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PAD4 Functions Upstream from Salicylic Acid to Control Defense Responses in Arabidopsis

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The Arabidopsis *PAD4* gene was previously shown to be required for synthesis of camalexin in response to infection by the virulent bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326 but not in response to challenge by the non-host fungal pathogen *Cochliobolus carbonum*. In this study, we show that *pad4* mutants exhibit defects in defense responses, including camalexin synthesis and pathogenesis-related *PR-1* gene expression, when infected by *P. s. maculicola* ES4326. No such defects were observed in response to infection by an isogenic avirulent strain carrying the avirulence gene *avrRpt2*. In *P. s. maculicola* ES4326–infected *pad4* plants, synthesis of salicylic acid (SA) was found to be reduced and delayed when compared with SA synthesis in wild-type plants. Moreover, treatment of *pad4* plants with SA partially reversed the camalexin deficiency and *PR-1* gene expression phenotypes of *P. s. maculicola* ES4326–infected *pad4* plants. These findings support the hypothesis that PAD4 acts upstream from SA accumulation in regulating defense response expression in plants infected with *P. s. maculicola* ES4326. A working model of the role of PAD4 in governing expression of defense responses is presented.

INTRODUCTION

Plants respond to pathogen attack by activating expression of a battery of defense responses. In some plant–pathogen interactions, host defense responses are triggered by specific recognition of the products of particular pathogen genes (called avirulence [*avr*] genes) by plant hosts carrying a corresponding resistance gene. Such gene-for-gene responses generally occur within 24 hr after infection and result in inhibition of pathogen growth and failure of the pathogen to cause disease. If the pathogen does not carry an *avr* gene recognized by the plant, defense responses are activated more slowly, the pathogen multiplies, and disease ensues. In this case, the pathogen is said to be virulent. Plants can acquire resistance to normally virulent pathogens through a phenomenon called systemic acquired resistance (SAR). Infection of part of a plant can result in expression of some defense-related genes in uninfected parts of the plant, with concomitant resistance to subsequent attack by virulent pathogens. Genetic analyses of plant responses to pathogen attack are being used to reveal the components of the signal transduction pathways controlling defense gene expression in response to avirulent and virulent pathogens as well as during SAR.

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Two virulent *Pseudomonas syringae* pathogens, *P. syringae* pv *maculicola* ES4326 and *P. syringae* pv *tomato* DC3000, have been found to infect Arabidopsis (Crute et al., 1994). Introduction of plasmids carrying any one of several *avr* genes into either of these strains yields isogenic avirulent strains that trigger gene-for-gene defense responses dependent on the presence of corresponding resistance genes in the host (Crute et al., 1994). Defense responses activated by infection with any of these strains include synthesis of the antimicrobial compound camalexin and expression of defense-related genes, including *PR-1*, *PR-5*, *BGL2* (*PR-2*; β-glucanase), and *ASA1* (anthranilate synthase) (Crute et al., 1994). For many of these responses, including camalexin accumulation, *PR-1* gene expression, and *ASA1* gene expression, activation occurs more rapidly in response to the avirulent strains than it does in response to the virulent ones (Niyogi and Fink, 1992; Glazebrook and Ausubel, 1994; Greenberg et al., 1994).

Expression of *PR-1*, *PR-5*, and *BGL2* is also associated with SAR. Plants that demonstrate SAR exhibit systemic expression of these genes and elevated levels of salicylic acid (SA) (Ryals et al., 1996). Treatment of plants with exogenous SA is sufficient to induce expression of *PR-1*, *PR-5*, and *BGL2* and to cause SAR, suggesting that SA is required for signaling during SAR (Ryals et al., 1996). This idea 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-1*, *PR-5*, or *BGL2* (Gaffney et al., 1993; Delaney et al., 1994). SA is required in the systemic tissue for expression of defense genes and SAR (Vernooij et al., 1994), but there is some debate as to whether it is also the signal molecule that is translocated from the infected leaves to the rest of the plant (Vernooij et al., 1994; Beffa et al., 1995; Shulaev et al., 1995; Mölders et al., 1996).

An Arabidopsis gene that has been variously named *NPR1* (for nonexpresser of *PR* genes; Cao et al., 1994), *NIM1* (for noninducible immunity; Delaney et al., 1995), and *SAI1* (for salicylic acid–insensitive; Shah et al., 1997) is required for SA-mediated disease resistance. When infected with a pathogen that induces SAR in wild-type plants, *npr1*/*nim1*/*sai1* mutants accumulate high levels of SA but do not activate expression of *PR-1*, *PR-5*, or *BGL2* and do not develop SAR (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). *NPR1*/*NIM1*/*SAI1* was recently cloned and shown to encode a protein containing ankyrin repeats (Cao et al., 1997; Ryals et al., 1997). The precise role of this protein in mediating responses to SA is not known, but it was recently demonstrated that it functions in the nucleus in response to SA treatment (M. Kinkema and X. Dong, personal communication).

The phenotypes of *nahG* and *npr1*/*nim1*/*sai1* plants indicate that SA-dependent responses are required for local defense against virulent and avirulent pathogens as well as for SAR. In response to local infections, *nahG* plants display reduced *PR-1* expression and enhanced growth of virulent bacterial pathogens, avirulent bacterial pathogens, and avirulent *Peronospora parasitica* isolates (Delaney et al., 1994; Lawton et al., 1995; Glazebrook et al., 1996). Although camalexin synthesis is not inducible by SA, *nahG* plants synthesize greatly reduced amounts of camalexin in response to *P. s. maculicola* ES4326 infection, suggesting that SA is necessary, but not sufficient, for full activation of camalexin synthesis (Zhao and Last, 1996). Mutations in *npr1*/*nim1*/*sai1* cause enhanced susceptibility to avirulent and virulent *P. syringae* strains and avirulent Peronospora isolates (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). The expression of *PR-1* in *npr1*/*nim1*/*sai1* plants in response to local infections or SA treatment is reduced relative to that in wild-type plants (Glazebrook et al., 1996; Shah et al., 1997). However, *npr1*/*nim1*/*sai1* mutations have no effect on camalexin synthesis, suggesting that although camalexin synthesis requires SA, this requirement is mediated by an NPR1/NIM1/SAI1–independent pathway (Glazebrook et al., 1996; Zhao and Last, 1996).

In previous work, we isolated an Arabidopsis mutant, *pad4*, displaying reduced camalexin synthesis in response to *P. s. maculicola* ES4326 infection, enhanced susceptibility to *P. s. maculicola* ES4326, and susceptibility to Peronospora isolates that are avirulent on wild-type plants (Glazebrook et al., 1996, 1997b). Significantly, *pad4* was unaffected in camalexin synthesis in response to challenge with the non-host fungal pathogen *Cochliobolus carbonum*, strongly suggesting that *pad4* is a mutation affecting a regulatory factor rather than a camalexin biosynthetic enzyme (Glazebrook et al., 1997b).

In this report, we further characterize *pad4* plants. We show that *pad4* affects expression of some defense-related genes, including *PR-1*, in response to infection by *P. s. maculicola* ES4326, but does not affect responses to *P. s. maculicola* ES4326 carrying *avrRpt2.* Responses to SA treatment were unaffected by *pad4. P. s. maculicola* ES4326–infected *pad4* plants displayed reduced synthesis and accumulation of SA and its glucoside (SAG), and the *PR-1* expression and camalexin synthesis defects of *pad4* plants were reversible by SA treatment. Taken together, the phenotypes of *pad4* plants argue that PAD4 is required upstream from SA in the signal transduction pathway leading from *P. s. maculicola* ES4326 infection to activation of defense responses.

RESULTS

Camalexin Induction in Response to Some Elicitors Is Unaffected by *pad4*

Our previous demonstration that *pad4* plants show a defect in camalexin synthesis in response to infection by the virulent bacterial pathogen *P. s. maculicola* ES4326, but not in response to challenge by the non-host fungal pathogen *C. carbonum* (Glazebrook et al., 1997b), led us to examine the effect of *pad4* on camalexin synthesis in response to various elicitors. Wild-type and *pad4* plants were infected with *P. s. maculicola* ES4326 or an isogenic strain carrying the avirulence gene *avrRpt2.* Camalexin levels in the infected leaves were determined at various intervals after infection. Figure 1A shows that when plants were infected with *P. s. maculicola* ES4326, camalexin levels in wild-type plants were much higher than those in *pad4* plants. In contrast, when plants were infected with *P. s. maculicola* ES4326 carrying *avrRpt2*, camalexin levels in wild-type and *pad4* plants were not significantly different. Similar results were obtained using a different virulent *P. syringae* strain, *P. s. tomato* DC3000, and *P. s. tomato* DC3000 carrying *avrRpt2* (data not shown). This suggests that the signal transduction pathway leading to camalexin synthesis in response to *P. s. maculicola* ES4326 or *P. s. tomato* DC3000 infection requires *PAD4*, whereas camalexin synthesis in response to strains carrying *avrRpt2* is mediated by a *PAD4*-independent pathway. Two other treatments known to trigger camalexin synthesis are infection with *Xanthomonas campestris* pv *campestris* BP109 and spraying with silver nitrate. Figures 1B and 1C show that *pad4* did not affect camalexin synthesis in response to either of these treatments, suggesting that responses to these stimuli are also independent of *PAD4.*

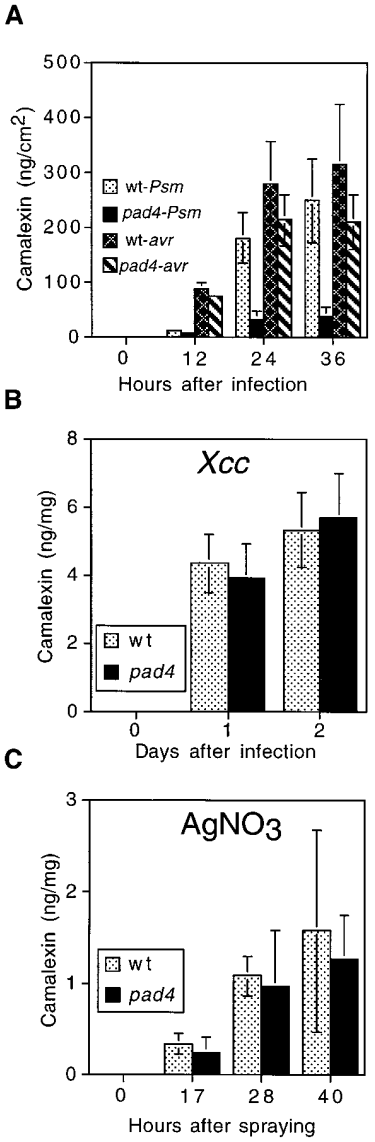

Figure 1. Camalexin Levels in Wild-Type and *pad4* Plants after Various Treatments.

Each data point represents the mean and standard deviation of six replicate samples.
(A) Infection with *P. s. maculicola* ES4326 or *P. s. maculicola* ES4326 carrying *avrRpt2.* wt-*Psm*, wild-type plants infected with *P. s. maculicola* ES4326; *pad4-Psm*, *pad4* plants infected with *P. s. maculicola* ES4326; wt-*avr*, wild-type plants infected with *P. s. maculicola* ES4326 carrying *avrRpt2*; *pad4-avr*, *pad4* plants infected with *P. s. maculicola* ES4326 carrying *avrRpt2.*
(B) Infection with *X. c. campestris* (*Xcc*) BP109.
(C) Spraying with 5 mM silver nitrate.

Expression of the Defense Gene *PR-1* Is Impaired in *pad4* Plants

The finding that *PAD4* is required for activation of camalexin synthesis in response to infection by *P. s. maculicola* ES4326 raised the possibility that *PAD4* might encode a regulator of defense responses. To test whether *pad4* affects activation of other defense responses in addition to camalexin synthesis, mRNA levels of several defense-related genes were examined by RNA gel blot hybridization. Figure 2A shows that the mRNA levels of *PR-1* were greatly reduced in infected *pad4* leaves relative to the levels in wild-type plants. Expression of *PR-5*, *BGL2*, and *ASA1* were affected less strongly, if at all, by the *pad4* mutation. *ASA1* encodes anthranilate synthase (Niyogi and Fink, 1992), which is required for camalexin synthesis because anthranilate is a precursor to camalexin (Tsuji et al., 1993). Zhao and Last (1996) have observed a tight correlation between camalexin levels and *ASA1* expression; however, this correlation is not maintained in *pad4* plants. Clearly, *pad4* has effects on defense gene expression, but these effects are not consistent among all genes induced by *P. s. maculicola* ES4326 infection.

Infection with *P. s. maculicola* ES4326 carrying *avrRpt2* also leads to increases in expression of defense genes, including *PR-1* (Greenberg et al., 1994). Figure 2B shows that *PR-1* mRNA levels in plants infected with *P. s. maculicola*

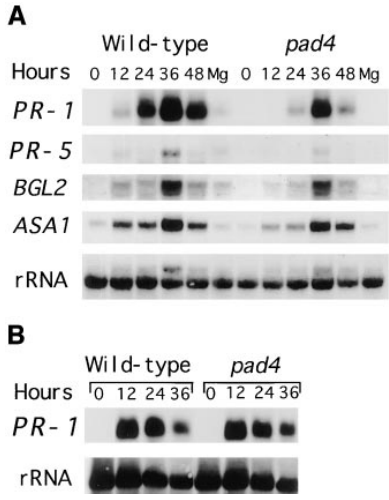

Figure 2. Defense Gene Expression in Leaves Infected with *P. s. maculicola* ES4326 or *P. s. maculicola* ES4326 Carrying *avrRpt2.*

Leaves were excised at 0, 12, 24, 36, or 48 hr after infection. Mg indicates leaves mock inoculated with 10 mM $MgSO_4$ sampled at 36 hr. rRNA indicates hybridization with an 18S rRNA probe used to evaluate equal loading.
(A) Infection with *P. s. maculicola* ES4326.
(B) Infection with *P. s. maculicola* ES4326 carrying *avrRpt2.*

ES4326 carrying *avrRpt2* were unaffected by *pad4.* Taken together with the results in Figure 1A, this suggests that host defense responses to *P. s. maculicola* ES4326 carrying *avrRpt2* are largely *PAD4* independent.

Responses to SA Are Unaffected in *pad4* Plants

SA is required for induction of *PR-1* gene expression in infected leaves (Gaffney et al., 1993). SA treatment is also sufficient for *PR-1* gene expression and SAR (White, 1979; Malamy et al., 1990). To determine whether *pad4* affects responses to SA, *pad4* plants were treated with SA and tested for *PR-1* gene expression and pathogen resistance. Figure 3A shows that SA treatment induced similar levels of *PR-1* expression in wild-type and *pad4* plants, with *PR-1* mRNA present at high levels 1 day after treatment and declining thereafter. Similarly, Figure 3B shows that SA treatment induced resistance to *P. s. maculicola* ES4326 in wild-type and *pad4* plants. Based on these experiments, it appears that the signal transduction pathway between SA and *PR-1* expression is intact in *pad4* plants.

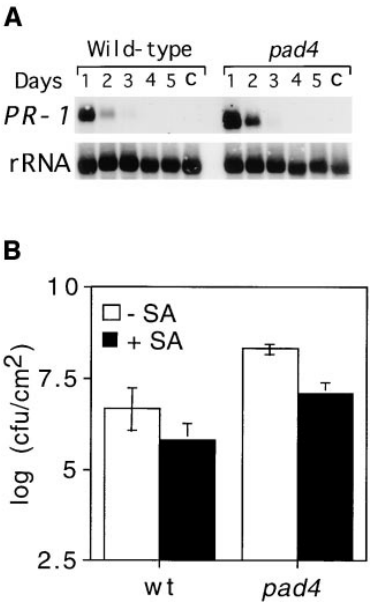

Figure 3. Exogenous SA Induces *PR-1* Expression and Enhanced Resistance to *P. s. maculicola* ES4326 in Wild-Type and *pad4* Plants.

Plants were sprayed with 5 mM SA in 0.02% [v/v] Silwet L-77 or with 0.02% Silwet alone until uniformly wet.

(A) *PR-1* expression in response to SA. Samples were taken daily after spraying. The c indicates control; plants were sprayed with 0.02% Silwet alone and sampled at 3 days after spraying.

(B) Effect of spraying plants with SA on *P. s. maculicola* ES4326 growth. One day after treatment, plants were infected with *P. s. maculicola* ES4326 at a dose of 10^3 cfu/cm^2. Bacterial titer was determined 3 days after infection. Each bar represents the mean and standard deviation of eight replicates. Similar results were obtained in three other independent experiments. −SA, plants not treated with SA before infection; +SA, plants treated with SA before infection; wt, wild type.

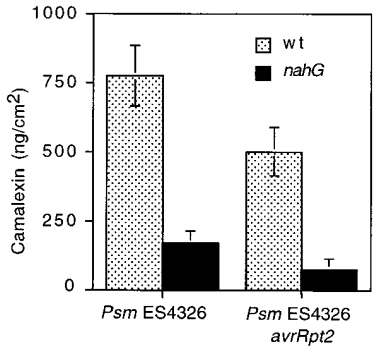

Figure 4. Camalexin Accumulation in Wild-Type and *nahG* Plants Infected with Pathogens.

Wild-type (wt) L*er* and L*er nahG* transgenic plants were infected with either *P. s. maculicola* (*Psm*) ES4326 or *P. s. maculicola* ES4326 carrying *avrRpt2* at a dose of 10^5 cfu/cm^2. Camalexin in the infected leaves was determined at the times that camalexin levels are high in wild-type plants: 24 hr after infection for *P. s. maculicola* ES4326 carrying *avrRpt2* and 32 hr after infection for *P. s. maculicola* ES4326. Each bar represents the mean and standard deviation of eight replicate samples.

SA Accumulation in Response to *P. s. maculicola* ES4326 Infection Is Compromised in *pad4* Plants

SA is required both for *PR-1* gene expression and for camalexin synthesis. In the case of *PR-1*, SA is both necessary and sufficient for increased *PR-1* expression in response to pathogen attack (Ryals et al., 1996). In the case of camalexin accumulation, SA is not sufficient, because SA treatment does not lead to significant camalexin accumulation. Nevertheless, SA is required, because *nahG*-transformed plants fail to accumulate camalexin in response to *P. s. maculicola* ES4326 infection (Zhao and Last, 1996). We tested the accumulation of camalexin in wild-type and *nahG* plants infected with *P. s. maculicola* ES4326 or *P. s. maculicola* ES4326 carrying *avrRpt2.* As shown in Figure 4, camalexin levels in *nahG* plants infected with either pathogen were much lower than the levels in wild-type plants. Thus, SA is required for full induction of camalexin synthesis in response to either *P. s. maculicola* ES4326 or *P. s. maculicola* ES4326 carrying *avrRpt2.*

One possible explanation for the phenotypes of *pad4* plants is that they accumulate reduced amounts of SA in re-

sponse to *P. s. maculicola* ES4326 infection, resulting in reduced camalexin synthesis and reduced *PR-1* expression. To test this idea, wild-type and *pad4* plants were infected with *P. s. maculicola* ES4326 or *P. s. maculicola* ES4326 carrying *avrRpt2*, and both SA and its glucoside (SAG) were monitored over the course of the infection. Figure 5A shows that the SA levels in *pad4* plants infected with *P. s. maculicola* ES4326 were lower than the levels in infected wild-type plants at all time points tested. In wild-type plants, SA levels were highest at 12 hr after infection, whereas in *pad4* plants, SA levels were highest at 24 hr after infection. Figure 5C shows that after infection with *P. s. maculicola* ES4326, SAG levels were greatly reduced in *pad4* plants. In contrast, when plants were infected with *P. s. maculicola* ES4326 carrying *avrRpt2*, there was very little difference between SA levels in *pad4* and wild-type plants, as shown in Figure 5B. Figure 5D shows that after infection with *P. s. maculicola* ES4326 carrying *avrRpt2*, the SAG levels in *pad4* plants were significantly lower than those in wild-type plants, but these differences were not as large as those observed in *P. s. maculicola* ES4326–infected plants (Figure 5C). Based on these results, it seems likely that the camalexin and *PR-1* defects of *pad4* mutants result from reduced SA accumulation in response to *P. s. maculicola* ES4326 infection.

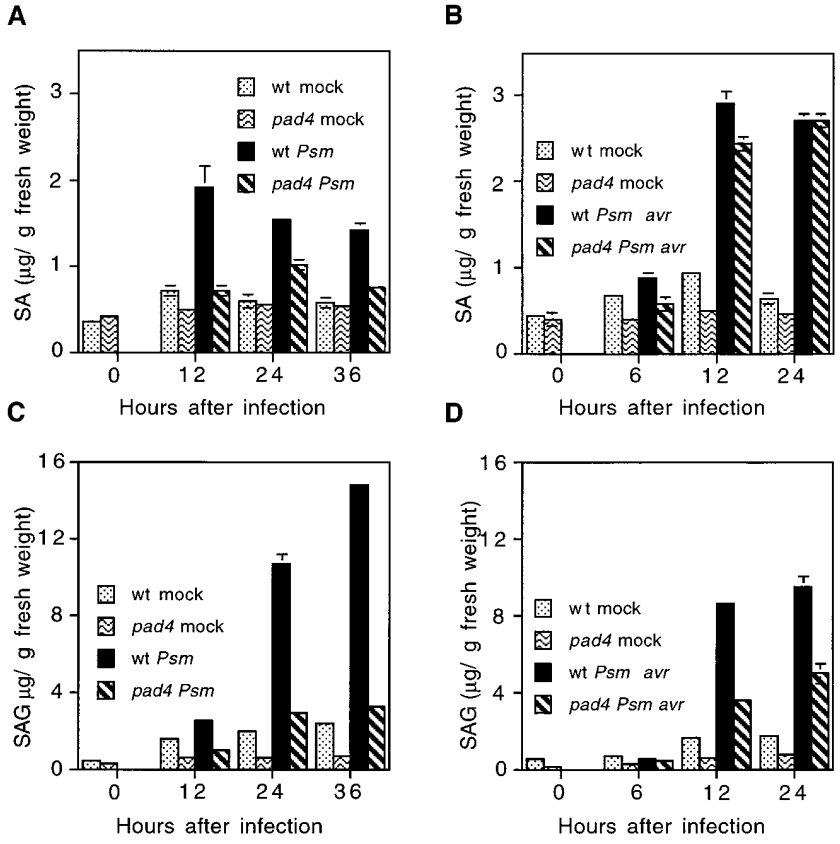

Figure 5. SA and SAG Levels in Infected Wild-Type and *pad4* Plants.

Wild-type (wt) and *pad4* plants were infected with *P. s. maculicola* ES4326 (*Psm*), *P. s. maculicola* ES4326 carrying *avrRpt2* (*Psm avr*), or mock infected with 10 mM $MgSO_4$ (mock). Each column represents the mean of three replicate samples. Error bars representing the standard deviation are shown where they are large enough to be visible. SA and SAG were assayed on the same samples. The experiments with *P. s. maculicola* ES4326 and with *P. s. maculicola* ES4326 carrying *avrRpt2* were conducted at different times; therefore, results should not be compared directly.

(A) SA levels in plants infected with *P. s. maculicola* ES4326.

(B) SA levels in plants infected with *P. s. maculicola* ES4326 carrying *avrRpt2.*

(C) SAG levels in plants infected with *P. s. maculicola* ES4326.

(D) SAG levels in plants infected with *P. s. maculicola* ES4326 carrying *avrRpt2.*

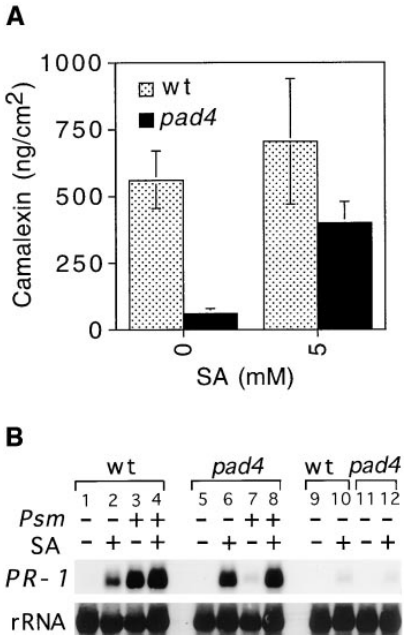

Figure 6. SA Application before *P. s. maculicola* ES4326 Infection Restores Camalexin Accumulation and *PR-1* Expression in *pad4* Plants.

Wild-type (wt) and *pad4* plants were treated either with 5 mM SA in 0.02% [v/v] Silwet L-77 or with 0.02% Silwet alone 1 day before infection with *P. s. maculicola* ES4326.

(A) Effect of exogenous SA on camalexin levels in *P. s. maculicola* ES4326–infected plants. Infected leaves were sampled 2 days after infection. Each data point represents the mean and standard deviation of six replicates.

(B) Effect of exogenous SA on *PR-1* expression in *P. s. maculicola* ES4326–infected plants. Leaves infected with *P. s. maculicola* ES4326 were sampled from mock-treated wild-type and *pad4* plants (lanes 3 and 7) and SA-treated plants (lanes 4 and 8) 2 days after infection. For comparison, mock-treated plants were sampled 1 and 2 days after treatment (lanes 1 and 9, respectively, for the wild type; lanes 5 and 11, respectively, for *pad4*), as were SA-treated plants (lanes 2 and 10, respectively, for the wild type; lanes 6 and 12, respectively, for *pad4*). Similar results were obtained in another independent experiment. (+) and (−) in the row labeled SA indicate the presence or absence of SA treatment, repectively. (+) and (−) in the row labeled *Psm* indicate the presence or absence of *P. s. maculicola* ES4326 infection, respectively. Because of the strong *PR-1* signal in leaves infected with *P. s. maculicola* ES4326, the exposure for this blot was shorter than was the exposure for the blot shown in Figure 3A; consequently, the expression of *PR-1* 48 hr after SA treatment appears lower than it does in Figure 3A.

Exogenous SA Restores Camalexin Accumulation and *PR-1* Expression in *P. s. maculicola* ES4326–Infected *pad4* Plants

If the reduction in SA accumulation in response to *P. s. maculicola* ES4326 infection observed in *pad4* plants is the cause of the camalexin accumulation and *PR-1* gene expression defects, then it should be possible to restore camalexin synthesis and *PR-1* gene expression to *P. s. maculicola* ES4326–infected *pad4* plants by supplying them with SA. Figure 6A shows that *pad4* plants treated with SA before infection displayed much higher camalexin levels than did *pad4* plants that were not pretreated with SA. In the experiment shown in Figure 6A, SA treatment of wild-type plants had little effect on camalexin levels. In some experiments, we observed significant reductions in camalexin levels in wild-type plants treated with SA, whereas SA always caused a large increase in camalexin levels in *pad4* plants.

The possibility that SA treatment might also rescue the *PR-1* expression defect of *pad4* plants was tested. Wild-type and *pad4* plants were sprayed with either 5 mM SA or water. After 1 day, plants were infected with *P. s. maculicola* ES4326, and *PR-1* mRNA levels were assessed 2 days after infection. Figure 6B shows that 1 day after SA treatment, *PR-1* mRNA levels were elevated in both wild-type and *pad4* plants (lanes 2 and 6) and declined to barely detectable levels by day 2 (lanes 10 and 12). In plants that were treated with SA before infection, the *PR-1* mRNA level in *pad4* plants was comparable to that in wild-type plants (Figure 6B, lanes 4 and 8). The *PR-1* expression in SA-treated, *P. s. maculicola* ES4326–infected *pad4* plants cannot be solely due to the SA treatment, because the *PR-1* mRNA level in *pad4* plants treated with SA only had declined by 2 days after treatment. Rather, the SA treatment must have restored the ability of the *pad4* plants to respond to *P. s. maculicola* ES4326, demonstrating that the *PR-1* gene expression defect caused by *pad4* is also reversed by SA treatment. The observations that SA treatment can reverse the camalexin accumulation and *PR-1* gene expression defects of *P. s. maculicola* ES4326–infected *pad4* plants provide strong support for the idea that reduced SA accumulation in *pad4* plants is the cause of the camalexin and *PR-1* defects.

Genetic Map Position of *PAD4*

The *pad4* mutant (ecotype Columbia [Col] background) was crossed with an ecotype Landsberg *erecta* (L*er*) plant to map the *pad4* mutation in the F_2 progeny. However, segregation of the camalexin-deficient phenotype of *pad4* in the F_2 progeny was found to be 1 Pad^-:9 Pad^+ (21 Pad^- plants and 190 Pad^+ plants), rather than the 1:3 ratio observed in backcrosses to Col (Glazebrook et al., 1996). Apparently, there is a gene in L*er* that masks the Pad^- phenotype of *pad4* homozygotes. Consequently, the L*er* mapping strategy was abandoned. The *pad4* mutant was then crossed with wild-type ecotype Keswick (Ksk). The camalexin-deficient phenotype segregated 1 Pad^-:3 Pad^+ in this cross; therefore, these plants were used for cleaved amplified polymorphic sequence (CAPS) mapping (Konieczny and Ausubel, 1993). *PAD4* was found to lie on chromosome 3, between the markers *GL1* and *BGL2.* (Of 64 chromosomes tested,

seven were recombinant between *PAD4* and *BGL2*, yielding a recombination frequency of 11%. Fifteen were recombinant between *GL1* and *PAD4*, yielding a recombination frequency of 23%.)

DISCUSSION

We have previously described five complementation groups of *pad* mutants, defined as mutants displaying defects in camalexin synthesis in response to *P. s. maculicola* ES4326 infection (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997b). Mutations in *PAD1*, *PAD2*, or *PAD4* cause enhanced susceptibility to *P. s. maculicola* ES4326, whereas mutations in *PAD3* or *PAD5* have no effect on *P. s. maculicola* ES4326 growth (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997b). One model (the regulatory model) explaining this result postulates that camalexin does not play a crucial role in limiting *P. s. maculicola* ES4326 growth but that *PAD1*, *PAD2*, and *PAD4* are pleiotropic defense response regulators affecting expression of other defense responses, in addition to camalexin accumulation, that are important for limiting *P. s. maculicola* ES4326 growth (Glazebrook and Ausubel, 1994). An alternative model (the biochemical model) postulates that camalexin does play a crucial role in limiting *P. s. maculicola* ES4326 growth but that the *pad3* and *pad5* mutations block the camalexin biosynthetic pathway at a point such that antimicrobial camalexin precursors accumulate, compensating for the loss of camalexin itself (Glazebrook and Ausubel, 1994).

The results reported in this study demonstrating that *PAD4* has pleiotropic effects on resistance responses are most consistent with the regulatory model for explaining the phenotypes of *pad* mutants. Mutations that affect regulation of defense responses are useful in dissecting the signal transduction pathways controlling expression of defense responses. The studies reported here strongly suggest that the *pad4* mutation affects SA accumulation, thereby causing pleiotropic effects on expression of defense responses.

Figure 7 shows our working model of the role of *PAD4* in defense response signaling. When plants are infected with *P. s. maculicola* ES4326, *PAD4* is needed for SA concentrations to reach the level required for camalexin synthesis and expression of *PR-1.* In contrast, when plants are infected with *P. s. maculicola* ES4326 carrying *avrRpt2*, SA accumulates in a *PAD4*-independent manner. The accumulation of SA causes *PR-1* expression in an *NPR1*/*NIM1*/*SAI1*–dependent manner. SA accumulation is necessary, but not sufficient, to activate camalexin synthesis. *NPR1*/*NIM1*/*SAI1* is not required for camalexin synthesis. If the *pad4* allele used in this work does not cause complete loss of function, then a single-pathway model could explain our results. In such a model, responses to *P. s. maculicola* ES4326 carrying *avrRpt2* might be triggered by a strong signal that requires PAD4 function for its transmission but is adequately transmitted by a partially functional PAD4. In response to *P. s. maculicola* ES4326, this signal might be much weaker and inadequately transmitted by a partially functional PAD4.

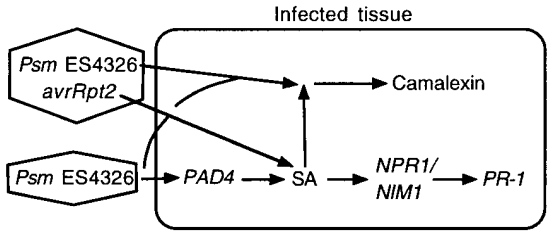

Figure 7. Model for the Role of *PAD4* in Signal Transduction.

When plants are infected with *P. s. maculicola* (*Psm*) ES4326, PAD4 function is needed for SA accumulation and activation of defense responses that require SA, such as camalexin synthesis and *PR-1* expression. When plants are infected with *P. s. maculicola* ES4326 carrying *avrRpt2*, SA accumulation occurs by a PAD4-independent mechanism.

In leaves infected with *P. s. maculicola* ES4326 carrying *avrRpt2*, *PAD4* does not play a major role in activation of defense responses. However, it is likely that *PAD4* is required for activation of responses during some other gene-for-gene resistance responses. In previous work, we showed that *pad4* mutants fail to demonstrate resistance to several different avirulent isolates of the oomycete pathogen Peronospora. Although wild-type plants challenged with these parasites allowed little or no sporulation, *pad4* plants allowed profuse sporulation (Glazebrook et al., 1997b).

Mutations in *EDS1* (for enhanced disease susceptibility) also cause increased susceptibility to virulent pathogens (*P. s. tomato* DC3000 and compatible Peronospora isolate Emwa1) and to some but not all avirulent pathogens (many avirulent Peronospora isolates but not *P. s. tomato* DC3000 carrying *avrB*) (Parker et al., 1996). Recent results suggest that *EDS1* is required for gene-for-gene resistance responses mediated by the TIR-NBS-LRR (for Toll/Interleukin-1/resistance–nucleotide binding site–leucine rich repeat) class of resistance genes (J. Parker, personal communication). The spectrum of pathogens affected by the *pad4* and *eds1* mutations differs in that the *eds1-1* mutant is susceptible to the non-host Peronospora isolate P-006, whereas *pad4* is not (Parker et al., 1996; Glazebrook et al., 1997a). Comparison of the Arabidopsis–pathogen interactions affected by *pad4* and *eds1* is complicated by the fact that *pad4* is in the Col background, whereas the *eds1-1* allele is in the Wassilewskija (Ws) background, and there are many differences in genes affecting disease resistance between the two ecotypes. In the future, analysis of *pad4* and *eds1* alleles that recently were isolated in the L*er* background will allow comparison of *pad4* and *eds1* phenotypes in the absence of potentially interfering differences between ecotypes, thereby helping to resolve the issue of whether EDS1 and

PAD4 act in the same or different pathways (J. Parker, personal communication).

The phenotypes of *pad4* and *eds1* mutants support the idea that different gene-for-gene resistance responses lead to resistance via different signaling pathways. Presumably, these pathways converge at some point, but the observation that gene expression patterns in response to bacteria carrying the avirulence gene *avrRpt2* are quite different from the patterns in response to bacteria carrying the avirulence gene *avrRpm1* suggests that the differences between pathways may be extensive (Reuber and Ausubel, 1996; Ritter and Dangl, 1996). Furthermore, both *pad4* and *eds1* mutants affect growth of compatible pathogens in addition to growth of certain incompatible pathogens, suggesting that elements of the signal transduction pathway(s) activated by recognition of certain avirulent pathogens are also involved in signal transduction in response to infection by certain virulent pathogens.

The genes *PR-1*, *PR-5*, and *BGL2* are coordinately induced in response to treatment with SA. When plants were infected with *P. s. maculicola* ES4326, the phenotypes of *pad4* plants revealed differential regulation of defense responses. Synthesis of camalexin and expression of *PR-1* were reduced in *pad4* plants. Expression of *PR-5*, *BGL2*, and *ASA1* were less strongly affected. The *eds5* mutation also causes reduced *PR-1* expression in response to *P. s. maculicola* ES4326 infection but has no effect on the expression of *PR-5* and *BGL2* or on camalexin synthesis (Rogers and Ausubel, 1997). The *npr1*/*nim1*/*sai1* mutations cause similar effects in that mRNA levels of *PR-1*, but not those of *PR-5* or *BGL2*, are reduced in response to pathogen infection. This does not seem to be due to "leakiness" of the alleles, because expression of *PR-1*, *PR-5*, and *BGL2* in response to SA treatment was completely abolished in the *npr1*/*nim1*/*sai1* mutants (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997).

These results are difficult to reconcile with the idea that expression of *PR-1*, *PR-5*, and *BGL2* in infected leaves is due solely to coordinate regulation through the SA pathway. One possible explanation is that an SA-independent pathway acts in conjunction with the SA-dependent pathway to control expression of *PR-1*, *PR-5*, and *BGL2*, and the relative contribution of each pathway varies among the different genes (this potential pathway is not shown in Figure 7). An alternative explanation is that expression of each gene could be variably affected by different mutations because of quantitative differences in their induction. Such differences could result partly from the fact that in infected leaves, both the fold induction and the absolute expression level of *PR-1* are much higher than those of *PR-5* or *BGL2.*

Camalexin synthesis in response to *X. c. campestris* BP109 infection or silver nitrate treatment was not affected by *pad4.* These stimuli induce much less camalexin synthesis than does infection by *P. s. maculicola* ES4326. Possibly, such low levels of camalexin synthesis do not require *PAD4* function. The quantities of camalexin synthesized by *pad4* plants in response to *P. s. maculicola* ES4326 infection are greater than are the amounts in wild-type plants infected with *X. c. campestris* BP109 or treated with silver nitrate.

The observation that SA treatment can reverse the defense response expression defects of *pad4* plants raises interesting questions about the relationship between SA levels and *PR-1* gene expression. Treatment of *pad4* plants with SA 1 day before *P. s. maculicola* ES4326 infection restored *PR-1* gene expression 2 days after infection (3 days after SA treatment). In contrast, by 2 days after SA treatment, *PR-1* expression in uninfected leaves was very low. Does this imply that SA levels at this time are also very low? If so, how does SA treatment restore *PR-1* expression in *pad4* plants? If SA levels are still high 3 days after treatment, then why are *PR-1* mRNA levels low? Low levels of SA potentiate responses to pathogen attack (Shirasu et al., 1997), and evidence supports the idea that there is a feedback loop in the SAR signal transduction pathway (Ryals et al., 1996), further complicating analysis of the role of SA in signal transduction.

In conclusion, we have shown that *PAD4* is a regulator of defense responses and acts upstream from SA to affect expression of *PR-1* and camalexin synthesis. *PAD4* function does not seem to be required for these defense responses when plants are infected with *P. s. maculicola* ES4326 carrying *avrRpt2*, suggesting that there is a *PAD4*-independent mechanism that supplies SA in response to this avirulent strain. Further analysis of the responses of *pad4* plants to pathogen attack is likely to reveal more interesting features of the regulatory mechanisms controlling expression of defense responses in plants.

METHODS

Plants and Growth Conditions

Wild-type plants (*Arabidopsis thaliana* ecotype Columbia [Col]) and *pad4* plants from a line that had been backcrossed four times to the wild-type parent (Col) (Glazebrook et al., 1996) were used in this study. Arabidopsis ecotype Keswick (Ksk) was a gift from E. Holub (Horticulture Research International, Warwick, UK). Landsberg *erecta* (*Ler*) plants carrying the *nahG* transgene were a gift from X. Dong (Duke University, Durham, NC) (Bowling et al., 1994). Plants were grown in pots in Metro-Mix 200 soil (Scotts-Sierra Horticultural Products Company, Marysville, OH) in a growth chamber (22 ± 2°C, 85% relative humidity, 100 μE m^{-2} sec^{-1} fluorescent illumination) on a 12-hr-light and 12-hr-dark cycle. Fully expanded leaves of 4-week-old plants were used for all experiments.

Inoculations with Bacteria and Treatment with Chemicals

Pseudomonas syringae pv *maculicola* ES4326 (Dong et al., 1991), *P. s.* pv *tomato* DC3000 (Cuppels, 1986), and *Xanthomonas campestris* pv *campestris* BP109 (Weiss et al., 1994) have been described previously. The avirulence gene *avrRpt2* was carried on plasmid pLH12, as described previously (Dong et al., 1991; Whalen et al., 1991). *P.*

syringae strains were grown in King's B medium supplemented with appropriate antibiotics (Glazebrook and Ausubel, 1994), and *X. c. campestris* BP109 was grown in Luria-Bertani medium supplemented with 50 μg/mL rifampicin. Bacteria were infiltrated into Arabidopsis plants, as described by Glazebrook and Ausubel (1994). Unless stated otherwise, for camalexin assays, the bacterial dose for *P. syringae* strains was 3×10^4 colony-forming units (cfu)/cm^2 leaf area (equivalent to $OD_{600} = 0.006$); for *X. c. campestris* BP109, it was 5×10^5 cfu/cm^2 ($OD_{600} = 0.1$). For experiments involving extraction of total RNA from infected leaves, *P. s. maculicola* ES4326 and *P. s. maculicola* ES4326 carrying *avrRpt2* were introduced at a dose of 10^4 cfu/cm^2 leaf area (equivalent to $OD_{600} = 0.002$). For determination of *P. s. maculicola* ES4326 growth, plants were infected with *P. s. maculicola* ES4326 at a dose of 10^3 cfu/cm^2 leaf area (equivalent to $OD_{600} = 0.0002$). After 3 days, leaves were excised and bacterial growth was assayed, as described by Glazebrook et al. (1996). Data are reported as means and standard deviations of the log (cfu/cm^2) of six replicates. For chemical treatment, plants were sprayed with 5 mM SA or 5 mM silver nitrate with 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX) to reduce surface tension. Control plants were sprayed with water containing 0.02% Silwet L-77.

Camalexin Quantitation

For *P. syringae*–infected leaves, fresh weight changes markedly over the course of infection because of water loss from the infected tissue. Therefore, for *P. syringae*–infected leaves, samples consisted of four leaf discs cut with a number 3 cork borer (1.1 cm^2 total), and camalexin concentrations were normalized to leaf area. For leaves infected with *X. c. campestris* BP109 or treated with silver nitrate, samples consisted of ~100 mg of tissue, and camalexin concentrations were normalized to fresh weight. Camalexin assays were performed as described by Glazebrook et al. (1996). For each data point, the results are reported as the mean and standard deviation from six replicates.

RNA Gel Blot Analysis

Tissue samples consisting of three to five 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 (Ausubel et al., 1995; Reuber and Ausubel, 1996). After blotting onto a Hybond-N$^+$ membrane (Amersham Corp., Arlington Heights, IL), hybridizations were performed using various digoxigenin-labeled probes described below, followed by washes in $0.5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C and chemiluminescent detection with CSPD, according to instructions provided by the supplier (Boehringer Mannheim). For hybridization with more than one probe, blots were stripped and reprobed. Single-stranded DNA probes were prepared by amplification of appropriate sequences from cDNA clones (*PR-1* and *PR-5*; Uknes et al., 1992) and from plasmid pATBG12 (*BGL2*; Dong et al., 1991), as described by Glazebrook et al. (1996), except that digoxigenin-11-dUTP was used as the label, according to the instructions of the supplier (Boehringer Mannheim). For the *ASA1* probe, a single-stranded probe was made from plasmid pKN41 (Niyogi and Fink, 1992) by using sense primer 5′-GCTTACCGTTGTTTGGTC-3′ and antisense primer 5′-AGCAATGTCCATGTCACC-3′. For the 18S rRNA probe, a single-stranded probe was made from the clone JHD2-15A (stock no. CD3-197; Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH) by using sense primer 5′-CTGGTTGATCCTGCCAGTAG-3′ and antisense primer 5′-CAGGTTCACCTACGGAAACC-3′. Blots were stripped and reprobed with the 18S rRNA probe to assess equal loading of RNA samples.

Determination of Endogenous Levels of SA and SAG

Mature leaves of 4-week-old wild-type and *pad4* plants were infected with *P. s. maculicola* ES4326 at a dose of 10^4 cfu/cm^2 (equivalent to $OD_{600} = 0.002$) or mock infected with 10 mM $MgSO_4$. At intervals after infection, samples were collected (1 g of tissue per sample, from approximately seven plants) and frozen in liquid nitrogen. SA and SAG were determined as described by Bowling et al. (1994). Raw data were multiplied by 2 to reflect 50% recovery of SA and SAG in this assay.

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