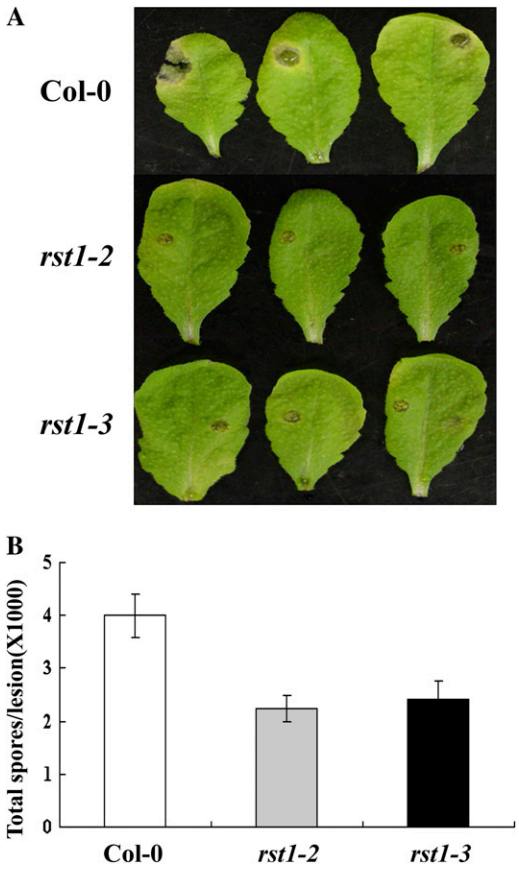

Figure 5. The *rst1* mutants exhibit enhanced susceptibility to the necrotrophic pathogen *A. brassicicola*. A, Disease symptoms on leaves of drop-inoculated wild-type and *rst1* plants at 6 dpi. B, Spore count on *A. brassicicola*-inoculated plants at 4 dpi. Data represent means ± SD. The mean values were determined from three independent experiments. Each experiment contained an average spore count from 20 inoculated leaves per genotype.

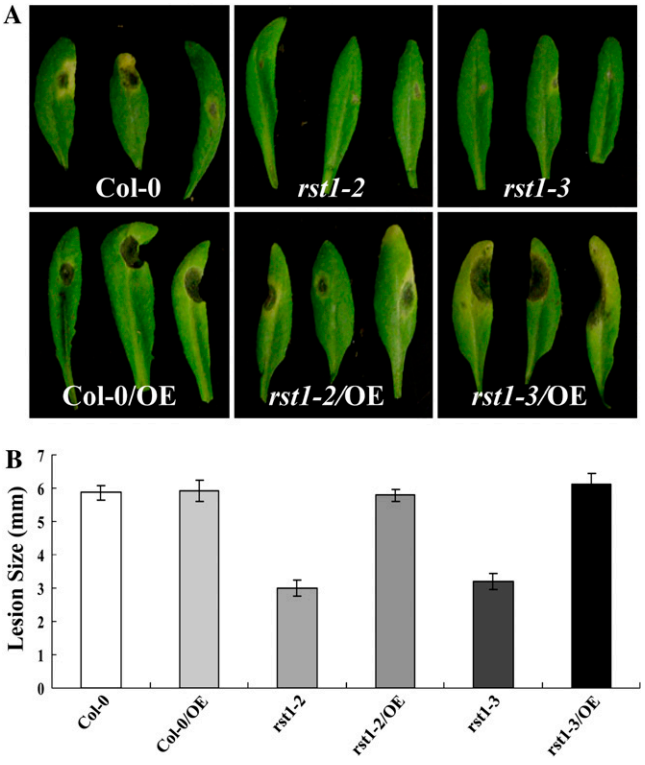

Figure 6. The *rst1* mutants show enhanced resistance to the necrotrophic pathogen *B. cinerea*. A, *rst1* mutants show fewer disease symptoms on drop-inoculated leaves. Top row, Col-0, *rst1-2*, and *rst1-3* plants at 3 dpi. Bottom row, rescued *rst1* mutant phenotype of plants harboring *CaMV 35Spro:RST1*. B, Mean disease lesion size at 3 dpi with *B. cinerea*. Data represent means ± SD. The mean values were determined from three independent experiments. Each experiment contained the average lesion size from 20 inoculated leaves per genotype. OE, Overexpression.

albeit in the lower range (data not shown). Interestingly, the *BG1* gene, whose product cleaves abscisic acid (ABA) from a glycosyl conjugate, shows extremely high expression in *rst1*, indicating an association of the general stress-responsive ABA with *RST1* function (Table II). Furthermore, the *PAD3* gene that encodes the cytochrome P450 protein required for the synthesis of the Arabidopsis phytoalexin camalexin was also up-regulated in *rst1* (Table II). As such, the enhanced resistance of *rst1* to *B. cinerea* and *A. brassicicola* may also involve enhanced phytoalexin accumulation. Furthermore, the *FAD6* gene, whose product is involved in synthesis of fatty acids leading to the synthesis of JA and other lipid-related products, shows increased expression in *rst1* compared with the wild type (Ferrari et al., 2007; Chaturvedi et al., 2008). Arabidopsis *fad* mutants show increased susceptibility to pathogen infection and insect attack (McConn et al., 1997; Staswick et al., 1998; Vijayan et al., 1998) providing further evidence for a complex regulatory function of *RST1* in disease response.

To further determine how *RST1* interacts within the JA/ET-dependent defense pathways, we constructed double mutants *rst1-2 coi1-1* and *rst1-2 ein2-1*. The *coi1-1* and *ein2-1* mutants are impaired in JA perception and ET signaling, respectively (Xie et al., 1998; Alonso et al., 1999). Both *coi1* and *ein2* show enhanced susceptibility to *B. cinerea* (Thomma et al., 1998, 1999). *B. cinerea* disease assays of double mutants reveal that *coi1* is completely epistatic to *rst1*, whereas *ein2* is partially epistatic to *rst1* (Fig. 9). Thus, the resistance observed in the *rst1* mutant to *B. cinerea* is dependent on functional *COI1* and *EIN2* genes. Taken together, these results implicate *RST1* as a negative regulator for JA synthesis or signaling whose down-regulation enhances Arabidopsis resistance to *B. cinerea* and *A. brassicicola*.

RST1 Has No Role in Plant Response to the Bacterial Pathogen *Pseudomonas syringae*

To determine whether the *rst1* mutation affects responses to a bacterial pathogen, we inoculated *rst1* plants with the virulent *P. syringae* pv *tomato* (*Pst*) strain DC3000 and the avirulent *P. syringae* DC3000 strain expressing *avrRps4*. No significant differences in bacterial growth and disease symptoms were observed between the wild types and their isogenic *rst1* mutants (Supplemental Fig. S8). As previously reported, enhanced susceptibility was exhibited in *pad4-1* and *NahG* plants to both the virulent *Pst* DC3000 and avirulent *Pst* DC3000 (*avrRps4*) strains (Feys et al., 2001; Lee et al., 2007). Thus, *RST1* appears to have no role in basal defense response to *P. syringae* and *RPS4*-mediated race-specific resistance, indicating that *RST1* has a certain level of pathogen specificity rather than being a general defense regulator.

The *RST1* Transcript Is Expressed in Vascular and Anther Tissues, and the RST1 Protein Is Localized to the Plasma Membrane

To determine the spatial and temporal expression of *RST1*, we expressed a *GUS* reporter gene in Arabidopsis Col-0 under the control of a 1,200-bp fragment of the *RST1* promoter region. The *RST1pro:GUS* construct was transformed into Arabidopsis using the floral dip method, and a total of eight independent transformants were used for expression analysis. Strong GUS activity was detected in the veins of leaves, petioles, and hypocotyls from 1-week-old seedlings and anthers of mature flowers (Fig. 10A). GUS activity was not easily detected in the inflorescence stem, root, cauline leaves, siliques, and seeds, consistent with a previous report (Chen et al., 2005) demonstrating that *RST1* expression in those tissues is very low and best detected using PCR-based methods. The *35Spro:GUS* vector control showed blue staining in essentially all tissues (data not shown). The expression of *RST1* in anther tissues is consistent with its role in JA-related functions, as JA has been implicated in male fertility (Feys et al., 1994; Park et al., 2002).

The *RST1* cDNA was fused to the *GFP* to examine the subcellular localization of the RST1 protein in root

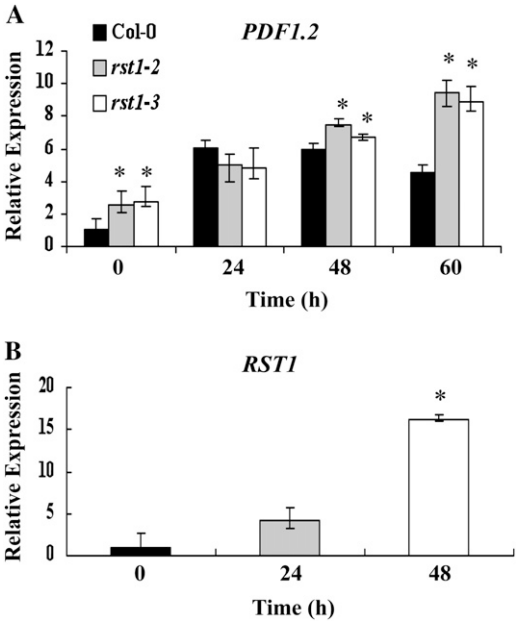

Figure 7. The expression of *PDF1.2* and *RST1* genes in *B. cinerea*-inoculated plants. A, The expression of *PDF1.2* in wild-type, *rst-2*, and *rst1-3* plants before inoculation and at 24, 48, and 60 hpi with *B. cinerea*. Asterisks indicate significant differences from wild-type Col-0 at each time point as determined by Student's *t* test ($P < 0.05$). B, The expression of *RST1* in wild-type Col-0 plants before and 24 and 48 hpi with *B. cinerea*. The data represent means ± SD. Mean values were determined from three independent experiments. Arabidopsis *TUBULIN* was used as an internal control. Control samples were normalized to 1 hpi. The asterisks indicates a significant difference from the 0-h time point as determined by Student's *t* test ($P < 0.05$).

cells of transgenic plants. The cauliflower mosaic virus (CaMV) 35Spro:RST1:GFP (containing full-length RST1 cDNA) was transformed into *rst1-2*, and then transgenic plants were isolated from the T1 generation. Stem glossiness and seed abortion phenotypes were observed as being reverted to the wild type in the complemented lines. GFP localization within the T2 generation of these fully complemented lines was verified using the confocal microscope. The rescued phenotypes of *rst1-2* provide strong evidence that the recombinant RST1 protein localized to the normal in situ location. Visualization of RST1:GFP root cell expression using confocal light microscopy provided results consistent with RST1 protein localization to the plasmalemma (Fig. 10B). To exclude autofluorescence signal from the cell wall (due to phenolics), we confirmed that green fluorescence was undetectable in Col-0 under the same conditions (Fig. 10B).

DISCUSSION

We describe the unique role of the Arabidopsis *RST1* gene in regulating plant immunity to an obligate biotrophic pathogen and two species of necrotrophic fungi. Compared with the isogenic wild-type parents, the *rst1* mutant is more resistant to the two necrotrophic fungi, *B. cinerea* and *A. brassicicola*, but more susceptible to the biotrophic fungus *E. cichoracearum*. By comparison, the response of *rst1* to virulent and avirulent strains of the bacterial *P. syringae* did not differ from the wild type. Although many Arabidopsis mutants have been reported showing increased resistance to biotrophs but increased susceptibility to necrotrophs, *rst1*, to our knowledge, is the first plant mutant to show, in contrast, a clearly elevated resistance to necrotrophs but susceptibility to biotrophs**.** An Arabidopsis mutant like *rst1* with a comparable disease phenotype, *lacs2*, in a similar way shows elevated resistance to the necrotroph *B. cinerea* and higher susceptibility to *P. syringae* (Tang et al., 2007). The *P. syringae* pathogen, however, is not a strict biotroph or an obligate parasite, as is *E. cichoracearum*, and as such, *rst1* defines a unique defense response in this regard. Consistent with a role for SA in mediating resistance to biotrophs, many of the previously reported biotroph-resistant but necrotroph-susceptible mutants exhibit elevated levels of SA and enhanced cell death prior to or after infection (Epple et al., 2003; Bohman et al., 2004; Veronese et al., 2004; Nandi et al., 2005). The *eds4* and *pad4* mutants show enhanced susceptibility to biotrophs similar to *rst1* and are also similarly deficient in SA accumulation, defective in SA responses, and exhibit enhanced expression of JA-mediated genes (Penninckx et al., 1996; Gupta et al., 2000). However, *eds4* and *pad4* do not exhibit increased resistance to necrotrophic pathogens (Ferrari et al., 2003; Dhawan et al., 2009), as does *rst1*. Several Arabidopsis mutations, including *mpk4*, *bik1*, and *wrky33*, impair JA- and ET- dependent plant defense responses and cause susceptibility to the necrotrophic pathogens *A. brassicicola* and/or *B. cinerea* (Petersen et al., 2000; Wiermer et al., 2005; Veronese et al., 2006; Zheng et al., 2006). As such, *MPK4*, *BIK1*, and *WRKY33* appear, along with *RST1*, to play a role in the antagonistic cross talk regulating the SA and JA pathogen-defense signaling pathways. The *rst1* mutation, however, uniquely suppresses SA through the up-regulation of JA levels,

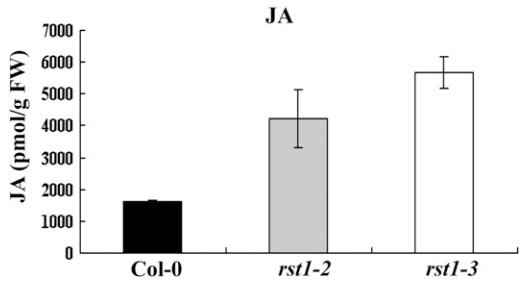

Figure 8. The *rst1* mutants show increased basal JA accumulation. The basal amounts of JA in *rst1-2* and *rst1-3* is higher than that of Col-0. The levels of JA in leaves from 4-week-old soil-grown plants of Col-0, *rst1-2*, and *rst1-3* were analyzed using HPLC-mass spectrometry. Values represent means ± SD ($n = 3$). FW, Fresh weight.

Table III. *JA synthesis, perception, and response genes up-regulated in rst1 mutants*

Annotation was based on The Arabidopsis Information Resource database (http://Arabidopsis.org). The expression fold change of probe sets is indicated only when change is significant ($P \leq 0.01$ and $P \geq 2.0$). Collected data were from three independent experiments and analyzed as indicated in "Results."

Description	AGI Code	Fold Change
JA biosynthesis		
LOX2	At3g45140	4.3
LOX3	At1g17420	2.2
Putative LOX	At1g72520	2.1
OPR3/DDE1	At2g06050	2.1
4CL-like	At4g05160	2.4
KAT2	At2g33150	2.2
JA-mediated signaling pathway		
RCD1	At1g32230	4.3
SGT1B	A4g11260	2.4
Response to JA stimulus		
JAZ2	At1g74950	2.1
JAZ5	At1g17380	2.2
JAZ6	At1g71030	2.0
JAZ7	At2g34600	2.0
JAZ9	At1g70700	2.2
ATPRB1	At2g14580	3.0
TAT3	At2g24850	3.5
MYB2	At1g52030	5.5
CPL3/ETC3	At4g01060	2.2
F2N1_20	At4g01280	2.0
GSH1	At4g23100	2.4
JR2/CORI3	At4g23600	3.9
VSP2	At5g24770	4.7
PDF1.2a	At5g44420	5.4
PDF1.2c	At5g44430	5.4
PDF1.2b	At2g26020	5.1
PDF1.3	At2g26010	4.6
Other pathogen-related genes		
BG1	At1g52400	16
Chitinase	At2g43570	5.4
Peroxidase50	At4g37520	5.0
PAD3	At3g26830	4.8
FAD6	At4g30950	2.3

rather than the reverse. A third group of Arabidopsis mutants show increased resistance to necrotrophic fungi without an apparent effect on SA defense responses (Penninckx et al., 2003; Coego et al., 2005). The Arabidopsis response to *P. syringae* is mediated by SA-dependent defense pathways. However, we observed no effect of the *rst1* mutation on plant response to *P. syringae* despite *rst1*'s reduced SA levels and SA-dependent responses. Whether the differences in cuticle lipids between the wild type and *rst1* influence any of the differential responses to the bacterial pathogen *P. syringae* (a nonobligate pathogen) and the obligate biotrophic fungus and necrotrophic fungi examined here, either through physical or chemical cues, requires further investigation. The specificity of the susceptible responses in the *rst1* mutant to an obligate biotrophic fungus, however, suggests that there may be a unique RST1-regulated pathogen response linked to cuticle production.

In spite of cross talk between these pathways, plant defense to biotrophs is primarily modulated by SA-dependent signaling, whereas defense to necrotrophs is primarily modulated by ET/JA-dependent signaling (Thomma et al., 1999; Dewdney et al., 2000; Diaz et al., 2002; Ferrari et al., 2003). Based on our results, *RST1* appears to be most closely associated with direct regulation of the JA-dependent defense pathways but likely has a strong indirect effect on SA signaling pathways as well. In support of a regulatory role of *RST1* in JA signaling, the *rst1* mutant exhibits increased basal expression of the *PDF1.2* gene and enhanced *PDF1.2* transcription upon necrotrophic infection. By comparison, the *PR1* and *PR2* genes are suppressed in *rst1* after inoculation with the biotrophic pathogen *E. cichoracearum*. In addition, *rst1* mutants have much lower SA levels compared with the wild type during biotrophic infection, indicating a negative

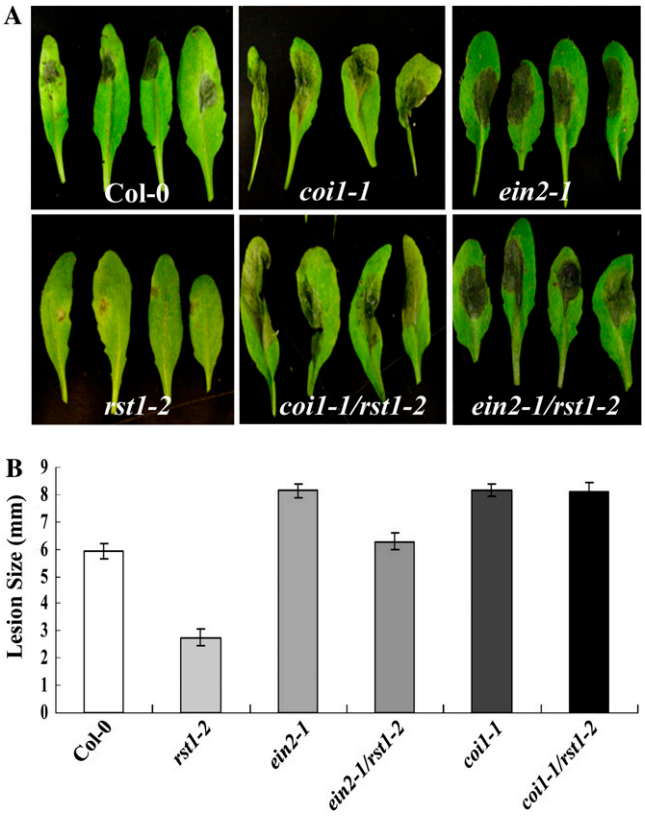

Figure 9. Effects of *coi1* and *ein2* mutations on the *B. cinerea* resistance of the *rst1* mutant. A, Disease symptoms on drop-inoculated leaves of wild-type, single mutant, and double mutant plants at 3 dpi with *B. cinerea*. B, Mean disease lesion size in *B. cinerea*-inoculated plants at 3 dpi. Data represent means ± SD. The values were determined from three independent experiments. Each experiment contained measurements from lesions of 20 inoculated leaves per genotype. Disease assays were performed by drop inoculation of leaves on whole plants, and representative leaves were detached for pictures.

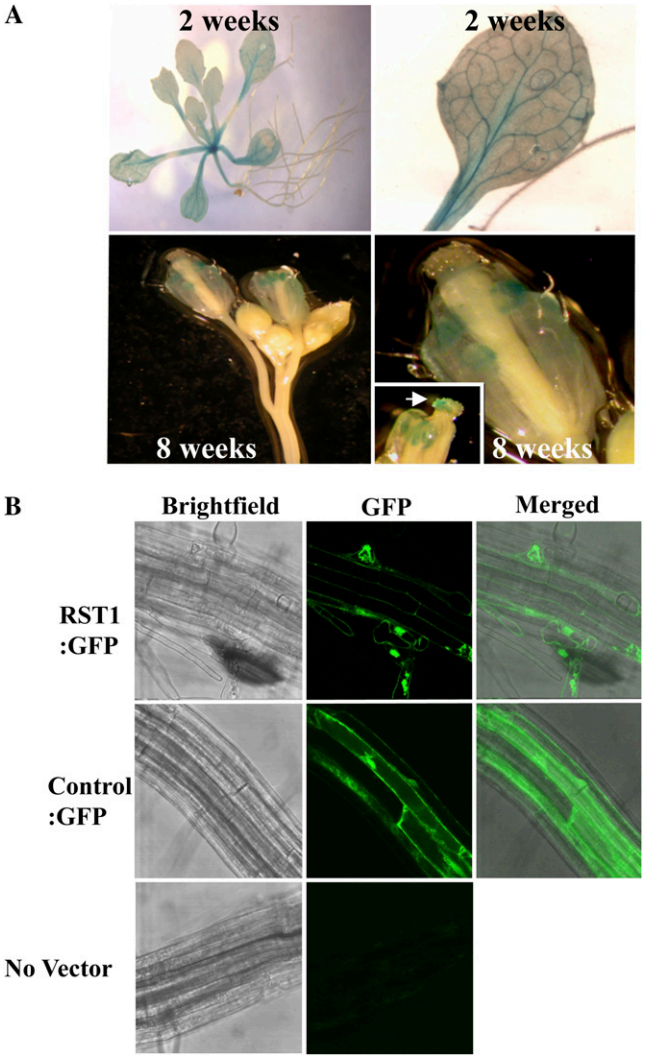

Figure 10. Tissue-specific expression of RST1pro:GUS-derived fusion protein and subcellular localization of RST1:GFP-derived fusion protein in Arabidopsis roots. A, Series of images showing GUS activity in transgenic Arabidopsis plants expressing GUS under the control of the RST1 promoter. Two-week-old seedlings and leaves (top row) and the flowers from 8-week-old plants (bottom row) are shown. The white arrow indicates a pollen tube on the stamen of a flower. B, Subcellular localization of the CaMV 35Spro:RST1:GFP-derived fusion protein in the roots of transgenic Arabidopsis plants. Subcellular localizations of RST1 (top row), GFP control (middle row), and no-vector control (bottom row) are shown.

effect of the *rst1* mutation on SA signaling or synthesis. Transcriptome analysis reveals a surprisingly high induction of numerous JA synthesis genes in the *rst1* mutant, suggesting that the wild-type RST1 protein likely serves as a suppressor of JA synthesis (just as *RST1* appears to suppress cutin and wax synthesis). Previous reports show that activation of the JA defense pathway can have a suppressive effect on SA synthesis and associated gene expression (Petersen et al., 2000). Based on this, we hypothesize that in the absence of RST1, JA synthesis is elevated, leading to the suppression of SA synthesis and/or responses (Fig. 11). The increased susceptibility of *rst1* to *E. cichoracearum* is consistent with this hypothesis, as resistance to obligate pathogens is primarily mediated through an SA-dependent pathway. Impaired or reduced SA synthesis or responses is known to reduce *PR1* and *PR2* gene expression and is associated with increased susceptibility to biotrophic pathogens. In this way, our results are consistent with the previously reported antagonistic interactions between the JA/ET- and SA-dependent pathways (Kunkel and Brooks, 2002; Spoel et al., 2003).

A previous report on the *rst1* mutant revealed that the *RST1* gene is associated with cuticle wax synthesis and embryo development (Chen et al., 2005). The *rst1* mutant exhibited a deficiency in stem cuticle waxes of 59% but an increase in rosette leaf waxes of 43% (primarily due to increased leaf alkanes of 31 and 33 carbon chain length). In this report, we show that the level of total leaf cutin monomers was increased as much as 32% above wild-type levels (due primarily to increased C18:2 dicarboxylic acids), indicating that the functional *RST1* acts as a suppressor of both cutin monomer and wax production pathways. Similarly, the Arabidopsis *bdg* mutant (defective in an α/β-hydrolase fold-containing protein) had increased amounts of leaf cutin monomers and waxes and, like *rst1*, exhibited enhanced resistance to *B. cinerea* (Kurdyukov et al., 2006; Tang et al., 2007). The *lacs2* mutant (defective in an acyl-CoA synthetase protein) also has increased *B. cinerea* resistance, but in contrast to *rst1* and *bdg*, it has decreased cutin monomers, especially C18:2 dicarboxylic acids (Bessire et al., 2007; Chassot et al., 2007). In previous studies, it was speculated that the increased cuticle permeability of *bdg* and *lacs2* may cause elevated secretion of antifungal compounds through a less restrictive cuticle, resulting in the increased *B. cinerea* resistance observed in *bdg* and *lacs2* (Bessire et al., 2007). However, the *rst1* mutant, in contrast to *bdg* and *lacs2*, does not show

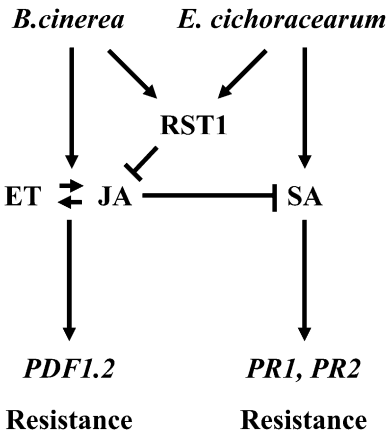

Figure 11. A model for the function of RST1 as a modulator of plant defense through the suppression of JA biosynthesis.

elevated permeability of its leaf cuticles relative to the wild type, indicating the existence of some other mechanism for *rst1*'s resistance to necrotrophic infection. It is interesting that transcriptome analyses of another wax mutant, *cer6* (defective in 3-ketoacyl-CoA synthase required for very long-chain wax synthesis), revealed that many ET/JA- and SA-dependent defense genes were differentially expressed (Fiebig et al., 2000; Garbay et al., 2007) and indicated that cuticle metabolic pathways may play a direct role in modulating plant pathogen-defense-responsive pathways. Whether this cuticle modulation of plant defense occurs via physical factors or chemical signaling is still uncertain. Potentially, the increased amounts of cutin and cuticular wax on *rst1* leaves could protect against fungal necrotrophs by creating a more significant physical barrier that restricts pathogen turgor- and cutinase/esterase-driven infection and penetration processes (Pascholati et al., 1992; Fric and Wolf, 1994; Nicholson and Kunoh, 1994; Zimmerli et al., 2004; Skamnioti and Gurr, 2007). The perturbation of SA signaling, JA-mediated responses, and SA and JA levels also raises the possibility that *rst1*'s altered cuticle lipids could serve themselves as elicitors of plant defense responses (Lin and Kolattukudy, 1978; Woloshuk and Kolattukudy, 1986; Podila et al., 1988; Trail and Koeller, 1993; Francis et al., 1996; Li et al., 2002; Chassot and Métraux, 2005). Still, it cannot be dismissed that *RST1* or its protein product could also act more directly as a regulatory or otherwise fungus-active compound, with cuticle modifications being a secondary effect of the *RST1* defect.

Recent reports demonstrate that the 18:1 free fatty acid products of SSI2, a stearoyl-acyl carrier protein desaturase, are important signaling determinants conferring resistance to *B. cinerea* through the JA signaling pathway (Kachroo et al., 2001; Chandra-Shekara et al., 2007; Chaturvedi et al., 2008). Although our microarray analysis of *rst1* does not show altered *SSI2* expression, a fatty acid desaturase gene, *FAD6*, is significantly induced in the *rst1* background, indicating that lipid synthesis pathways other than cuticle lipid pathways may also be affected by the *rst1* mutation. Furthermore, a previous report showed that the *rst1* mutation blocks the synthesis of seed storage lipids derived from triacylglycerols (Chen et al., 2005). The effect of *RST1* expression on multiple lipid synthetic pathways raises intriguing questions about the function of *RST1* in generating lipids that might have signaling roles in plant pathogen interactions. Further studies are clearly needed, however, to dissect the mechanism of RST1 function and determine the point of action at which it regulates lipid biosynthesis.

Finally, our microarray analysis revealed another possible role for *RST1* in ABA-associated defense response pathways. Of note, the *rst1* mutant shows a 16-fold increase in transcription of the *BG1* gene, a gene that encodes a glycosyl hydrolase known to cleave ABA from its glycosyl conjugate (Lee et al., 2006). Previous reports show that ABA affects plant defense responses negatively or positively depending on the plant-pathogen combination (Mauch-Mani and Mauch, 2005). ABA has been shown to suppress SA signaling (Audenaert et al., 2002; Mohr and Cahill, 2007), and antagonistic cross talk has been observed between SA- and ABA-mediated signaling in certain environmental stress responses (Yasuda et al., 2008). In addition, *ENHANCED DISEASE RESISTANCE1*-mediated resistance to powdery mildew is mediated, in part, by enhanced ABA signaling (Wawrzynska et al., 2008). As such, the down-regulation of SA synthesis in *rst1* could be due to alterations in *rst1*'s ABA levels. Further studies are thus needed to assess ABA amounts and other disease-associated ABA-related phenotypes in the *rst1* mutant.

We report here the first plant mutant, to our knowledge, to exhibit resistance to necrotrophic pathogens but susceptibility to biotrophic pathogens, in contrast to previously reported mutants that exhibit increased susceptibility to necrotrophs but resistance to biotrophs (Veronese et al., 2006). *RST1* modulates defense responses by affecting interactions between JA and SA synthesis and response pathways. Our findings here also implicate lipid pathways and ABA as players in *RST1*-mediated defense responses. Although the localization and bioinformatic analyses presented here indicate that RST1 is a plasma membrane-bound protein having 11 predicted transmembrane domains (Hofmann and Stoffel, 1993), RST1 does not show significant identity to any protein of known function. Notwithstanding, the predicted three-dimensional protein structure of RST1 reveals similarity to the peroxisomal ATP-binding cassette transporter COMATOSE (CTS), a protein involved in JA synthesis via its role in transporting JA precursors into the peroxisomal lumen (Theodoulou et al., 2005). In contrast to *rst1*, the basal JA levels were greatly reduced in the *cts* mutant, and JA accumulates much less in response to wounding in *cts* plants compared with the wild type. If the RST1 protein is in fact a transporter similar to CTS, it is unclear why its deficiency would increase rather than decrease JA levels, as does a deficiency of CTS. Further studies are needed to shed light on the exact role of RST1 in the complex signaling networks leading to plant disease resistance.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) plants were grown on soilless medium (Metro-Mix200; Grace-Sierra) in growth chambers under a 12-h-light (23°C)/12-h-dark (22°C) or a 16-h-light (23°C)/8-h-dark (22°C) cycle at 70% relative humidity. Arabidopsis accessions Col-0 and C24 were used as a wild type. The T-DNA insertion mutant *rst1-1* (C24 background) was screened as described previously (Chen et al., 2005) and was selected from backcross populations to remove additional T-DNA inserts. *rst1-2* and *rst1-3* (Col-0 background) were obtained from the SALK T-DNA insertion collection at the Arabidopsis Biological Resource Center and had been backcrossed one time. *rst1-2* and *rst1-3* were crossed reciprocally. The *pad4-1* line was described previously (Feys et al., 2005).

Pathogen Infections

Erysiphe cichoracearum strain UCSC1 was provided by Dr. Roger Innes (Indiana University, Bloomington). *E. cichoracearum* UCSC1 was maintained by inoculation of 4- or 5-week-old *pad4-1* plants by tapping conidia from two or three infected leaves. Actively growing conidia (7–10 dpi) were used for inoculation of plants for experiments. Two methods of inoculation were used. High-density inoculations (20–50 conidia mm^{-2}) were conducted by gently touching infected leaves to target plants. This method was used to determine disease resistance score with various ecotypes of Arabidopsis and in initial observation. Low-density inoculations were conducted with a modified settling tower (Adam and Somerville, 1996), a square metal tower 71 cm high covered with nylon mesh (40-μm openings) to break up the conidial chains. The more uniform low-density method was used for quantitative analysis of fungal development.

Cultures of *Botrytis cinerea* strain BO5-10 and *Alternaria brassicicola* strain MUCL 20297 were grown and disease assays were performed as described previously (Veronese et al., 2006). The bacterial pathogen *Pseudomonas syringae* pv *tomato vir* (*Pst* DC3000) and *avr* (*Pst* DC3000 *avrRps4*) were grown at 30°C on King's B agar plates or in liquid medium (King et al., 1954) containing 50 μg mL^{-1} kanamycin and 50 μg mL^{-1} rifampicin. Cultured bacteria were resuspended in 10 mM $MgCl_2$ and then adjusted to 1×10^5 colony-forming units mL^{-1}. Suspended cells were infiltrated into leaves using a 1-mL syringe without a needle. Three leaf discs were collected from three independent samples and then ground in 10 mM $MgCl_2$, serial diluted at 1:10, and plated on King's agar plates. Colonies were counted 48 h after incubation at 30°C.

Quantification of *E. cichoracearum* Growth

For quantitative analysis, the leaves were detached from 4- to 5-week-old plants grown in a growth chamber and placed on a 1.5% water agar plate with petioles embedded in the medium. The leaves could be sustained for at least 6 d under these conditions. The plates were inoculated using a settling tower as described above. The agar plates were placed in a Percival growth chamber at 20°C. High humidity was maintained by covering the plates with a plastic lid. The germination of spores was determined at 1 d after inoculation. Secondary hyphal length (4 d after inoculation) and conidiophore number (5 d after inoculation) were obtained from a minimum of six stained leaves from independent experiments. The number of conidiophores was counted per colony. Leaves were stained by boiling for 2 min in alcoholic lactophenol trypan blue (20 mL of ethanol, 10 mL of phenol, 10 mL of water, 10 mL of lactic acid, and 10 mg of trypan blue). The stained leaves were mounted under coverslips with 50% glycerol and examined using standard light microscopy images. Well-separated colonies in the central part of upper leaf surface were selected for analysis.

RNA Preparation and Quantitative RT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen). Two micrograms of total RNA was used as a template for first-strand cDNA synthesis with SuperScript II (Invitrogen) and an oligo(dT) primer. One microliter of cDNA was used as a template for the following primer sets: RST1-F (5′-TGGATGCCTACACTGTGGTT-3′), RST1-R (5′-GTACATGAGGAGAAGCGCAA-3′), PR1-F (5′-CATACACTCTGGTGGGCCTT-3′), PR1-R (5′-GACCACAAACTCCATTGCAC-3′), PR2-F (5′-ATCTCCCTTGCTCGTGAATC-3′), PR2-R (5′-TCGAGATTTGCGTCGAATAG-3′), PDF1.2-F (5′-GTTTGCGGAAACAGTAATGC-3′), PDF1.2-R (5′-CACACGATTTAGCACCAAAGA-3′), Tublin-F (5′-CGTGGATCACAGCAATACAGAGCC-3′), and Tublin-R (5′-CCTCCTGCACTTCCACTTCGTCTTC-3′). Gene-specific primers were designed using PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/). Hairpin stability and compatibility were analyzed using OligoAnalyzer 3.0 (http://www. idtdna. com/ analyzer/ Applications/OligoAnalyzer/). The PCR products were 130 to 150 bp in length. Quantitative RT-PCR was performed in 20-μL reactions containing 20 ng of template obtained from first-strand cDNA synthesis. Amounts were 0.3 μM each primer and 2× QuantiTect SYBR Green PCR Master Mix (Qiagen). The following PCR program was used to amplify: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s (denaturing), 58°C for 1 min (annealing), and 72°C for 1 min (extension). Primer efficiencies and relative expression levels were calculated using the comparative C_T method (User Bulletin 2, ABI Prism 7700 Sequence Detection System). $2^{-\Delta\Delta C}{}_T$ values of control samples were normalized to 1.

Microarray Hybridization and Statistical Analysis

Total RNA (70 μg) was extracted from each sample using the Qiagen RNeasy Plant RNA Miniprep kit, RNA samples were reverse transcribed using SuperScript III (Invitrogen), and cDNAs were labeled with Cy3 or Cy5 by indirect labeling (Gong et al., 2005). Microarray slides over 26,000 probes (70-mer oligonucleotides) were used (http//ag.arizona.edu/microarray). To eliminate bias in the microarrays as a consequence of dye-related differences in labeling efficiency, dye labeling for each paired sample (mutant/wild type) was swapped. Three biological repeats were performed. Signal intensities were collected by GenePix 4000B (Axon Instruments), and images were analyzed using GenePix Pro 4.0. Lower intensities of the spots than background or with an aberrant spot shape were flagged by the GenePix software and confirmed manually, and original raw data of GPR files were analyzed by the TIGR-TM4 package (http://www.tm4.org; Saeed et al., 2003). Statistical analyses were performed using TM4-MEV (version 3.0.3). In MEV, a one-way *t* test with $P = 0.01$ was carried out to determine the different expression (mutant/wild type).

SA Measurements

Leaf tissues were collected from 4-week-old soil-grown plants. Tissue (0.3 g fresh weight) was extracted in 6 mL of ice-cold methanol for 24 h at 4°C and then in a solution of 3.6 mL of water plus 3 mL of chloroform with 20 μL of 5 mM 3,4,5-trimethoxy-trans-cinnamic acid (internal standard) for 24 h at 4°C. Supernatants were dried by speed vacuum. The residue was resuspended in 0.6 mL of ice-cold water:methanol (1:1, v/v), and SA was quantified by HPLC as described previously (Freeman et al., 2005).

Quantification of JA Levels

Leaf tissue (300 mg fresh weight per sample) was collected and immediately frozen at −80°C. The leaves were then extracted using 6 mL of cold methanol for 24 h at 4°C. At the time of methanol addition, 60 ng of dihydro-JA was added as an internal standard for quantitation. The methanol was separated from the plant tissue. The methanol solution was added to 3.6 mL of water and 3 mL of chloroform. After shaking, samples were allowed to sit for 24 h at 4°C. The supernatants were dried by speed vacuum. The residue was resuspended in 0.5 mL of an 80% methanol:20% water solution. The solution was centrifuged at 16,000*g* for 5 min. The supernatant was transferred to a new vessel and dried by speed vacuum. The remaining residue was redissolved in 50 μL of 50% mobile phase A and 50% mobile phase B prior to analysis by HPLC-mass spectrometry. Separations were performed on an Agilent 1100 system using a Waters Xterra MS C8 column (5 μm, 2.1 × 150 mm). A binary mobile phase consisting of solvent systems A and B was used in gradient elution where A was 0.1% (v/v) formic acid in double distilled water and B was 0.1% (v/v) formic acid in acetonitrile. The mobile phase flow rate was 0.3 mL min^{-1}. Initial conditions were set at 75:25 A:B with a linear gradient to 20:80 from 0 to 30 min. Gradient conditions were reset to 75:25 A:B from 30 to 32 min, then the column was equilibrated for 10 min at initial conditions prior to the next run. Following separation, the column effluent was introduced by negative mode electrospray ionization into an Agilent MSD-TOF spectrometer. Electrospray ionization capillary voltage was −3.5 kV, nitrogen gas temperature was set to 350°C, drying gas flow rate was 9.0 L min^{-1}, nebulizer gas pressure was 35 psig, fragmentor voltage was 135 V, skimmer was 60 V, and octopole radio frequency was 250 V. Mass data (mass-to-charge ratio from 65 to 800) were collected and analyzed using Agilent MassHunter software. JA quantification was accomplished using a multilevel calibration curve.

Cutin Polyester Analysis

Leaf polyester content was analyzed on 20-d-old plants based on modification of depolymerization methods described previously (Bonaventure et al., 2004; Franke et al., 2005). Ground leaf tissues were delipidated in a Soxhlet extractor for 72 h with chloroform:methanol (1:1, v/v) containing 50 mg L^{-1} butylated hydroxytoluene. After delipidation, tissues were washed with methanol containing 50 mg L^{-1} butylated hydroxytoluene and dried in a vacuum desiccator for 4 d to 1 week before chemical analysis. Depolymerization reactions consisted of 6 mL of 3 N methanolic hydrochloride (Supelco) containing 0.45 mL (7%, v/v) of methyl acetate at 60°C. Methyl heptadecanoate was used as an internal standard. After 16 h, reactions were allowed to

cool to room temperature and terminated by the addition of 6 mL of saturated, aqueous NaCl followed by two extractions (10 mL) with distilled dichloromethane to remove methyl ester monomers (Bonaventure et al., 2004). The organic phase was washed three times with 0.9% (w/v) aqueous NaCl, dried with 2,2-dimethoxypropane, and dried under nitrogen gas. Monomers were derivatized in pyridine and BSTFA (1:1, v/v) for 15 min at 100°C. Excess pyridine:BSTFA was removed with nitrogen gas, and the sample was dissolved in heptane:toluene (1:1, v/v) prior to analysis with a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector and a 12-m, 0.2-mm i.d. HP-1 capillary column with helium as the carrier gas. The gas chromatograph was programmed with an initial temperature of 80°C and increased at 15°C min^{-1} to 200°C, then increased at 2°C min^{-1} to 280°C. Quantification was based on uncorrected flame ionization detection peak areas relative to the internal standard methyl heptadecanoate peak area. Areas of rosette leaves were determined by ImageJ software (http://rsb.info.nih.gov/ij/) using digital images of flattened leaves. Cutin data were analyzed using SAS 9.1.3 software (SAS Institute). Student's *t* tests were used to detect significant differences between cutin monomer means. Five replicates of each line were used for leaf cutin monomer analysis, and α was set at 0.05.

Generation of *RST1* Overexpression and Complemented Transgenic Plants

To generate the *CaMV 35Spro*:*RST1* construct, *RST1* genomic DNA was amplified by PCR with primer sets (including an *Xba*I restriction site [boldface] in the first part of the forward primer) F (5′-GC**TCTAGA**TTGGGCCAAATCG-GACGGC-3′) and R (5′-GTGGCGACAATTTAAGGAG-3′) for the first part and F2 (5′-GACCTTTCAGCGTCCGGCG-3′) and R2 (5′-GGCTACTATGTC-GATGTACC-3′) for the second fragment. Two fragments were amplified, as few applicable multiple cloning sites were present in the binary vector pCAMBIA99-1 (a pCAMBIA 1200-based vector containing modified enzyme sites). The first fragment (3,858 bp) consists of the 50 bp from the start codon to about 50 bp downstream of the *Pst*I site in the middle of the *RST1* genomic sequence. The second part (4,410 bp) consists of about 30 bp upstream of *Pst*I to about 100 bp downstream of the stop codon. Amplified PCR fragments were subcloned into pGEM-T Easy vector (Promega). Subcloned first and second fragments in T Easy vector were digested by *Xba*I and *Pst*I or by *Pst*I and *Eco*RI, respectively, and then two fragments were subcloned into pCAMBIA99-1 between the *Xba*I and *Eco*RI sites. The construct was introduced into *rst1-2*, *rst1-3*, and wild-type Col-0 using an *Agrobacterium tumefaciens*-mediated (strain GV3101) floral dipping transformation method (Clough and Bent, 1998).

Construction of the RST1 Promoter:GUS Reporter and GUS Activity Assay

To generate *RST1 promoter*:*GUS*, a 1,200-bp upstream region including the initiation codon of *RST1* was amplified by PCR with the following primers containing *Bam*HI and *Spe*I restriction sites: 5′-GAATTCCGCGGCCCCTC-CACTAACC-3′ and 5′-CCATGGCGTATGAGGCCATCGCTTTGG-3′, respectively. The PCR product was digested with *Bam*HI and *Spe*I and subcloned at *Bam*HI and *Spe*I sites of the pCAMBIA 1303 vector, which harbors *GUS* and *GFP* reporter genes. The *RST1pro*:*GUS*:*GFP* clone, along with a *35Spro*:*GUS*:*GFP* control, were transformed into Arabidopsis Col-0 using *Agrobacterium*-mediated transformation. Various developmental stages of transgenic plants were incubated overnight in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Rose Scientific) and 0.1 M potassium phosphate buffer (pH 7.5 with 0.1% [v/v] Triton X-100). Chlorophyll was removed by washing samples two to three times with 70% (v/v) ethanol. Samples were monitored and captured using a Nikon E 800 microscope.

Subcellular Localization of RST1

To generate *CaMV* $35S_{pro}$:*RST1*:*GFP*, the full-length *RST1* open reading frame without stop codon was synthesized with the following primer set including *Xho*I and *Spe*I restriction sites: F (5′-CTCGAGATGGCCTCA-TACGCTACG-3′) and R (5′-ACTAGTAGACATGTCCATAGAAGCAA-3′), respectively. The PCR products were subcloned in pGEM-T Easy vector (Promega), digested with *Xho*I, produced as a blunted end using Klenow fragment polymerase (Roche), and then digested with *Spe*I. The fragment including a 5′ blunted end and a 3′ cohesive end was subcloned in frame with pCAMBIA 1302 prepared as an insert fragment except using *Nco*I instead of *Xho*I. Plasmids were purified using the Qiagen Plasmid Mini Purification kit according to the manufacturer's protocol. The plasmids were introduced into *rst1-2*. Five rescued plants in the T3 generation were screened from the *rst1-2* background. Plants were grown on a Murashige and Skoog solid plate for 3 to 4 d. Images were taken using a Radiance 2100 MP Rainbow (Bio-Rad) on a TE2000 (Nikon) inverted microscope using a 60 × 1.4 numerical aperture lens. The 488-nm line of the four-line argon laser (National Laser) was used to excite the GFP, and the fluorescence emitted between 500 and 540 nm was collected. The transformants in the Col-0 background were confirmed as controls.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AY307371.

Supplemental Data

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

Supplemental Figure S1. Characterization of *rst1* mutations and the predicted structure of the RST1 protein.

Supplemental Figure S2. Response of Arabidopsis wild-type C24 and the *rst1-1* mutant to *E. cichoracearum* inoculation.

Supplemental Figure S3. Transpiration rate in the dark of wild-type Col-0, *rst1-2*, and *pad 4-1*.

Supplemental Figure S4. Sensitivity of Col-0, *rst1-3*, *pad4-1*, and C24 plants to BASTA.

Supplemental Figure S5. Permeability assay on leaf cuticles of wild-type Col-0, *rst1-2*, *rst1-3*, *pad4-1*, and *lacs2-3*.

Supplemental Figure S6. The *rst1* mutants show slightly high basal expression of *PR1* and *PR2*.

Supplemental Figure S7. The *rst1* mutants show higher basal expression of *BG1* (At1g52400), *CHITINASE* (At2g43570), and *ATHILA RETROELEMENT* (At5g32475).

Supplemental Figure S8. The *rst1* mutants display normal bacterial growth after inoculation with *Pst* strains DC3000 and DC3000 expressing *avrRps4*.

ACKNOWLEDGMENTS

We thank Shisong Ma and Dong-ha Oh for statistical data analyses. We thank Dr. David Salt's laboratory for help with the SA assay and Dr. Roger Innes for providing *E. cichoracearum* strain UCSC1. We also thank the World Class University Program (R32-10148) of the Ministry of Education, Science, and Technology in Korea for their support. Lastly, we thank the SALK Institute Genomic Analysis Laboratory and the Arabidopsis Biological Resource Center for providing the sequence-indexed Arabidopsis T-DNA mutants (SALK 070359 and 129280).

Received May 28, 2009; accepted July 17, 2009; published July 22, 2009.

LITERATURE CITED

Adam L, Somerville SC (1996) Genetic characterization of five powdery mildew disease resistance loci in *Arabidopsis thaliana*. Plant J **9:** 341–356

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. Science **284:** 2148–2152

Audenaert K, De Meyer GB, Hofte MM (2002) Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiol **128:** 491–501

Bessire M, Chassot C, Jacquat AC, Humphry M, Borel S, Petéot J, Méraux JP, Nawrath C (2007) A permeable cuticle in *Arabidopsis* leads to a strong resistance to *Botrytis cinerea*. EMBO J **26:** 2158–2168

Bohman S, Staal J, Thomma BP, Wang M, Dixelius C (2004) Characterization of an Arabidopsis-*Leptosphaeria maculans* pathosystem: resistance

partially requires camalexin biosynthesis and is independent of salicylic acid, ethylene and jasmonic acid signalling. Plant J **37:** 9–20

Bonaventure G, Beisson F, Ohlrogge J, Pollard M (2004) Analysis of the aliphatic monomer composition of polyesters associated with Arabidopsis epidermis: occurrence of octadeca-*cis*-6, *cis*-9-diene-1,18-dioate as the major component. Plant J **40:** 920–930

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88:** 57–63

Chandra-Shekara AC, Venugopal SC, Barman SR, Kachroo A, Kachroo P (2007) Plastidial fatty acid levels regulate resistance gene-dependent defense signaling in *Arabidopsis*. Proc Natl Acad Sci USA **104:** 7277–7282

Chassot C, Métraux JP (2005) The cuticle as source of signal for plant defense. Plant Biosyst **139:** 28–31

Chassot C, Nawrath C, Métraux JP (2007) Cuticular defects lead to full immunity to a major plant pathogen. Plant J **49:** 972–980

Chaturvedi R, Krothapalli K, Makandar R, Nandi A, Sparks AA, Roth MR, Welti R, Shah J (2008) Plastid ω3-fatty acid desaturase-dependent accumulation of a systemic acquired resistance inducing activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid. Plant J **54:** 106–117

Chen X, Goodwin SM, Liu X, Bressan RA, Jenks MA (2005) Mutation of the *RESURRECTION1* locus of Arabidopsis reveals an association of cuticular wax with embryo development 1. Plant Physiol **139:** 909–919

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Coego A, Ramirez V, Gil MJ, Flors V, Mauch-Mani B, Vera P (2005) An *Arabidopsis* homeodomain transcription factor, OVEREXPRESSOR OF CATIONIC PEROXIDASE 3, mediates resistance to infection by necrotrophic pathogens. Plant Cell **17:** 2123–2137

Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. Nature **411:** 826–833

Dewdney J, Reuber TL, Wildermuth MC, Devoto A, Cui J, Stutius LM, Drummond EP, Ausubel FM (2000) Three unique mutants of *Arabidopsis* identify eds loci required for limiting growth of a biotrophic fungal pathogen. Plant J **24:** 205–218

Dhawan R, Luo H, Foerster AM, Abuqamar S, Du HN, Briggs SD, Mittelsten Scheid O, Mengiste T (2009) HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in *Arabidopsis*. Plant Cell **21:** 1000–1019

Diaz J, ten Have A, van Kan JAL (2002) The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. Plant Physiol **129:** 1341–1351

Doares SH, Narvaez-Vasquez J, Conconi A, Ryan CA (1995) Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol **108:** 1741–1746

Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc Natl Acad Sci USA **89:** 2480–2484

Epple P, Apel K, Bohlmann H (1995) An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol **109:** 813–820

Epple P, Mack AA, Morris VR, Dangl JL (2003) Antagonistic control of oxidative stress-induced cell death in Arabidopsis by two related, plant-specific zinc finger proteins. Proc Natl Acad Sci USA **100:** 6831–6836

Fauth M, Schweizer P, Buchala A, Markstadter C, Riederer M, Kato T, Kauss H (1998) Cutin monomers and surface wax constituents elicit H_2O_2 in conditioned cucumber hypocotyl segments and enhance the activity of other H_2O_2 elicitors. Plant Physiol **117:** 1373–1380

Ferrari S, Galletti R, Denoux C, De Lorenzo G, Ausubel FM, Dewdney J (2007) Resistance to *Botrytis cinerea* induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires *PHYTOALEXIN DEFICIENT3*. Plant Physiol **144:** 367–379

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) Arabidopsis local resistance to Botrytis cinerea involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. Plant J **35:** 193–205

Feys BJ, Moisan LJ, Newman MA, Parker JE (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J **20:** 5400–5411

Feys BJ, Wiermer M, Bhat RA, Moisan LJ, Medina-Escobar N, Neu C, Cabral A, Parker JE (2005) *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. Plant Cell **17:** 2601–2613

Feys BJF, Benedetti CE, Penfold CN, Turner JG (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6:** 751–759

Fiebig A, Mayfield JA, Miley NL, Chau S, Fischer RL, Preuss D (2000) Alterations in *CER6*, a gene identical to *CUT1*, differentially affect long-chain lipid content on the surface of pollen and stems. Plant Cell **12:** 2001–2008

Francis SA, Dewey FM, Gurr SJ (1996) The role of cutinase in germling development and infection by *Erysiphe graminis* f. sp. *hordei*. Physiol Mol Plant Pathol **49:** 201–211

Franke R, Briesen I, Wojciechowski T, Faust A, Yephremov A, Nawrath C, Schreiber L (2005) Apoplastic polyesters in *Arabidopsis* surface tissues: a typical suberin and a particular cutin. Phytochemistry **66:** 2643–2658

Freeman JL, Garcia D, Kim D, Hopf A, Salt DE (2005) Constitutively elevated salicylic acid signals glutathione-mediated nickel tolerance in Thlaspi nickel hyperaccumulators 1. Plant Physiol **137:** 1082–1091

Fric F, Wolf G (1994) Hydrolytic enzymes of ungerminated and germinated conidia of Erysiphe graminis DC. f.sp. hordei Marchal. J Phytopathol **140:** 1–10

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754–756

Garbay B, Tautu MT, Costaglioli P (2007) Low level of pathogenesis-related protein 1 mRNA expression in 15-day-old *Arabidopsis cer6-2* and *cer2 eceriferum* mutants. Plant Sci **172:** 299–305

Gilbert RD, Johnson AM, Dean RA (1996) Chemical signals responsible for appressorium formation in the rice blast fungus *Magnaporthe grisea*. Physiol Mol Plant Pathol **48:** 335–346

Gong Q, Li P, Ma S, Indu Rupassara S, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana*. Plant J **44:** 826–839

Goodwin SM, Jenks MA (2005) Plant cuticle function as a barrier to water loss. *In* MA Janks, PM Hasegawa, eds, Plant Abiotic Stress. Blackwell Publishing, Oxford, pp 14–36

Gupta V, Willits MG, Glazebrook J (2000) *Arabidopsis thaliana* EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: evidence for inhibition of jasmonic acid signaling by SA. Mol Plant Microbe Interact **13:** 503–511

Hofmann K, Stoffel W (1993) TMbase: a database of membrane spanning protein segments. Biol Chem Hoppe Seyler **374:** 166

Jenks MA, Joly RJ, Peters PJ, Rich PJ, Axtell JD, Ashworth EN (1994) Chemically induced cuticle mutation affecting epidermal conductance to water vapor and disease susceptibility in Sorghum bicolor (L.) Moench. Plant Physiol **105:** 1239–1245

Kachroo P, Kachroo A, Lapchyk L, Hildebrand D, Klessig DF (2003a) Restoration of defective cross talk in *ssi2* mutants: role of salicylic acid, jasmonic acid, and fatty acids in *SSI2*-mediated signaling. Mol Plant Microbe Interact **16:** 1022–1029

Kachroo A, Lapchyk L, Fukushige H, Hildebrand D, Klessig D, Kachroo P (2003b) Plastidial fatty acid signaling modulates salicylic acid- and jasmonic acid-mediated defense pathways in the *Arabidopsis ssi2* mutant. Plant Cell **15:** 2952–2965

Kachroo P, Shanklin J, Shah J, Whittle EJ, Klessig DF (2001) A fatty acid desaturase modulates the activation of defense signaling pathways in plants. Proc Natl Acad Sci USA **98:** 9448–9453

King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med **44:** 301–307

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Curr Opin Plant Biol **5:** 325–331

Kurdyukov S, Faust A, Nawrath CBS, Voisin D, Efremova N, Franke R, Schreiber L, Saedler H, Méraux JP (2006) The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. Plant Cell **18:** 321–339

Lee J, Nam J, Park HC, Na G, Miura K, Jin JB, Yoo CY, Baek D, Kim DH, Jeong JC (2007) Salicylic acid-mediated innate immunity in Arabidopsis is regulated by SIZ1 SUMO E3 ligase. Plant J **49:** 79–90

Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee IJ (2006) Activation of glucosidase via stress-induced poly-

merization rapidly increases active pools of abscisic acid. Cell **126:** 1109–1120

Li DX, Sirakova T, Rogers L, Ettinger WF, Kolattukudy PE (2002) Regulation of constitutively expressed and induced cutinase genes by different zinc finger transcription factors in Fusarium solani f. sp. pisi (Nectria haematococca). J Biol Chem **277:** 7905–7912

Lin TS, Kolattukudy PE (1978) Induction of a polyester hydrolase (cutinase) by low levels of cutin monomers in Fusarium solani f.sp. pisi. J Bacteriol **133:** 942–951

Loake G, Grant M (2007) Salicylic acid in plant defence: the players and protagonists. Curr Opin Plant Biol **10:** 466–472

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science **250:** 1002–1004

Malamy J, Hennig J, Klessig DF (1992) Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. Plant Cell **4:** 359–366

Mauch-Mani B, Mauch F (2005) The role of abscisic acid in plant-pathogen interactions. Curr Opin Plant Biol **8:** 409–414

McConn M, Creelman RA, Bell E, Mullet JE (1997) Jasmonate is essential for insect defense in *Arabidopsis*. Proc Natl Acad Sci USA **94:** 5473–5477

Métraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science **250:** 1004–1006

Mohr PG, Cahill DM (2007) Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in *Arabidopsis* infected with *Pseudomonas syringae* pv. *tomato*. Funct Integr Genomics **7:** 181–191

Nandi A, Moeder W, Kachroo P, Klessig DF, Shah J (2005) *Arabidopsis ssi2*-conferred susceptibility to *Botrytis cinerea* is dependent on *EDS5* and *PAD4*. Mol Plant Microbe Interact **18:** 363–370

Nawrath C (2002) The biopolymers cutin and suberin. *In* CR Somerville, EM Meyerowitz, eds, The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD, doi/, http://www.aspb.org/publications/arabidopsis/

Nawrath C (2006) Unraveling the complex network of cuticular structure and function. Curr Opin Plant Biol **9:** 281–287

Nicholson RL, Kunoh H (1994) Early interactions, adhesion, and establishment of the infection court by Erysiphe graminis. Can J Bot **73:** S609–S615

Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J **31:** 1–12

Pascholati SF, Yoshioka H, Kunoh H, Nicholson RL (1992) Preparation of the infection court by *Erysiphe graminis* f. sp. *hordei*: cutinase is a component of the conidial exudates. Physiol Mol Plant Pathol **41:** 53–59

Penninckx I, Eggermont K, Schenk PM, Van den Ackerveken G, Cammue BP, Thomma BP (2003) The Arabidopsis mutant *iop1* exhibits induced overexpression of the plant defensin gene *PDF1.2* and enhanced pathogen resistance. Mol Plant Pathol **4:** 479–486

Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW, Buchala A, Metraux JP, Manners JM, Broekaert WF (1996) Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. Plant Cell **8:** 2309–2323

Penninckx IAMA, Thomma BPHJ, Buchala A, Metraux JP, Broekaert WF (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. Plant Cell **10:** 2103–2114

Petersen M, Brodersen P, Naested H, Andreasson E, Lindhart U, Johansen B, Nielsen HB, Lacy M, Austin MJ, Parker JE (2000) *Arabidopsis* MAP Kinase 4 negatively regulates systemic acquired resistance. Cell **103:** 1111–1120

Podila GK, Dickman MB, Kolattukudy PE (1988) Transcriptional activation of a cutinase gene in isolated fungal nuclei by plant cutin monomers. Science **242:** 922–925

Rasmussen JB, Hammerschmidt R, Zook MN (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. Plant Physiol **97:** 1342–1347

Reina-Pinto JJ, Yephremov A (2009) Surface lipids and plant defenses. Plant Physiol Biochem **6:** 540–549

Reuber TL, Plotnikova JM, Dewdney J, Rogers EE, Wood W, Ausubel FM (1998) Correlation of defense gene induction defects with powdery mildew susceptibility in *Arabidopsis* enhanced disease susceptibility mutants. Plant J **16:** 473–485

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, et al (2003) TM4: a free, open-source system for microarray data management and analysis. Biotechniques **34:** 374–378

Schweizer P, Jeanguéat A, Möinger E, Méraux JP (1994) Plant protection by free cutin monomers in two cereal pathosystems. *In* MJ Daniels, JA Downie, AE Osbourn, eds, Advances in Molecular Genetics of Plant-Microbe Interactions. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 371–374

Schweizer P, Jeanguenat A, Whitacre D, Métraux J (1996) Induction of resistance in barley against *Erysiphe graminis* f. sp. *hordei* by free cutin monomers. Physiol Mol Plant Pathol **49:** 103–120

Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Méraux JP, Nawrath C (2000) Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell **12:** 721–738

Skamnioti P, Gurr SJ (2007) *Magnaporthe grisea* cutinase2 mediates appressorium differentiation and host penetration and is required for full virulence. Plant Cell **19:** 2674–2689

Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, Van Pelt JA, Mueller MJ, Buchala AJ, Metraux J-P, Brown R, Kazan K, et al (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15:** 760–770

Staswick PE, Yuen GY, Lehman CC (1998) Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. Plant J **15:** 747–754

Tang D, Simonich MT, Innes RW (2007) Mutations in LACS2, a long-chain acyl-coenzyme A synthetase, enhance susceptibility to avirulent *Pseudomonas syringae* but confer resistance to *Botrytis cinerea* in Arabidopsis. Plant Physiol **144:** 1093–1103

Theodoulou FL, Job K, Slocombe SP, Footitt S, Holdsworth M, Baker A, Larson TR, Graham IA (2005) Jasmonic acid levels are reduced in COMATOSE ATP-binding cassette transporter mutants: implications for transport of jasmonate precursors into peroxisomes. Plant Physiol **137:** 835–840

Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci USA **95:** 15107–15111

Thomma BPHJ, Eggermont K, Tierens KFMJ, Broekaert WF (1999) Requirement of functional *Ethylene-Insensitive 2* gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol **121:** 1093–1101

Trail F, Koeller W (1993) Diversity of cutinases from plant pathogenic fungi: purification and characterization of two cutinases from *Alternaria brassicicola*. Physiol Mol Plant Pathol **42:** 205–220

Veronese P, Chen X, Bluhm B, Salmeron J, Dietrich RA, Mengiste T (2004) The *BOS* loci of Arabidopsis are required for resistance to *Botrytis cinerea* infection. Plant J **40:** 558–574

Veronese P, Nakagami H, Bluhm B, Abuqamar S, Chen X, Salmeron J, Dietrich RA, Hirt H, Mengiste T (2006) The membrane-anchored *BOTRYTIS-INDUCED KINASE1* plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. Plant Cell **18:** 257–273

Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J (1998) A role for jasmonate in pathogen defense of *Arabidopsis*. Proc Natl Acad Sci USA **95:** 7209–7214

Wawrzynska A, Christiansen KM, Lan Y, Rodibaugh NL, Innes RW (2008) Powdery mildew resistance conferred by loss of the ENHANCED DISEASE RESISTANCE1 protein kinase is suppressed by a missense mutation in *KEEP ON GOING*, a regulator of abscisic acid signaling. Plant Physiol **148:** 1510–1522

Wiermer M, Feys BJ, Parker JE (2005) Plant immunity: the EDS1 regulatory node. Curr Opin Plant Biol **8:** 383–389

Woloshuk CP, Kolattukudy PE (1986) Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of *Fusarium solani* f.sp. *pisi*. Proc Natl Acad Sci USA **83:** 1704–1708

Xia Y, Gao QM, Yu K, Lapchyk L, Navarre D, Hildebrand D, Kachroo A,

Kachroo P (2009) An intact cuticle in distal tissues is essential for the induction of systemic acquired resistance in plants. Cell Host Microbe **5:** 151–165

Xiao F, Goodwin SM, Xiao Y, Sun Z, Baker D, Tang X, Jenks MA, Zhou JM (2004) *Arabidopsis CYP86A2* represses *Pseudomonas syringae* type III genes and is required for cuticle development. EMBO J **23:** 2903–2913

Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science **280:** 1091–1094

Yalpani N, Silverman P, Wilson TMA, Kleier DA, Raskin I (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell **3:** 809–818

Yasuda M, Ishikawa A, Jikumaru Y, Seki M, Umezawa T, Asami T, Maruyama-Nakashita A, Kudo T, Shinozaki K, Yoshida S, et al (2008) Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. Plant Cell **20:** 1678–1692

Zheng Z, Qamar SA, Chen Z, Mengiste T (2006) Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J **48:** 592–605

Zimmerli L, Stein M, Lipka V, Schulze-Lefert P, Somerville S (2004) Host and non-host pathogens elicit different jasmonate/ethylene responses in Arabidopsis. Plant J **40:** 633–646