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Arabidopsis Enhanced Disease Susceptibility Mutants Exhibit Enhanced Susceptibility to Several Bacterial Pathogens and Alterations in *PR-1* Gene Expression

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To identify plant defense responses that limit pathogen attack, Arabidopsis *eds* mutants that exhibit enhanced disease susceptibility to the virulent bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326 were previously identified. In this study, we show that each of four *eds* mutants (*eds5-1*, *eds6-1*, *eds7-1*, and *eds9-1*) has a distinguishable phenotype with respect to the degree of susceptibility to a panel of bacterial phytopathogens and the ability to activate pathogenesis-related *PR-1* gene expression after pathogen attack. None of the four *eds* mutants exhibited observable defects in mounting a hypersensitive response. Although all four *eds* mutants were also capable of mounting a systemic acquired resistance response, enhanced growth of *P. s. maculicola* ES4326 was still apparent in the secondarily infected leaves of three of the *eds* mutants. These data indicate that *eds* genes define a diverse set of previously unknown defense responses that affect resistance to virulent pathogens.

INTRODUCTION

Plants respond in a variety of ways to pathogenic microorganisms (Lamb et al., 1989; Lamb, 1994). In the case of so-called avirulent pathogens, the defense response involves specific recognition of the pathogen mediated by an avirulence (*avr*) gene(s) in the pathogen and corresponding resistance gene(s) in the plant. Infected plant cells then undergo rapid programmed cell death, which is termed the hypersensitive response (HR). In addition, a variety of biochemical and physiological responses are rapidly induced, including a membrane-associated oxidative burst that results in the NADPH-dependent production of O_2^- and H_2O_2. These responses lead to extensive growth limitation of the invading pathogen, which is termed avirulent.

In contrast to avirulent pathogens, virulent pathogens do not elicit an HR and are able to multiply and cause disease, even though many of the defense responses that are induced are the same as those induced after infection of an avirulent pathogen (Dixon and Lamb, 1990). In the case of a virulent pathogen, however, many of the induced host defense responses appear more slowly or are less extensive than after infection by an avirulent pathogen. Previously characterized defense responses that have been observed after infection by either virulent or avirulent pathogens include the strengthening of cell walls by lignification, suberization, callose deposition, cross-linking of hydroxyproline-rich proteins, induction of a variety of hydrolytic enzymes, including so-called pathogenesis-related (PR) proteins, and synthesis of low molecular weight antibiotics (called phytoalexins) (Kauffmann et al., 1987; Legrand et al., 1987; Lamb et al., 1989; Dixon and Lamb, 1990; Ponstein et al., 1994).

Particular plant defense responses may play a direct role in conferring resistance to pathogens. For example, many PR proteins, such as chitinases and β-1,3-glucanases and phytoalexins, directly inhibit pathogen growth in vitro (Paxton, 1981; Schlumbaum et al., 1986; Mauch et al., 1988; Woloshuk et al., 1991; Terras et al., 1992; Sela-Buurlage et al., 1993; Ponstein et al., 1994). In some cases, it has also been shown that constitutive expression in transgenic plants of *PR* genes or certain phytoalexin biosynthetic genes decreases disease susceptibility (Broglie et al., 1991; Alexander et al., 1993; Hain et al., 1993; Liu et al., 1994; Zhu et al., 1994; Terras et al., 1995).

Isolation of plant defense response mutants would not only help to elucidate the roles of known pathogen-induced responses in combating particular pathogens but would also facilitate the identification of plant defense mechanisms not already correlated with a known biochemical or molecular genetic response. Unfortunately, however, most of the plant hosts that have been used in the past for host–pathogen studies are not suitable for genetic analysis because of large or polyploid genomes and long generation times.

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However, with the development of well-characterized host–pathogen systems involving the model plant Arabidopsis as the host, comprehensive genetic analysis of host defense responses has recently become feasible (Crute et al., 1994; Kunkel, 1996).

Two features of plant defense that have been subjected to genetic analysis in Arabidopsis are systemic acquired resistance (SAR) and phytoalexin production. SAR occurs after infection of a plant with an avirulent pathogen (which elicits an HR) and is characterized by the accumulation of PR proteins in the uninfected leaves and concomitant resistance to a variety of normally virulent pathogens (Enyedi et al., 1992; Malamy and Klessig, 1992). Treatment of plants with salicylic acid (SA) also leads to PR protein accumulation and pathogen resistance (Enyedi et al., 1992; Malamy and Klessig, 1992), indicating that SA plays an important role as a signaling compound in SAR (Gaffney et al., 1993). SAR has been best characterized in tobacco and cucumber; however, all of the important features of SAR observed in these systems have also been observed in Arabidopsis (Uknes et al., 1992, 1993). Arabidopsis *npr1* (Cao et al., 1994) and *nim1* (Delaney et al., 1995) mutants (which are most likely allelic) fail to activate *PR* gene expression after treatment with SA, do not perform SAR, and exhibit enhanced susceptibility to *Pseudomonas syringae* and *Peronospora parasitica*.

In other studies, a series of Arabidopsis phytoalexin-deficient (*pad*) mutants that accumulate reduced levels of camalexin was isolated (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997). As in the case of *npr1* mutants, *P. s.* pv *maculicola* ES4326 formed disease lesions and grew to higher titers on certain *pad* mutants when inoculated at doses below the threshold dose required to confer disease symptoms in wild-type plants. Some *pad* mutants also exhibited increased susceptibility to *P. parasitica* (Glazebrook et al., 1997).

The enhanced susceptibility phenotype of *npr1* and certain *pad* mutants to *P. s. maculicola* ES4326 indicates that Arabidopsis actively limits the growth of virulent *P. syringae* strains, despite the fact that *P. syringae* is capable of causing severe disease lesions. To obtain additional Arabidopsis mutants with an enhanced disease susceptibility (*eds*) phenotype that are not allelic to previously identified *npr1* or *pad* mutants, a direct screen was performed for mutants that exhibited increased susceptibility to *P. s. maculicola* ES4326 (Glazebrook et al., 1996). This screen led to the isolation of 10 *eds* mutants that defined at least seven new complementation groups (Glazebrook et al., 1996).

Here, we report further characterization of four *eds* mutants. Importantly, each of these *eds* mutants exhibits a distinctive phenotype with respect to either *PR-1* mRNA accumulation or susceptibility to a panel of phytopathogenic bacterial species. However, none of the *eds* mutants showed significant alteration in the HR or SAR responses. Our data indicate that *eds5-1*, *eds6-1*, *eds7-1*, and *eds9-1* define a variety of previously uncharacterized defense-related functions limiting the severity of virulent bacterial infections.

RESULTS

eds-12 Defines a New Complementation Group

Three of the 10 *eds* mutants described in Glazebrook et al. (1996) were not subjected to complementation analysis because their phenotypes either were not robust enough for complementation testing or did not segregate as single locus mutations. One of these three *eds* mutants, *eds-12*, appears to contain two independent mutations: one results in an *eds* mutant phenotype and the other results in a *pad* phenotype. In the M_3 generation of the original *eds-12* line, we observed segregation of a *pad* phenotype (data not shown). Therefore, individual F_2 plants from a backcross of the original M_2 *eds-12* mutant to wild-type Arabidopsis ecotype Columbia were scored both for an *eds* mutant phenotype (by visually scoring disease symptoms 3 days after infiltration with *P. s. maculicola* ES4326 at a dose of $\sim 10^3$ colony-forming units [cfu] per cm^2 leaf area) and for a *pad* phenotype (by isolation and quantitation of camalexin 2 days after infiltration with *P. s. maculicola* ES4326 at a dose of $\sim 10^5$ cfu/cm^2 leaf area). Among the 64 F_2 individuals scored, 20 were Pad^-, 15 were Eds^-, and eight were both Pad^- and Eds^-, suggesting that the two phenotypes were caused by mutations at unlinked loci. One F_3 family derived from an individual F_2 generation plant confirmed to be *PAD/PAD eds/eds* was chosen for further characterization. A second backcross confirmed that the *eds* phenotype segregated as a single, recessive locus (35 Eds^+: 10 Eds^-; $\chi^2 = 0.19$; $0.6 < P < 0.7$ for 3:1 segregation), and complementation testing showed that this *eds* mutation failed to complement any of the known *eds* complementation groups (*eds2*, *eds3*, *eds4*, *eds5*, *eds6*, *eds7*, and *eds8*) previously defined in our laboratory (data not shown). As a result of this genetic analysis, the *eds* mutation in mutant line *eds-12* was renamed *eds9-1*. In addition to *eds9-1*, three *eds* mutants, *eds5-1*, *eds6-1*, and *eds7-1*, that all display severe mutant phenotypes were chosen for further analysis, as described below.

Response of *eds* Mutants to Avirulent Bacteria

Infection of wild-type Arabidopsis ecotype Columbia with avirulent bacterial pathogens, such as *P. s. maculicola* ES4326 carrying the cloned *avr* gene *avrRpt2*, results in the elicitation of an HR. The HR is characterized by the rapid induction of a number of inducible plant defense responses and results in a 100- to 1000-fold limitation of the growth of the infecting bacteria as compared with an isogenic *P. syringae* strain lacking the cloned *avrRpt2* gene. Because *eds* mutants permit enhanced growth of the virulent strain *P. s. maculicola* ES4326, it was of interest to determine whether they also permit enhanced growth of the isogenic avirulent strain carrying *avrRpt2*. However, Figure 1 shows that none of the four *eds* mutants (*eds5-1*, *eds6-1*, *eds7-1*, and *eds9-1*)

allowed significantly more growth of *P. s. maculicola* ES4326 carrying *avrRpt2* than did wild-type plants after infiltration of a relatively low dose of $\sim 10^3$ cfu/cm^2 leaf area. In addition, the tissue collapse characteristic of the HR was observed in all four of these *eds* mutants within 24 hr after infection with a high dose (10^6 cfu/cm^2 leaf area) of *P. s. maculicola* ES4326 carrying *avrRpt2* (data not shown). Therefore, neither the ability to generate an HR nor the ability to limit bacterial growth associated with the HR appears to be compromised in any of these four *eds* mutants.

The initial inoculum of *P. s. maculicola* ES4326 and *P. s. maculicola* ES4326 carrying *avrRpt2* (Figure 1; determined by measuring bacterial density 30 min after infiltration) in all five genotypes tested was 3.2 ± 0.1 log (cfu/cm^2 leaf area; data not shown). This demonstrates that the enhanced disease susceptibility phenotypes of these *eds* mutants cannot simply be a consequence of morphological changes that allow the delivery of larger initial inocula into *eds* mutants compared with that of the wild type. Although the data shown in Figure 1 suggest that *eds5-1* may allow somewhat more growth of *P. s. maculicola* carrying *avrRpt2* than does the wild type, this difference was not always observed and is not statistically significant when the data are analyzed using an independent *t* test.

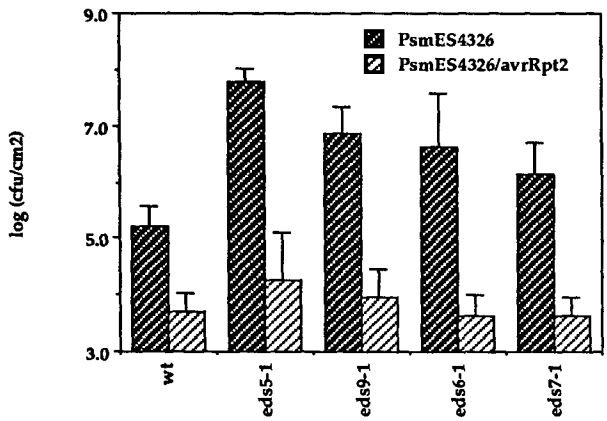

Figure 1. Growth of Virulent and Isogenic Avirulent Strains of *P. s. maculicola* ES4326 in Leaves of Arabidopsis Ecotype Columbia and Isogenic *eds* Mutants.

Three days after infiltration with *P. s. maculicola* (Psm) ES4326 or *P. s. maculicola* ES4326 carrying *avrRpt2* at a density of 3.0 log (cfu/cm^2), leaves were assayed for bacterial density. The *t* values and confidence limits are as follows: for each genotype, *P. s. maculicola* ES4326 compared with *P. s. maculicola* ES4326 carrying *avrRpt2*, wild type (wt), 6.8 (>99.5%); *eds5-1*, 9.1 (>99.5%); *eds6-1*, 6.7 (>99.5%); *eds7-1*, 8.7 (>99.5%); *eds9-1*, 9.8 (>99.5%); for *P. s. maculicola* ES4326 only, wt–*eds5-1*, 13.6 (>99.5%); wt–*eds6-1*, 3.2 (>99.0%); wt–*eds7-1*, 3.2 (>99.0%); wt–*eds9-1*, 6.3 (>99.5%); for *P. s. maculicola* ES4326 carrying *avrRpt2* only, wt–*eds5-1*, 1.4 (>90%); wt–*eds6-1*, 0.29 (<90%); wt–*eds7-1*, 0.23 (<90%); wt–*eds9-1*, 1.0 (<90%). Data points are the means of six replicate samples, and this experiment was repeated with similar results.

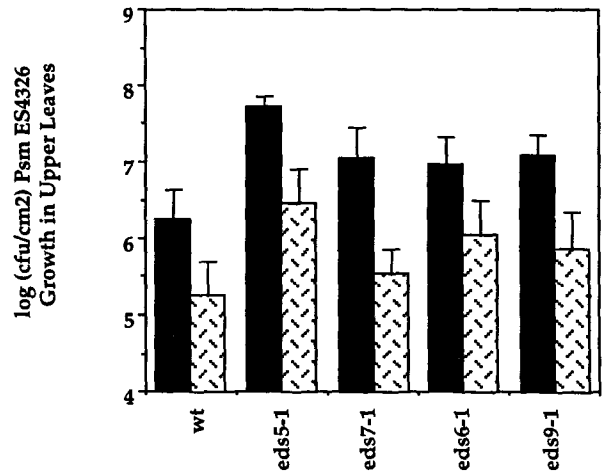

Figure 2. Induction of SAR in Arabidopsis Ecotype Columbia and Isogenic *eds* Mutants.

Eight days before infection with *P. s. maculicola* (Psm) ES4326 at 3.0 log (cfu/cm^2), three lower leaves per plant were inoculated with either 10 mM $MgSO_4$ as a control (black bars) or 6.0 log (cfu/cm^2) *P. s. phaseolicola* 3121 carrying *avrRpt2* (ticked bars). Three days after inoculation with *P. s. maculicola* ES4326, infected leaves were assayed for bacterial density. The *t* values and confidence limits are as follows: for each genotype, control compared with SAR induced, wild type (wt), 3.6 (>99.5%); *eds5-1*, 5.9 (>99.5%); *eds6-1*, 2.3 (>97.5%); *eds7-1*, 3.8 (>99.5%); *eds9-1*, 5.0 (>99.5%); for control plants only, wt–*eds5-1*, 9.5 (>99.5%); wt–*eds6-1*, 2.0 (>95.0%); wt–*eds7-1*, 3.1 (>99.0%); wt–*eds9-1*, 4.8 (>99.5%); for SAR induced only, wt–*eds5-1*, 3.9 (>99.5%); wt–*eds6-1*, 2.6 (>97.5%); wt–*eds7-1*, 0.70 (<90%); wt–*eds9-1*, 1.9 (>95.0%). Data points are the means of six replicate samples, and this experiment was repeated with similar results.

SAR Response in *eds* Mutants

In wild-type Arabidopsis, infection by an avirulent, necrotizing pathogen results in the induction of SAR. The SAR is characterized by enhanced expression of *PR* genes and the subsequent limitation of the growth of a virulent pathogen throughout the plant. Because *npr1* mutants exhibit an *eds* phenotype and are blocked in the SAR response downstream of SA (Cao et al., 1994), it was of interest to determine whether other *eds* mutants also exhibited a debilitated SAR response. We used *P. s.* pv *phaseolicola* 3121 carrying *avrRpt2* to induce SAR because this strain elicits a strong HR (Yu et al., 1993) and because it can be readily distinguished from *P. s. maculicola* ES4326 on the basis of antibiotic resistance.

Three lower leaves per plant were inoculated with either 10^6 cfu/cm^2 *P. s. phaseolicola* 3121 carrying *avrRpt2* or with 10 mM $MgSO_4$. Eight days later, upper leaves that were not infected previously were challenged with 10^3 cfu/cm^2 *P. s. maculicola* ES4326. Figure 2 shows that in the wild type and

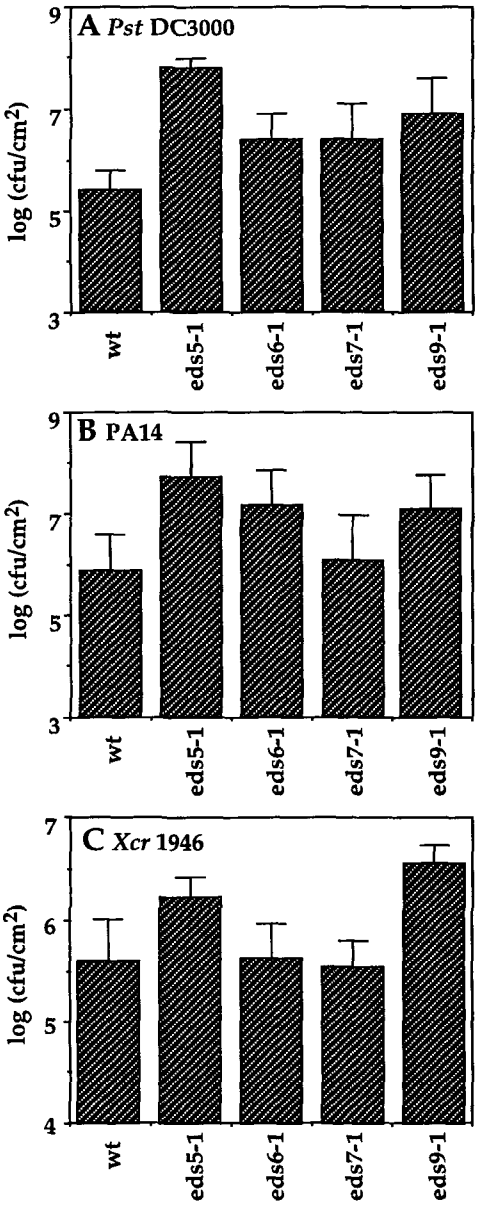

Figure 3. Growth of Bacterial Pathogens in Arabidopsis Ecotype Columbia and Isogenic *eds* Mutants.

(A) Growth of *P. s. tomato* (*Pst*) DC3000. Three days after infection with *P. s. tomato* DC3000 at a dose of 3.0 log (cfu/cm^2), leaves were assayed for bacterial density. The *t* values and confidence limits are as follows: wild type (wt)–*eds5-1*, 11.1 (>99.5%); wt–*eds6-1*, 2.0 (>95.0%); wt–*eds7-1*, 1.8 (>95.0%); wt–*eds9-1*, 2.9 (>99.0%). Data points are the means of six replicate samples, and this experiment was repeated twice with similar results.

(B) Growth of *P. aeruginosa* UCBPP-PA14 (PA14). Three days after infection with *P. aeruginosa* UCBPP-PA14 at a dose of 3.0 log (cfu/cm^2), leaves were assayed for bacterial density. The *t* values and confidence limits are as follows: wt–*eds5-1*, 4.1 (>99.5%); wt–*eds6-1*, 2.8 (>99.0%); wt–*eds7-1*, 0.43 (<90.0%); wt–*eds9-1*, 2.3 (>97.5%).

all four *eds* mutants, plants that were preinfected with *P. s. phaseolicola* 3121 carrying *avrRpt2* allowed less growth of *P. s. maculicola* ES4326 after a subsequent infection than did plants that were preinoculated with 10 mM $MgSO_4$. Therefore, these four *eds* mutants (*eds5-1*, *eds6-1*, *eds7-1*, and *eds9-1*) are capable of mounting at least a partial SAR response. Interestingly, however, *eds5-1* and *eds6-1* allowed significantly more growth of *P. s. maculicola* ES4326 in upper, not previously infected leaves after the induction of SAR than did wild-type Columbia. *eds9-1* allowed somewhat more growth of *P. s. maculicola* ES4326 in upper, not previously infected leaves after the induction of SAR; the difference between *eds9-1* and the wild type is just barely above the 95% confidence limit by an independent *t* test (refer to Figure 2 for *t* values). *eds6-1* did not allow significantly more growth of *P. s. maculicola* ES4326 in upper, not previously infected leaves after the induction of SAR. These results, with the exception of *eds6-1*, mirror the *eds* phenotype observed after infecting non-SAR–induced plants with *P. s. maculicola* ES4326.

Sensitivity of *eds* Mutants to Other Bacterial Phytopathogens

The *eds* mutants that we have isolated were identified because they are more susceptible to *P. s. maculicola* ES4326. Therefore, it was of interest to determine whether these *eds* mutants defined genes that were involved in conferring host resistance to other pathogens as well. Figure 3A shows that all four *eds* mutants are also more susceptible to *P. s.* pv *tomato* DC3000, a tomato pathogen that is virulent on ecotype Columbia (Whalen et al., 1991). This result was not surprising because *P. s. maculicola* ES4326 and *P. s. tomato* DC3000 are closely related to phytopathogens (Davis et al., 1991). This phenotype with *P. s. tomato* DC3000 also mirrors the *eds* phenotype in that *eds5-1* is the most severely affected mutant with respect to both *P. s. maculicola* ES4326 and *P. s. tomato* DC3000, whereas *eds6-1* and *eds7-1* are the least severely affected. However, as shown by the *t* values in the legend to Figure 3A, the growth difference between both *eds6-1* and *eds7-1* and the wild type are above the 95% confidence limit for an independent *t* test.

Data points are the means of eight replicate samples, and this experiment was repeated with similar results.

(C) Growth of *X. c. raphani* (*Xcr*) 1946. Three days after infection with *X. c. raphani* 1946 at a dose of 4.0 log (cfu/cm^2), leaves were assayed for bacterial density. The *t* values and confidence limits are as follows: wt–*eds5-1*, 1.1 (<90.0%); wt–*eds6-1*, 0.07 (<90.0%); wt–*eds7-1*, 0.35 (<90.0%); wt–*eds9-1*, 2.2 (>97.5%). Data points are the means of 12 replicate samples, and this experiment was repeated three times with similar results.

Figure 3B shows that *eds5-1*, *eds6-1*, and *eds9-1* but not *eds7-1* are more susceptible to *P. aeruginosa* UCBPP-PA14, a human clinical isolate of this opportunistic pathogen that has also been shown to be an ecotype-specific Arabidopsis pathogen (Rahme et al., 1995). Again, this phenotype mirrors the *eds* phenotype demonstrated with *P. s. maculicola* ES4326 in that *eds5-1* shows the greatest difference in growth of *P. aeruginosa* UCBPP-PA14, whereas *eds6-1* and *eds9-1* show smaller but significant differences.

Figure 3C shows that only *eds9-1* is significantly more sensitive to *Xanthomonas campestris* pv *raphani* 1946, a leaf-spotting pathogen that is virulent on Arabidopsis ecotype Columbia. *eds5-1* allows somewhat more growth of *X. c. raphani* 1946 than does the wild type. Although this difference appeared to be reproducible, it was never quite large enough to be above the 95% confidence limit by an independent *t* test (see legend to Figure 3C for *t* values).

All four *eds* mutants were also tested for their susceptibility to the vascular pathogen *X. c.* pv *campestris* 8004; however, none exhibited increased susceptibility to *X. c. campestris* 8004 (data not shown). Finally, the four *eds* mutants were tested to determine whether any showed increased susceptibility to *P. s. phaseolicola* 3121, a bean pathogen that is nonpathogenic on Arabidopsis. None of the four mutants allowed any growth of *P. s. phaseolicola* 3121 (data not shown) and were indistinguishable from the wild type.

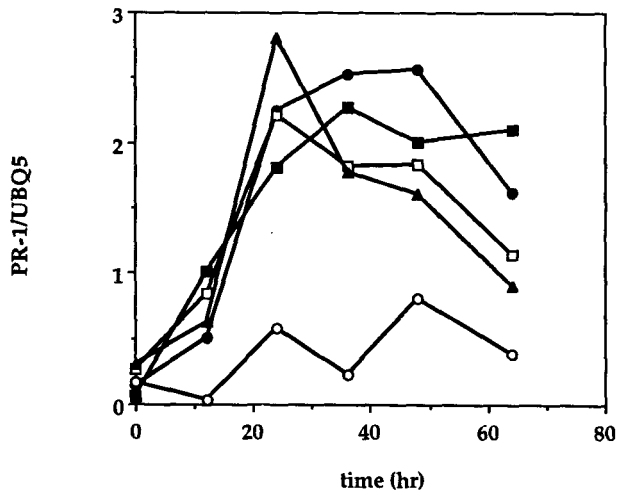

Figure 4. Induction of *PR-1* in Arabidopsis Ecotype Columbia and Isogenic *eds* Mutants.

At the indicated times after infection with *P. s. maculicola* ES4326 at a dose of 5.0 log (cfu/cm^2), infected leaves were harvested for RNA isolation. *PR-1* RNA was quantified and normalized to counts obtained for the ubiquitin *UBQ5* control probe. This experiment was repeated twice with similar results. ●, the wild type; ○, *eds5-1*; □, *eds6-1*; ▲, *eds7-1*; ■, *eds9-1*.

Defense Gene Induction in *eds* Mutants

Infection of Arabidopsis with *P. s. maculicola* ES4326 results in the accumulation of mRNAs corresponding to a variety of defense-related genes (Davis et al., 1991; Dong et al., 1991; Kiedrowski et al., 1992; Niyogi and Fink, 1992; Uknes et al., 1992; Reuber and Ausubel, 1996; G.-I. Yu, T.L. Reuber, and F.M. Ausubel, manuscript in preparation). To determine whether defense gene induction was impaired in the four *eds* mutants, we collected leaf tissue samples after infection with *P. s. maculicola* ES4326, and RNA was isolated and subjected to gel blot analysis. These RNA gel blots were probed sequentially with labeled DNA fragments corresponding to 11 different defense-related genes (*PR-1*, *PR-5*, *BGL2*, phenylalanine ammonia-lyase *PAL1*, glutathione S-transferase *GST1*, pathogen-induced genes 2 and 18 [*PIG2* and *PIG18*], anthranilate synthase *ASA1*, elicitor-induced *ELI3*, and *avr*-induced genes *AIG1* and *AIG2*). As shown in Figure 4, *PR-1* mRNA accumulation in *eds5-1* was ~10% that of wild-type levels. *PR-1* was previously shown to be induced to high levels in Arabidopsis after infection with virulent pathogens (Uknes et al., 1992). Interestingly, only *PR-1* mRNA levels were affected in *eds5-1* (Figure 4). The other *eds* mutants showed wild-type levels of mRNA accumulation corresponding to all 11 defense-related genes examined (data not shown).

PR-1 has been shown to be induced more rapidly and to higher levels in Arabidopsis after infection with *P. s. maculicola* ES4326 carrying *avrRpt2* than after infection with *P. s. maculicola* ES4326 (Greenberg et al., 1994). Figure 5 shows that *PR-1* induction is also impaired in *eds5-1* after infection with *P. s. maculicola* ES4326 carrying *avrRpt2*, only accumulating to ~10% of wild-type levels. These data suggest that a common signal transduction pathway (mutated in *eds5-1*) mediates the induction of *PR-1* downstream of both *P. s. maculicola* ES4326 and *P. s. maculicola* ES4326 carrying *avrRpt2*. Moreover, because *eds5-1* exhibits an HR indistinguishable from that of the wild type after infection with *P. s. maculicola* ES4326 carrying *avrRpt2* and because *eds5-1* limits the growth of *P. s. maculicola* ES4326 carrying *avrRpt2* similar to the wild type (Figure 1), it can be concluded that high levels of *PR-1* gene induction are not necessary for the HR in response to or for growth limitation of *P. s. maculicola* ES4326 carrying *avrRpt2*.

X. c. raphani 1946 induces *PR-1* in wild-type Columbia to levels comparable with *P. s. maculicola* ES4326 (data not shown). Although *eds5-1* is not more susceptible to *X. c. raphani* 1946 (Figure 3C), *PR-1* induction in *eds5-1* after *X. c. raphani* 1946 infection is reduced to ~10 to 20% of wild-type levels (data not shown) and is similar to the reduction observed after infection of *eds5-1* with *P. s. maculicola* ES4326 carrying or not carrying *avrRpt2*. These data suggest that *PR-1* does not play a significant role in limiting the growth of *X. c. raphani* 1946.

PR-1 has also been shown to be induced by mechanical damage (Grosset et al., 1990). Interestingly, *eds5-1* was also impaired in its ability to induce *PR-1* after mechanical damage (data not shown). Therefore, *eds5-1* is deficient in the induction of *PR-1* after a variety of treatments.

Cosegregation of Multiple Phenotypes

Because *eds* mutants have multiple, related phenotypes, it was important to determine whether at least some of these multiple *eds* phenotypes cosegregated with enhanced susceptibility to *P. s. maculicola* ES4326. The growth of both *P. s. maculicola* ES4326 and *P. aeruginosa* UCBPP-PA14 was examined in F_3 *eds5-1/eds5-1* families and in F_3 *eds9-1/eds9-1* families. Both were derived from crosses of the *eds* mutants to wild-type ecotype Columbia plants. Figure 6 demonstrates that all four *eds5-1/eds5-1* families examined show increased sensitivity to both *P. s. maculicola* ES4326 and *P. aeruginosa* UCBPP-PA14; similar data were obtained for four *eds9-1/eds9-1* families (data not shown). Based on the probability that two recessive unlinked mutations would cosegregate, the probability that the mutations causing *P. s.*

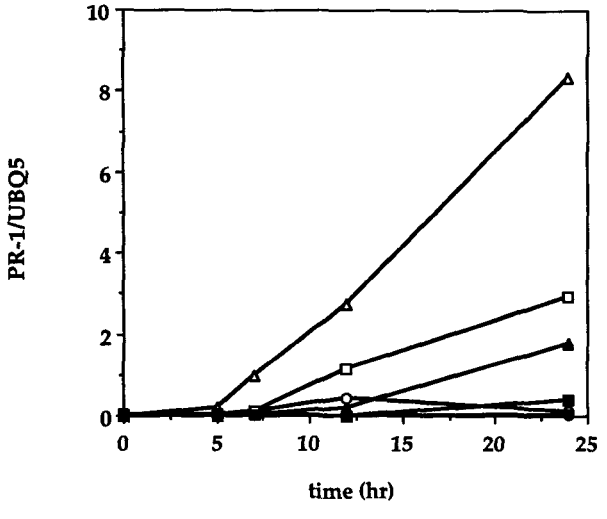

Figure 5. Induction of *PR-1* in Arabidopsis Ecotype Columbia and *eds5-1*.

At the indicated times after infection with *P. s. maculicola* ES4326 at a dose of 7.0 log (cfu/cm^2) or *P. s. maculicola* ES4326 carrying *avrRpt2* at a dose of 7.0 log (cfu/cm^2) or mock infection with 10 mM $MgSO_4$ solution, treated leaves were harvested for RNA isolation. *PR-1* RNA was quantitated and normalized to counts obtained for the *UBQ5* control probe. This experiment was repeated with similar results. ○, wild type inoculated with 10 mM $MgSO_4$; □, wild type inoculated with *P. s. maculicola* ES4326; △, wild type inoculated with *P. s. maculicola* ES4326 carrying *avrRpt2*; ●, *eds5-1* inoculated with 10 mM $MgSO_4$; ■, *eds5-1* inoculated with *P. s. maculicola* ES4326; ▲, *eds5-1* inoculated with *P. s. maculicola* ES4326 carrying *avrRpt2*.

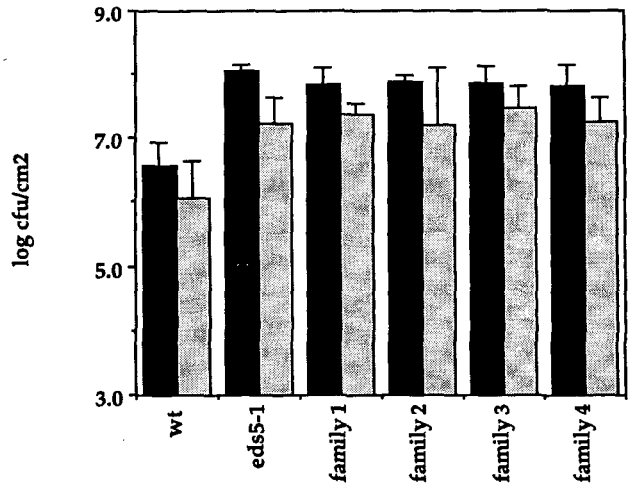

Figure 6. Cosegregation of Enhanced Susceptibility to *P. syringae* and *P. aeruginosa* in *eds5-1*.

Three days after infection with either *P. s. maculicola* ES4326 (black bars) or *P. aeruginosa* UCBPP-PA14 (shaded bars), both at a dose of 3.0 log (cfu/cm^2), infected leaves were assayed for bacterial density. The *t* values and confidence limits are as follows: for *P. s. maculicola* ES4326 growth, wild type (wt)–*eds5-1*, 5.2 (>99.5%); wt–family 1, 3.8 (>99.5%); wt–family 2, 4.5 (>99.5%); wt–family 3, 3.9 (>99.5%); wt–family 4, 3.3 (>99.0%); for *P. aeruginosa* UCBPP-PA14 growth, wt–*eds5.1*, 3.4 (>99.0%); wt–family 1, 4.9 (>99.5%); wt–family 2, 2.4 (>97.5%); wt–family 3, 4.7 (>99.5%); wt–family 4, 3.4 (>99.0%). Data points are the means of four replicate samples, and similar data were obtained in a similar experiment with *eds9-1*.

maculicola ES4326 sensitivity and *P. aeruginosa* UCBPP-PA14 sensitivity in either one of these *eds* mutants are unlinked is 0.25[4] or 3.9×10^{-3}. This does not eliminate the possibility that these two phenotypes are caused by two linked mutations; however, it seems unlikely that this would have occurred in two independently isolated *eds* mutants.

As described above, *eds5-1* has the additional phenotype of only inducing *PR-1* to ~10% of wild-type levels. Table 1 shows that the *eds5-1* sensitivity to *P. s. maculicola* ES4326 cosegregated with the noninduction of *PR-1* phenotype in four F_3 *eds5-1/eds5-1* families.

DISCUSSION

The genetic and phenotypic characterization of four Arabidopsis *eds* mutants (*eds5-1*, *eds6-1*, *eds7-1*, and *eds9-1*) suggests that the mutations in these lines correspond to previously unknown defense-related functions. We reached this conclusion because none of these *eds* mutations affects the HR response, the synthesis of camalexin, or, except for *eds5-1*, the expression of 11 different defense-related genes. In addition, all of these *eds* mutants are capable of mounting

Table 1. Cosegration of Enhanced Susceptibility to *P. syringae* and Decreased Levels of *PR-1* Induction in *eds5-1*

Family	Growth of *P. s. maculicola* ES4326 in Log (cfu/cm^2)[a]	Induction of *PR-1* (Arbitrary Units)[b]
Wild type	5.4 ± 0.5	12.4
eds5-1	7.5 ± 0.1	2.4
Family 2	6.7 ± 0.4	2.5
Family 12	7.4 ± 0.1	1.5
Family 5	7.1 ± 0.4	1.3
Family 6	7.4 ± 0.4	2.1

[a] Three days after infection with *P. s. maculicola* ES4326 at a dose of 3.0 log (cfu/cm^2), infected leaves were assayed for bacterial density. The *t* values are as follows: wt–*eds5-1*, 8.2; wt–family 2, 4.0; wt–family 12, 7.9; wt–family 5, 5.5; wt–family 6, 5.9. Data points are the means of six replicate samples.

[b] Forty-eight hours after infection with *P. s. maculicola* ES4326 at a dose of 5.0 log (cfu/cm^2), infected leaves were harvested for RNA preparation and gel blot analysis. *PR-1* RNA was quantitated and normalized to counts obtained for the *UBQ5* control probe.

at least a partial SAR response, unlike the *npr1* and *nim1* mutants (Cao et al., 1994; Delaney et al., 1995). One possibility, however, is that these mutations correspond to a biochemically defined gene, such as *PR-1*, but do not affect the level of mRNA accumulation after pathogen attack. Only one of these mutants (*eds5-1*) has been mapped with enough precision to rule out this latter possibility (data not shown).

Importantly, as summarized in Table 2, the four *eds* mutations characterized here probably affect diverse defense functions because mutations at each of these four loci result in distinguishable phenotypes. *eds6-1*, *eds7-1*, and *eds9-1* exhibit unique patterns of susceptibility to three different bacterial pathogens, and *eds5-1* is the only *eds* mutant among these four that fails to accumulate wild-type levels of *PR-1* mRNA after pathogen attack. Mutant *eds7-1* contains the most pathogen-specific mutation, allowing enhanced growth only of the closely related pseudomonads *P. s. maculicola* ES4326 and *P. s. tomato* DC3000. Mutants *eds5-1* and *eds6-1* are also more susceptible to the distantly related *P. aeruginosa* UCBPP-PA14. *eds9-1* is the most globally affected mutant of this group, allowing enhanced growth of *P. s. maculicola* ES4326, *P. s. tomato* DC3000, *P. aeruginosa* UCBPP-PA14, and *X. c. raphani* 1946. Although *eds5-1* reproducibly allowed ~0.5 log additional growth of *X. c. raphani* 1946 compared with the wild type, this was not a large enough difference to be statistically significant at the 95% confidence limit by using an independent *t* test because of the high degree of variance inherent in this type of data.

The observation that the four *eds* mutants exhibited differential susceptibilities to these *Pseudomonas* and *Xanthomonas* species implies that the defense responses defined by these *EDS* genes are not equally effective against different pathogens. Mutants, such as *eds9-1*, for example, that are susceptible to a variety of pathogens are more likely to contain lesions in a general defense response rather than in a pathogen-specific response.

Previous evidence from both in vitro and transgenic plant studies also indicates that several defense responses are not equally effective against different pathogens. For example, chitinase and β-1,3-glucanase show differing inhibitory activities toward different fungal pathogens (Mauch et al., 1988; Broglie et al., 1991), depending largely on the presence or absence of a chitin-containing fungal cell wall. The constitutive expression of the *PR-1a* and *PR-1b* genes in tobacco confers increased tolerance to two fungal pathogens of tobacco but does not confer resistance to tobacco mosaic virus or a variety of other viral or bacterial pathogens (Cutt et al., 1989; Alexander et al., 1993). The antibiotic activity of two proteins isolated from radish seed shows a high degree of specificity to filamentous fungi (Terras et al., 1992). Purified osmotin inhibited *Phytophthora infestans* to a greater degree than it inhibited *P. parasitica*, and the overexpression of osmotin in potato has been shown to delay the development of symptoms after *P. infestans* but not *P. parasitica* infection (Liu et al., 1994). These data are consistent with the observation that different *eds* mutants show differential susceptibilities to different bacterial phytopathogens.

Interestingly, none of the four *eds* mutants was more susceptible to the vascular pathogen *X. c. campestris* 8004, at least when it was infiltrated via the stomata. This result is not necessarily surprising because stomatal infiltration is a very artificial mode of infection for this particular pathogen and

Table 2. Phenotypes of Arabidopsis *eds* Mutants

Conditions	*npr1*[a]	*eds5-1*	*eds6-1*	*eds7-1*	*eds9-1*
Growth of *P. syringae*	Enhanced	Enhanced	Enhanced	Enhanced	Enhanced
Growth of *P. aeruginosa*	ND[b]	Enhanced	Enhanced	Same as in the wild type	Enhanced
Growth of *X. c. raphani*	ND	May be partially enhanced	Same as in the wild type	Same as in the wild type	Enhanced
PR-1 induction by SA[c]	0%	100%	100%	100%	100%
PR-1 induction by *P. syringae*	10%	10%	100%	100%	100%

[a] *npr1* data are from Cao et al. (1994) and Glazebrook et al. (1996).

[b] ND, not determined.

[c] *PR-1* induction by SA is from Cao et al. (1994) and Glazebrook et al. (1996).

because, unlike the leaf-spotting pathogens *P. s. maculicola*, *P. s. tomato*, and *X. c. raphani*, *X. c. campestris* does not normally replicate in the leaves (Parker et al., 1993). Also, none of the four *eds* mutants tested was more susceptible to the bean pathogen *P. s. phaseolicola* 3121, indicating that the *eds* mutants are most likely not defective in defense-related responses (or other responses) necessary for containment of this non-host pathogen.

As observed with the wild type, all four *eds* mutants allowed ~100-fold less growth of *P. s. maculicola* ES4326 in upper leaves of plants preinoculated with a necrotizing pathogen as compared with the growth of *P. s. maculicola* ES4326 in control plants. This result indicates that the signal transduction pathway leading to the induction of SAR appears to be at least partially intact in all four mutants, in contrast to *npr1* (Cao et al., 1994). On the other hand, *eds5-1*, *eds9-1*, and *eds6-1* allowed significantly more growth of *P. s. maculicola* ES4326 after the induction of SAR than did wild-type Columbia, suggesting that at least some of the defense-related factors that are responsible for limiting pathogen growth at the site of infection are also important components of SAR.

One model that is consistent with the induction of SAR in the *eds* mutants is that SAR consists entirely of the activation at a distance of the same defense-related factors that are normally elicited locally in response to virulent pathogens. According to this model, the growth limitation observed in a secondary challenge after induction of SAR can be attributed to the pre-induction of these defense-related factors. Alternatively, the growth limitation observed in SAR could be due to specific SAR-induced defense-related factors or due to a combination of SAR-specific factors and defense-related factors induced locally after infection by virulent pathogens. If this is the case, it seems likely that the postulated SAR-specific factors would be intact in the *eds* mutants and that the *eds* phenotype observed in the secondary challenge is due to the defect in the local induction of defense-related factors in the secondarily infected leaves. Irrespective of which of these models is correct, it seems unlikely that the *eds* mutants contain any specific defect in their ability to generate a systemic SAR induction signal; however, it is possible that the signal transduction pathway leading to the activation of local defense responses and SAR are the same or contain common components and that such a common component is only partially functional in the *eds* mutants, leading to lower levels of both a local and systemic signal.

There are several possible explanations for why the expression of the majority of *PR* genes in these *eds* mutants was indistinguishable from that of the wild type. It is possible that these mutants have changes in pathogen-induced genes that have not been discovered yet. It is also possible that these *eds* mutants have defects in the biochemical function but not expression levels of a known *PR* gene. The one significant alteration in gene expression that we observed was in *eds5-1*, in which *PR-1* mRNA only accumulated to ~10% that of wild-type levels after a variety of treatments. This is additional circumstantial evidence, along with the results of Alexander et al. (1993) and others, that *PR-1* may actually be one of several factors responsible for some of the growth limitation observed in wild-type plants. In Arabidopsis, the *PR-1* structural gene maps to chromosome 2 (E. Drenkard and F.M. Ausubel, unpublished results), and *eds5-1* is linked to the *fah1-2* mutation (Chapple et al., 1992) on chromosome 4 (E.E. Rogers and F.M. Ausubel, unpublished results). Therefore, *eds5-1* is not the structural gene for *PR-1*, and it is tempting to speculate that the *EDS5* gene product is involved in controlling the expression of *PR-1*.

One goal of this work is to elucidate the signal transduction cascades involved in pathogen recognition and defense response activation. After infection with *P. s. maculicola* ES4326, *PR-5* and *BGL2* mRNA accumulation in *npr1-2*, *npr1-3*, and *eds5-1* was indistinguishable from that of the wild type. In contrast, *PR-1* mRNA only accumulated to ~10% that of wild-type levels after *P. s. maculicola* ES4326 infection in these three mutants (Glazebrook et al., 1996; Figure 4). Because *npr1* mutants and *eds5-1* exhibit similar phenotypes with respect to *PR* gene accumulation, it is possible that *EDS5* could be upstream of *NPR1* in the same signaling pathway. On the other hand, because *eds5-1* is capable of mounting a limited but significant SAR response whereas *npr1* mutants are severely compromised in their ability to induce SAR (Cao et al., 1994), it is possible that there are multiple signal transduction pathways leading to *PR-1* gene expression and that *EDS5* and *NPR1* are in distinct pathways.

The result that all four *eds* mutants are capable of limiting the growth of *P. s. maculicola* ES4326 containing the cloned *avr* gene *avrRpt2* suggests that the HR pathway is at least partially distinct from the plant defense responses necessary for defending Arabidopsis from virulent pathogens. It also indicates that these mutants belong to a different class than that of the previously reported Arabidopsis resistance gene mutants. Arabidopsis mutants have been isolated that show defects in limiting the growth of both virulent and avirulent bacterial pathogens, for example, *eds1* (Parker et al., 1996). However, the *eds* mutants described here appear to be in a different class because their pathogen interaction phenotypes affect virulent pathogens.

One concern in assigning *EDS* genes to distinct defense-related pathways or responses on the basis that they exhibit distinct phenotypes is that some *EDS* genes may be represented by weaker alleles than are other *EDS* genes and therefore may show only a subset of the phenotypes of the stronger alleles. This caveat applies to many of the conclusions that we have reached in this study. There is no straightforward experimental way to address this concern in the absence of isolating multiple mutant alleles corresponding to each *EDS* gene or in the absence of determining the molecular basis of individual mutants by cloning *EDS* genes.

In conclusion, we have shown that four *eds* mutants are more susceptible to at least one other virulent bacterial pathogen, demonstrating that these mutations are not spe-

Table 3. Reagents Used for the Generation of Probes

Gene	Sense Primer	Antisense Primer	Template
BGL2	CTACAGAGATGGTGTCA	AGCTGAAGTAAGGGTAG	pATBG12[a]
PR-1	GTAGGTGCTCTTGTTCTTCCC	CACATAATTCCCACGAGGATC	Genomic DNA[b]
PR-5	CACATTCTCTTCCTCGTGTTC	TAGTTAGCTCCGGTACAAGTG	Genomic DNA[b]
UBQ5	GTGGTGCTAAGAAGAGGAAGA	TCAAGCTTCAACTCCTTCTTT	Genomic DNA[c]
PAL1	—[d]	GGGACCAGTAGCTTTG	pSKPAL[e]
GST1	—	CAAGACTCAATTATCGAAGATTAC	pGST1[f]
PIG2	—	—	pPIG2[f]
PIG18	—	—	pPIG18[f]
ELI3	GACATTGATGTGAGGTCC	ATCGGCAGTTATGTTGTG	pELI1[g]
ASA1	GCTTACCGTTGTTTGGTC	AGCAATGTCCATGTCACC	pKN1[h]
LOX1	GGATGGACTCACTGTTG	CATGAAGAGCAGAAG	pLOXR1.5[i]
AIG1	CTAACTCTAGCGGATGGA	CTTCCATTTCAGCACGCA	pLR2[j]
AIG2	GTGAAAATAAAAACAATGACAAGC	GGACTAAAATCAAACTTGTTCTTA	pAIG2-3[j]

[a] Dong et al. (1991).
[b] Uknes et al. (1992).
[c] Greenberg et al. (1994).
[d] Dash indicates that no primer was used.
[e] Davis et al. (1991).
[f] G.-L. Yu, T.L. Reuber, and F.M. Ausubel, manuscript in preparation.
[g] Kiedrowski et al. (1992).
[h] Niyogi and Fink (1992).
[i] K. Peterman (Wellesley College, Wellesley, MA).
[j] Reuber and Ausubel (1996).

cific to *P. s. maculicola* ES4326; *eds9-1* is more susceptible to three additional bacterial pathogens. In addition, none of these four mutants has defects in the HR, and all four mutants mount at least a partial SAR response. Taken together, these data indicate that these four *eds* mutants probably define several diverse, previously unknown defense responses involved in limiting the growth of virulent pathogens. Finally, because *eds5-1* only induces *PR-1* to ~10% that of wild-type levels, this mutant may help to elucidate the signal transduction cascade leading to *PR-1* gene induction.

METHODS

Bacteria, Plants, Growth Conditions, and Genetic Crosses

Pseudomonas syringae pv *maculicola* ES4326 (Dong et al., 1991), *P. s.* pv *phaseolicola* 3121 (Rahme et al., 1992), and *P. s.* pv *tomato* DC3000 (Cuppels, 1986) have been described previously. The plasmid pLH12 carries *avrRpt2* (Whalen et al., 1991). *P. syringae* strains were cultured at 28°C in King's B medium (pH 7.0, 10 mg/mL protease peptone, 15 mg/mL glycerol, 1.5 mg/mL K_2HPO_4, and 4 mM $MgSO_4$) supplemented with 100 μg/mL streptomycin for *P. s. maculicola* ES4326, 50 μg/mL rifampicin for *P. s. phaseolicola* 3121 and *P. s. tomato* DC3000, or 10 μg/mL tetracycline for pLH12. *P. aeruginosa* UCBPP-PA14 (Rahme et al., 1995) was grown at 37°C in Luria-Bertani medium (Difco) containing 50 μg/mL rifampicin. *Xanthomonas campestris* pv *campestris* 8004 (Parker et al., 1993) and *X. c.* pv *raphani* 1946 (Parker et al., 1993) were both grown at 28°C in NYG media (Parker et al., 1993) containing 50 μg/mL rifampicin. Colonies were plated for counting on the above media containing 15 g/L Bacto agar (Difco) and grown at the same temperature as the corresponding liquid cultures.

Arabidopsis thaliana ecotype Columbia was grown in Metromix 2000 (Scott, Marysville, OH) soil, in a Conviron growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) (20 ± 2°C, 90% relative humidity) on a 12-hr-light/12-hr-dark cycle under 150 μE m^{-2} sec^{-1} of fluorescent illumination. All experiments were performed in a Conviron growth chamber , with the exception of experiments involving *P. aeruginosa* UCBPP-PA14, in which, after infection, plants were placed in a growth chamber (Percival, Boone, IA) at 30°C and 100% humidity (Rahme et al., 1995). Plants were infected with suspensions of bacterial cells in 10 mM $MgSO_4$ by using a 1-mL syringe (without a needle) to force the suspension through the stomata by pressing the syringe against the abaxial side of the leaves. Genetic crosses were performed by dissecting immature flowers of the pollen recipient before anther dehiscence and applying pollen from the pollen donor to the recipient pistil. The extraction and quantitation of camalexin were performed as previously described (Glazebrook and Ausubel, 1994).

Bacterial Growth Assays

Plants grown in a growth chamber were infected with the relevant bacterium at the indicated dose. In practice, the dose was determined by OD_{600}, with the dose of 10^3 colony-forming units (cfu) per cm^2 leaf area being equivalent to OD_{600} of 0.0002. After 3 days, growth was assayed by excising a sample consisting of two 0.18-cm^2 disks from each infected leaf by using a cork borer, grinding the sample in 10 mM $MgSO_4$ by using a plastic pestle, and plating

appropriate dilutions on the appropriate bacterial growth medium containing the relevant antibiotic(s). Data are reported as means and standard deviations of the log of the number of colony-forming bacterial units per square centimeter of leaf tissue, log (cfu/cm^2), of four to 12 replicates. Differences in bacterial growth between the wild type and the mutants were judged significant if they were above the 95% confidence limit using an independent *t* test in more than one experiment. In general, the significance of differences between means depends not only on the actual difference between the means and the variance but also on the number of replicate data points and the number of times the experiment was repeated. Therefore, the degree of overlap between error bars was not always a reliable indicator of the significance of the difference between means. For example, in Figure 2, although the error bars for the wild type and *eds9-1* overlap, the difference between the means is significant at a 95% confidence limit by an independent *t* test.

RNA Gel Blot Analysis

Total RNA was purified from Arabidopsis leaves as described previously (Reuber and Ausubel, 1996). Samples (5 μg) were separated on formaldehyde–agarose gels (Ausubel et al., 1996), transferred to a GeneScreen hybridization membrane, hybridized with various probes (described below), and washed according to the instructions of the supplier (New England Nuclear, Boston, MA). Blots were stripped by boiling in 1% SDS and 0.1 × SSC (Ausubel et al., 1996) for 10 min before hybridization with a subsequent probe.

Probes were prepared by amplification of appropriate sequences by using polymerase chain reaction (PCR), purification of the products on agarose gels, and labeling of single-stranded probes by PCR using these purified products, antisense primers, and α-^{32}P-dCTP. The exceptions were the phenylaline ammonia-lyase *PAL1* and glutathione *S*-transferase *GST1* probes in which pSKPAL and pGST1 were cut with BamHI and ClaI, respectively, and used rather than purified PCR products in the labeling of single-stranded probes by PCR. Also, for pathogen-induced genes 2 and 18 (*PIG2* and *PIG18*), the plasmids pPIG2 and pPIG18 were cut with BamHI to remove the inserts. The inserts were then gel purified and labeled by random priming (Ausubel et al., 1996). Sequences of the oligonucleotide primers, the templates used, and the reference for each probe are shown in Table 3.

Blots were analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The data shown in Figures 4 and 6 and Table 1 were obtained by volume integration of the signals in the bands hybridizing with the pathogenesis-related *PR-1* probe and then by normalization of these values to the values obtained by volume integration of the signals in the bands hybridizing with the ubiquitin *UBQ5* probe to compensate for lane-to-lane variations in the amounts of RNA.

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