Copyright © 1997 by the Genetics Society of America

Phytoalexin-Deficient Mutants of Arabidopsis Reveal That *PAD4* Encodes a Regulatory Factor and That Four *PAD* Genes Contribute to Downy Mildew Resistance

Jane Glazebrook,*,† Michael Zook,‡ Figen Mert,§ Isabelle Kagan,‡ Elizabeth E. Rogers,† Ian R. Crute,§ Eric B. Holub,§ Raymond Hammerschmidt‡ and Frederick M. Ausubel†

**Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, Maryland 20742, †Department of Genetics, Harvard Medical School and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, ‡Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824, and §Plant Pathology and Weed Science Department, Horticulture Research International, Wellesbourne, Warwick CV35 9EF, United Kingdom*

Manuscript received October 18, 1996
Accepted for publication January 7, 1997

ABSTRACT

We are working to determine the role of the Arabidopsis phytoalexin, camalexin, in protecting the plant from pathogen attack by isolating phytoalexin-deficient (*pad*) mutants in the accession Columbia (Col-0) and examining their response to pathogens. Mutations in *PAD1, PAD2,* and *PAD4* caused enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* strain ES4326 (PsmES4326), while mutations in *PAD3* or *PAD5* did not. Camalexin was not detected in any of the double mutants *pad1-1 pad2-1, pad1-1 pad3-1* or *pad2-1 pad3-1*. Growth of PsmES4326 in *pad1-1 pad2-1* was greater than that in *pad1-1* or *pad2-1* plants, while growth in *pad1-1 pad3-1* and *pad2-1 pad3-1* plants was similar to that in *pad1-1* and *pad2-1* plants, respectively. The *pad4-1* mutation caused reduced camalexin synthesis in response to PsmES4326 infection, but not in response to *Cochliobolus carbonum* infection, indicating that *PAD4* has a regulatory function. *PAD1, PAD2, PAD3* and *PAD4* are all required for resistance to the eukaryotic biotroph *Peronospora parasitica*. The *pad4-1* mutation caused the most dramatic change, exhibiting full susceptibility to four of six Col-incompatible parasite isolates. Interestingly, each combination of double mutants between *pad1-1, pad2-1* and *pad3-1* exhibited additive shifts to moderate or full susceptibility to most of the isolates.

PLANT resistance to microbial pathogens is often mediated by "gene-for-gene" interactions, in which specific pathogen genes, called avirulence genes, result in specific recognition by plants carrying corresponding resistance (*R*) genes (FLOR 1971). Gene-for-gene recognition often leads to expression of the hypersensitive response (HR), a programmed cell death response that is the hallmark of many of these interactions (MITTLER and LAM 1996; RYERSON and HEATH 1996; WANG *et al.* 1996), and to rapid activation of other defense responses in the infected tissue. With the exception of the HR, host defense responses can also be induced during interactions with virulent pathogens, although this generally occurs more slowly than in interactions with avirulent pathogens.

Inducible plant defense responses include antimicrobial compounds called phytoalexins, lytic enzymes such as chitinases and glucanases, oxidizing agents, cell wall lignification, and a number of pathogenesis related proteins and transcripts of unknown function (DIXON and LAMB 1990; LAMB *et al.* 1989). For many of these responses, evidence that they are causally responsible for limiting pathogen growth has been obtained from studies of their toxicity *in vitro* and from increased disease resistance displayed by transgenic plants constitutively expressing one or more pathogen-inducible defense-related genes (SCHLUMBAUM *et al.* 1986; BROGLIE *et al.* 1991; WOLOSHUK *et al.* 1991; TERRAS *et al.* 1992, 1995; ALEXANDER *et al.* 1993; HAIN *et al.* 1993; SELA-BUURLAGE *et al.* 1993; LIU *et al.* 1994; PONSTEIN *et al.* 1994; ZHU *et al.* 1994; WU *et al.* 1995).

The antimicrobial properties of phytoalexins suggest that they are an important component of plant defensive arsenals. However, there is evidence both for and against this idea, and it seems likely that phytoalexins contribute to resistance in some plant-pathogen interactions, but not in others. The supporting evidence includes observations that avirulent pathogen strains induce phytoalexin accumulation in the host, whereas similar virulent strains do not (SMITH 1982; DARVILL and ALBERSHEIM 1984; ESSENBERG *et al.* 1992), inverse correlations between degree of pathogen growth and host phytoalexin levels (*e.g.*, LONG *et al.* 1985; CONN *et al.* 1988), and increased host resistance resulting from constitutive expression of a phytoalexin biosynthetic gene (HAIN *et al.* 1993). On the other hand, resistance of several Brassica species to *Leptosphaeria maculans* showed no correlation with phytoalexin levels (ROUXEL *et al.* 1991; PEDRAS and SEGUIN-SWARTZ 1992), and phy-

Corresponding author: Jane Glazebrook, Center for Agricultural Biotechnology, 5128 Plant Sciences Bldg., University of Maryland, College Park, MD 20742. E-mail: glazebro@umbi.umd.edu

toalexins sometimes accumulate to high levels in response to infection by virulent pathogens (*e.g.*, PUEPPKE and VANETTEN 1976; DENNY and VANETTEN 1981; BRINDLE *et al.* 1988; LUCY *et al.* 1988; PEDRAS and SEGUIN-SWARTZ 1992; GLAZEBROOK and AUSUBEL 1994).

The general lack of strong experimental evidence for a role for phytoalexins in disease resistance is due in part to the lack of knowledge of the relative contribution that various chemical and structural barriers make to the resistance response. To provide more definitive evidence for or against the role of phytoalexins in particular host/parasite interactions, new experimental approaches must be found. Recently, we reported the isolation of phytoalexin deficient (*pad*) mutants of *Arabidopsis thaliana* (GLAZEBROOK and AUSUBEL 1994). This genetic approach has the potential to provide definitive evidence for the role of the Arabidopsis phytoalexin, camalexin, in disease resistance. Like other phytoalexins produced by brassicas, camalexin is an indole derivative (3-thiazol-2′-yl-indole) with a sulfur-containing moiety (TSUJI *et al.* 1992; CHAPPLE *et al.* 1994). It inhibits the the growth of both fungi and bacteria *in vitro,* although the concentration required for inhibition of fungal growth is generally about fivefold lower than that required to inhibit bacterial growth (BROWNE *et al.* 1991; ROGERS *et al.* 1996; TSUJI *et al.* 1992).

The initial screen for *pad* mutants yielded mutations in three different genes, *PAD1, PAD2,* and *PAD3* (GLAZEBROOK and AUSUBEL 1994). Infection by *Pseudomonas syringae* pv. *maculicola* strain ES4326 (PsmES4326) induced camalexin in the *pad1-1, pad2-1,* and *pad3-1* mutants to 30, 10 and <1% (undetectable) of the level in wild-type plants, respectively. None of the *pad* mutants were compromised in their ability to resist infection by *P. syringae* strains carrying the avirulence genes *avrRpt2* or *avrRpm1,* as judged by observation of an HR and by reduction of growth of strains carrying avirulence genes relative to growth of isogenic virulent strains. This strongly suggested that camalexin does not make an important contribution to gene-for-gene resistance to these *P. syringae* strains. However, the *pad1-1* and *pad2-1* mutants did display enhanced susceptibility to the virulent strains PsmES4326 and *Pseudomonas syringae* pv. *tomato* strain DC3000 (PstDC3000). In contrast, growth of these strains in the *pad3-1* mutant was not significantly different from that in wild-type plants (GLAZEBROOK and AUSUBEL 1994). Subsequent to the isolation of *pad1-1,pad2-1,* and *pad3-1,* two additional *pad* alleles, *pad2-2* and an allele of a new *pad* gene, *PAD4,* were identified in a screen for mutants displaying enhanced susceptibility to PsmES4326. The *pad4-1* mutation was shown to be a recessive allele of a single gene, to cause reduced camalexin synthesis in response to PsmES4326 infection, and to cause strongly enhanced susceptibility to PsmES4326 (GLAZEBROOK *et al.* 1996).

We have proposed two models to explain why some *pad* mutations cause enhanced susceptibility to PsmES4326 while the *pad3-1* mutant does not (GLAZEBROOK and AUSUBEL 1994). In the biosynthetic model, the *pad3-1* mutation blocks the camalexin biosynthetic pathway at a point such that a precursor which also has antimicrobial activity accumulates, compensating for the loss of camalexin. The other mutations block the pathway at other points such that antimicrobial precursors do not accumulate. Attempts to test this model by searching for antimicrobial compounds synthesized by *pad* mutants have been unsuccessful (J. GLAZEBROOK and F. M. AUSUBEL, unpublished results). In the regulatory model, camalexin is not primarily responsible for limiting growth of PsmES4326 in wild-type plants. The phenotypes of the *pad1-1, pad2-1* and *pad4-1* mutants result from pleiotropic regulatory effects on defense gene expression. Failure to express defense responses other than camalexin causes the enhanced susceptibility. Examination of the mRNA levels of several defense related genes following PsmES4326 infection did not reveal any defects in the *pad* mutants (J. GLAZEBROOK and F. M. AUSUBEL, unpublished results). Studies of the effects of camalexin on growth of PsmES4326 *in vitro* suggested that camalexin levels in infected tissues may not be high enough to restrict pathogen growth in infected leaves, lending support to the regulatory model (ROGERS *et al.* 1996).

In the work described here, we have extended the characterization of the *pad* mutants in an effort to gain insight into the role of camalexin in various Arabidopsis-pathogen interactions and to help explain the varying phenotypes of *pad* mutants in interactions with virulent *P. syringae* strains. We describe the isolation of two additional *pad* mutations and construction of several double *pad* mutants. The single and double *pad* mutants were characterized with respect to their effects on camalexin synthesis in response to *P. syringae* or *Cochliobolus carbonum* infection, and their effects on growth of *P. syringae,* a virulent *Xanthomonas campestris* strain, and six incompatible isolates of the biotrophic oomycete *Peronospora parasitica* (downy mildew; referred to hereafter as Peronospora). The major results of this analysis are that *PAD4* has a regulatory, rather than a biosynthetic function, and that *PAD1, PAD2, PAD3,* and *PAD4* are required for resistance to Peronospora.

MATERIALS AND METHODS

Arabidopsis mutants: Phytoalexin-deficient mutants *pad1-1, pad2-1, pad3-1,* and *pad4-1* were derived from EMS treatment of accession Columbia, followed by backcrosses to wild-type Columbia. (GLAZEBROOK and AUSUBEL 1994; GLAZEBROOK *et al.* 1996). The *pad5-1* mutant was backcrossed twice to wild-type Columbia before it was used for camalexin accumulation or pathogen-susceptibility studies.

Inoculations with pathogens

***Pseudomonas syringae*:** Strains PsmES4326 and PsmES4326(*avrRpt2*), carrying the avirulence gene *avrRpt2* on plasmid pLH12, have been described (DONG *et al.* 1991; WHALEN *et al.* 1991). Bacteria were grown in King's B medium supple-

mented with appropriate antibiotics and infiltrated into Arabidopsis plants as described previously (GLAZEBROOK and AUSUBEL 1994). For camalexin assays, the bacterial dose was 10^5 cfu/cm^2 of leaf area, and for bacterial growth assays, it ranged from 10^2 to 10^3 cfu/cm^2, as indicated in the figures. Bacterial growth was assayed as described (GLAZEBROOK *et al.* 1996).

***Xanthomonas campestris*:** Strain XccBP109 (WEISS *et al.* 1994) was grown in LB medium (AUSUBEL *et al.* 1995) supplemented with 50 μg/ml rifampicin. Plants were infected as described for *P. syringae*. For camalexin assays, the bacterial dose was 10^5 cfu/cm^2, and for bacterial growth assays, it was 10^3 cfu/cm^2.

***Cochliobolus carbonum*:** *C. carbonum* (Race 1) was grown on V-8 juice agar (per liter: 200 ml V-8 juice, 2 g $CaCO_3$, 14 g agar). Leaves to be inoculated with *C. carbonum* were detached and placed in a Petri dish lined with moistened filter paper. *C. carbonum* spores were washed into water from 1-wk-old cultures grown on V-8 brand vegetable juice agar, adjusted to 5×10^5 spores/ml, and 0.5 ml droplets of the suspension were placed on the abaxial sides of the leaves.

***Peronospora parasitica*:** A previously described cotyledon assay was modified slightly for inoculations with Peronospora (DANGL *et al.* 1992; HOLUB *et al.* 1994). Inoculum was adjusted to 5×10^4 conidiosporangia per milliliter, and applied in a fine mist onto seedlings using an atomizer driven by compressed air rather than by manual drop inoculations onto each cotyledon. Approximately 1-μl drops were retained on each cotyledon. All of the isolates, except for *P-006*, were originally derived and bulked from oospore infection (sexual inoculum) of a single Arabidopsis seedling. The isolate *P-006* was derived from *Brassica oleracea* (MOSS *et al.* 1994). The degree of parasite reproduction was determined by counting the number of sporangiophores produced per seedling with the aid of a hand-held magnifying lens (×3) or dissecting microscope (×6–10).

Fluorometric screen for *pad* mutants: M2 seeds derived from selfing of EMS-mutagenized plants (Columbia accession, homozygous for *gl1*, a mutation resulting in leaves and cotyledons lacking leaf hairs) were obtained from Lehle seeds (Tucson, AZ). For screening, two leaves from each of ten plants were placed in a 15-cm Petri dish lined with moistened filter paper. The leaves were inoculated with *C. carbonum* as described above. After 24 hr at room temperature, the spore droplets were removed from the leaves and placed in a test tube. Two milliliters of water was added, and the solution was analyzed for camalexin using a Hitachi model F-2000 fluorometer (330 nm excitation, 393 nm emission). Individual M2 plants with low camalexin levels were retested in the M3 generation.

Construction of double *pad* mutants

***Construction of pad1-1 pad2-1*:** Previous genetic mapping of *pad1-1* and *pad2-1* showed that they lay ~10 cM apart from each other on chromosome 4 (J. GLAZEBROOK and F. M. AUSUBEL, unpublished results). Consequently, the expected frequency of *pad1-1 pad2-1* double homozygotes in the F_2 of a cross between *pad1-1* and *pad2-1* was expected to be very small. The strategy used for isolation of the double homozygotes was based on the fact that the *pad1-1* mutant displays an altered leaf morphology, characterized by serrated edges and a flat, as opposed to the wild-type convex, topology. This leaf phenotype cosegregates with the phytoalexin deficiency phenotype at a resolution of 10 kb (J. GLAZEBROOK and F. M. AUSUBEL, unpublished results). The F_2 progeny of a cross between *pad1-1* and *pad2-1* were screened for *pad2-1* homozygotes based on their low camalexin levels and wild-type leaf morphology. The F_3 progeny of these plants were examined for segregation of plants with *pad1-1* leaf morphology. Among 35 F_3 families, four families included plants with altered leaf morphology. These plants were considered to be *pad1-1 pad2-1* homozygotes. This assignment was confirmed by crossing the putative double mutants to *pad1-1* and *pad2-1* single mutants, and testing the F_1 progeny (eight from each cross) for phytoalexin-deficiency. In all cases, no complementation for phytoalexin deficiency was observed, demonstrating that the candidate plants were indeed *pad1-1 pad2-1* double mutants.

***Construction of pad1-1 pad3-1*:** We predicted that the phenotypes of the *pad1-1 pad3-1* double mutant would include *pad1-1* leaf morphology and undetectable camalexin levels. The F_2 progeny of a cross between *pad1-1* and *pad3-1* were screened for these phenotypes. A candidate plant with *pad1-1* leaf morphology and undetectable camalexin was identified and crossed to *pad1-1* and *pad3-1* single mutants. All of the resulting F_1 plants (eight from each cross) displayed camalexin-deficiency, demonstrating that the candidate plant was a *pad1-1 pad3-1* double mutant.

Construction of *pad2-1 pad3-1*: We assumed that the *pad2-1 pad3-1* double mutant would have an undetectable-camalexin phenotype. F_2 progeny from a cross between *pad2-1* and *pad3-1* were screened for those with undetectable camalexin. Nine such plants were then crossed to both *pad2-1* and *pad3-1* mutants. Camalexin assay of the resulting F_1 plants (eight from each cross) demonstrated that three of the original nine plants were homozygous for *pad2-1* and *pad3-1*.

Camalexin assays: In the large-scale assay, each sample consisted of five leaves from 3- to 4-wk-old plants. For leaves infected with *C. carbonum*, the spore droplet was included in the sample. Samples were boiled in 30 ml of 80% (v/v) methanol until the volume of the solution was reduced to ~10 ml. Three volumes of water were added to the methanolic extract, and the solution was extracted twice with an equal volume of chloroform. The chloroform extracts were pooled, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The remaining residue was dissolved in chloroform, applied to a silica gel G60 TLC plate and developed using chloroform:methanol (9:1, v/v). Camalexin was visualized under short wave ultraviolet (UV) light and scraped into a scintered glass funnel. Camalexin was eluted from the silica gel using ethyl acetate. The ethyl acetate eluant was evaporated to dryness under a stream of nitrogen, and dissolved in 100 μl of hexane:isopropanol (93:7, v/v) just before HPLC analysis. The HPLC mobile phase (hexane:isopropanol 93:7, v/v) was pumped through a 5-μm Econosphere 150 × 4.6 mm silica column (Alltech Associates, Deerfield, IL) at a flow rate of 1.0 ml/min. The eluant from the column was monitored at 215 nm using a variable wavelength detector. The amount of camalexin in each sample was determined by comparison to the HPLC detector response to injection of known amounts of pure camalexin.

The small-scale assay for camalexin was performed as described previously (GLAZEBROOK and AUSUBEL 1994). For scoring of *pad* mutants in segregating populations, the small-scale assay was used, but camalexin levels were judged by visual examination of the TLC plates, rather than by quantitative measurement.

RESULTS

Isolation of *pad* mutants by fluorometric screening: *C. carbonum* is a maize pathogen that is not an Arabidopsis pathogen but does induce camalexin accumulation. The fluorometric assay for camalexin accumulation is based on the observation that camalexin will diffuse into the inoculation droplet of a spore sus-

pension of *C. carbonum* placed on an Arabidopsis leaf. Twenty-four hours after inoculation, the amount of camalexin in the inoculation droplet is measured directly with a flourometer. EMS-mutagenized M2 seed from plants of the Col-0 accession carrying the *gl1* mutation was obtained from Lehle seeds. The *gl1* mutation causes lack of trichomes (leaf hairs) and is useful as a marker. Leaves of 5500 plants grown from this seed were inoculated with *C. carbonum* to induce camalexin synthesis, and camalexin was assayed fluorometrically. Approximately 30 plants that had camalexin levels significantly lower than the average were identified and allowed to set seed. Among these M3 families, two displayed reduced camalexin levels in response to PsmES4326 infection. In one family, No. 4648, no camalexin was detected in any of the plants. In the other family, No. 2120, of six plants tested, one plant appeared to have as much camalexin as wild-type, three had slightly less, and two had very little. We hypothesized that the original M2 plant was heterozygous for a semi-dominant *pad* mutation, and chose one of the plants (No. 2120A) with very little camalexin to establish a line for further analysis.

Genetic analysis of the new *pad* mutants: Complementation testing was used to determine whether Nos. 4648 and 2120A defined new *pad* genes or whether they were alleles of previously identified *PAD* genes. Numbers 4648 and 2120A were crossed to wild-type, *pad1-1*, *pad2-1*, *pad3-1*, and *pad4-1*. In response to PsmES4326 infection, all of the F_1 progeny from the crosses with No. 4648 displayed high levels of camalexin, except the progeny of the No. 4648 × *pad3-1* cross, in which camalexin was undetectable. The progeny of the No. 4648 × *pad3-1* cross could not have been *pad3-1* self-progeny because one-quarter of the F_2 progeny displayed the Gl^- phenotype caused by the *gl1* mutation in No. 4648. These results showed that the No. 4648 mutation is recessive and that it is an allele of *PAD3*. The No. 4648 mutation was renamed as *pad3-2*. The No. 2120A mutation complemented all of the other *pad* mutations, demonstrating that it is a recessive allele of a previously unidentified *pad* gene. Therefore, No. 2120A was renamed as *pad5-1*.

The *pad3-2* mutant was crossed to wild-type plants of the Landsberg-*erecta* accession. Segregation of the phytoalexin-deficient phenotype was examined in the F_2 progeny. Among 439 plants tested, camalexin was undetectable in 102 plants, while 337 plants displayed wild-type camalexin levels. This segregation ratio is approximately 3:1 ($\chi^2 = 0.729$, $0.6 < P < 0.7$), indicating that the no-camalexin phenotype of *pad3-2* results from a recessive mutation in a single nuclear gene. The segregation of the low-camalexin phenotype was also examined in the F_2 progeny of the cross between *pad5-1* and wild-type Col-0. Among 52 plants tested, 15 displayed low camalexin while 37 displayed high camalexin levels. It is not clear why the intermediate phenotype of *pad5-1/PAD5* heterozygotes was not observed in this, or any subsequent experiments. The segregation ratio is approximately 3:1 ($\chi^2 = 0.412$, $0.4 < P < 0.5$), confirming that the *pad5-1* low-camalexin phenotype also results from a recessive mutation in a single nuclear gene. In the course of this experiment, we observed that *pad5-1* is linked to *gl1* (13 of the 15 Pad^- plants were also Gl^-, and there were no Gl^- plants that were Pad^+) on chromosome *3*.

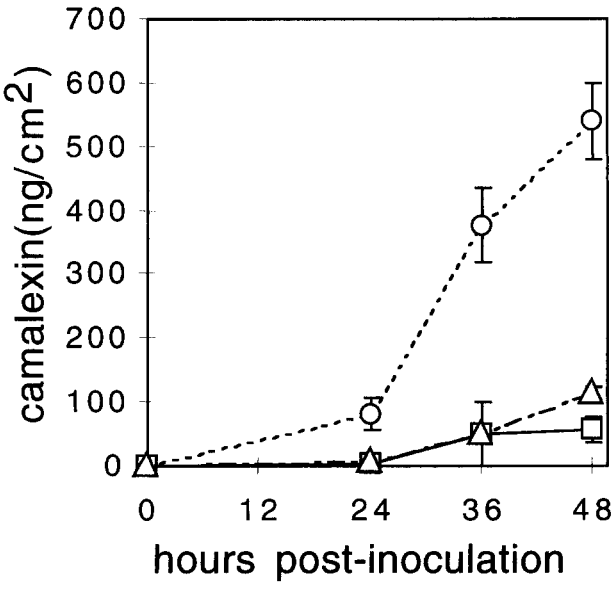

FIGURE 1.—Camalexin accumulation in *pad4-1* and *pad5-1* mutants. Plants were infected with PsmES4326 at a dose of 10^5 cfu/cm^2. At the indicated times, 1 cm^2 samples were excised from the leaves with a cork borer, and camalexin was assayed using the small-scale assay described in MATERIALS AND METHODS. Each point represents the mean and standard deviation of four replicate samples. ○, Col-0; □, *pad4-1*; △, *pad5-1*.

Effects of the *pad3-2*, *pad4-1* and *pad5-1* mutations on interactions with PsmES4326: The previously isolated *pad4-1* mutant was not thoroughly characterized with respect to its phenotypes during infection by PsmES4326 (GLAZEBROOK *et al.* 1996), so it was included with these studies of *pad3-2* and *pad5-1*. To measure the effects of *pad4-1* and *pad5-1* mutations on camalexin synthesis in response to PsmES4326 infection, wild-type, *pad4-1*, and *pad5-1* plants were infected with PsmES4326 at a dose of 10^5 cfu/cm^2, and camalexin was assayed at various times using the small-scale camalexin assay described in MATERIALS AND METHODS. Figure 1 shows that camalexin levels in *pad4-1* and *pad5-1* mutants were ~10 and 20% of the wild-type level, respectively. The time course of camalexin accumulation was similar in wild-type and *pad* mutant plants. Consistent with our previous characterization of *pad3-1* (GLAZEBROOK and AUSUBEL 1994), no camalexin was detected in *pad3-2* mutant plants following infection with PsmES4326 (data not shown).

The *pad3-2*, *pad4-1* and *pad5-1* mutants were tested for *P. syringae* growth phenotypes. The *pad3-2* mutant did not

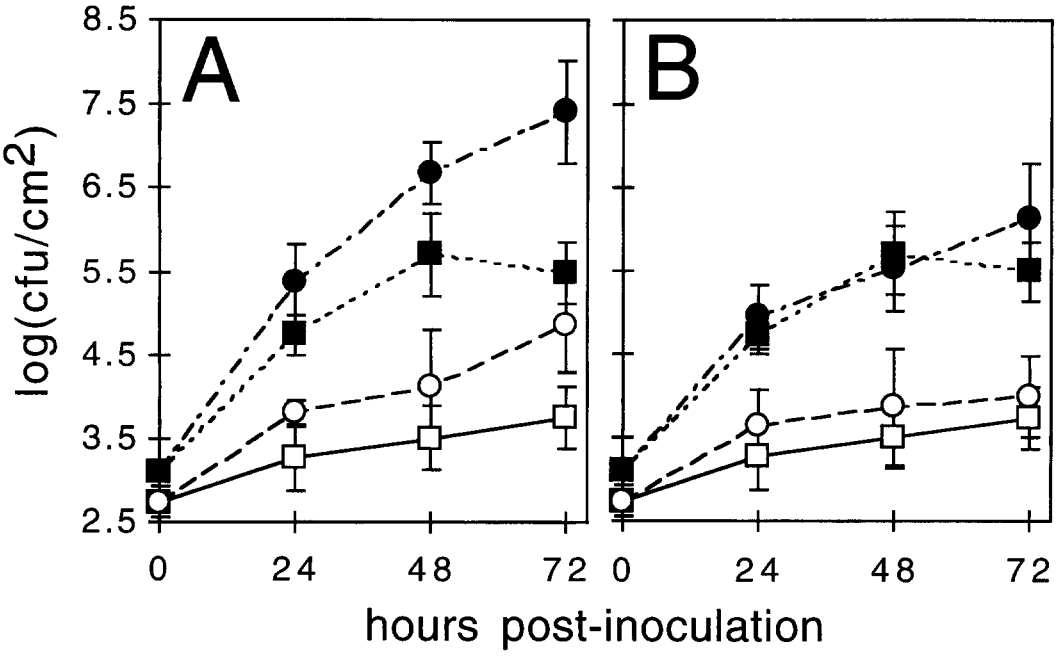

FIGURE 2.—Growth of *P. syringae* strains in *pad4-1* and *pad5-1* mutants. Wild-type, *pad4-1*, and *pad5-1* plants were inoculated with PsmES4326 or PsmES4326(*avrRpt2*). At the indicated times, samples were cut from infected leaves and bacterial titers were determined. Each point represents the mean and standard deviation of six to eight replicate samples. (A) Wild-type and *pad4-1* plants. ■, PsmES4326 in Col-0; □, PsmES4326(*avrRpt2*) in Col-0; ●, PsmES4326 in *pad4-1*; ○, PsmES4326(*avrRpt2*) in *pad4-1*. (B) Wild-type and *pad5-1* plants. ■, PsmES4326 in Col-0; □, PsmES4326(*avrRpt2*) in Col-0; ●, PsmES4326 in *pad5-1*; ○, PsmES4326(*avrRpt2*) in *pad5-1*.

display enhanced susceptibility to PsmES4326 (data not shown). This phenotype is similar to that of the *pad3-1* mutant described previously (GLAZEBROOK and AUSUBEL 1994). Col-0 wild-type, *pad4-1*, and *pad5-1* plants were infected with the virulent strain, PsmES4326, or an isogenic strain carrying the avirulence gene *avrRpt2*. Bacteria carrying *avrRpt2* trigger a rapid gene-for-gene resistance response mediated by the resistance gene *RPS2* and consequently grow to much lower titers in infected leaves than strains lacking *avrRpt2* (DONG *et al.* 1991; WHALEN *et al.* 1991; BENT *et al.* 1994; MINDRINOS *et al.* 1994). Figure 2A shows that in the *pad4-1* mutant, the final titer of PsmES4326 was 50 times higher than it was in wild-type plants, indicating that the *pad4-1* mutation has a deleterious effect on the capacity of Arabidopsis to restrict PsmES4326 growth. The titer of PsmES4326(*avrRpt2*) was also somewhat higher, but the differences in titer between PsmES4326 and PsmES4326(*avrRpt2*) were comparable in wild-type and *pad4-1* mutant plants, indicating that the *pad4-1* mutation does not have a significant effect on the gene-for-gene resistance to PsmES4326(*avrRpt2*). In contrast, Figure 2B shows that the *pad5-1* mutation did not have a significant effect on growth of either PsmES4326 or PsmES4326(*avrRpt2*).

Cosegregation of the phytoalexin-deficient and enhanced susceptibility phenotypes of *pad4-1* was examined to test whether these phenotypes were caused by the same or different mutation(s). A F_2 population derived from the third backcross of *pad4-1* to Col-0 wild-type was screened for phytoalexin deficiency. Eight plants that were phytoalexin deficient and eight plants that were not phytoalexin deficient were tested for enhanced susceptibility to strain PsmES4326 by determining the titer of the pathogen in infected leaves 3 days after infection at a dose of 10^3 cfu/cm^2. For each plant, samples were taken from two leaves, and the titers were averaged. For the eight phytoalexin deficient plants, the titers, as mean of the log(cfu/cm^2) ± half the difference between the samples, ranged from 6.66 ± 0.32 to 7.70 ± 0.05. For the eight plants that were not phytoalexin deficient, titers ranged from 4.71 ± 0.67 to 5.42 ± 0.27. Thus, a perfect correlation was observed between phytoalexin deficiency and enhanced susceptibility among these sixteen plants. The probability of obtaining such a result if the two phenotypes were caused by separate unlinked recessive mutations is $0.25^8 \times 0.75^8$ or 1.5×10^{-6}. While we cannot rule out the possibility that the two phenotypes are caused by closely linked mutations, we consider this highly unlikely because the phytoalexin deficiency and enhanced susceptibility phenotypes of the *pad1-1* and *pad2-1* mutations also cosegregate (GLAZEBROOK and AUSUBEL 1994).

Phenotypes of double *pad* mutants: In view of the differences in susceptibility to PsmES4326 observed among various *pad* mutants, it was of interest to construct double *pad* mutants and determine their phenotypes during infection by PsmES4326. Three lines homozygous for two different *pad* mutations, *pad1-1 pad2-1*, *pad1-1 pad3-1*, and *pad2-1 pad3-1*, were constructed as described in MATERIALS AND METHODS. The *pad1-1*

FIGURE 3.—Morphological phenotypes of *pad* mutants. Top left, Columbia wild-type; top right, *pad1-1*; bottom left, *pad1-1 pad2-1*; bottom right, *pad2-1*.

mutant displays an altered leaf morphology characterized by serrated edges and a flat, as opposed to the wild-type convex, topology (GLAZEBROOK and AUSUBEL 1994). This morphological phenotype cosegregates with the phytoalexin-deficient phenotype at a resolution of 10 kb (J. GLAZEBROOK and F. M. AUSUBEL, unpublished results), suggesting that the two phenotypes result from the same mutation. Interestingly, all four of the *pad1-1 pad2-1* lines obtained displayed an altered leaf morphology, as shown in Figure 3. This morphology was similar to that of *pad1-1*, except that the plants were more compact than *pad1-1* plants, and the leaves were less green with slightly yellow veins. The morphologies of the *pad1-1 pad3-1* and *pad2-1 pad3-1* plants were indistinguishable from those of *pad1-1* and wild-type plants, respectively.

Camalexin accumulation in the double mutants was tested by infecting leaves with PsmES4326 at a dose of 10^5 cfu/cm^2 and assaying for camalexin 40 hr after infection. In contrast to *pad1-1* and *pad2-1* single mutants, no camalexin was detected in any of the double mutants. The effects of the double mutations on growth of PsmES4326 were examined by infecting plants with a very low dose of PsmES4326 and determining bacterial titer in infected leaves daily. As shown in Figure 4, *pad1-1 pad2-1* plants allowed more growth of the bacteria than either *pad1-1* or *pad2-1* single mutants, growth in *pad1-1 pad3-1* was similar to that in *pad1-1*, and growth in *pad2-1 pad3-1* was similar to that in *pad2-1*. Thus, while the *pad1-1 pad2-1* double mutant exhibited phytoalexin deficiency and pathogen susceptibility phenotypes that were more extreme than those of either single mutant, addition of the *pad3-1* mutation to either the *pad1-1* or *pad2-1* backgrounds did not increase the level of PsmES4326 susceptibility.

The *pad4-1* mutation interferes with regulation of camalexin biosynthesis: The *pad* mutations could lie in genes encoding camalexin biosynthetic enzymes, in genes involved in pathogen recognition or in genes involved in signal transduction leading to activation of camalexin synthesis. It is possible that some factors are required for activation of camalexin synthesis in response to some stimuli but not in response to others. To test these ideas, we inoculated wild-type and *pad* mutant plants with *C. carbonum* and compared the amounts of camalexin that accumulated with the amounts that accumulated in response to PsmES4326 infection. The *pad1-1* (Figure 5, A and B), *pad2-1* (Figure 5, A and B), and *pad5-1* (Figure 5, E and F) mutations reduced camalexin levels to comparable extents regardless of how camalexin synthesis was induced. In contrast, the *pad4-1* mutation (Figure 5, C and D) did not cause a reduction in camalexin levels when *C. carbonum* induced camalexin synthesis, but reduced camalexin levels to 10% of wild-type when *P. syringae* induced camalexin synthesis. No camalexin was detected in the *pad3-1* mutant in response to either pathogen or in mock-inoculated controls (not shown). These results demonstrate that the *pad4-1* mutant retains the ability to synthesize wild-type levels of camalexin, but fails to do so in response to PsmES4326 infection. Therefore, it is unlikely that the protein encoded by *PAD4* is a camalexin biosynthetic enzyme. Rather, these data suggest that *PAD4* encodes a protein that is required for either recognition of or response to PsmES4326, but that is not required for recognition of or response to *C. carbonum*.

The *pad* mutations have no effect on growth of *Xanthomonas campestris* pv. *campestris* BP109: If the increased susceptibility of the *pad1-1*, *pad2-1*, and *pad4-1* mutants is due to their effects on camalexin synthesis or other defense responses coordinately regulated with camalexin synthesis, then these mutants should not show enhanced susceptibility to pathogens that do not induce camalexin synthesis. It was reported previously that strain Xcc8004 did not induce camalexin synthesis in Arabidopsis (TSUJI *et al.* 1991). Consistent with this observation, Figure 6A shows that infection by strain XccBP109 induces camalexin to only 1% of the level induced by PsmES4326 infection. Wild-type, *pad1-1*, *pad2-1*, *pad3-1*, *pad4-1*, and *pad5-1* mutants were infected with XccBP109, and bacterial growth was monitored over a period of 4 days. As shown in Figure 6, B and C, none of these *pad* mutations significantly affected XccBP109 growth. This result supports the idea that the enhanced-susceptibility phenotypes of some *pad* mutants result from direct effects on defense responses, rather than from secondary effects leading to reduction in plant vigor.

***PAD1*, *PAD2*, *PAD3*, and *PAD4* are required for downy mildew resistance:** The amount of camalexin required to completely inhibit growth in culture is five- to 10-fold lower for the fungi *Fusarium oxysporum* and *Cladosporium*

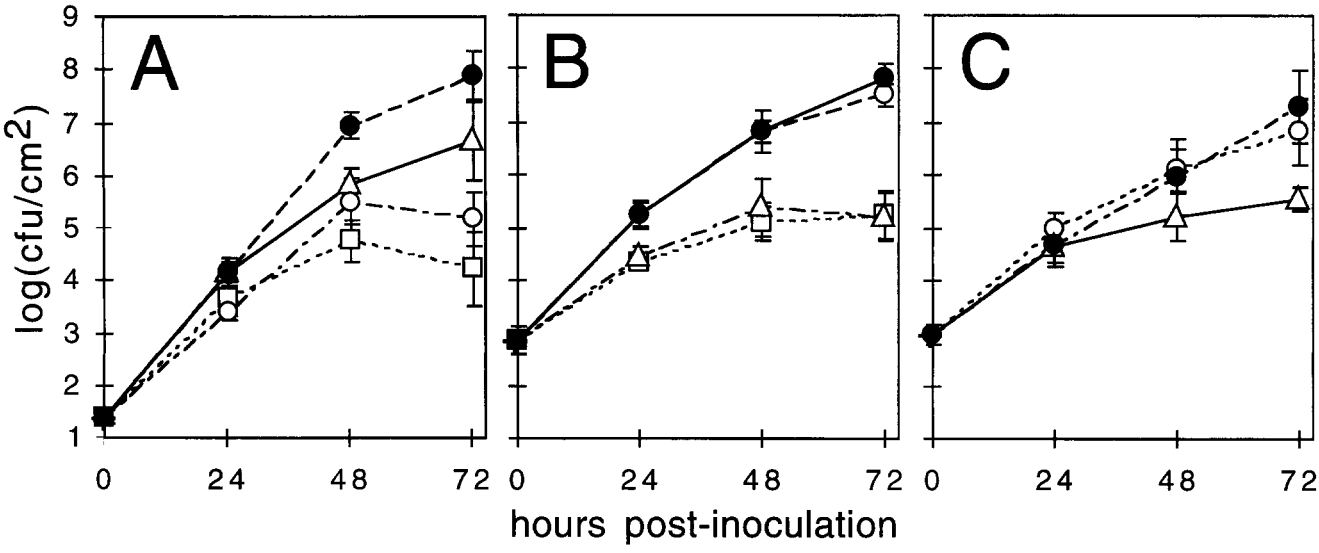

FIGURE 4.—Growth of PsmES4326 in double *pad* mutants. Plants were infected with PsmES4326, and at the indicated times, samples were cut from infected leaves and bacterial titers were determined. Each point represents the mean and standard deviation of six to eight replicate samples. (A) Bacterial growth in *pad1-1 pad2-1*. □, Col-0; △, *pad1-1*; ○, *pad2-1*; ●, *pad1-1 pad2-1*. (B) Bacterial growth in *pad1-1 pad1-3*. □, Col-0; ○, *pad1-1*; △, *pad3-1*; ●, *pad1-1 pad3-1*. (C) Bacterial growth in *pad2-1 pad3-1*. ○, *pad2-1*; △, *pad3-1*; ●, *pad2-1 pad3-1*.

cucumerinum than for the bacteria *P. syringae* (several strains) and *E. coli* (TSUJI *et al.* 1992; ROGERS *et al.* 1996). For this reason, it seems likely that camalexin could play an important role in conferring resistance to eukaryotic pathogens. To investigate this hypothesis, we examined the effects of *pad* mutations on resistance to several Peronospora isolates. Peronospora is a biotrophic parasite that causes downy mildew in members of the Cruciferae (CHANNON 1981; KOCH and SLUSARENKO 1990), and it has become a model organism for investigating the molecular genetics of resistance to a eukaryotic parasite in Arabidopsis (HOLUB and BEYNON 1996). Numerous isolates have been characterized by their ability to reproduce asexually and incite host responses in several Arabidopsis accessions. Molecular genetic analyses have revealed more than twenty *RPP* (Recognition of *P. parasitica*) loci that determine pathotype-specific resistance to particular isolates of the parasite (reviewed by HOLUB and BEYNON 1996).

Six Columbia-incompatible isolates of Peronospora were used in this investigation. The corresponding *RPP* gene(s) responsible for specific recognition of each parasite isolate were determined previously (HOLUB *et al.* 1994; HOLUB and BEYNON 1996). Seedlings of wild-type Columbia and a set of single and double *pad* mutants were inoculated with each isolate, and the degree of susceptibility was measured as the number of sporangiophores produced per cotyledon on the seventh day after inoculation (Table 1).

Among the single mutants, *pad1-1* and *pad5-1* exhibited no change in susceptibility to any of the isolates. However, the *pad2-1* mutant showed moderate or low susceptibility to Emoy2 and weak susceptibility to Hind4, and the *pad3-1* mutant showed weak susceptibility to Hind4. The *pad4-1* mutant exhibited the most dramatic phenotype among the single mutants with a change to full susceptibility following inoculations with four of the five parasite isolates that were originally derived from Arabidopsis.

Among the double *pad* mutants, it was clear that the *PAD1, PAD2,* and *PAD3* genes are required in an additive manner for expression of full downy mildew resistance. Interestingly, each double mutant appeared to exhibit a different pattern of response to the different Peronospora isolates. The *pad1-1 pad2-1* double mutant showed greater susceptibility to Cala2 than did either of the *pad1-1* or *pad2-1* single mutants, the *pad1-1 pad3-1* double mutant showed greater susceptibility to Emoy2, Cala2, Hiks1, and Hind4 than did either of the *pad1-1* or *pad3-1* single mutants, and the *pad2-1 pad3-1* double mutants showed greater susceptibility to all of the Peronospora isolates, except Hiks1, as compared with either of the *pad2-1* or *pad3-1* single mutants. This evidence contrasts with our finding from infection of the double mutants with PsmES4326, that the *pad3-1* mutation had no effect on bacterial growth.

DISCUSSION

We set out to further characterize Arabidopsis *pad* mutants in an effort to clarify the role of camalexin in restricting pathogen growth. As we observed previously for the *pad1-1, pad2-1,* and *pad3-1* mutants (GLAZEBROOK and AUSUBEL 1994), none of the *pad* mutants were compromised for their ability to restrict the growth of PsmES4326 carrying the avirulence gene *avrRpt2,* providing further support for our previous conclusion that camalexin does not play a major role in

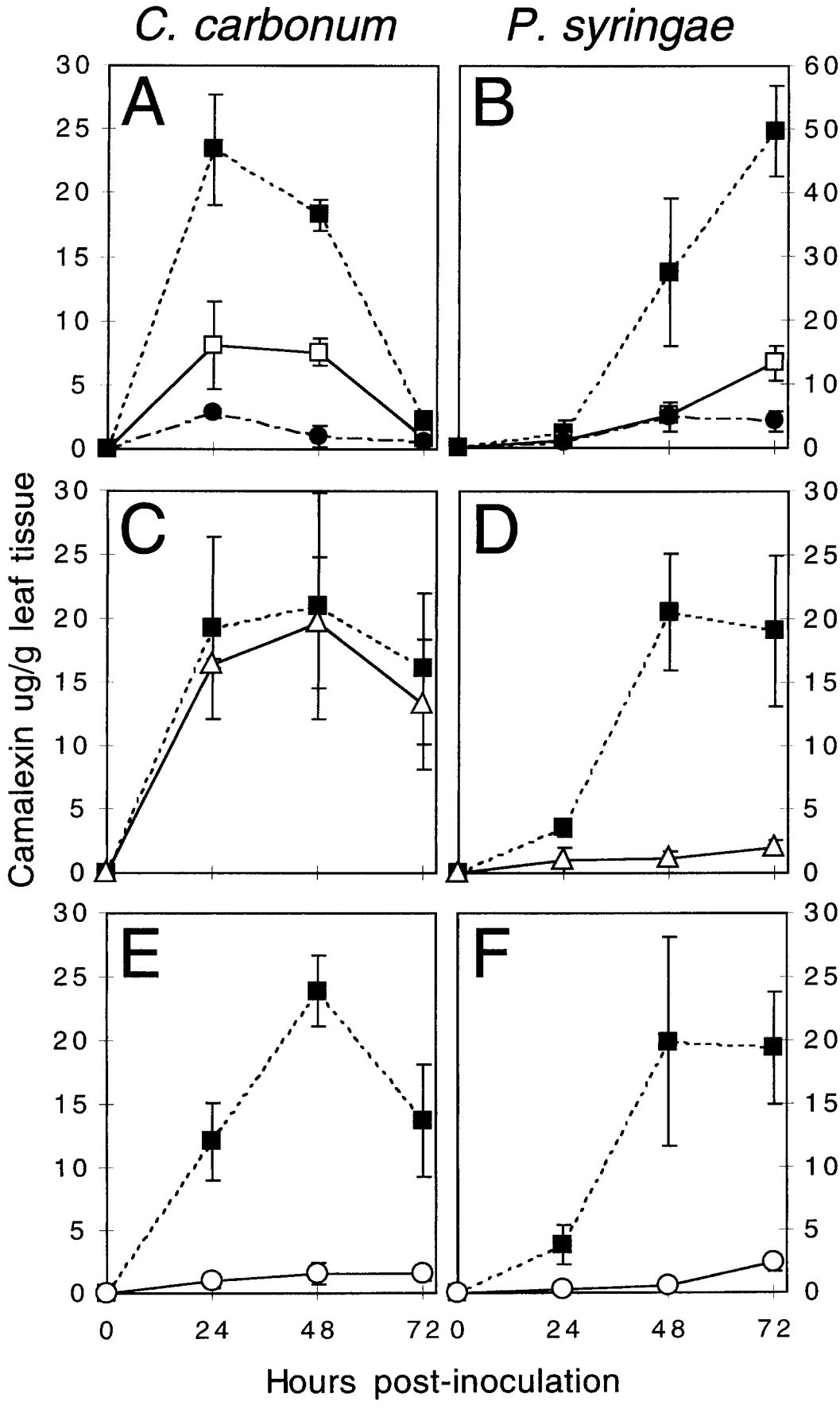

FIGURE 5.—Camalexin accumulation in wild-type and *pad* mutant plants in response to infection by *C. carbonum* or *P. syringae*. Plants were infected and samples were assayed at various times using the large-scale camalexin assay as described in MATERIALS AND METHODS. Each point represents the mean of three replicate samples. Error bars, representing standard deviation, are shown where they are larger then the symbols. ■, wild-type Col-0; □, *pad1-1*; ●, *pad2-1*; △, *pad4-1*; ○, *pad5-1*. The experiments shown in A and B, C and D, and E and F were performed at different times, so the data cannot be directly compared. No camalexin was detected in *pad3-1* mutants infected with either pathogen, or in the mock-inoculated controls included in all the experiments.

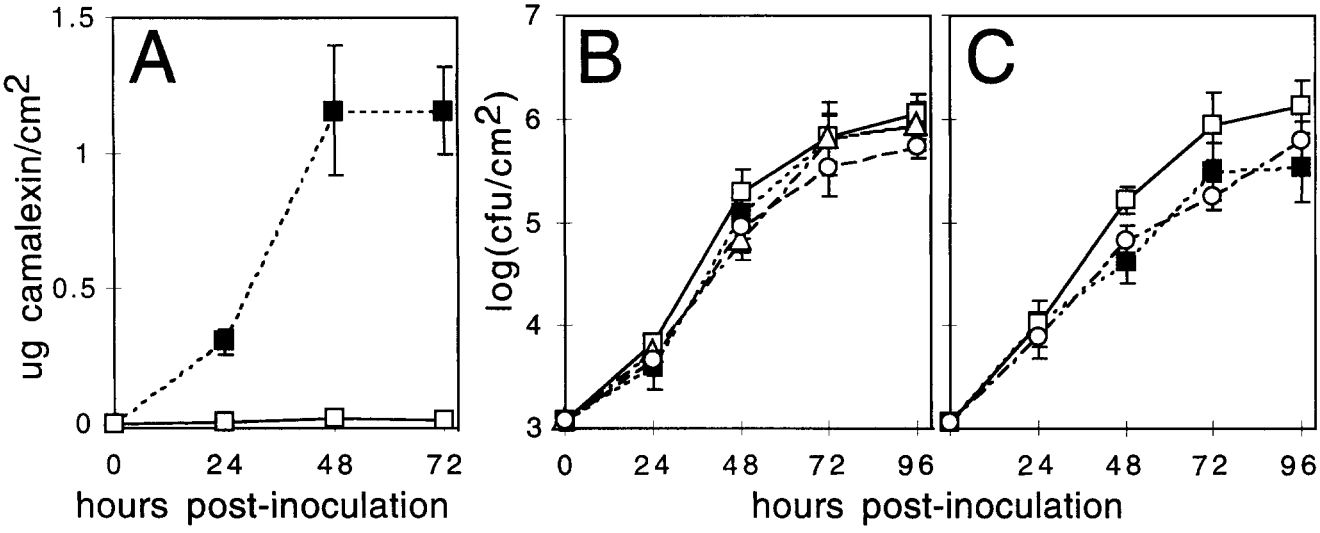

FIGURE 6.—Induction of camalexin by *X. campestris* pv. *campestris* BP109 (A) and growth of XccBP109 in wild-type and *pad* mutant plants (B and C). (A) Wild-type plants were infected with PsmES4326 or XccBP109 at a dose of 10^5 cells/cm^2 leaf area. At the indicated times, samples were cut from infected leaves and camalexin was assayed using the small-scale assay described in MATERIALS AND METHODS. Each point represents the mean and standard deviation of six replicate samples. ■, PsmES4326; □, XccBP109. (B) Wild-type, *pad1-1*, *pad2-1*, or *pad3-1* plants were infected with XccBP109. At the indicated times, samples were cut from infected leaves, and bacterial densities were determined. Each point represents the mean and standard deviation of six replicate samples. ■, Col-0; □, *pad1-1*; △, *pad2-1*; ○, *pad3-1*. (C) Wild-type, *pad4-1*, or *pad5-1* plants were infected with XccBP109, and bacterial growth was determined as described for B. ■, Col-0; □, *pad4-1*; ○, *pad5-1*. The experiments shown in panels B and C were performed at different times, therefore the results cannot be directly compared.

gene-for-gene resistance to *P. syringae* strains carrying either of the avirulence genes *avrRpt2* or *avrRpm1* (GLAZEBROOK and AUSUBEL 1994). *PAD4*, like the *PAD1* and *PAD2* genes described previously (GLAZEBROOK and AUSUBEL 1994), is involved in restricting growth of PsmES4326. In contrast, no significant effect of mutations in *PAD3* or *PAD5* on bacterial growth were detected. The finding that *pad4-1* has no effect on camalexin synthesis in response to *C. carbonum* inoculation revealed that the product of the *PAD4* gene is probably involved in pathogen recognition or signal transduction leading to activation of camalexin synthesis. With the exception of *PAD5*, all of the *PAD* genes were required for full expression of resistance to Peronospora infection.

If *PAD4* encodes a regulatory factor, this factor is required for activation of defense mechanism(s) in response to PsmES4326 or most incompatible Peronospora isolates, but not in response to *C. carbonum* or Peronospora isolate Hiks1. It is known that signaling in response to avirulent, or incompatible pathogens, is very different from signaling in response to virulent pathogens (LAMB *et al.* 1989). However, the *pad4-1* phenotype does not fit this pattern, since PsmES4326 is virulent, while *C. carbonum* and the Peronospora isolates we studied are not. This observation is consistent with the intriguing possibility that several different signaling pathways may lead to activation of defense mechanisms and that the determination of which pathways operate in response to different pathogens is not necessarily correlated with pathogen virulence or phylogeny. Other recent findings have been explained similarly. For example, the Arabidopsis *eds1* mutant is extremely susceptible to many Peronospora isolates that are incompatible on the wild-type parent, exhibits enhanced susceptibility to the virulent *P. syringae* strain PstDC3000, but is unaffected in resistance to PstDC3000 carrying the avirulence gene *avrB* (PARKER *et al.* 1996). *EDS1* and *PAD4* are almost certainly different genes, since *eds1* has no defect in camalexin synthesis in response to PsmES4326 (E. E. ROGERS and F. M. AUSUBEL, unpublished results). Another example of multiple pathogen response pathways in plants is the differential response to *P. syringae* strains carrying different avirulence genes. Recognition of *P. syringae* strains carrying the avirulence gene *avrRpt2*, mediated by the plant *R* gene *RPS2*, leads to expression of different transcripts than recognition of *P. syringae* strains carrying the avirulence gene *avrRpm1*, mediated by the *R* gene *RPM1* (REUBER and AUSUBEL 1996; RITTER and DANGL 1996). This is despite the fact that *RPS2* and *RPM1* encode similar proteins (BENT *et al.* 1994; MINDRINOS *et al.* 1994; GRANT *et al.* 1995).

We have now characterized mutations in three *PAD* genes, *PAD1*, *PAD2*, and *PAD4*, that cause increased susceptibility to PsmES4326, and mutations in two *PAD* genes, *PAD3* and *PAD5*, that have no significant effect. As described in *Introduction*, an explanation for these results hinges on whether the *PAD1*, *PAD2*, and *PAD4* genes encode camalexin biosynthetic enzymes or pleiotropic regulators of camalexin synthesis and other defense responses. The results of this study strongly suggest that *PAD4* encodes a regulator. We have not yet

TABLE 1

Asexual reproduction of *Peronospora parasitica* isolates in *pad* mutants

Arabidopsis Accession	Peronospora isolates[a]					
	Emoy2 (*4-IV*)	Emwa1 (*4-IV*)	Cala2 (*2-IV*)	Hiks1 (*7-I*)	Hind4 (*19-II, 4?-IV*)	P-006 ND
Col-wildtype	R/L[b]	R	R	N	N	N
Col-*pad1-1*	N	N	N	N	N	N
	O(—)[c]	O(—)	O(—)	O(—)	O(—)	
Col-*pad2-1*	L-M	N	N	N	R	N
	11 (2.4)	O(—)	O(—)	O(—)	3 (0.5)	
Col-*pad3-1*	L	N	N	N	R	N
	9 (1.1)	O(—)	O(—)	O(—)	3 (0.5)	
Col-*pad4-1*	H	H	H	N	M-H	N
	20 (3.0)	22 (2.6)	22 (1.5)	O(—)	15 (0.7)	
Col-*pad5-1*	N	N	N	N	N	N
Col-*pad1-1 pad2-1*	L	N	L-M	N	R	N
	6 (2.6)	O(—)	6 (2.3)	O(—)	3 (0.6)	
Col-*pad1-1 pad3-1*	M	N	L	L-M	M	N
	14 (1.0)	O(—)	6 (2.6)	5 (0.5)	13 (1.7)	
Col-*pad2-1 pad3-1*	H	M	L-M	N	M	N
	20 (2.4)	13.2 (1.5)	8 (2.8)	O(—)	18 (1.0)	

[a] Isolate and the corresponding *RPP* gene indicated in parentheses (arabic numerals refer to the resistance locus number, roman numerals refer to the chromosome to which the resistance locus has been mapped). ND = not designated. For Hind4, different *RPP* loci were identified between two inbred Arabidopsis mapping populations: a single locus *RPP19* was mapped on chromosome *II* using F9 Col-5 × Nd-1, and a single unnumbered locus was mapped near *RPP4* on chromosome *IV* using F8 Ler-0 × Col-4 (C. CAN and E. B. HOLUB, unpublished results). *P-006* represents a different *forma specialis* as an isolate obtained from *Brassica oleracea.*

[b] Qualitative rating of asexual reproduction: H, heavy; M, medium; L, low; R, rare; N, none. Assessments were made from pots that each contained 10–20 seedlings; ca. 200 seedlings were observed overall for each combination of mutant and parasite isolate in four separate experiments, including two blind experiments.

[c] Quantitative measure of reproduction measured as the mean number of sporangiophores per cotyledon and standard error (in parentheses). Data was only obtained from the fourth experiment in which five to nine seedlings were sampled for each combination of host and parasite.

investigated if *PAD4* affects expression of other defense responses; this work is planned. It will also be necessary to determine the function of the *PAD1* and *PAD2* genes to assess whether the biochemical model or the regulatory model is a better explanation of the phenotypes of *pad1, pad2, pad3,* and *pad5* mutants. If *PAD1, PAD2,* and *PAD4* are pleiotropic regulators, then camalexin probably does not play a major role in limiting growth of PsmES4326. While it may seem unlikely that *PAD1, PAD2,* and *PAD4* all encode pleiotropic regulators, it is a realistic possibility in light of the apparent complexity of the signal transduction pathways leading to defense gene expression.

Our study of the effects of *pad* mutations on *RPP* genes for resistance to Peronospora isolates revealed a complex pattern. The *pad1-1, pad2-1,* and *pad3-1* single mutants displayed only weakly increased susceptibility to Peronospora. However, the three double mutants, *pad1-1 pad2-1, pad1-1 pad3-1,* and *pad2-1 pad3-1,* all showed strongly enhanced susceptibility. Since additon of *pad1-1* to a *pad2-1* or *pad3-1* background causes increased susceptibility, *PAD1* must be important for Peronospora resistance. Analagous arguments can be made for *PAD2* and *PAD3.* Therefore, the increased susceptibility of the double *pad* mutants to Peronospora demonstrates that *PAD1, PAD2,* and *PAD3* all play significant roles in restricting sporulation of these parasites, even though this was not readily apparent from the phenotypes of the single mutants. This is an important observation, because it suggests that there may be sufficient functional redundancy in resistance responses that the effects of some mutations on resistance are only revealed when two or more such mutations are present. The presumptive regulatory gene *PAD4* also plays an important role in limiting Peronospora sporulation. We did not detect any effect of *PAD5* on Peronospora sporulation. By analogy with the phenotypes of the *pad1-1, pad2-1,* and *pad3-1* mutants, it is possible that an effect of *pad5-1* would be detected in a double mutant containing *pad5-1* together with another *pad* mutation.

One explanation for the Peronospora susceptibility phenotypes of the *pad* mutants is that camalexin does play an important role in inhibiting Peronospora sporulation but that the single *pad* mutants retain sufficient quantities of camalexin to inhibit Peronospora sporulation, whereas the double mutants do not. Because this explanation would have to apply to *pad3-1,* in which we

cannot detect any accumulation of camalexin, a more likely alternative may be that the *pad3-1* mutant accumulates a camalexin biosynthetic precursor, which has sufficient antimicrobial activity to inhibit Peronospora, but this biosynthetic intermediate is less toxic to Peronospora than the level of camalexin present in wild-type plants. Addition of the *pad1-1* or *pad2-1* mutations might reduce the level of this precursor, causing increased susceptibility to the parasite. If camalexin, or related antimicrobial compounds, do play a significant role in downy mildew resistance of Arabidopsis, there would be a contrast with our results using avirulent *P. syringae* strains, which show that camalexin synthesis does not play a major role in resistance. It is possible that Peronospora is more sensitive to camalexin than is PsmES4326 and that this accounts for the results. This hypothesis will be difficult to test because Peronospora is an obligate parasite so definitive experiments must be conducted *in planta*.

An alternative explanation for the Peronospora data is that camalexin is not important for resistance and that all of the *PAD* genes that contribute to resistance do so as a consequence of pleiotropic effects on other defense responses. This model requires that *PAD3*, as well as *PAD1*, *PAD2*, and *PAD4*, is a pleiotropic regulator of defense responses. To explain the *P. syringae* data, it would be necessary to postulate that camalexin synthesis is regulated via at least two pathways, one including *PAD1*, *PAD2*, and *PAD4* that is required for camalexin synthesis and other responses important for limiting growth of PsmES4326 and one including *PAD3* that is required for camalexin synthesis and other responses that are not important for limiting PsmES4326 but are important for Peronospora resistance. This model is quite complicated, but there do not appear to be any simple models that explain all of the phenotypes of the *pad* mutants. The alternative models clearly demonstrate that mutational analyses alone will not determine the role of camalexin in disease resistance; the genes must be cloned as the next step in resolving the issue.

The Peronospora isolates examined in this study varied in the level of sporulation they exhibited on particular plant genotypes. As these isolates are not isogenic lines, this variation could be explained by several factors, including differences among the isolates in their ability to induce camalexin synthesis and/or other defense responses (*e.g.*, functional differences among the host's *RPP* genes) and differences among isolates in their sensitivity to camalexin and/or other defense responses. Such genetic variability of host and parasite will be particularly interesting in light of recent observations that different bacterial resistance genes can activate different host response genes (REUBER and AUSUBEL 1996; RITTER and DANGL 1996). Such phenomena may explain, for example, why resistance to the Peronospora isolate Hiks1 appears to be largely unaffected by the *pad* mutations; perhaps the *RPP7* gene responsible for recognition of this isolate involves a different signaling pathway for host responses than other *RPP* genes.

Genetic dissection of host defense responses using the Arabidopsis model system is proving to be a powerful tool for identifying components of the plant defense system that would be difficult to detect by other means. Our analysis of the *pad* mutants has resulted in the identification of *PAD4*, a gene involved in control of expression of defense mechanisms, and revealed considerable complexity in the control of camalexin synthesis in response to different pathogens. The rapid progress in technologies supporting map-based cloning of Arabidopsis genes will facilitate cloning and sequencing of the *PAD* genes. These experiments should enable us to predict whether the *PAD* genes encode regulatory factors or biosynthetic enzymes, thus clarifying the role of camalexin in Arabidopsis/pathogen interactions.

This work was supported by the National Research Initiatives Competitive Grants Program grant 940–1199 and National Institutes of Health grant 48707 awarded to F.M.A., by University of Maryland Biotechnology Institute start-up funds awarded to J.G., and by a Ph.D. fellowship to F. M. from the Turkish government. E.B.H. was supported by the strategic competitive grant to Horticulture Research International from the UK Biotechnology and Biological Sciences Research Council. M.Z., I.K., and R.H. were supported by the Michigan State Agricultural Experiment Station and grant IBN-9220912 from the National Science Foundation.

LITERATURE CITED

ALEXANDER, D., R. M. GOODMAN, M. GUT-RELLA, C. GLASCOCK, K. WEYMANN *et al.*, 1993 Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. USA **90:** 7327–7331.

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN *et al.*, 1995 *Current Protocols in Molecular Biology*. Greene Publishing Associates/Wiley Interscience, New York.

BENT, A. F., B. N. KUNKEL, D. DAHLBECK, K. L. BROWN, R. SCHMIDT *et al.*, 1994 *RPS2* of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. Science **265:** 1856–1860.

BRINDLE, P. A., P. J. KUHN and D. R. THRELFALL, 1988 Biosynthesis and metabolism of sesquiterpenoid phytoalexins and triterpenoids in potato cell suspension cultures. Phytochemistry **27:** 133–150.

BROGLIE, K., I. CHET, M. HOLLIDAY, R. CRESSMAN, P. BIDDLE *et al.*, 1991 Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science **254:** 1194–1197.

BROWNE, L. M., K. L. CONN, W. A. AYER and J. P. TEWARI, 1991 The camalexins: new phytoalexins produced in the leaves of *Camelina sativa* (Cruciferae). Tetrahedron **47:** 3909–3914.

CHANNON, A. G., 1981 Downy mildew of *Brassicas*., pp. 321–339 in *The Downy Mildews*, edited by D. M. SPENCER. Academic Press, London.

CHAPPLE, C. C. S., B. W. SHIRLEY, M. ZOOK, R. HAMMERSCHMIDT and S. C. SOMERVILLE, 1994 Secondary metabolism in Arabidopsis, pp. 989–1030 in *Arabidopsis*, edited by E. M. MEYEROWITZ, and C. R. SOMERVILLE. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

CONN, K. L., J. P. TEWARI and J. S. DAHIYA, 1988 Resistance to *Alternaria brassicae* and phytolaexin elicitation in rapeseed and other crucifers. Plant Sci. **56:** 21–25.

DANGL, J. L., E. B. HOLUB, T. DEBENER, H. LEHNACKERS, C. RITTER *et al.*, 1992 Genetic definition of loci involved in *Arabidopsis*-pathogen interactions, pp. 393–417 in *Methods in Arabidopsis Research*, edited by C. KONCZ, J. SCHELL and N.-H. CHUA. World Scientific Publishing, Singapore.

DARVILL, A. G., and P. ALBERSHEIM, 1984 Phytoalexins and their elicitors-a defense against microbial infection in plants. Annu. Rev. Plant Physiol. **35:** 243–275.

DENNY, T. P., and H. D. VANETTEN, 1981 Tolerance by *Nectria haematococca* MP VI of the chickpea (*Cicer arietinum*) phytoalexins medicarpin and maackiain. Physiol. Plant Pathol. **19:** 419–437.

DIXON, R. A., and C. J. LAMB, 1990 Molecular communication in interactions between plants and microbial pathogens. Ann. Rev. Plant Physiol. Plant Mol. Biol. **41:** 339–367.

DONG, X., M. MINDRINOS, K. R. DAVIS and F. M. AUSUBEL, 1991 Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell **3:** 61–72.

ESSENBERG, M., M. L. PIERCE, B. HAMILTON, E. C. COVER, V. E. SCHOLES *et al.*, 1992 Development of fluorescent, hypersensitively necrotic cells containing phytoalexins adjacent to colonies of *Xanthomonas campestris* pv *malvacearum* in cotton leaves. Phys. Mol. Plant. Path. **41:** 85–99.

FLOR, H. H., 1971 Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. **9:** 275–296.

GLAZEBROOK, J., and F. M. AUSUBEL, 1994 Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc. Natl. Acad. Sci. USA **91:** 8955–8959.

GLAZEBROOK, J., E. E. ROGERS and F. M. AUSUBEL, 1996 Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics **143:** 973–982.

GRANT, M. R., L. GODIARD, E. STRAUBE, T. ASHFIELD, J. LEWALD *et al.*, 1995 Structure of the Arabidopsis *RPM1* gene which enables dual-specificity disease resistance. Science **269:** 843–846.

HAIN, R., H.-J. REIF, E. KRAUSE, R. LANGEBARTELS, H. KINDL *et al.*, 1993 Disease resistance results from foreign phytoalexin expression in a novel plant. Nature **361:** 153–156.

HOLUB, E. B., and J. L. BEYNON, 1996 Symbiology of mouse-ear cress (*Arabidopsis thaliana*) and oomycetes. Adv. Bot. Res. **24:** 227–272.

HOLUB, E. B., J. L. BEYNON and I. R. CRUTE, 1994 Phenotypic and genotypic characterization of interactions between isolates of *Peronospora parasitica* and accessions of *Arabidopsis thaliana*. Mol. Plant-Microbe Interact. **7:** 223–239.

KOCH, E., and A. J. SLUSARENKO, 1990 *Arabidopsis* is susceptible to infection by a downy mildew fungus. Plant Cell **2:** 437–445.

LAMB, C. J., M. A. LAWTON, M. DRON and R. A. DIXON, 1989 Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56:** 215–224.

LIU, D., K. G. RAGHOTHAMA, P. M. HASEGAWA and R. A. BRESSAN, 1994 Osmotin overexpression in potato delays development of disease symptoms. Proc. Natl. Acad. Sci. USA **91:** 1888–1892.

LONG, M., P. BARTON-WILLIS, B. J. STASKAWICZ, D. DAHLBECK and N. T. KEEN, 1985 Further studies on the relationship between glyceollin accumulation and the resistance of soybean leaves to *Pseudomonas syringae* pv. *glycinea*. Phytopathology **75:** 235–239.

LUCY, M., P. S. MATTHEWS and H. D. VANETTEN, 1988 Metabolic detoxificiation of the phytoalexins maackiain and medicarpin in *Nectria hematococca* field isolates: relationship to virulence on chickpea. Physiol. Mol. Plant Pathol. **33:** 187–199.

MINDRINOS, M., F. KATAGIRI, G.-L. YU and F. M. AUSUBEL, 1994 The *A. thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell **78:** 1856–1860.

MITTLER, R., and E. LAM, 1996 Identification, characterization and purification of a tobacco endonuclease activity induced upon hypersensitive cell death. Plant Cell **7:** 1951–1962.

MOSS, A. N., I. R. CRUTE and J. A. LUCAS, 1994 Laboratory production of oospores of *Peronospora parasitica* (crucifer downy mildew) and the recovery and characterization of sexual progeny from crosses between isolates with different host specificity. Plant Pathol. **43:** 713–725.

PARKER, J. E., E. B. HOLUB, L. N. FROST, A. FALK, N. D. GUNN *et al.*, 1996 Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. Plant Cell **8:** 2033–2046.

PEDRAS, M. S. C., and G. SEGUIN-SWARTZ, 1992 The blackleg fungus: phytotoxins and phytoalexins. Can. J. Plant Pathol. **14:** 67–75.

PONSTEIN, A. S., S. A. BRES-VLOEMANS, M. B. SELA-BUURLAGE, P. J. M. VANDENELZEN, L. S. MELCHERS *et al.*, 1994 A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. Plant Physiol. **104:** 109–118.

PUEPPKE, S. G., and H. D. VANETTEN, 1976 The relation between pisatin and the development of *Aphanomyces euteiches* in diseased *Pisum sativum*. Phytopathology **66:** 1174–1185.

REUBER, T. L., and F. M. AUSUBEL, 1996 Isolation of *Arabidopsis* genes that differentiate between disease resistance responses mediated by the *RPS2* and *RPM1* disease resistance genes. Plant Cell **8:** 241–249.

RITTER, C., and J. L. DANGL, 1996 Interference between two specific pathogen recognition events mediated by distinct plant disease resistance genes. Plant Cell **8:** 251–257.

ROGERS, E. E., J. GLAZEBROOK and F. M. AUSUBEL, 1996 Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis*-pathogen interactions. Mol. Plant-Microbe Interact. **9:** 748–757.

ROUXEL, T., A. KOLLMANN, L. BOULIDARD and R. MITHEN, 1991 Abiotic elicitation of indole phytoalexins and resistance to *Leptosphaeria maculans* with *Brassicaceae*. Planta **184:** 271–278.

RYERSON, D. E., and M. C. HEATH, 1996 Cleavage of DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. Plant Cell **8:** 393–402.

SCHLUMBAUM, A., F. MAUCH, U. VOEGLI and T. BOLLER, 1986 Plant chitinases are potent inhibitors of fungal growth. Nature **324:** 365–367.

SELA-BUURLAGE, M. B., A. S. PONSTEIN, S. A. BRES-VLOEMANS, L. S. MELCHERS, P. J. M. VANDENELZEN *et al.*, 1993 Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol. **101:** 857–863.

SMITH, D. A., 1982 Toxicity of phytoalexins, pp. 218–252 in *Phytoalexins*, edited by J. A. BAILEY and J. W. MANSFIELD. John Wiley and Sons, New York.

TERRAS, F. R. G., H. M. E. SCHOOFS, M. F. C. DEBOLLE, F. V. LEUVEN, S. B. REES *et al.*, 1992 Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J. Biol. Chem. **267:** 15301–15309.

TERRAS, F. R. G., K. EGGERMONT, V. KOVALEVA, N. V. RAIKHEL, R. W. OSBORN *et al.*, 1995 Small cysteine-rich antifungal proteins from radish: Their role in host defense. Plant Cell **7:** 573–588.

TSUJI, J., S. C. SOMERVILLE and R. M. HAMMERSCHMIDT, 1991 Identification of a gene in *Arabidopsis thaliana* that controls resistance to *Xanthomonas campestris* pv. *campestris*. Physiol. Mol. Plant Pathol. **38:** 57–65.

TSUJI, J., E. P. JACKSON, D. A. GAGE, R. HAMMERSCHMIDT and S. C. SOMERVILLE, 1992 Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv. *syringae*. Plant Physiol. **98:** 1304–1309.

WANG, H., J. LI, R. M. BOSTOCK and D. G. GILCHRIST, 1996 Apoptosis: a functional paradigm for programmed cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell **8:** 375–391.

WEISS, B. D., M. A. CAPAGE, M. KESSEL and S. A. BENSON, 1994 Isolation and characterization of a generalized transducing phage for *Xanthomonas campestris* pv. *campestris*. J. Bacteriol. **176:** 3354–3359.

WHALEN, M. C., R. W. INNES, A. F. BENT and B. J. STASKAWICZ, 1991 Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell **3:** 49–59.

WOLOSHUK, C. P., J. S. MEULENHOFF, M. SELA-BUURLAGE, P. J. M. VANDENELZEN and B. J. C. CORNELISSEN, 1991 Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell **3:** 619–628.

WU, G., B. J. SHORTT, E. B. LAWRENCE, E. B. LEVINE, K. C. FITZSIMMONS *et al.*, 1995 Disease resistance conferred by expression of a gene encoding H2O2-generating glucose oxidase in transgenic potato plants. Plant Cell **7:** 1357–1368.

ZHU, Q., E. A. MAHER, S. MASOUD, R. DIXON and C. J. LAMB, 1994 Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Biotechnology **12:** 807–812.

Communicating editor: V. SUNDARESAN