The Arabidopsis RESURRECTION1 gene regulates a novel antagonistic interaction in plant defense to biotrophs and necrotrophs

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The Arabidopsis RESURRECTION1 gene regulates a novel antagonistic interaction in plant defense to biotrophs and necrotrophs

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Abstract
We report a role for the Arabidopsis *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 eleven 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 (SA) 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 *coil* 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 SA dependent defense and enhances JA mediated defense, through a mechanism that also controls cuticle synthesis.

*Keywords:* Powdery mildew, *Erysiphe cichoracearum, Botrytis cinerea, Alternaria brassicicola*, jasmonic acid, ethylene, salicylic acid, cuticular wax, cutin monomer.
INTRODUCTION

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., 2003; 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 (HR) (Dangl and Jones, 2001). Pathogen induced HR 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 (GST1), 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; Métraux et al., 1990; Rasmussen et al., 1991; Yalpani et al., 1991; Enyedi et al., 1992; Malamy 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; Diaz et al., 2002; Ferrari et al., 2003; Kachroo et al., 2003). Arabidopsis mutants with altered JA and/or ET signaling or biosynthesis, or the synthesis of OA, show increased susceptibility to the necrotrophs B. cinerea or A. brassicicola (Thomma et al., 1999; Kachroo et al., 2003; 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; Penninckx et al., 1998; Kachroo et al., 2003). 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., 2003). 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 epi- and intra-cuticular 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; Goodwin and Jenks, 2005; Nawrath, 2006). 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 pathogens (Xiao et al., 2004); 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 and rice resistance to *Erysiphe graminis* and *Magnaporthe grisea*, respectively (Schweizer et al., 1994; Schweizer et al., 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 *Botrytis 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 *Botrytis 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 anti-fungal or effector compounds, or 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 obtained from the Arabidopsis Biological Research Center (Columbus, Ohio) (Chen et al., 2005). Transcript analysis by reverse transcription polymerase chain reaction (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 Columbia wild type (Fig. 1, A and B). 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 days post inoculation (dpi) (Table I). At 1 dpi, no significant difference in the asexual spore germination and development of appressorial germ tubes for 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 to wild type (Fig. 2A; Table I). At 5 dpi, E. cichoracearum produced 2 to 4 times more conidiophores on the rst1 mutants than 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 the rst1 mutants with all mutants displaying more rapid pathogen development than wild type (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 ~200 bp of both up- and down-stream 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 to wild-type plants (Table 2). 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 C\textsubscript{18:2} dicarboxylic acids were significantly higher than in wild-type levels, rising from 47.5% of the total cutin monomers in wild type to 56.2% and 66.3% of total cutin monomers for *rst1-2* and *rst1-3*, respectively (Table 2). Other cutin monomers changed very little in these allelic mutants (Table 2). As such, both cuticular wax (Chen et al., 2005) and cutin amount per leaf area is significantly elevated in the *rst1* mutants. Since the leaf area of *rst1-2* and *rst1-3* is unchanged from its 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, and 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-media grown *rst1* plants did not differ from those of the wild type. Moreover, sensitivity to herbicide (BASTA) was not significantly different between wild type and *rst1*, nor did the leaves of *rst1* and wild type show differences in the rate of uptake of toluidine blue stain (Supplemental Fig. S3, S4, and S5). Additionally, no difference was observed in stomatal index or trichome number of the adaxial and abaxial leaf surfaces on *rst1* compared to wild type (Chen et al., 2005). These results provide strong evidence that, although *rst1* has an increased amount 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* were slightly elevated in non-inoculated *rst1*-2 and *rst1*-3 compared to 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 21-26% and 11-14%, respectively, of wild-type induction levels. The lower expression of these pathogenesis related genes in *rst1*-3 than *rst1*-2 corresponds well with the relatively greater increase in susceptibility of *rst1*-3 than *rst1*-2 to *E. cichoracearum* (Table I). Both non-inoculated 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 to wild type following *E. cichoracearum* infection (Fig. 3). The *RST1* gene is also responsive to pathogen infection, with expression in wild type being slightly induced in response to *E. cichoracearum* (Fig. 3). The expression of SA signaling genes N*PR1* and P*AD4* are significantly reduced in the *rst1* mutants relative to 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 to wild type in non-inoculated 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 \textit{rst1} Mutant Displays Enhanced Resistance to Necrotrophic Pathogens \textit{Alternaria brassicicola} and \textit{Botrytis cinerea}

To determine the effects of impaired \textit{RST1} function on plant responses to necrotrophic pathogens, we examined the response of \textit{rst1} to \textit{A. brassicicola} and \textit{B. cinerea}. The \textit{rst1}-2 and \textit{rst1}-3 mutants displayed elevated resistance to both necrotrophs relative to the wild type based on disease symptoms and pathogen growth (Fig. 5A and B, and 6A). The size of disease lesions on inoculated leaves of \textit{B. cinerea} was approximately 3 fold smaller than on wild type (Fig. 6B). The inoculated \textit{rst1} mutant alleles supported significantly lower pathogen sporulation and showed confined disease lesions suggesting that \textit{rst1} suppresses pathogen growth and disease symptoms. Consistent with restoration of \textit{E. cichoracearum} resistance in \textit{rst1} plants expressing the wild-type \textit{RST1} gene (Fig. 1B), the \textit{B. cinerea} susceptibility was restored to wild-type levels in \textit{rst1} mutants expressing the 35S\textsubscript{pro}:\textit{RST1} construct (as was the wild-type wax phenotype), further confirming that expression of the wild-type \textit{RST1} gene promotes infection by the necrotrophic fungi tested (Fig. 6B).

Expression of the plant defensin gene \textit{PDF1.2} positively correlates with activation of JA/ET dependent defenses and resistance to necrotrophic pathogens (Penninckx et al., 1996; Penninckx et al., 1998). In an effort to further examine the role of \textit{RST1} in JA/ET-mediated defenses we determined the expression levels of \textit{PDF1.2} in wild-type and \textit{rst1} plants at various time points after inoculation with \textit{B. cinerea}, as previously described (Veronese et al., 2006). The expression of \textit{PDF1.2} in uninoculated \textit{rst1} plants was significantly higher than in wild-type plants. After inoculation, the expression continued to increase in both the wild-type and \textit{rst1} plants at comparable levels up to 24 hours post inoculation (hpi). However, in the \textit{rst1} mutants, the expression of \textit{PDF1.2} surpassed that of wild type by 48 hpi (Fig. 7A). After 48 h, \textit{PDF1.2} expression began to decline in wild-type plants, but continued to increase in the \textit{rst1} mutants (Fig. 7A). The induced expression of the \textit{PDF1.2} gene was sustained in a manner consistent with the sustained resistance response to the pathogen. The highly induced expression of \textit{RST1} in wild-type plants at 48 hpi preceded the observed increase in \textit{PDF1.2} expression (Fig. 7B). Consistent with their
observed disease responses, the rst1-2 and rst1-3 mutants had 2.6-3.5 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 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 to 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 family 19 (Chitinase), and Athila retroelement to find similar high expression (Fig. 3, 7A, Supplemental Fig. S6, 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 II). By comparison, ethylene synthesis related genes were not altered in rst1, and only three ethylene specific signaling or stimulus genes had elevated expression, albeit in the lower range (data not shown). Interestingly, the BG1 gene, whose product cleaves 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). Further, 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 to 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-dependant 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; Thomma et al., 1999). B. cinerea disease assays of double mutants reveal that coi1 is completely epistatic to rst1, whereas ein2 is partially epistatic to rst1 (Fig. 9A and B). 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 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 PstDC3000 and avirulent PstDC3000 (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 1200 bp fragment of the **RST1** promoter region. The **RST1pro:**GUS construct was transformed into Arabidopsis using the floral dip method, and a total of 8 independent transformants were used for expression analysis. Strong GUS activity was detected in the veins of leaves, petioles, and hypocotyls from one-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 green fluorescent protein (**GFP**) to examine the subcellular localization of the RST1 protein in root cells of transgenic plants. CaMV35Spro:**RST1:**GFP (containing full length RST1 cDNA) were transformed into **rst1-2**, and then isolated transgenic plants were isolated from the T1 generation. Stem glossiness and seed abortion phenotypes were observed as being reverted to wild type in the complemented lines. GFP localization within the T2 generation of these fully complemented lines were verified using 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 to 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 wild type. Although many Arabidopsis mutants have been reported showing increased resistance to biotrophs but increased susceptibility to necrotrophs, rst1 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, nor 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 (Veronese et al 2004; Nandi et al 2005, Bohmann et al 2004; Epple et al., 2003). 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 2003; Dhawan 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, rather than the reverse. A third group of Arabidopsis
mutants show increased resistance to necrotrophic fungi without an apparent effect on SA defense responses (Coego et al., 2005; Penninckx et al, 2003). 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 wild type and *rst1* influence any of the differential responses to the bacterial pathogen *P. syringae* (a non-obligate 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 to the wild type during biotrophic infection, indicating a negative 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 PRI 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 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 elevated permeability of its leaf cuticles relative to wild type, indicating the existence of some other mechanism for rst1’s resistance to necrotrophic infection. It is interesting to note 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; Frick and Wolf, 1994; Nicholson et al., 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 Köller, 1993; Francis et al., 1996; Li et al., 2002; Chassot and Metraux, 2005). Still, it cannot be dismissed that RST1 or its protein product could also act more directly as a regulatory or otherwise fungal-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., 2003; 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 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 crosstalk has been observed between SA and ABA-mediated signalling in certain environmental stress responses (Yasuda et al., 2008). In addition, *ENHANCED DISEASE RESISTANCE 1* (*EDR1*) 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, that we are aware of, 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 ABC 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 to 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 plants were grown on soilless media (Metro-Mix200; Grace-Sierra, Malpitas, CA, USA) in growth chambers under 12 h light (23°C)/12 h dark (22°C) or 16 light (23°C)/8h dark (22°C) cycle at 70% relative humidity (RH). Arabidopsis accessions Columbia (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 performed to remove additional T-DNA inserts. rst1-2 and rst1-3 (Columbia background) were obtained from SALK T-DNA insertion collection at the Arabidopsis Biological Research Center (ABRC in Ohio university) 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, IN, USA). E. cichoracearum UCSC1 was maintained by inoculation of 4 or 5 weeks old pad4-1 plants by tapping conidia from two or three infected leaves. Actively growing conidia (7-10 dpi; Days Post Infection) 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 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 performed as described previously (Veronese
et al., 2006). The bacterial pathogen _Pseudomonas syringae_ pv. Tomato _vir_ (Pst DC3000) and _avr_ (Pst DC3000 _avr Rps4_) 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 MgCl₂, and then adjusted to $1 \times 10^5$ cfu ml⁻¹. Suspended cells were infiltrated into leaves by using a 1ml syringe without a needle. Three leaf discs collected from three independent samples, and then ground in 10 mM MgCl₂, serial diluted 1:10, and plated on King’s agar plate. 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 growth chamber, and placed on a 1.5% water agar plate with petioles imbedded in the medium. The leave could be sustained for at least 6 days 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 day after inoculation. Secondary hyphal length (4 days after inoculation) and conidiophore number (5 days after inoculation) were obtained from a minimum of six stained leaves from independent experiment. 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 phenol, 10 mL water, 10 ml lactic acid, and 10 mg trypan blue). The stained leaves were mounted under cover slips 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 following primer sets: RST1-F(TGGATGCCTACACTGTGGTT), RST1-R(GTACATGAGGAGGACGCAA), PR1-F(CATACACTCTGGGCGCTT), PR1-R(GACCACAAACTCCATTGCAC), PR2-F(ATCTCCTTGTGGTGTT), PR2-R(TCGAGATTTGCGTCGAATAG), PDF1.2-F(GTTTGCGCAGCAGTAATGC), PDF1.2-R (CACACGATTAGCAGAA-AGA). Tublin-F(5'-CGTGGATACAGCAATACAGAGCC-3'), Tublin-R(5'-CCTCCTG-CACTTCAGAGGAGCC-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 130-150 bp in length. Quantitative RT-PCR was performed in 20 µl reactions containing 20 ng of template obtained from first-cDNA strand synthesis. 0.3 µM each primer, and 2X 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; 40 cycle of 95°C for 15 sec (denaturing), 58°C for 1 min (annealing), and 72°C for 1 min (extension). Primer efficiencies and relative expression level calculated using comparative C_T method (User Bulletin 2; ABI Prism 7700 Sequence Detection System). 2^-∆∆C_T of control samples were normalized to 1.

Microarray Hybridization and Statistical Analysis

Total RNA (70µg) was extracted from each sample using the Qiagen RNeasy Plant RNA miniprep kit (Quagen), RNA samples were reverse transcribed by using SuperScrip III (Invitrogen) and cDNAs labeled with Cy3 or Cy5 by indirect labeling (Gong et al., 2005). Microarray slides over 26,000 probes (70-mer oligo nucleotides) were used (http://ag.arizona.edu/microarray). To eliminate bias in microarray as a consequence of dye-related differences in labeling efficiency, dye labeling for each paired sample (mutant/WT) was swapped. Three biological repeats performed. Signal intensities were collected by GenePix 4000B (Axon Instruments) and images 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 conformed manually, original raw data of GPR files were analysed.
by the TIGR-TM4 package (http://www.tm4.org; (Saeed et al., 2003). Statistical analyses performed using TM4-MEV (ver. 3.0.3). In MEV, a one-way $t$ test with $P = 0.01$ was carried out to determine the different expression (mutant/WT).

**Salicylic Acid Measurement**

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 $\mu$L of 5 mM 3,4,5-trimethoxy-trans-cinamic 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 hours 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 3.6 mL of water and 3 mL of chloroform. After shaking, samples were allowed to sit for 24 hours at 4°C. The supernatants were dried by speed vacuum. The residue was resuspended in 0.5 mL of a 80% methanol: 20% water solution. The solution was centrifuged at 16,000g for 5 minutes. The supernatant was transferred to a new vessel and dried by speed vacuum. The remaining residue was redissolved in 50 $\mu$L of 50% mobile phase A and 50% mobile phase B prior to analysis by HPLC-MS. Separations were performed on an Agilent 1100 system (Palo Alto, CA) using a Waters Xterra MS C8 column (5 $\mu$m, 2.1 x 150 mm). A binary mobile phase consisting of solvent systems A and B were used in gradient elution where A was 0.1% formic acid (v/v) in ddH2O and B was 0.1% formic acid (v/v) in acetonitrile. The mobile phase flow rate was 0.3 mL/min. 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 minutes at initial conditions prior to the next run. Following separation the column effluent was introduced by negative mode electrospray ionization (ESI) into an Agilent MSD-TOF spectrometer. ESI capillary voltage was $-3.5$ kV, nitrogen gas temperature was set to $350 \, ^\circ$C, drying gas flow rate was $9.0$ L/min, nebulizer gas pressure was $35$ psig, fragmentor voltage was 135 V, skimmer was 60 V, and OCT RF was 250 V. Mass data (from m/z 65-800) were collected and analyzed using Agilent MassHunter software. JA quantification was accomplished using a multi-level calibration curve.

**Cutin Polyester Analysis**

Leaf polyester content was analyzed on 20-day old plants based on modification of depolymerization methods described by (Bonaventure et al., 2004; Franke et al., 2005). Ground leaf tissues were delipidated in a Soxhlet extractor for 72 hours with chloroform:methanol (1:1, v/v) containing $50$ mg L-1 of butylated hydroxytoluene (BHT). After delipidation tissues were washed with methanol containing $50$ mg L-1 BHT and were dried in a vacuum desiccator for 4 days 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) methyl acetate at $60^\circ$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% aqueous NaCl (w/v), 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 $100^\circ$C. Excess pyridine:BSTFA was removed with nitrogen gas, and the sample dissolved in heptane:toluene (1:1 v/v) prior to analysis with a Hewlett-Packard 5890 series II GC equipped with a flame ionization detector (FID) and 12 m, 0.2 mm id HP-1 capillary column with helium as the carrier gas. The GC was programmed with an initial temperature of $80^\circ$C and increased at $15^\circ$C min$^{-1}$ to 200$^\circ$C, then increased at 2$^\circ$C min$^{-1}$ to 280$^\circ$C. Quantification was based on uncorrected FID peak areas relative to internal standard methyl heptadecanoate peak area. Areas of rosette leaves were
determined by ImageJ software (http://rsb.info.nih.gov/ij/) by using digital images of flattened leaves. Cutin data were analyzed using SAS 9.1.3 software (SAS Institute Inc., Cary, NC, USA). 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 \( \alpha \) was set at 0.05.

**Generation of RST1 Overexpression and Complemented Transgenic Plants**

To generate the CaMV35Spro:RST1 construct, RST1 genomic DNA was amplified by PCR with primer sets (including an XbaI restriction site (Italic) on 1\(^{st}\) part of the forward primer), having F (5’- GCCTTAGATTGGGCGCAAATCGGACGGC-3’) R (5’- GTGGCGACAATTTAAGG AG-3’) for 1\(^{st}\) part, F2 (5’-GACCTTTCAGCGTCCGCG-3’) R2 (5’-GGCTACTATGTCG ATGTACC-3’) for 2\(^{nd}\) fragment. Two fragments were amplified as few applicable multi-cloning sites were present in the binary vector pCAMBIA 99-1 (pCAMBIA 1200-based vector containing modified enzyme sites). The first fragment (3858 bp) consists of the 50 bp from start codon to about 50 bp downstream of the PstI site on the middle of RST1 genomic sequence. The second part (4410bp) consists of about 30 bp upstream of pst1 to about 100 bp downstream of the stop codon. Amplified PCR fragments were subcloned into pGEM-T easy vector (Promega). Subcloned 1\(^{st}\) and 2\(^{nd}\) fragments in T-easy vector were digested by XbaI and PstI or by PstI and EcoRI, respectively, and then two fragments were subcloned into pCAMBIA99-1 between the XbaI and EcoRI 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 1200 bp upstream region including initiation codon of RST1 was amplified by PCR with the following primers containing BamHI and SpeI restriction sites (5’-GAATTCCGCGGCCCTCCACTAACC -3’) and (5’-CCATGGGC-
GTATGAGGCCATCGCTTTGG-3’) respectively. The PCR product was digested with BamHI and SpeI and subcloned at BamHI and SpeI sites of the pCAMBIA 1303 vector which harbors a GUS and GFP reporter genes. The RST1pro:GUS:GFP clone, along with a 35Spro:GUS:GFP control, was transformed into Arabidopsis Col-0 using Agrobacterium mediated transformation. Various developmental stages of transgenic plants were incubated overnight in 1 mM X-gluc(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 2-3 times with 70 % (v/v) ethanol. Samples were monitored and captured using a Nikon E 800 microscope.

**Subcellular Localization of RST1**

To generate CaMV35Spro:RST1:GFP, the full-length RST1 ORF without stop codon was synthesized with the following primer sets including XhoI and SpeI restriction site: F (5’-CTCGAGATGGCCTCATACGCTACG-3’) and R (5’-ACTAGTAGACATGTCCATA-GAAGCAA-3’), respectively, the PCR products were subcloned in pGEM-T easy vector (Promega) and then digested with XhoI and produced blunted end using Klenow fragment polymerase (Roche), and then digested with SpeI. The fragment including 5’ blunted end and 3’ cohesive end was subcloned in frame at the pCAMBIA 1302 prepared as insert fragment except using NcoI instead of XhoI. Plasmids were purified using Quiagen 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 an rst1-2 background. Plants were grown on the MS solid plate for 3-4 days. Images were taken using a Radiance 2100 MP Rainbow (Bio-Rad) on a TE2000 (Nikon) inverted microscope using 60 x 1.4 NA lens. The 488-nm line of the 4-line argon laser (National Laser) was used to excite the GFP, and the fluorescence was emitted between 500nm and 540nm was collected. The transformants of Col-0 background were confirmed as controls.
Legends for Supplemental Figures

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

(A) T-DNA insertion sites (top panel) and RST1 transcript abundance (lower panel) in the allelic mutants of *RST1*. Black boxes represent exons, and lines indicate introns. The insertion sites were confirmed by sequencing. *rst1-1* is in C24 background, *rst1-2* and *rst1-3* are in Col-0. Truncated *RST1* transcript is expressed in *rst1-1* and *rst1-3* but not in *rst1-2* (bottom panel). Primers were designed from 1st and 17th or 13th and 17th exons for full length or truncated *RST1* respectively. The primers are indicated as arrow in (A, top panel). Arabidopsis *Tubulin* was used as control (bottom panel).

(B) Predicted structure of RST1 protein suggests localization to the plasma membrane. RST1 has 11 membrane helices, the size of transcript is 5,865 bp and encodes polypeptide of 1,841 amino acid with a molecular mass 203.6 kD. PM; plasma membrane (Hofmann and Stoffel, 1993 and http://psort.nibb.ac.jp/).

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

(A) Wild-type C24.  
(B) *rst1-1* rosette.  
(C) Magnified view of *rst1-1* leaf showing heavy powdery mildew infection.

**Figure S3.** Transpiration rate in the dark of wild-type Col-0, *rst1-2*, and *pad4-1*. Aerial part of plants was detached from 4-5 week old soil-grown plants. The data represent mean ± SD (n=3). Similar values were observed in three independent experiments.

**Figure S4.** Sensitivity of Col-0, *rst1-3*, *pad4-1*, and C24 plants to BASTA. Plants were grown on soil for 4 weeks. Photographs were taken 48 h after various BASTA treatments. C24:BASTA indicates plants harboring a BASTA resistant gene in the C24 background.

**Figure S5.** Permeability assay on leaf cuticles of wild-type Col-0, *rst1-2*, *rst1-3*, *pad4-1* and *lacs2-3*. Droplets of a toluidine blue solution were placed on detached leaves for 2 h and then washed with water. Representative stained leaves from each genotype are shown. The *lacs2-3* mutant showing enhanced permeability is used as a control. Each leaf was detached from 30 day soil-grown plants under long-day (16 light/8 dark) conditions.

**Figure S6.** The *rst1* mutants show slightly high basal expression of *PR1* and *PR2*. Basal transcript levels of *PR1* (top panel) and *PR2* (bottom panel). The transcript levels were determined by Quantitative RT-PCR. Total RNA was extracted from leaves of 30-day-old plants. The data represent means ± SD (n=4). The mean values were similar in
three independent experiments. Tubulin was used as internal control. Control samples were normalized to 1.

**Figure S7.** The *rst1* mutants show higher basal expression of *BG1* (At1g52400), *Chitinase* (At2g43570), and *Athila retroelement* (At5g32475). The transcript levels were determined by RT-PCR. Total RNA was extracted from leaves of 30-day-old plants. Tubulin was used as internal control.

**Figure S8.** The *rst1* mutants display normal bacterial growth after inoculation with *Pseudomonas syringae* pv. *Tomato* strains DC3000 and DC3000 expressing *avrRps4*. The growth of *pst* DC3000 (top panel) and *pst* DC3000 expressing *avrRps4* (bottom panel) in leaves of wild-type Col-0, *rst1-2, rst1-3, pad4-1*, and *NahG* plants. Bacterial titers were measured at 0 and 3 dpi. The data indicate means ± SD derived from four independent replicates. *pad4-1* and *NahG* (expressing the SA-depleting enzyme, salicylic hydroxylase) plants showing enhanced susceptibility were used as controls.

**ACKNOWLEDGMENTS**

This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number #2006-35304-17323. We wish to thank Shisong Ma and Dong-ha Oh for statistical data analyses. We thank Dr. David Salt laboratory at Purdue University (West Lafayette, IN) for help with the salicylic acid assay, and Dr. Roger Innes at Indiana University (Bloomington, IN) for providing *Erysiphe cichoracearum* strain UCSC1. We also wish to thank the World Class University Program (R32-10148) of the Ministry of Education, Science and Technology in Korea for their support. Lastly, we would like to 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).
Figure Legends

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

Figure 2. Microscopic analyses showing the growth of \textit{E. cichoracearum} in inoculated plants. (A) \textit{E. cichoracearum} growth at 4 days post-inoculation (dpi) showing hyphal branching on leaves of wild-type Col-0, \textit{rst1-2}, \textit{rst1-3} and \textit{pad4-1} at 4 dpi. Bar = 100 µm. (B) \textit{E. cichoracearum} growth at 5 dpi showing sporulation in \textit{rst1-2} and \textit{rst1-3} leaves. Lower panels of (A) and (B) are magnified views inside boxes on respective upper panels. Representative samples are shown. The experiment was repeated at least three times. Bar = 100 µm.

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

Figure 4. The \textit{rst1} mutants accumulate less SA than wild type after \textit{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 \textit{E. cichoracearum} infection at 7 days post-inoculation (dpi). Values represent the mean ± SD (n=3).

Figure 5. The \textit{rst1} mutants exhibit enhanced susceptibility to the necrotrophic pathogen \textit{A. brassicicola}. (A) Disease symptoms on leaves of drop-inoculated wild-type and \textit{rst1} plants at 6 dpi. (B) Spore count on \textit{A. brassicicola} inoculated plants at 4 days post inoculation (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 less disease symptom on drop-inoculated leaves. Col-0, rst1-2 and rst1-3 plants at 3 days post inoculation, dpi (top row). Rescued rst1 mutant phenotype of plants harboring CaMV 35Spro:RST1 (bottom row).
(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.

**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 hours post inoculation (hpi) with *B. cinerea.* *Asterisks indicate significant difference from the wild-type Col-0 at each time point as determined by student’s t-tests (*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 internal control. Control samples were normalized to 1 hpi. *Asterisks indicate significant difference from the 0 hr time point as determined by student’s t-tests (*p* < 0.05).

**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-MS. Values represent the mean ± SD (n=3).

**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 and double mutant plants at 3 days post inoculation (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 root.
(A) Series of images showing GUS activity in transgenic Arabidopsis plants expressing GUS under control of the RST1 promoter. Two week-old seedlings and leaves (top row) and the flowers from 8 week-old plants (bottom row), White arrow indicates pollen tube on the stamen in flower (small figure in bottom row).
(B) Subcellular localization of the CaMV 35Spro:RST1:GFP derived fusion protein in the Arabidopsis root of transgenic plants. Subcellular localization of RST1 (top row), GFP control (middle row), and no vector control (bottom row).

**Figure 11.** A model for the function of RST1 as a modulator of plant defense through the suppression of JA biosynthesis.
Table 1. *E. cichoracearum* development on leaves of Arabidopsis wild-type Col-0, *rst1*-2, and *rst1*-3.

| Genotype | Germination (%) | Hyphal length\(c\) (mm) ± SD | Conidiophores/Colony\(d\) ± SD |
|----------|----------------|--------------------------------|-------------------------------|
| Col-0    | 62.1 (100)\(c\) | 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)               |

* Detached leaves were inoculated and incubated in petri plates as described in methods. Leaves were fixed and stained with tryphan blue.

* Asexual spore germination measured at 1 day post inoculation.

* The length of secondary hyphae per germling measured 4 days post inoculation. Values indicates mean ± SD

* Conidiophores were counted on 5-6 randomly selected single fungal colonies per leaf on 3-5 leaves after 5 days post inoculation. Values indicates mean ± SD

* The number within parentheses indicate the number of replicates.
Table 2. Effects of the *rst1* mutation on Arabidopsis leaf cutin monomers. The amount (µg/dm²) of total cutin monomers was higher in rosette leaves of the two isogenic allelic mutants *rst1*-2 and *rst1*-3 than wild-type Col-0 due primarily to an increase in C18:2 dicarboxylic acids. Values represent the mean ± SD (n=5). *Asterisks indicate significant difference from the wild-type Col-0 amount as determined by student’s t-tests (p < 0.05).

| Cutin Monomers<sup>a</sup> | 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*|

<sup>a</sup> 16-OH C16:0 is 16-hydroxy hexadecanoic acid, 10,16-diOH C16 is 10(9),16-dihydroxy hexadecanoic acid, C16:0 dioic is hexadecane-1,16-dioic acid, 18-OH C18:0 is 18-hydroxy octadecanoic acid, 18-OH C18:1 is 18-hydroxy octadec-9-enoic- acid, 18-OH C18:2 is 18-hydroxy octadeca-9,12-dienoic- acid, C18:0 dioic is octadecane-1,18-dioic acid, C18:1 dioic is octadecene-1,18-dioic acid, and C18:2 dioic is octadecadien-1,18-dioic acid. OH denotes a hydroxyl functional group.
Table 3. Jasmonic acid synthesis, perception and response genes up-regulated in rst1 mutants.

| Description                                      | AGI ID   | Fold-Change |
|--------------------------------------------------|----------|-------------|
| **Jasmonic Acid 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         |
| **Jasmonic Acid mediated signaling pathway**     |          |             |
| RCD1                                             | At1g32230| 4.3         |
| SGT1B                                            | At4g11260| 2.4         |
| **Response to Jasmonic Acid 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         |
| Peroxidase 50                                     | At4g37520| 5.0         |
| PAD3                                             | At3g26830| 4.8         |
| FAD6                                             | At4g30950| 2.3         |

Annotation based on The Arabidopsis Information Resource (TAIR) data base (http://arabidopsis.org). The expression fold-change of probe sets is indicated only when change is significant (P ≤ 0.01) and ≥ 2.0. Collected data were from three independent experiments, and analyzed as indicated in results.
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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 days and infected with *E.cichor acearum*. At least 10 plants were tested per genotype. Photographs were taken 10-14 days post-inoculation (dpi). The experiment was repeated at least 3 times and representative results are shown. OE (overexpression) indicates plants expressing *RST1* genomic region including the introns from the CaMV 35S promoter (*CaMV 35Spro:RST1*).
Figure 2. Microscopic analyses showing the growth of *E. cichoracearum* in inoculated plants. (A) *E. cichoracearum* growth at 4 days post-inoculation (dpi) showing hyphal branching on leaves of wild-type Col-0, *rst1-2*, *rst1-3* and *pad4-1* at 4 dpi. Bar = 100 µm. (B) *E. cichoracearum* growth at 5 dpi showing sporulation in *rst1-2* and *rst1-3* leaves. Lower panels of (A) and (B) are magnified views inside boxes on respective upper panels. Representative samples are shown. The experiment was repeated at least three times. Bar = 100 µm.
**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 days post-inoculation (dpi). Arabidopsis *tubulin* was used as internal control. Control samples were normalized to 1. Values represent the mean ± SD (n=4).
**Figure 4.** The *rst1* mutants accumulate less SA than 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 days post-inoculation (dpi). Values represent the mean ± SD (n=3).
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 days post inoculation (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 less disease symptom on drop-inoculated leaves. Col-0, *rst1-2* and *rst1-3* plants at 3 days post inoculation, dpi (top row). Rescued *rst1* mutant phenotype of plants harboring *CaMV 35S pro:RST1* (bottom row). (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.
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 hours post inoculation (hpi) with B. cinerea. *Asterisks indicate significant difference from the wild-type Col-0 at each time point as determined by student’s t-tests ($p < 0.05$). 
(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 internal control. Control samples were normalized to 1 hpi. *Asterisks indicate significant difference from the 0 hr time point as determined by student’s t-tests ($p < 0.05$).
**Figure 8.** The *rstl* mutants show increased basal JA accumulation. The basal amounts of JA in *rstl*-2 and *rstl*-3 is higher than that of Col-0. The levels of JA in leaves from 4 week old soil-grown plants of Col-0, *rstl*-2, and *rstl*-3 were analyzed using HPLC-MS. Values represent the mean ± SD (n=3).
Figure 9. Effects of *coil* and *ein2* mutations on the *B. cinerea* resistance of the *rst1* mutant.  
(A) Disease symptoms on drop-inoculated leaves of wild-type, single and double mutant plants at 3 days post inoculation (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 *RST1*<sub>pro</sub>:*GUS* derived fusion protein and subcellular localization of *RST1*:GFP derived fusion protein in Arabidopsis root.

(A) Series of images showing *GUS* activity in transgenic Arabidopsis plants expressing *GUS* under control of the *RST1* promoter. Two week-old seedlings and leaves (top row) and the flowers from 8 week-old plants (Bottom row), White arrow indicates pollen tube on the stamen in flower (small figure in bottom row).

(B) Subcellular localization of the *CaMV 35Spro*:RST1:GFP derived fusion protein in the Arabidopsis root of transgenic plants. Subcellular localization of RST1 (top row), GFP control (middle row), and no vector control (bottom row).
Figure 11. A model for the function of RST1 as a modulator of plant defense through the suppression of JA biosynthesis.