Isolation of phytoalexin-deficient mutants of Arabidopsis thaliana and characterization of their interactions with bacterial pathogens

(Pseudomonas syringae/avirulence/camalexin/plant defense)

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ABSTRACT A genetic approach was used to assess the extent to which a particular plant defense response, phytoalexin biosynthesis, contributes to Arabidopsis thaliana resistance to Pseudomonas syringae pathogens. The A. thaliana phytoalexin, camalexin, accumulated in response to infection by various P. syringae strains. No correlation between pathogen avirulence and camalexin accumulation was observed. A biochemical screen was used to isolate three mutants of A. thaliana ecotype Columbia that were phytoalexin deficient (pad mutants). The mutations pad1, pad2, and pad3 were found to be recessive alleles of three different genes. pad1 and pad2 were mapped to chromosome IV and pad3 was mapped to chromosome III. Infection of pad mutant plants with strains carrying cloned avirulence genes revealed that the pad mutations did not affect the plants' ability to restrict the growth of these strains. This result strongly suggests that in A. thaliana, phytoalexin biosynthesis is not required for resistance to avirulent P. syringae pathogens. Two of the pad mutants displayed enhanced sensitivity to isogenic virulent P. syringae pathogens, suggesting that camalexin may serve to limit the growth of virulent bacteria.

The development of a model plant/pathogen system that allows facile genetic analyses of both host and pathogen has made it feasible to take a genetic approach to the question of which plant defense responses are important for resistance to pathogens. The plant host is Arabidopsis thaliana and the pathogens are Pseudomonas syringae pathovars tomato and maculicola (Pst and Psm, respectively). Gram-negative bacteria that cause "bacterial speck" diseases in many crop plants (1). The virulent strains Psm ES4326, Pst DC3000, and Psm M4 cause a disease characterized by chlorosis and water-soaking of infected leaves, while multiplying by a factor of up to 10⁴ within the intercellular spaces (2–4). Introduction of one of the avirulence genes, avrRpt2, avrRpm1, or avrB, into any of the virulent strains causes loss of virulence, evidenced by their failure to cause disease symptoms and multiplication by only a factor of 10² in infected leaves (2–5). Two resistance genes, Rp32 and RpM1, which cosegregate with resistance to strains carrying avrRpt2 or avrRpm1, respectively, have been identified (3, 6, 7). Thus, this system shows the kind of gene-for-gene specificity observed in other plant–pathogen interactions, in which pathogens carrying particular avirulence genes are avirulent on host plants carrying the corresponding resistance gene (8, 9).

In many cases, defense responses are found to be induced earlier in interactions with avirulent strains than they are in interactions with virulent ones. This rapid response is thought to cause the observed failure of avirulent strains to grow and cause disease. The slower induction of defense responses observed in interactions with virulent strains may serve to restrict pathogen growth in the later stages of the infection (8, 9). A large number of plant defense responses have been identified, based mainly on observations that they are induced in response to pathogen attack. Relatively little is known concerning which of these responses actually contribute to resistance against particular pathogens. This is partly due to the fact that since no plant mutants with defects in individual components of the defense response have been isolated, it has been difficult to study the effectiveness of defense responses in vivo.

One defense response which has been studied extensively is phytoalexin synthesis. Phytoalexins are small molecules synthesized by plants in response to pathogen attack which have antimicrobial activity (10). Several lines of evidence support the hypothesis that phytoalexins are important components of plants' defensive arsenals. Phytoalexins are broad-spectrum antibiotics and inhibit the growth of fungal and bacterial phytopathogens in vitro (11). In many plant/pathogen systems, phytoalexins accumulate rapidly in response to avirulent pathogen races, but not in response to virulent ones (9, 11). Introduction of a gene encoding stilbene synthase, a phytoalexin biosynthetic enzyme from grape, into tobacco conferred increased resistance against a fungal pathogen of tobacco (12). In contrast, mutants of Nectria hematotheca that have lost the ability to detoxify the pea phytoalexin pisatin remain virulent but may cause smaller disease lesions than wild-type fungi (H. D. VanEtten, personal communication).

A. thaliana produces a phytoalexin with the structure of 3-thiazol-2-ylindole (13). This compound is commonly referred to as camalexin, because it was first identified as a phytoalexin produced by Camelina sativa (14). Camalexin appears to be the only phytoalexin that is produced in significant quantities by A. thaliana (13). Infection of A. thaliana by avirulent P. syringae bacteria induced camalexin biosynthesis, while infection by unrelated virulent Xanthomonas campestris bacteria did not (13). Camalexin was shown to inhibit the growth of a phytopathogenic fungus, Cladosporium cucumerium, and P. syringae in vitro (13).

In this work, the question of the role of phytoalexins in combating phytopathogens was approached by removing the phytoalexin from a plant–pathogen interaction by genetic mutation and analyzing the effect on pathogen growth. Specifically, mutants of A. thaliana with defects in camalexin biosynthesis were isolated. These mutants were used to analyze the role of camalexin in interactions between A. thaliana and isogenic pairs of P. syringae strains differing only in the presence or absence of cloned avr genes by examining the consequences of phytoalexin deficiency on the ability of the host to resist pathogen attack.

Abbreviations: Psm, Pseudomonas syringae pv. maculicola; Pst, Pseudomonas syringae pv. tomato; cfu, colony-forming unit(s).

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MATERIALS AND METHODS

Strains, Media, and Growth Conditions. *Psm* ES4326 (4) and *Pst* DC3000 (2) have been described. Plasmids pH12 and K48 carried *avrRpt2* (2) and *avrRpm1* (3), respectively. For simplicity, strains carrying one of these avirulence genes on a plasmid are referred to as strain/avr (e.g., *Psm* ES4326/avrRpt2 is *Psm* ES4326 carrying pH12). Bacteria were grown at 28°C in King’s B medium (protease peptone, 10 mg/ml; K$_2$HPO$_4$, 1.5 mg/ml; glycerol, 15 mg/ml) (15) supplemented with appropriate antibiotics (at 100 μg/ml streptomycin for *Psm* ES4326, rifampicin at 25 μg/ml for *Pst* DC3000, and tetracycline at 10 μg/ml for strains carrying pH12 or K48). *A. thaliana* was grown in Metromix 2000 (W. R. Grace) soil, either in a climate-controlled greenhouse (20 ± 2°C; relative humidity, 70 ± 5%) or an 16-hr light/8-hr dark cycle or in a Conviron growth chamber (20 ± 2°C, relative humidity 90%) on a 12-hr light/dark cycle under 125 microeinsteins of fluorescent illumination. Plants grown in the growth chamber were used for experiments involving determination of bacterial symptoms and growth. Other experiments were conducted on plants grown in the greenhouse. Plants were infected with suspensions of bacterial cells in 10 mM MgSO$_4$ by pressing a 1-ml syringe (without a needle) against the abaxial side of the leaves and forcing the suspension through the stomata into the intercellular spaces.

**Camalexin Determination.** For each sample, three leaf disks cut with a no. 2 cork borer (1.2-cm$^2$ total) were combined and heated in 350 μl of 80% methanol for 20 min. The tissue was removed, and the methanol was evaporated under vacuum. The aqueous residue was extracted with two 50-μl aliquots of chloroform, which were combined and evaporated to dryness. The residue was dissolved in a small volume of chloroform, applied to silica thin-layer chromatography (TLC) plates (J. T. Baker/VWR Scientific), and developed in 9:1 (vol/vol) ethyl acetate/hexane. Camalexin (R$_f$ 0.81) was visualized by its blue fluorescence under a hand-held long-wave ultraviolet lamp (365 nm). The silica containing camalexin was scraped off the plate, and camalexin was extracted into 3 ml of methanol. The emission at 385 nm after excitation at 315 nm was measured with a Perkin-Elmer LS-3 fluorimeter, and camalexin concentration was calculated by comparison with a standard curve obtained by using purified camalexin kindly provided by W. A. Ayer (University of Alberta, Edmonton, Canada). For each data point, this assay was performed on six samples, and the results are reported as the mean and standard deviation.

**Mutant Screening.** Seven thousand fifty-eight plants grown from several independent lots of M2 seed obtained from ethyl methanesulfonate-mutagenesis of the Columbia ecotype (16) were screened for those which failed to accumulate camalexin in response to *Psm* ES4326 infection. One leaf of each plant was infiltrated with a suspension of *Psm* ES4326 (OD$_{600}$ of 0.02, resulting in 10$^5$ cells per cm$^2$ in leaves) to induce camalexin accumulation. After 40 hr, these leaves were excised, and camalexin was extracted and visualized on TLC plates as described above, except that only one 50-μl aliquot of chloroform was used for extraction.

**Determination of Bacterial Growth in Plants.** Mature, fully expanded leaves of 4- to 6-week-old plants were inoculated with bacteria suspended in 10 mM MgSO$_4$. For each data point, 6–8 infected leaves were excised, and a 0.28-cm$^2$ leaf disc was cut from each leaf with a no. 2 cork borer. Each disc was ground with a plastic pestle in a microcentrifuge tube containing 0.5 ml of 10 mM MgSO$_4$. This material was diluted and samples were spread on King’s B plates containing appropriate antibiotics. Plates were incubated for 2 days at 28°C, and colonies were counted. Means and standard deviations were determined from the logarithm of the number of colony-forming units (cfu) per cm$^2$.

Genetic Analysis. Crosses were performed by dissecting immature flowers prior to anther dehiscence and applying pollen to the exposed pistils. Genetic mapping was performed by the CAPS procedure (17). Homozygous pad mutant plants (Columbia ecotype) were crossed to La-er (Landsberg ecotype). The F$_2$ progeny were screened for phytoalexin deficiency to identify homozygous pad/pad plants. DNA was prepared from these plants and used to determine whether they were homozygous for Columbia alleles, heterozygous, or homozygous for Landsberg alleles at several marker loci. Map distances were calculated from the recombination frequencies by using Haldane’s mapping function as described (18). The order of loci was determined by examination of three-point data.

RESULTS

There Is No Correlation Between Pathogen Avirulence and Phytoalexin Accumulation in *A. thaliana*–*P. syringae* Interactions. To test whether camalexin biosynthesis was a specific response to avirulent pathogens or a more general response to pathogen attack, camalexin accumulation in wild-type plants of ecotype Columbia (Col-0) following infection with various *P. syringae* strains at a dose of 10$^8$ cfu/cm$^2$ was monitored (Fig. 1A). Camalexin accumulated to similar high levels in response to the virulent strain *Psm* ES4326 and the isogenic strains *Psm* ES4326/avrRpt2 (Fig. 1A) and *Psm* ES4326/avrRpm1 (data not shown), suggesting that the presence of avirulence genes had little effect on camalexin accumulation. However, camalexin accumulated more rapidly (compare the levels at 24 hr) in response to strain *Pst* DC3000/avrRpt2 than in response to the avirulent virulent strain *Pst* DC3000 (Fig. 1A), suggesting that in the *Pst* DC3000 strain background, there is an effect of the avirulence gene in inducing camalexin accumulation. Resistance of Col-0 to avrRpt2–carrying strains requires the resistance gene *RPS2* (6, 7). To test whether the difference in camalexin induction between strains *Pst* DC3000/avrRpt2 and *Pst* DC3000 was an *RPS2*-dependent response to the *avrRpt2* gene, camalexin accumulation in the Columbia ecotype *RPS2* mutant *rps2-10IC* (7) was examined. In *rps2-10IC* plants, camalexin induction was similar in response to either *Pst* DC3000/avrRpt2 or *Pst* DC3000 (Fig. 1B), indicating that the effect of *avrRpt2* (in the *Pst* DC3000 strain background) on camalexin accumulation is mediated by *RPS2*. No camalexin was detected in plants treated with a buffer control (data not shown). Evidently, camalexin levels are affected by multiple factors, including the genetic background of virulent strains and the presence of avirulence genes.

Strains *Psm* ES4326, *Pst* DC3000, and *Psp* NPS3121 (which is not a pathogen of *A. thaliana*) all displayed similar

![Fig. 1. Accumulation of camalexin over time after infection of wild-type (A) or *rps2-10IC* (B) mutant plants with *Psm* ES4326 (●), *Psm* ES4326/avrRpt2 (○), *Pst* DC3000 (□), or *Pst* DC3000/avrRpt2 (□) at 10$^8$ cfu/cm$^2$. Similar results were obtained in an independent experiment.](image-url)
levels of camalexin sensitivity in vitro. Incubation of logarithmic-phase cells with camalexin at 500 μg/ml in 5% dimethyl sulfoxide/95% King’s B medium caused a 10-fold reduction in viability relative to incubation in 5% dimethyl sulfoxide/95% King’s B medium. Incubation with camalexin at 100 μg/ml had little effect on viability. This level of camalexin sensitivity was also observed for Escherichia coli (Elizabeth Rogers and F.M.A., unpublished data). These data suggest that Psm ES4326 and Pst DC3000 are not particularly tolerant to camalexin.

**Isolation of A. thaliana Mutants with Defects in Camalexin Synthesis in Response to Psm ES4326 Infection.** A genetic approach was used to investigate the importance of camalexin in defense against *P. syringae*. Mutants of ecotype Col-0 with defects in camalexin synthesis in response to pathogen attack were isolated. Approximately 7000 M2 generation plants from an ethyl methanesulfonate-mutagenized population were screened by a biochemical assay. One leaf of each plant was infected with Psm ES4326 at a concentration of 10^5 cfu/cm^2 40 hr before screening, to induce camalexin biosynthesis. The infected leaves were excised and assayed for camalexin as described in Materials and Methods. Plants which appeared to have reduced camalexin levels were retested by the same procedure.

Four putative mutants were obtained in this screen. All of the M3 progeny tested from three of these putative mutants showed the phytoalexin-deficient phenotype, whereas phytoalexin deficiency was not observed in the progeny of the fourth putative mutant. The three phytoalexin-deficient mutants thus identified were named pad1, pad2, and pad3. We assayed the accumulation of camalexin in the pad mutants in response to Psm ES4326 infection (Fig. 2). Plants were infiltrated with Psm ES4326 at 10^5 cfu/cm^2 and camalexin was determined. In the pad1 and pad2 mutants, camalexin accumulated to approximately 30% and 10% of the levels reached in Col-0, respectively. No camalexin was detected in the pad3 mutant. The time course of camalexin accumulation in the pad1 and pad2 mutants was similar to that in Col-0. When wild-type and pad mutant plants were infected with Psm ES4326/avrRpt2, the reduction in camalexin levels in the three pad mutants relative to wild-type plants was as great or greater than when plants were infected with Psm ES4326 (data not shown).

**The pad Mutations Are Recessive Alleles of Single Nuclear Genes.** To determine whether the phytoalexin-deficient phenotypes of the pad mutants resulted from single mutations, each pad mutant was crossed to wild-type plants of the Col-0 or La-er ecotypes. The resulting F1 progeny all accumulated camalexin to wild-type levels (Pad+ phenotype), indicating that the pad mutations were recessive. These plants were allowed to self, and the F2 generation was tested for phytoalexin deficiency. In each cross approximately one-fourth of the F2 progeny were Pad− (Table 1), demonstrating that pad1, pad2, and pad3 are all recessive alleles of single nuclear genes. To remove unlinked secondary mutations that might interfere with characterization of the responses of the pad mutants to pathogens, all three pad mutants were backcrossed to wild-type Col-0. Except for complementation tests and mapping crosses, all of the experiments described in this report either were originally performed or were repeated using pad lines resulting from at least two backcrosses.

**The pad Mutations Define Three Complementation Groups.** Complementation tests were performed to determine whether the pad mutations affected the same or different genes. The pad mutants were crossed to each other in pairwise combinations, and the F1 progeny were tested for camalexin accumulation in response to Psm ES4326 infection (Table 2). All of the F1 progeny from each cross were Pad+, demonstrating that the three pad mutations complemented each other. Therefore, we concluded that pad1, pad2, and pad3 defined three different genes.

**Map Positions of the pad Mutations.** The CAPS mapping technique was used to place the pad mutations on the A. thaliana genetic map (17). The data showed that pad1 and pad2 were located on chromosome IV, between markers AG and DHS, whereas pad3 was located on chromosome III, between BGL2 and gl-1 (Table 3).

**Effects of pad Mutations on Pathogen Growth.** The growth of virulent strains and isogenic strains carrying cloned avirulence genes in wild-type and pad mutant plants was examined to determine whether the pad mutations caused defects in the ability of plants to resist infection. Three isogenic pairs of strains were used in order to control for effects specific to particular avirulence genes or strain backgrounds. These were Psm ES4326 and Psm ES4326/avrRpt2, Pst DC3000 and Pst DC3000/avrRpt2, and Psm ES4326 and Psm ES4326/avrRpm1. When Col-0 was infected with virulent strains at a high initial inoculum (10^6 cfu/cm^2), the pathogen grew to a higher density (10^8 cfu/cm^2) than it did when the inoculum was lower (10^3 cfu/cm^2) grew to 10^6 cfu/cm^2. This result indicates that Col-0 has some ability to resist infection by these virulent pathogens. In studying pathogen growth in pad mutants, a low inoculum was used, so that there would be a

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**Table 1. Segregation of pad mutations in crosses to wild type**

<table>
<thead>
<tr>
<th>Pollen donor</th>
<th>Recipient</th>
<th>F1 Pad*</th>
<th>F1 Pad*</th>
<th>F2 Pad*</th>
<th>F2 Pad*</th>
</tr>
</thead>
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<tr>
<td>pad1/pad1</td>
<td>Col-0</td>
<td>6</td>
<td>0</td>
<td>218</td>
<td>62</td>
</tr>
<tr>
<td>pad2/pad2</td>
<td>La-er</td>
<td>6</td>
<td>0</td>
<td>268</td>
<td>92</td>
</tr>
<tr>
<td>pad3/pad3</td>
<td>La-er</td>
<td>6</td>
<td>0</td>
<td>197</td>
<td>63</td>
</tr>
</tbody>
</table>

χ^2 = (Pad*:Pad* = 3:1) 1.21 (0.2 < P < 0.3) 0.02 (0.8 < P < 0.9) 0.08 (0.7 < P < 0.8)

**Table 2. Complementation testing**

<table>
<thead>
<tr>
<th>Pollen donor</th>
<th>Recipient</th>
<th>Pad*</th>
<th>Pad*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAD2/pad2</td>
<td>pad3/pad3</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>PAD1/pad3</td>
<td>pad1/pad1</td>
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<td>0</td>
</tr>
<tr>
<td>pad2/pad2</td>
<td>pad1/pad1</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>
possibility of observing deleterious effects of the pad mutations on limiting growth of virulent strains, as well as strains carrying avr genes. As a consequence of this choice of low inoculum, the differences between the growth in wild-type plants of strains carrying or lacking avr genes were not as great as they were in experiments where higher inoculum concentrations were used, as in previous publications from this laboratory (4, 7).

In Fig. 3, row A, the growth of various strains in pad1 mutants is compared with that in Col-0. In all three pairs of strains, the growth in the pad1 mutant of the strain carrying an avr gene was much lower than that of the virulent strain, indicating that the pad1 mutation did not interfere with the plants' ability to resist bacteria carrying avr genes. However, the growth of the virulent strains in pad1 was much higher than that in Col-0, indicating that the pad1 mutation did interfere with the plants' ability to restrict the growth of virulent pathogens.

Pathogen growth in pad2 mutants as shown in Fig. 3, row B. The effect of the pad2 mutation was similar to that of the pad1 mutation. It did not interfere with limiting the growth of the strains carrying avr genes, but it did cause an increase in the growth of the virulent strains. In experiments where the growth of virulent pathogens in the pad1 and pad2 mutants was directly compared, the pad1 mutant consistently allowed somewhat more growth of the virulent strains than the pad2 mutant did (data not shown).

The pad3 mutant behaved differently from the pad1 and pad2 mutants, as shown in Fig. 3, row C. The growth of both the virulent strains and the strains carrying avr genes was similar in Col-0 and pad3 mutant plants, indicating that the pad3 mutation did not interfere with the plants' ability to resist either type of strain.

In summary, none of the pad mutations caused defects in the plants' ability to limit the growth of strains carrying avr genes relative to that of avirulent ones. The results strongly suggest that camalexin is not required for effective resistance to avirulent P. syringae pathogens. Two of the pad mutations caused increased sensitivity to virulent strains, while the third did not. Therefore, a definitive conclusion regarding the role of camalexin in interactions with virulent P. syringae pathogens cannot be drawn from these data alone.

Segregation of Phytoalexin Deficiency and Pathogen Sensitivity. To determine whether the pad1 and pad2 mutations cosegregated with increased sensitivity to Psm ES4326, bacterial growth in F2 pad/pad families derived from crosses to Col-0 was measured. Nine of nine pad1/pad1 families and nine of nine pad2/pad2 families allowed significantly more bacterial growth than Col-0. Both phenotypes were recessive, so the probability that phytoalexin deficiency was caused by a mutation unlinked to that causing pathogen sensitivity is 0.25, or 3.8 × 10⁻⁶. Of course, it is not possible to rule out the possibility that two closely linked mutations are responsible for the phenotypes observed, but it seems highly unlikely that this would have occurred in two different independently isolated pad mutants.

pad1 and pad2 were found to complement the pathogen sensitivity phenotype. Forty-eight hours after inoculation at a dose of 5 × 10⁶ cfu/cm², the densities of Psm ES4326 (log[cfu/cm²]) were as follows; F1, PAD1/pad1 PAD2/pad2 (Table 2, cross 4), 5.72 ± 0.26; Col-0, 5.55 ± 0.39; pad1/pad1, 6.84 ± 0.62; pad2/pad2, 6.73 ± 0.26. We concluded that pad1 and pad2 complemented each other for limitation of bacterial growth as well as for camalexin biosynthesis.

**Discussion**

A major question in plant pathology is, Which plant defense responses are required for the limitation of pathogen growth? This question is particularly interesting, because it can be investigated when host recognition of a pathogen avirulence gene results in dramatic restriction of the growth of an otherwise virulent pathogen. In the work described in this paper, a genetic approach was taken to test the hypothesis that phytoalexin biosynthesis is an important factor in limiting bacterial pathogen growth. Three A. thaliana mutants that accumulated less phytoalexin in response to P. syringae attack than did wild-type plants were isolated. To the authors' knowledge, these are the first phytoalexin mutants to be isolated in any plant. These mutants were used to test whether phytoalexin deficiency interfered with either the ability of the plant to severely restrict the growth of strains carrying avr genes or its ability to limit the growth of isogenic virulent strains inoculated at low concentration. In the case of the strains carrying avr genes the differences between the
growth of isogenic strains carrying or lacking **avr** genes were the same in wild-type and all three **pad** mutant plants, demonstrating that camalexin accumulation is not required for resistance of **A. thaliana** to **P. syringae** strains carrying **avr** genes. This resistance must be due to some other defense response(s).

The behavior of the virulent strains **Psm** ES4326 and **Pst** DC3000 in the **pad** mutants is less easily explained. The **pad1** and **pad2** mutants, which synthesized reduced levels of camalexin, allowed significantly more growth of virulent pathogens than wild-type plants did, but **pad3** mutants, which did not synthesize detectable levels of camalexin, did not differ from wild-type plants with respect to pathogen growth (Fig. 3). Since the apparently complete loss of camalexin in the **pad3** mutant did not result in increased pathogen growth, camalexin itself must not be required for limiting the growth of **Psm** ES4326 and **Pst** DC3000 in planta. This leaves the question of why the **pad1** and **pad2** mutations caused an increase in pathogen sensitivity.

One possible explanation is that phytoalexins are not required for limitation of **P. syringae** growth in **A. thaliana**, but the **pad1** and **pad2** mutations exert pleiotropic effects on other defense responses which are required for limiting pathogen growth. This could occur if **pad1** and **pad2** are lesions in genes encoding components of the signal transduction pathway leading to activation of plant defense responses. Preliminary experiments, however, demonstrated no difference in the activation of a variety of defense-related genes in **pad** mutants compared with wild-type plants (J.G., unpublished data).

Another possible explanation for the pathogen growth phenotypes of the **pad** mutants is that phytoalexin is required for limitation of **Psm** ES4326 and **Pst** DC3000 growth. In this model, the increased pathogen growth in **pad1** and **pad2** is explained by the reduced camalexin levels in these mutants. The absence of a pathogen growth phenotype in the **pad3** mutant is explained by hypothesizing that the **pad3** mutation blocks the camalexin biosynthetic pathway at a point such that a precursor accumulates which is itself a phytoalexin (i.e., has antimicrobial activity). The presence of this intermediate compound limits pathogen growth. If this model is correct, the **pad1** and **pad2** mutations must affect camalexin biosynthesis at a point in the pathway such that any intermediates which accumulate do not have antimicrobial activity. In a fungal bioassay similar to that described in ref. 14 no pathogen-inducible antimicrobial compounds were observed in the **pad3** mutant by using the extraction and assay protocol that detects camalexin from wild-type plants. In preliminary experiments using different extraction protocols (J.G., unpublished data), no phytoalexins were observed in the **pad3** mutant, but such a molecule may eventually be found by means of additional extraction and assay procedures.

To understand why the **pad** mutants vary with respect to their susceptibility to virulent **P. syringae** strains, it will be necessary to characterize more **pad** mutants and to elucidate the biochemical pathway leading to camalexin biosynthesis. Each of the **pad** mutants defined a different locus, so there is a high probability that there are more genes required for camalexin biosynthesis. Analysis of the existing **pad** mutants, as well as any others identified in the future, should be helpful in determining how camalexin is synthesized. At present, none of the biosynthetic pathways leading to any of the indole-based phytoalexins of the Brassicaceae have been elucidated.

While our work has shown that camalexin is not required for resistance to avirulent **P. syringae** strains in **A. thaliana**, it is still possible that camalexin will prove to be important for resistance to other avirulent pathogens. The similarity of camalexin to the commercial fungicide thiabendazole suggests that camalexin could play an important role in interactions with phytopathogenic fungi (14). Various species of fungi are known to infect **A. thaliana**, and several gene-for-gene resistance responses have been identified in these systems (19–23).

In summary, this work has shown that the plant defense response can be successfully dissected by using a genetic approach in the **A. thaliana** model for plant–pathogen interactions.

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