***Arabidopsis thaliana* EDS4 Contributes to Salicylic Acid (SA)-Dependent Expression of Defense Responses: Evidence for Inhibition of Jasmonic Acid Signaling by SA**

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The *Arabidopsis* enhanced disease susceptibility 4 (*eds4*) mutation causes enhanced susceptibility to infection by the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326). Gene-for-gene resistance to bacteria carrying the avirulence gene *avrRpt2* is not significantly affected by *eds4*. Plants homozygous for *eds4* exhibit reduced expression of the pathogenesis-related gene *PR-1* after infection by *Psm* ES4326, weakened responses to treatment with the signal molecule salicylic acid (SA), impairment of the systemic acquired resistance response, and reduced accumulation of SA after infection with *Psm* ES4326. These phenotypes indicate that *EDS4* plays a role in SA-dependent signaling. SA has been shown to have a negative effect on activation of gene expression by the signal molecule jasmonic acid (JA). Two mutations that cause reduced SA levels, *eds4* and *pad4*, cause heightened responses to inducers of JA-dependent gene expression, providing genetic evidence to support the idea that SA interferes with JA-dependent signaling. Two possible working models of the role of *EDS4* in governing activation of defense responses are presented.

When pathogenic microorganisms attack plants, the plants activate a battery of defense responses to fight the spread of infection. In some plant-pathogen interactions, products of pathogen genes called avirulence (*avr*) genes are recognized by products of corresponding resistance (*R*) genes in the host, resulting in rapid activation of host defense responses. Typically, an *R* gene recognizes only a single *avr* gene, so this form of resistance is called gene-for-gene resistance (Baker et al. 1997). During gene-for-gene resistance responses, the infected plant cells undergo a form of rapid, programmed cell death called the hypersensitive response (HR) (Hammond-Kosack and Jones 1996). Pathogens that trigger gene-for-gene resistance are called avirulent pathogens. If the pathogen does not carry an *avr* gene recognized by the

plant, it does not elicit an HR, defense responses are activated more slowly, and disease ensues. Such pathogens are called virulent pathogens.

Infection by an avirulent pathogen causes the dying cells in the locally infected tissue to emit a signal that activates systemic acquired resistance (SAR) in distant tissues of the plant. Plants can acquire resistance to normally virulent pathogens through SAR. SAR is characterized by the enhanced expression of defense genes such as pathogenesis-related (*PR*) genes like *PR-1*, *PR-5*, and *BGL-2* in the systemic tissues. Salicylic acid (SA) plays a key role in activation of SAR (Hammond-Kosack and Jones 1996; Durner et al. 1998). Exogenous application of SA is sufficient to induce SAR and activate *PR-1* defense gene expression (Ryals et al. 1996). The requirement for SA as a signal in SAR was proven by construction of transgenic plants expressing a bacterial salicylate hydroxylase gene (*nahG*) that converts SA to catechol (Gaffney et al. 1993). When infected with pathogens that induce SAR in wild-type plants, *nahG* plants fail to develop SAR and do not exhibit systemic expression of *PR* genes (Delaney et al. 1994). While it is clear that SA is required in the systemic tissues for *PR* gene expression, it does not appear to be the translocated signal (Vernooij et al. 1994; Willits and Ryals 1998). SA has also been found to play an important role in the induction of defense gene expression in the local tissues (Delaney et al. 1994). *Arabidopsis nahG* plants are compromised in local resistance, displaying enhanced susceptibility to a variety of virulent and avirulent pathogens (Delaney et al. 1994; Lawton et al. 1995; Reuber et al. 1998).

Genetic analysis has revealed some components of the signal transduction pathways that control activation of defense responses. Several mutants with defects in genes required for the activation of the SA signaling pathway have been characterized. Plants carrying *pad4* mutations display reduced SA accumulation, reduced *PR-1* expression, and low levels of the antimicrobial metabolite camalexin in leaves infected with the virulent bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326) (Zhou et al. 1998). SA is necessary and sufficient for activation of *PR-1* expression, and necessary but not sufficient for camalexin synthesis (Zhou et al.

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1998). The defense response defects in *pad4* plants appear to be due to inadequate SA accumulation in response to infection because they can be suppressed by exogenous application of SA (Zhou et al. 1998). Consequently, *PAD4* is thought to act by promoting SA accumulation, leading to activation of SA-dependent defense responses (Zhou et al. 1998).

When plants carrying *eds5* (*sid1*, salicylic acid induction-deficient 1) or *sid2* mutations are infected with *P. syringae*, they accumulate very little SA and *PR-1* transcript (Nawrath and Metraux 1999; Rogers and Ausubel 1997). Unlike *pad4* plants, *eds5* and *sid2* plants do not have defects in camalexin synthesis (Nawrath and Metraux 1999). Treatment with SAR activators BTH (benzothiadiazole) or INA (2,6-dichloroisonicotinic acid) activates *PR-1* expression in *eds5* and *sid2* plants. Taken together, these phenotypes suggest that *EDS5* and *SID2* act upstream from SA accumulation in a way that does not interfere with activation of camalexin synthesis (Nawrath and Metraux 1999).

Mutants with defects in a gene called *npr1* (non-inducer of *PR* proteins) or *nim1* (non-inducible immunity) do not express *PR* genes in response to exogenous SA (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). Mutations in *NPR1* cause increased susceptibility to *P. syringae*, reduced *PR-1* expression in infected leaves, and failure to establish SAR (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). In contrast to *pad4*, *eds5*, and *sid2* plants, in *npr1* plants SA accumulation is higher than it is in wild-type plants. These phenotypes indicate that *NPR1* acts downstream from SA (Cao et al. 1994). Yeast two-hybrid experiments showed that *NPR1* interacts with bZIP transcription factors that bind to the *PR-1* promoter, suggesting that *NPR1* promotes defense gene expression by altering the activities of transcription factors (Zhang et al. 1999).

Arabidopsis has also been used to study the role of jasmonic acid (JA)-dependent signaling in disease resistance. Chemicals such as methyl jasmonate (MeJA), an ester that releases JA, or rose bengal, an oxidizing agent, act in a JA-dependent manner to induce local and systemic production of a set of plant defense proteins including thionins (Epplé et al. 1995) and defensins (Penninckx et al. 1996). Infection by the fungal pathogen *Alternaria brassicicola* activates the expression of the defensin gene *PDF1.2* in a JA-dependent manner (Penninckx et al. 1996). JA-dependent responses must be important for resistance to *A. brassicicola*, since pre-treatment with MeJA enhances resistance (Penninckx et al. 1996), and the *coi1* mutation, which blocks responses to JA, compromises resistance (Feys et al. 1994).

SA-dependent signaling and JA-dependent signaling can be mutually inhibitory. In tobacco, SA inhibits transcription of genes encoding enzymes required for JA synthesis (Harms et al. 1998; Nishitani et al. 1997). Moreover, SA treatment inhibits JA-mediated activation of basic *PR* gene expression while JA treatment inhibits SA-mediated activation of acidic *PR* gene expression (Niki et al. 1998). Several Arabidopsis mutants with defects in SA signaling display enhanced sensitivity to inducers of JA-dependent expression of *PDF1.2*. Plants carrying *nahG*, which fail to accumulate SA, and *npr1* plants, which fail to respond to SA, express *PDF1.2* at higher levels than wild-type plants when infected with *A. brassicicola* or treated with rose bengal (Clarke et al. 1998; Penninckx et al. 1996). Conversely, constitutive expression of *PDF1.2* in

cpr6 plants is inhibited by treatment with INA, an inducer of SA signaling (Clarke et al. 1998).

A collection of mutants that includes plants with defects in genes required for activation of defense responses was obtained by screening a mutagenized population of Arabidopsis for plants that showed an enhanced disease susceptibility (*eds*) phenotype in response to infection by *Psm* ES4326 (Glazebrook et al. 1996). The *eds* mutations included alleles of *npr1*, *pad4*, and *eds5* (Glazebrook et al. 1996). We now describe the characterization of *eds4*, another mutant obtained in the *eds* screen (Glazebrook et al. 1996). We have found that *eds4* plants exhibit reduced accumulation of SA after infection by *Psm* ES4326, attenuated responses to exogenous SA, and heightened responses to stimulation of JA signaling. These phenotypes led to the conclusion that *EDS4* contributes to SA accumulation in response to pathogen attack, as well as to SA-induced activation of defense responses.

RESULTS

Gene-for-gene resistance to *Psm* ES4326 carrying *avrRpt2* is not affected by *eds4*.

Previous work showed that susceptibility to the virulent strain *Psm* ES4326 is enhanced in *eds4* plants (Glazebrook et al. 1996). To test for effects of *eds4* on gene-for-gene resistance, we compared growth of *Psm* ES4326 and an isogenic avirulent strain carrying the avirulence gene *avrRpt2*. Wild-type and *eds4* plants were infected with each strain, and bacterial titers in infected leaves were determined daily. Figure 1 shows that the titers of both bacterial strains in *eds4* plants were five- to 10-fold higher than in wild-type plants. In both wild-type plants and *eds4* plants, the titer of the avirulent strain was approximately 100-fold lower than the titer of the virulent strain. In plants that are defective in gene-for-gene resistance due to a mutation in the resistance gene *RPS2*, which confers resistance to strains carrying *avrRpt2*, both *Psm* ES4326 and *Psm* ES4326 carrying *avrRpt2* reach the same

Fig. 1. Bacterial growth in wild type and *eds4* plants. Wild-type (open symbols) and *eds4* (closed symbols) plants were infected with *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 (squares) or an isogenic strain carrying the avirulence gene *avrRpt2* (circles). Bacterial growth was determined at 24, 36, and 72 h after infection. Each point represents the mean and standard deviation of six replicates.

titer as *Psm* ES4326 does in wild-type plants (Mindrinos et al. 1994). Since titers of *Psm* ES4326 carrying *avrRpt2* were much lower than *Psm* ES4326 titers in *eds4* plants, we concluded that *eds4* does not cause a significant defect in gene-for-gene resistance to strains carrying *avrRpt2*.

Expression of *PR-1* is impaired in *eds4* plants.

Some mutants that show enhanced susceptibility to *Psm* ES4326 also show defects in expression of defense genes after infection by this pathogen (Cao et al. 1994; Zhou et al. 1998; Rogers and Ausubel 1997). To test whether defense gene expression was impaired in *eds4* mutants, we infected wild-type and *eds4* plants with *Psm* ES4326 and monitored mRNA levels of several defense genes at intervals after infection. The levels of *PR-1* mRNA were reduced in infected *eds4* leaves, compared with the levels in infected wild-type leaves (Fig. 2). This experiment was done three times. The *PR-1* hybridization signals from all three experiments were measured and normalized to the 18S rRNA signals. The ratios of *PR-1* signal in wild-type plants to *PR-1* signal in *eds4* plants were calculated. The mean and standard deviation of this value was 1.85 ± 0.17 at 24 h and 2.22 ± 0.05 at 36 h. Expression of three other genes induced by *Psm* ES4326 infection—*BGL2*, *PR-5*, and *ASAI*—was not significantly affected by *eds4* (data not shown). The finding that *eds4* causes a decrease in the expression of *PR-1* in response to infection suggests that *EDS4* plays a regulatory role in the defense response.

Responses to SA are impaired in *eds4* plants.

The observation that *eds4* plants have a defect in *PR-1* expression suggested that *eds4* might interfere with SA signaling. To determine whether *eds4* affects responses to SA, we treated wild-type and *eds4* plants with SA 1 day prior to infection with *Psm* ES4326. Bacterial growth was measured in the infected leaves at 1, 2, and 3 days after infection. When the plants were grown and infected in a Conviron growth chamber under conditions of 22°C temperature and 85% relative humidity, SA treatment reduced the bacterial growth in wild-type plants by about fivefold but did not affect growth in *eds4* plants (Fig. 3A). However, when plants were grown and infected in a constant temperature room under conditions of 23°C and 50% relative humidity, pre-treatment with SA reduced bacterial growth by six- to tenfold in both wild-type and *eds4* plants (Fig. 3B). These results suggested that *eds4* might cause a partial block in responses to SA-induced resis-

tance such that compromised SA-induced resistance is only apparent under some growth conditions.

To test for quantitative effects of *eds4* on responses to SA, wild-type and *eds4* seedlings were grown on medium containing various concentrations of SA. Two weeks after germination, *PR-1* mRNA levels were examined. At SA concentrations of 0.025 and 0.05 mM, *PR-1* expression was lower in *eds4* seedlings than it was in wild-type seedlings (Fig. 4). This result confirms that responses to SA are defective in *eds4* plants.

EDS4 is required for resistance to *Psm* ES4326 and *PR-1* expression associated with SAR.

SA in systemic tissues is required for SAR (Vernooij et al. 1994). Consequently, it is likely that a reduction in the ability

Fig. 2. Expression of *PR-1* in leaves infected with *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326. Leaves were excised at 0, 12, 24, 36, and 48 h after infection. "M" indicates leaves mock-infected with 10 mM $MgSO_4$ that were sampled at 36 h; 1.5 μ g of RNA was loaded in each lane; 18S rRNA was used as a control for equal loading.

Fig. 3. Effect of exogenous salicylic acid (SA) on the growth of *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326. Plants were sprayed with 5 mM SA in 0.02% Silwet or 0.02% Silwet, until uniformly wet. One day after treatment, plants were infected with bacteria at a dose of 10^3 CFU/cm². Bacterial titer was determined at 1, 2, and 3 days after infection. Each bar represents the mean and standard deviation of eight replicates. In four other independent experiments, bacterial titers were determined 3 days after infection and similar results were obtained. **A**, Plants were grown in a Conviron chamber maintained at 22°C and 85% relative humidity. **B**, Plants were grown in a constant temperature room, maintained at 23°C and 50% relative humidity.

to respond to SA would cause a defect in SAR. We tested *eds4* plants for defects in SAR induced by infection with an avirulent pathogen. To induce SAR, four to five leaves of each wild-type and *eds4* plant were infected with *P. syringae* pv. *tomato* (*Pst*) DC3000 carrying *avrRpt2* or mock-infected with 10 mM MgSO₄. Four days after infection, two previously uninfected leaves of each plant were challenged with *Psm* ES4326. In systemic wild-type leaves, the bacterial titer of the virulent strain was reduced by prior infection with *Pst* DC3000 carrying *avrRpt2* (Fig. 5A). Conversely, in *eds4* plants, prior infection by *Pst* DC3000 carrying *avrRpt2* had no effect on growth of *Psm* ES4326.

Systemic expression of *PR-1* is also associated with SAR. We tested *eds4* plants for defects in *PR-1* expression during SAR. SAR was induced by infecting three leaves of each plant with *Pst* DC3000 carrying *avrRpt2*. In both wild-type and *eds4* plants, the HR was observed in the infected leaves after 16 h. The HR was not observed in control plants mock-infected with 10 mM MgSO₄. To examine *PR-1* expression, uninfected leaves were collected 1, 2, 3, and 4 days after the SAR-inducing infection. *PR-1* mRNA levels in the uninfected leaves of *eds4* plants infected with *Pst* DC3000 carrying *avrRpt2* were much lower than they were in uninfected leaves of infected wild-type plants (Fig. 5B). Taken together, these results show that *eds4* plants are unable to establish SAR in response to infection by an avirulent pathogen.

SA accumulation in response to infection is reduced in *eds4* plants.

We have shown that *eds4* plants have a defect in responding to SA. To test for a defect in accumulating SA, we measured the levels of SA and its sugar conjugate, SA glucoside (SAG), in wild-type and *eds4* plants after infection with *Psm* ES4326. The SA levels in *eds4* plants infected with *Psm* ES4326 were 25, 60, and 50% of the levels in wild-type plants at 12, 24, and 36 h after infection, respectively (Fig. 6A), while the SAG levels in *eds4* plants were approximately 50% of the levels in wild-type plants at all time points tested (Fig. 6B). Taken together, our results suggest that *EDS4* is required for accumulation of SA after *Psm* ES4326 infection, as well as for the efficient activation of *PR-1* expression in response to SA.

Fig. 4. Effect of salicylic acid (SA) on *PR-1* expression in wild type and *eds4* seedlings. Expression of *PR-1* in 2-week-old wild-type and *eds4* seedlings grown in the presence of various concentrations of SA. 18S rRNA was used to evaluate even loading. This experiment was performed three times and similar results were obtained.

Mutations affecting SA signaling cause altered sensitivity to JA signaling.

The SA-dependent and JA-dependent signal transduction pathways are known to be mutually inhibitory. To test the strength of the correlation between reduced SA signaling and enhanced JA signaling, we examined *PDF1.2* expression in *pad4* plants. The *pad4* mutation causes reduced SA accumulation after infection with *Psm* ES4326 (Zhou et al. 1998). When *pad4* plants and wild-type plants were treated with rose bengal, the level of *PDF1.2* mRNA in *pad4* plants was much higher than it was in wild-type plants (Fig. 7). In *eds4* plants treated with either rose bengal or MeJA, *PDF1.2* mRNA levels were higher than they were in similarly treated wild-type plants, although these differences were not as large as the difference between *pad4* plants and wild-type plants (Fig. 7). These observations confirm and extend the correlation between reduced strength of SA signaling and increased strength of JA signaling. This correlation appears to be quantitative, since the defect in SA signaling in *pad4* plants is more severe

Fig. 5. A, Effect of systemic acquired resistance (SAR) induced by an avirulent pathogen on the growth of *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326. Four to five half-leaves of each plant were infected with *P. syringae* pv. *tomato* (*Pst*) DC3000 carrying *avrRpt2* at a dose of 10⁴ CFU/cm² leaf area or with 10 mM MgSO₄. Four days after infection, two secondary leaves of each plant were infected with *Psm* ES4326. Bacterial titer was measured 3 days after infection. Each bar represents the mean and standard deviation of six replicates. B, Systemic expression of *PR-1* in wild-type and *eds4* plants. One half of each of three leaves per plant was infected with *Pst* DC3000 carrying *avrRpt2* at a dose of 10⁴ CFU/cm². Control plants were mock-infected with 10 mM MgSO₄. Uninfected leaves were excised at 1, 2, 3, and 4 days after infection with the bacteria. Uninfected leaves from mock-infected samples were excised at 2 days after infection. 18S rRNA was used as the loading control. Similar results were obtained in replicate experiments.

than the defect in *eds4* plants (judged by *PR-1* expression in response to *Psm* ES4326 infection) and the enhanced expression of *PDF1.2* is also greater in *pad4* plants than it is in *eds4* plants.

Mapping and complementation of *eds4*.

The *eds4-1* mutation was previously shown to segregate as a single recessive mutation (Glazebrook et al. 1996). This observation was confirmed in the second and third backcrosses of *eds4-1* plants to wild type. Among the F₂ progeny of these crosses, 11 plants were scored as *eds* and 35 were scored as wild-type, based on the extent of bacterial growth 3 days after

infection. These data are consistent with a 1:3 ratio ($\chi^2 = 0.03$, $P > 0.8$), indicating that *eds4-1* is a single recessive mutation.

In an effort to place *EDS4* on the genetic map, *eds4* plants (Columbia ecotype) were crossed to wild-type Landsberg plants. One hundred and forty F₂ plants from this cross were scored for the *eds* phenotype. Unlike the case of backcrosses to Columbia, no clear segregation was observed. Rather, bacterial titers varied over a continuous range, indicating that genes other than *eds4* that are segregating in the Columbia $\times$ Landsberg cross affect the bacterial growth phenotype. The camalexin-deficient phenotype conferred by *pad4* also shows aberrant segregation in a Columbia $\times$ Landsberg cross, but it segregates 1:3 in a Columbia $\times$ Keswick cross (Zhou et al. 1998). Consequently, *eds4* was crossed to wild-type Keswick plants. Like F₂ plants from the Landsberg cross, 99 F₂ plants from this cross also displayed a continuous range of bacterial titers, indicating that there are genes other than *eds4* that affect bacterial growth segregating in this cross also. Not surprisingly, efforts to determine a map position for *eds4*, assuming that one-fourth of the F₂ plants with the highest bacterial titers were *eds4* homozygotes, were unsuccessful.

There are three other Arabidopsis mutants that accumulate low levels of SA in response to *P. syringae* infection: *pad4*, *eds5/sid1*, and *sid2* (Nawrath and Metraux 1999; Rogers and Ausubel 1997; Zhou et al. 1998). All four *pad4* mutations cause a reduction in camalexin synthesis, while camalexin synthesis in *eds4* plants is indistinguishable from that in wild-type plants, so it is extremely unlikely that *eds4* is an allele of *pad4*. Complementation testing of *eds5* and *eds4* (Glazebrook et al. 1996) and *sid2* and *eds4* (data not shown) for the *Psm* ES4326 growth phenotype demonstrated that *eds4* is not allelic to *eds5* or *sid2*. Like the *eds4* mutation, *npr1* mutations block activation of *PR-1* expression in response to SA. However, *npr1* plants accumulate even more SA in response to *P. syringae* infection than wild-type plants do, in contrast to the reduced SA levels in *eds4* plants. Complementation testing of *eds4* and *npr1-1* for the *Psm* ES4326 growth phenotype demonstrated that *eds4* is not an *npr1* allele (data not shown).

DISCUSSION

The phenotypes of *eds4* plants demonstrate that *EDS4* functions in SA-dependent control of defense gene expression. After infection with *Psm* ES4326, SA and SAG levels in *eds4* plants are much lower than in similarly treated wild-type plants. Presumably, this reduction in SA levels is the cause of

Fig. 6. Salicylic acid (SA) and SA glucoside (SAG) levels in infected wild-type and *eds4* plants. Wild-type and *eds4* plants were infected with *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 or mock-infected with 10 mM MgSO₄. Leaves were collected at 12, 24, and 36 h after infection. Each column represents the mean of three replicate samples. Error bars representing the standard deviations are shown where they are large enough to be visible. Free SA and total SA (SA + SAG) were assayed on the same samples. SAG values were obtained by subtracting free SA from total SA in each sample. **A**, Free SA. **B**, SAG.

Fig. 7. *PDF1.2* expression in wild-type, *pad4*, and *eds4* plants. Expression of *PDF1.2* (defensin) gene in the leaves of wild-type, *pad4*, and *eds4* plants treated with methyl jasmonate (MJA) or rose bengal (RB) or untreated (Control). The tissues were excised at 2 days after treatment. 18S rRNA was used as a control for equal loading.

the reduced *PR-1* transcript levels and increased bacterial growth in infected *eds4* plants. The *eds4* mutation also causes reduced sensitivity to SA. In *eds4* seedlings, the concentration of exogenous SA required to trigger *PR-1* expression is higher than the concentration required by wild-type seedlings. There are at least two possible explanations for these results, as shown in Figure 8. One possibility is that *EDS4* encodes a negative regulator of SA turnover (Fig. 8A). In this model, *eds4* mutations would cause reduced accumulation of SA in infected leaves and reduced responsiveness to SA by reducing the intracellular concentration of SA. Another possibility is that *EDS4* acts downstream from SA, explaining the observation that *eds4* plants respond weakly to SA (Fig. 8B). In this model, *EDS4* also participates in an SA amplification loop,

Fig. 8. Models of the role of *EDS4* in signal transduction. In response to infection by *Pseudomonas syringae* pv. *maculicola* (Psm) ES4326, PAD4 acts in a positive autoregulatory loop to increase salicylic acid (SA) concentrations. This increase is required for camalexin synthesis and *PR-1* expression. **A**, *EDS4* acts in elevating SA levels by either coding for a negative regulator of SA turnover, or **B**, by participating in an SA amplification loop and in responding to SA, in a manner that contributes to activation of *PR-1* expression but does not affect camalexin synthesis. NPR1 is required for activation of *PR-1* expression but not for elevation of SA levels. SA signaling and JA signaling are mutually inhibitory, yet both signals are required for camalexin production. The rationale for these models and alternative explanations for the phenotypes of *eds4* plants are presented in the Discussion.

explaining the observation that SA levels are low in infected *eds4* plants. The *eds4* mutation does not completely block accumulation of SA or responses to SA. In either model, these effects could be due to residual function of *eds4*, or due to *EDS4*-independent SA signaling. Other genes are known to be required for accumulation of SA after Psm ES4326 infection, including *PAD4* and *EDS5/SID1* (Zhou et al. 1998; Nawrath and Metraux 1999).

NPR1 is required for activation of *PR-1* expression in response to SA. When *npr1* plants are infected with Psm ES4326, SA levels are higher than they are in infected wild-type plants (Cao et al. 1994). This suggests that NPR1 is required only for responses to SA, and not for production of SA. Consequently, *EDS4* is placed upstream from NPR1 in the signaling pathway. There is a formal possibility that *EDS4* and NPR1 act in parallel pathways, rather than *EDS4* acting through NPR1 (Fig. 8B). This seems unlikely, since *npr1* mutations block SA-induced *PR-1* expression completely.

PAD4 is required for activation of SA signaling in response to Psm ES4326 infection. SA levels in infected *pad4* plants are lower than in infected wild-type plants, but *pad4* mutants do not show a defect in response to SA (Zhou et al. 1998). *PAD4* also seems to be involved in SA signal amplification, since SA is required for pathogen-induced *PAD4* expression, and *PAD4* is required for SA accumulation (Jirage et al. 1999). Consequently, we have placed *PAD4* upstream from SA, and postulated that it participates in amplification of SA levels in infected tissue.

The *eds4* and *pad4* mutations both cause enhanced expression of the defensin gene *PDF1.2* in response to treatment with rose bengal. Expression of *PDF1.2* is a JA- and ethylene-dependent process that does not require SA (Penninckx et al. 1996, 1998). As described above, it is known that SA-dependent signaling and JA-dependent signaling can be mutually inhibitory. Both *eds4* and *pad4* mutations interfere with SA-dependent signaling. Presumably, the reduction in SA-dependent signaling in *eds4* and *pad4* plants relieves inhibition of JA-dependent signaling, leading to increased *PDF1.2* expression in response to rose bengal treatment. This effect appears to be quantitative, since *eds4* has a weaker effect on SA signaling than *pad4* (judged by *PR-1* RNA levels after Psm ES4326 infection), and it has a correspondingly weaker effect on *PDF1.2* RNA levels after rose bengal treatment.

Unlike *eds4* plants, *pad4* plants have a defect in camalexin synthesis after Psm ES4326 infection. The camalexin defect seems to be due to the reduced SA level in infected *pad4* plants, since treatment with exogenous SA effectively restores camalexin synthesis. SA is necessary for camalexin synthesis, since *nahG* plants are severely compromised for camalexin synthesis after infection by Psm ES4326 or Psm ES4326 carrying *avrRpt2*. SA is not sufficient to induce camalexin synthesis, since plants treated with SA do not produce camalexin. The levels of free SA in Psm ES4326-infected *eds4* plants (25, 60, and 50% of wild type at 12, 24, and 36 h, respectively) are comparable to those in infected *pad4* plants (25, 66, and 55% of wild type at 12, 24, and 36 h, respectively), yet *eds4* plants do not have a camalexin defect. There are several possible explanations for this observation.

The first explanation is based on the observation that despite the similar free SA levels in *eds4* and *pad4* plants, the SA-signaling defect caused by *pad4* seems to be more severe.

The *PR-1* mRNA level in infected *pad4* plants is lower than in infected *eds4* plants, and the total SA level in infected *pad4* plants (30% of wild type) is lower than in infected *eds4* plants (60% of wild type). It is possible that the SA defect in *eds4* plants is not severe enough to cause a camalexin defect. The severity of the *pad4* mutation could be exacerbated by its effect on JA signaling. Camalexin synthesis after *Psm* ES4326 infection may require JA signaling as well as SA signaling, since JA-insensitive *coil* plants synthesize very little camalexin after *Psm* ES4326 infection (Zhou et al. 1999). Since SA and JA signaling are mutually inhibitory, a process that requires both SA and JA signaling would probably require a fine balance of both signals. In *pad4* plants, JA signaling seems strongly enhanced, while SA signaling is greatly weakened. Both effects are less severe in *eds4* plants. Perhaps the skewed ratio of SA and JA signal strength interferes with activation of camalexin synthesis in *pad4* plants.

The *eds5/sid1* and *sid2* mutants have very low levels of SA, very low *PR-1* expression, and normal camalexin levels in leaves infected with *P. syringae*. Since the severity of the *eds5/sid1* and *sid2* effects on SA and *PR-1* expression is comparable to that of the *nahG* transgene, it is difficult to argue that the reason that these mutations do not affect camalexin accumulation is that they are insufficiently severe. The *eds5/sid1* and *sid2* mutations did not cause hypersensitivity to activation of *PDF1.2* expression by *Alternaria brassicicola* infection, but it remains possible that such an effect would be observed if the inducer was rose bengal or MeJA. Nawrath and Metraux (1999) have proposed that *eds5/sid1* and *sid2* interfere with an SA-independent negative regulation of camalexin, presumably compensating for their negative effect on SA-dependent activation of SA biosynthesis. If this model is correct, *eds4* phenotypes could be explained similarly, or by using the reduced-severity explanation we propose above.

A second possible explanation is that *pad4* and the *nahG* transgene affect an as yet undiscovered signaling pathway that is required for activation of camalexin synthesis and is not affected by *eds4*, *eds5/sid1*, or *sid2*. If this is true, then it must be possible to overcome the requirement for the unknown signal by supplying large amounts of exogenous SA, since SA treatment restores camalexin synthesis in *pad4* plants. The phenotype of *nahG* plants is widely assumed to reflect the loss of SA, but of course it is not possible to exclude the possibility that it has additional effects. A mutation that blocked the SA biosynthetic pathway would be very useful for resolving these issues.

EDS4 is required for biologically induced SAR. Experiments with *nahG* plants demonstrated that SA is required in systemic tissue for SAR (Vernooij et al. 1994). The SAR defect in *eds4* plants could be due to reduced responsiveness to SA, inadequate SA concentration in the systemic tissue, or both. Mutations in *NPRI*, which block SA-induced gene expression, also abolish SAR (Cao et al. 1994), as do mutations in *eds5/sid1* and *sid2*, which block SA production (Nawrath and Metraux 1999).

Future work will be directed toward isolating *EDS4* and characterizing double mutants homozygous for both *eds4* and mutations that cause constitutive defense gene expression. These experiments should help to elucidate the mechanism of *EDS4* action, and to further define its position in the signal transduction network controlling activation of defense responses.

MATERIALS AND METHODS

Plants and growth conditions.

Wild-type plants were *Arabidopsis thaliana* accession Columbia (Col), *eds4* plants were *eds4-1* homozygotes that had been backcrossed to the wild-type parent (Col) at least twice, and *pad4* plants were *pad4-1* homozygotes that had been backcrossed to the wild-type parent (Col) four times (Glazebrook et al. 1996). Generally, plants were grown in pots in Metro-Mix 200 soil (Scotts-Sierra Horticultural Products, Marysville, OH) in a Conviron (Pembina, ND) growth chamber at $22 \pm 2^\circ\text{C}$, 85% relative humidity, $100 \mu\text{E s}^{-1} \text{m}^{-2}$ fluorescent illumination on a 12-h light/dark cycle. Fully expanded leaves of 4-week-old plants were used for the experiments. For measurement of the bacterial titer in response to SA, some plants were grown in a constant temperature room at 23°C and 50% relative humidity. For measurement of SA levels, plants were grown in a Conviron chamber as described above, except that an 8-h light, 16-h dark cycle was used to obtain more leaves per plant and fully expanded leaves from 8-week-old plants were used. Seedlings were grown on plates containing Murashige-Skoog (MS) medium (Life Technologies, Rockville, MD) and various concentrations of sodium salicylate. Seeds were vernalized at 4°C for 2 days before placement in a growth environment. The plates were incubated under fluorescent lamps ($100 \mu\text{E s}^{-1} \text{m}^{-2}$) on a 24-h photo-period and 23°C (room temperature).

Inoculations with bacteria and treatment with chemicals.

Strains *Psm* ES4326 (Dong et al. 1991) and *Pst* DC3000 (Cuppels 1986) have been described. The avirulence gene *avrRpt2* was carried on plasmid pLH12 as described (Dong et al. 1991). *P. syringae* strains were grown in King's B medium supplemented with appropriate antibiotics (Glazebrook and Ausubel 1994). Bacterial suspensions were infiltrated into *Arabidopsis* plants as described previously (Glazebrook and Ausubel 1994). For experiments involving extraction of total RNA from infected leaves, *Psm* ES4326 or *Psm* ES4326 carrying *avrRpt2* was introduced at a dose of 10^4 CFU/cm² leaf area (equivalent to $\text{OD}_{600} = 0.002$). For determination of bacterial growth, plants were infected with *Psm* ES4326 and *Psm* ES4326 carrying *avrRpt2* at a dose of 10^3 CFU/cm² leaf area (equivalent to $\text{OD}_{600} = 0.0002$). Leaves were excised and bacterial growth was assayed as described (Glazebrook et al. 1996). Data are reported as means and standard deviations of the log (CFU/cm²) of at least six replicates. For determination of bacterial growth during SAR, four to five half-leaves of each plant were infected with *Pst* DC3000 carrying *avrRpt2* at a dose of 10^4 CFU/cm² leaf area (equivalent to $\text{OD}_{600} = 0.002$). Four days after infection, bacterial growth was measured in the systemic leaves by infecting two leaves of each plant with *Psm* ES4326, as described above. For treatment with SA, plants were sprayed with 5 mM sodium salicylate with 0.02% Silwet L-77 to reduce surface tension. Control plants were sprayed with water containing 0.02% Silwet L-77. Rose bengal (20 mM) and MeJA (45 μM in 0.1% [vol/vol] ethanol) were applied as 5- μl droplets on leaves (five drops per leaf) (Penninckx et al. 1996).

RNA blot analysis.

Tissue samples consisting of five to six leaves were collected and frozen in liquid nitrogen and stored at -80°C . Total

RNA was extracted and 5 µg of RNA per sample was separated on formaldehyde-agarose gels (Reuber and Ausubel 1996). After the samples were blotted onto Hybond-N⁺ membrane (Amersham, Arlington Heights, IL), hybridizations were performed with various digoxigenin-labeled probes described below, followed by washes in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and chemiluminescent detection with CSPD or CDP star according to instructions provided by the supplier (Boehringer Mannheim, Indianapolis, IN). For hybridization with more than one probe, blots were stripped and re-probed. Single-stranded DNA probes for *PR-1*, *PR-5* (Uknes et al. 1992), and *BGL-2* (Dong et al. 1991) were prepared as described (Zhou et al. 1998). The single-stranded *PDF1.2* probe was made with SP6 primer (5'-CTATAGTGTACCTAAAT-3') and *EcoRI*-linearized plasmid pZL1 (Life Technologies, Rockville, MD) containing the expressed sequence tag (EST) with GenBank accession number T04323 (obtained from the Arabidopsis Biological Resource Center). For *ASA1* (Niyogi and Fink 1992) and 18S rRNA, single-stranded probes were made as described (Zhou et al. 1998). The 18S rRNA probe was used to assess equal loading of RNA samples.

For quantitation of hybridization signals, the alkaline phosphatase substrate Attophos (Amersham, Piscataway, NJ) was applied to the blots according to the instructions of the supplier. The fluorescent product was quantitated with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Determination of endogenous levels of SA.

Mature leaves of 7-week-old wild-type and *pad4* plants were infected with *Psm* ES4326 at a dose of 10⁴ CFU/cm² (equivalent to OD₆₀₀ = 0.002) or mock-infected with 10 mM MgSO₄. At intervals after infection, leaves were collected (1 g of tissue per sample, approximately seven plants) and frozen in liquid nitrogen. Free SA and total SA were determined as described previously (Bowling et al. 1994). SAG was determined by subtracting the free SA value from the total SA value.

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