MPMI Vol. 9, No. 8, 1996, pp. 748–757. Publication no. M-1996-0903-01R.

Mode of Action of the *Arabidopsis thaliana* Phytoalexin Camalexin and Its Role in *Arabidopsis*-Pathogen Interactions

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Received 2 May 1996. Accepted 30 July 1996.

The virulent *Arabidopsis thaliana* pathogen *Pseudomonas syringae* pv. *maculicola* strain ES4326 (*Psm* ES4326) and other gram-negative bacteria are sensitive to camalexin (3-thiazol-2′-yl-indole), the *Arabidopsis* phytoalexin. Furthermore, *Psm* ES4326 is unable to degrade camalexin or to become tolerant to it. Apparently, *Psm* ES4326 is a successful pathogen even though it elicits synthesis of a host phytoalexin to which it is sensitive. Assays of membrane integrity revealed that, like other phytoalexins, camalexin disrupts bacterial membranes, suggesting that camalexin toxicity is a consequence of membrane disruption. A screen for camalexin-resistant mutants of *Psm* ES4326 yielded only partially resistant mutants, which displayed partial resistance in both killing and membrane integrity assays. These mutants were also resistant to low concentrations of tetracycline and nalidixic acid, suggesting that they were affected in components of the outer membrane. The mutants were not distinguishable from *Psm* ES4326 in virulence assays. Camalexin was toxic to *Arabidopsis* cells growing in tissue culture. However, comparison of the extent of cell death associated with disease symptoms in infected leaves of wild-type *Arabidopsis* and a camalexin-deficient mutant suggested that camalexin does not contribute significantly to cell death in infected tissue.

Additional keyword: plant-pathogen interaction.

Phytoalexins are low molecular weight antibacterial and antifungal compounds produced by plants in response to either pathogen infection or various abiotic elicitors. Dozens of phytoalexins with a variety of structures have been identified from various plants (Coxon 1982; Ingham 1982; Kuc 1982). A relatively newly described class of phytoalexins from brassicas are sulfur-containing compounds; one of these phytoalexins, camalexin (3-thiazol-2′-yl-indole), was first isolated from *Camelina sativa* (Browne et al. 1991). Tsuji and Somerville (1992) showed that camalexin was also synthesized by the model crucifer *Arabidopsis thaliana*, where it accumulates to high levels after infection with an avirulent *Pseudomonas syringae* pv. *syringae* strain (Tsuji et al. 1992). Camalexin has been shown to interfere with conidia germination of *Alternaria brassicae* (Browne et al. 1991) and to be toxic to *P. syringae* and *Cladosporium cucumerinum* (Tsuji et al. 1992).

We are interested in the effect of camalexin on both the plant and the pathogen in *Arabidopsis–P. syringae* interactions. In its response to *P. syringae, Arabidopsis* displays the common symptomatic features of the plant defense response, including the hypersensitive response (HR) (Bent et al. 1991; Dangl et al. 1991; Dong et al. 1991; Whalen et al. 1991) and systemic acquired resistance (SAR) (Cameron et al. 1994; Dempsey et al. 1993; Mauch-Mani and Slusarenko 1994; Uknes et al. 1993). The similarity between the *Arabidopsis–P. syringae* interaction and other well-studied plant-pathogen interactions extends to the molecular level. For example, the encoded products of the *Arabidopsis* resistance genes *RPS2* (Bent et al. 1994; Mindrinos et al. 1994) and *RPM1* (Grant et al. 1995), which confer resistance to *P. syringae* carrying the avirulence genes *avrRpt2* and *avrRpm1*, respectively, are remarkably similar in structure to resistance gene products from tobacco (Whitham et al. 1994), tomato (Martin et al. 1993), and flax (Ellis et al. 1995).

The role of phytoalexins in the plant defense response has primarily been studied in plant-pathogen systems in which *virulent* pathogens that cause disease on a *susceptible* host (so-called *compatible* interaction) can be directly compared with *avirulent* pathogens that elicit a strong defense response on a *resistant* host (so-called *incompatible* interaction). In many of these cases, there is a good correlation between the degree of host resistance and the level of phytoalexin accumulation. Well-studied examples of phytoalexin accumulation in plant-fungal interactions include accumulation of wyerone and wyerone acid in *Vicia faba* infected with *Botrytis* spp. (Smith 1982), accumulation of phaseollin and other isoflavonoid phytoalexins in *Phaseolus vulgaris–Colletotrichum lindemuthianum* interactions (Hargreaves and Bailey 1978), and accumulation of rishitin in potato tissues infected with *Phytophthora infestans* (Smith 1982). In the case of bacterial pathogens, sesquiterpenoid phytoalexins produced by cotton accumulate around sites of infection by avirulent but not virulent isolates of *Xanthomonas campestris* pv. *malvacearum* (Essenberg et al. 1992). However, virulent pathogens, such as

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Sclerotinia sclerotiorum, can induce phytoalexin biosynthesis in bean leaves (Smith and Banks 1986).

Not all plant-pathogen interactions can be readily catalogued as being either compatible or incompatible. Rather, there is a continuum of interaction phenotypes. In some of these types of interactions, phytoalexin accumulation correlates with resistance. For example, levels of glyceollin accumulation in various cultivars of soybean after infection with *P. syringae* pv. *glycinea* were inversely correlated with bacterial multiplication (Long et al. 1985). *Brassica campestris* subsp. *rapifera* was less susceptible to and produced more phytoalexin in response to infection with *Alternaria brassicae* than four rapeseed cultivars (Conn et al. 1988). On the other hand, there are also examples of plant-pathogen interactions in which there is a continuum of interaction phenotypes but phytoalexin accumulation does not correlate with resistance. For example, Rouxel et al. found that the total accumulation of indole phytoalexins by various *Brassica* spp. could not be correlated to the resistance of each species to the fungal pathogen *Leptosphaeria maculans* (Rouxel et al. 1991). Similarly, no clear relationship between phytoalexin accumulation and resistance was observed in interactions between various cruciferous plants and virulent and avirulent isolates of *L. maculans* (Pedras and Seguin-Swartz 1992).

There are also examples of phytoalexin accumulation in compatible plant-pathogen interactions. These include the induction of pisatin by the compatible fungus *Aphanomyces euteiches* (Pueppke and VanEtten 1976), of rishitin by a compatible race of *Phytophthora infestans* (Brindle et al. 1988), the induction of pisatin by compatible strains of the fungus *Nectria hematococca* (Denny and VanEtten 1981; Lucy et al. 1988) and the induction of spirobrassinin by virulent races of *Leptosphaeria maculans* (Pedras and Seguin-Swartz 1992). Similarly, our laboratory previously reported that the virulent pathogen *P. syringae* pv. *maculicola* strain ES4326 (*Psm* ES4326) elicits the synthesis of high camalexin levels in the *Arabidopsis* ecotype Columbia (Glazebrook and Ausubel 1994).

To help elucidate the role(s) of phytoalexins in plant-pathogen interactions, our laboratory isolated three *p*hytoa*l*exin-*d*eficient *(pad) Arabidopsis* mutants (Glazebrook and Ausubel 1994). Whereas the response of all three *pad* mutants to avirulent strains of *P. syringae* was unchanged, two of the *pad* mutants, *pad1* and *pad2*, were more susceptible than wild-type *Arabidopsis* to infection by virulent *P. syringae* strains. This suggested that camalexin does not play a major role in limiting the growth of avirulent *P. syringae* strains during the HR, but may play a role in limiting the growth of virulent *P. syringae* strains. Before the isolation of these mutants, the only evidence that suggested a role for phytoalexins in compatible interactions was the induction of phytoalexins by virulent pathogens.

We sought to further characterize the role of camalexin in the compatible interaction of *Arabidopsis* ecotype Columbia with the virulent pathogen *Psm* ES4326 because most of the previous work concerning the roles of phytoalexins in plant-pathogen interactions has focused on incompatible interactions. Since the mode of toxicity of Brassica sulfur-containing phytoalexins is not known, we first investigated the mode of camalexin toxicity. Previous studies of many phytoalexins showed that relatively high concentrations (10^{-4} M) are necessary for in vitro inhibition, and that phytoalexins are multi-site toxins that disrupt membrane function (Smith 1982). There are several examples of phytoalexins that damage fungal and/or bacterial membranes, including kievitone (Smith 1982), phaseollin (Smith 1982), maackiain (Smith 1982), glycinol (Weinstein and Albersheim 1983), glyceollin (Giannini et al. 1988), coumestrol (Yoshikawa et al. 1987), capsidiol (Turelli et al. 1984), rishitin (Robertson et al. 1985), and oryzalexin D (Sekido and Akatsuka 1987). In support of the conclusion that phytoalexins do not target specific metabolic processes, we found no reports in the literature of pathogen mutants that are constitutively resistant to a phytoalexin.

We also investigated phytotoxic effects of camalexin. Rishitin, a potato phytoalexin, inhibits pollen germination and lyses tomato and potato protoplasts, and slows growth and causes cell death in suspension cultures of bean and tobacco (Smith 1982). Similarly, because pisatin-treated pea cells exhibit the same type of membrane damage as cells in infected tissue, it has been proposed that pisatin toxicity is the major cause of wilting in infected tissues (Shiraishi et al. 1975). Despite the cytotoxicity of some phytoalexins, it has been demonstrated that in the case of at least some incompatible interactions, phytoalexin toxicity is not a cause of hypersensitive cell death (Keen et al. 1981). It is not clear, however, if phytoalexins are a major cause of plant cell death associated with disease symptoms in tissue infected with virulent pathogens. There are several possible mechanisms by which a plant could protect itself from the toxic effects of its own phytoalexin, including sequestration of phytoalexins away from host tissues.

In the work reported here we show that, like previously studied phytoalexins, camalexin rapidly disrupts the integrity of *P. syringae* membranes. Despite repeated attempts, we were not able to isolate *P. syringae* mutants that exhibited resistance to high levels of camalexin, but we were able to isolate mutants that exhibited partial camalexin resistance. Taking advantage of the existence of *Arabidopsis pad* mutants, we demonstrate that camalexin does not play a major role in mediating *Arabidopsis* cell death during pathogen infection.

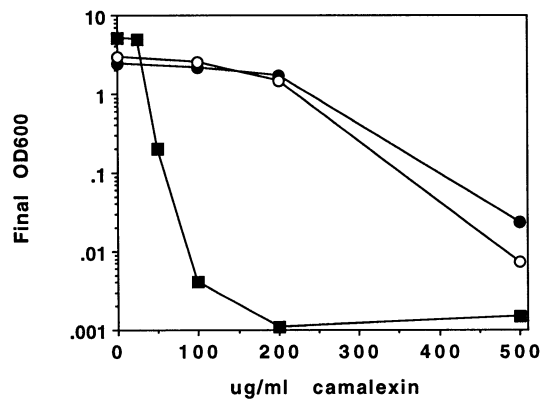

Fig. 1. Effect of camalexin on final density of a saturated microbial culture. Indicated concentrations of camalexin in King's medium B (for *Pseudomonas syringae* pv. *maculicola* strain ES4326), Luria-Bertani (for *Escherichia coli*), or YPD (for *Saccharomyces cerevisae*) with 5% (vol/vol) dimethyl sulfoxide were innoculated with approximately 1 × 10^6 bacterial cells/ml and incubated at 28°C for *Psm* ES4326 and 37°C for *E. coli*; after 24 h, the OD_{600} of each culture was measured as indication of bacterial density. Symbols: circles, *Psm* ES4326; triangles, *E. coli*; squares, *S. cerevisae*. This experiment was repeated with similar results.

RESULTS

Toxicity of camalexin to various microbes.

We observed that there is a critical threshold concentration of camalexin for killing bacteria and fungi, the particular concentration depending on the microorganism. Below the threshold concentration, camalexin had only a modest effect on viability; above the threshold, viability dropped by several orders of magnitude. For the gram-negative bacteria *E. coli* K12, *Psm* ES4326, *P. syringae* pv. *phaseolicola* 3121, and *Xanthomonas campestris* pv. *campestris* BP109, and the gram-positive bacteria *L. monocytogenes* 10403S, the threshold concentration was between 250 and 500 μg/ml (1.3 to 2.7 mM). For the gram-positive bacteria *B. subtilis* and for the fungi *F. oxysporum* and *S. cerevisiae*, the threshold concentration was significantly lower, approximately 20 to 50 μg/ml. Addition of subthreshold levels of camalexin (<200 μg/ml for *Psm* ES4326 or *E. coli* MM294; <25 μg/ml for *S. cerevisiae*) did not have a significant effect on viability (data not shown). Moreover, as illustrated in Figure 1, the final density to which each of these species would grow was not significantly affected by sublethal concentrations of camalexin. In addition, sublethal levels of camalexin (5 to 200 μg/ml) had no significant effect on the growth rate of *Psm* ES4326 (data not shown).

Interestingly, while King's medium B containing 500 μg of camalexin per ml was toxic to *Psm* ES4326, intracellular fluid from *Arabidopsis* leaves with camalexin added to a concentration of 500 μg/ml was not toxic to *Psm* ES4326. However, when *Psm* ES4326 cells were grown in intercellular fluid and then transferred to King's medium B containing camalexin, killing was indistinguishable from that observed when cells were grown in King's medium B, suggesting that intercellular fluid does not induce resistance to camalexin. Because camalexin was not degraded in intracellular fluid (data not shown), this result suggested that a component of intracellular fluid sequesters camalexin in a nontoxic form.

Mode of action of camalexin.

Many phytoalexins have been shown to damage cell membranes (Smith 1982). Therefore, we tested the effect of camalexin on the membrane integrity of *Psm* ES4326 with proline uptake, ion leakage, protein leakage, and supernatant serine deaminase assays. Failure of bacteria to take up labeled proline is good evidence for membrane disruption (Fraimow et al. 1991; Galvez et al. 1991; Ruhr and Sahl 1985; Schuller et al. 1989). Addition of 500 μg/ml camalexin not only immediately stopped proline uptake by *Psm* ES4326 (Fig. 2), but

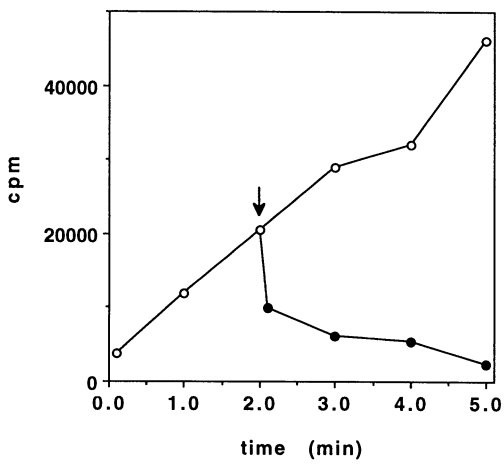

Fig. 2. Effects of camalexin on proline uptake by *Pseudomonas syringae* pv. *maculicola* strain ES4326. Counts per minute indicate ^{14}C-proline content of chloramphenicol-treated *Psm* ES4326. ^{14}C-proline was added at 0 min, and camalexin in dimethyl sulfoxide (DMSO), final concentrations 500 μg/ml and 5% (vol/vol), respectively (closed circles) or just 5% DMSO (open circles), was added as indicated by arrow. Cells were collected on a filter, washed, and counted. This experiment was repeated at least four times, with similar results.

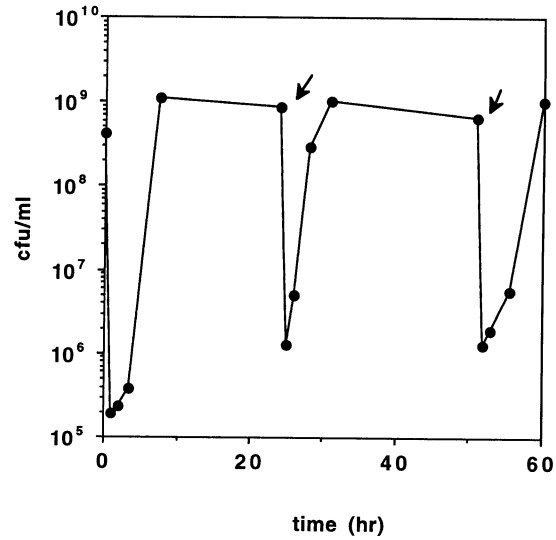

Fig. 3. Camalexin-mediated killing of *Pseudomonas syringae* pv. *maculicola*. Camalexin in dimethyl sulfoxide, final concentrations 500 μg/ml and 5% (vol/vol), respectively, was added to *P. syringae* pv. *maculicola* strain ES4326 in King's medium B. Culture was diluted 1:10 into the same media at times indicated by arrows. Colonies were plated on King's medium B plates containing streptomycin at indicated times. Similar results were obtained in an independent experiment.

Table 1. Assays demonstrating damage to *Pseudomonas syringae* pv. *maculicola* strain ES4326 membranes by camalexin[a]

Assay	No treatment	DMSO 5% (vol/vol)	Camalexin (100 μg/ml)	Camalexin (500μg/ml)	Toluene 1%(vol/vol)
Ion leakage	1.0 ± 0.1[b]	1.2 ± 0.2	2.0 ± 0.2	1.9 ± 0.1	2.2 ± 0.2
Serine deaminase activity	1.0 ± 0.1[c]	1.0 ± 0.1	1.9 ± 0.1	3.9 ± 0.1	3.3 ± 0.1
Protein leakage	1.0 ± 0.1[d]	1.0 ± 0.3	1.1 ± 0.1	2.7 ± 0.2	3.8 ± 1.1

[a] Values normalized by defining no treatment as 1.0. Assays performed as described in the Materials and Methods after 5-min incubations in dimethyl sulfoxide (DMSO) and/or camalexin. Values shown represent two or more independent experiments.
[b] Actual value: 20.5 ± 2.0 μS.
[c] Actual value: OD_{440} = 0.30 ± 0.03.
[d] Actual value: 612 ± 63 cpm.

also caused the efflux of most of the labeled proline taken up prior to camalexin treatment. In three other tests of membrane integrity, camalexin behaved similarly to toluene, a known membrane disruptive agent (De Smet et al. 1978; Jackson and DeMoss 1965). Camalexin caused increases in ion leakage, protein leakage and supernatant serine deaminase activity similar to the increases caused by toluene (Table 1). Treatment with solvent alone, 5% (vol/vol) dimethyl sulfoxide (DMSO), did not cause an increase in any of the three parameters measured. Taken together, these results show that camalexin exerts a deleterious effect on membrane integrity, and suggest that this effect is the cause of camalexin toxicity.

Kinetics of camalexin killing of *Psm* ES4326.

After the addition of a lethal dose of camalexin to a culture (≥250 μg/ml), *Psm* ES4326 CFU are reduced by several orders of magnitude within minutes (Fig. 3). Interestingly, however, as also shown in Figure 3, camalexin-treated cultures eventually regained their original titer, most likely due to the survival and subsequent growth of a few cells that escaped camalexin killing. This phenomenon is apparently due to camalexin becoming associated with the dead cells rather than remaining free in solution, which effectively reduces the camalexin concentration to a low enough level to allow some cells to survive. We based this conclusion on the following observations. First, ≥60% of the camalexin added to a culture can be found in pelleted cells; the camalexin concentration in the supernatant therefore drops below the threshold for killing. Second, the camalexin extracted from pelleted cells by the same hot methanol procedure used to extract camalexin from plant tissue retains full killing activity (data not shown). Third, the *Psm* ES4326 cells that escaped killing by the initial dose of camalexin and grew to a high titer were just as susceptible to camalexin killing as the original culture (Fig. 3). Moreover, as shown in Figure 3, the cycle of camalexin killing and subsequent grow-out could be repeated several times without any apparent increase in camalexin tolerance or resistance. These results suggest that camalexin becomes dissolved in the *Psm* ES4326 cell membranes and are consistent with the lipophilic nature of camalexin and the hypothesis that camalexin kills *Psm* ES4326 by disrupting membrane function.

***Psm* ES4326 responses to camalexin.**

Because *Psm* ES4326 is a vigorous *Arabidopsis* pathogen that elicits high levels of camalexin accumulation during a virulent infection, we determined whether *Psm* ES4326 can circumvent the toxic effect of camalexin. We tested whether *Psm* ES4326 can degrade camalexin by adding 50 μg of camalexin per ml (5- to tenfold less than the lethal dose) to a culture of *Psm* ES4326 at 1×10^8 CFU/ml. Over 5 days at 28°C, the total amount of camalexin in media with and without *Psm* ES4326 remained unchanged and the cells grew to a high density (data not shown). In addition, pre-exposure to low, noninhibitory concentrations of camalexin, 10 to 100 μg/ml, did not affect the threshold level of camalexin required to kill *Psm* ES4326 (data not shown). *Psm* ES4326 does not seem to have the capacity to degrade camalexin, or to become tolerant to it in response to low camalexin concentrations.

***P. syringae* camalexin-resistant mutants.**

We isolated *Psm* ES4326 mutants with decreased sensitivity to camalexin. *Psm* ES4326 was mutagenized with MNNG to 95% lethality, which gave a rate of approximately 1 in 1,000 Lac^- colonies (wild-type *Psm* ES4326 is Lac^+). Six independent, putatively camalexin-resistant mutants were selected from 5×10^9 mutagenized cells as described in Materials and Methods. We calculated that these six mutants arose at a frequency of 1 in 8×10^8 mutagenized cells, a much lower rate than the rate of mutation to a Lac^- phenotype. As shown in Figure 4, 500 μg of camalexin per ml reduced the CFU/ml of the six mutants by three to five orders of magnitude, in comparison to six orders of magnitude for the parent *Psm* ES4326. However, none of the six putative mutants were fully resistant to camalexin; the CFU/ml of the most resistant mutant was still reduced by three orders of magnitude within minutes after camalexin treatment. All six mutants were prototrophic and showed no obvious growth defects in rich or minimal media (data not shown). A similar mutant selection was carried out on 1×10^9 mutagenized *E. coli* MM294 cells; however, no camalexin-resistant mutants were obtained.

All the membrane integrity assays performed on the parent *Psm* ES4326 were repeated on two of the six mutants, #7 and #9. Figure 5A shows that camalexin still interfered with proline uptake in these two mutants. Importantly, however, both mutants were more resistant than wild-type *Psm* ES4326 to camalexin, as measured by their ability to take up ^{14}C-labeled proline in the presence of camalexin. Moreover, comparison of Figure 5A and B shows that the decreased sensitivity of mutants #7 and #9 closely parallels the decreased susceptibility to camalexin-mediated killing.

All six camalexin-resistant mutants were also resistant to low levels of nalidixic acid and tetracycline in comparison to *Psm* ES4326 (Table 2). As this indicates possible outer membrane alterations (Nikaido 1994), outer membranes and lipopolysaccharide were isolated from *Psm* ES4326 and all six mutants and analyzed following a variety of previously published methods (Hancock and Nikaido 1978; Poxton et al.

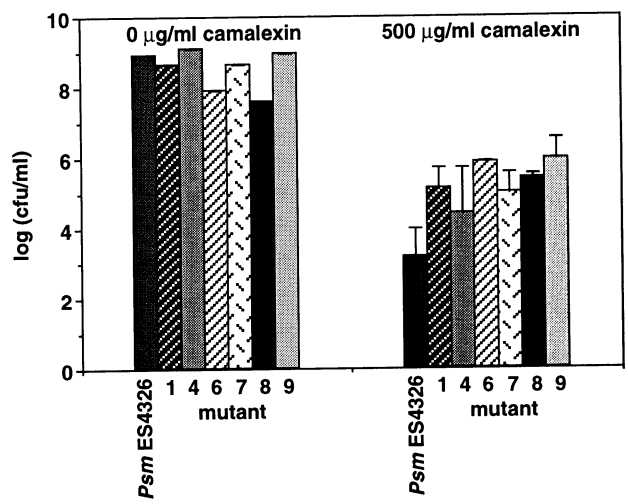

Fig. 4. Camalexin-mediated killing of *Pseudomonas syringae* pv. *maculicola* mutants partially resistant to camalexin. Cell density of *P. syringae* pv. *maculicola* strain ES4326 and all six mutants before and after addition of camalexin in dimethyl sulfoxide, final concentrations 500 μg/ml and 5% (vol/vol), respectively. Cells were plated on King's medium B containing streptomycin 10 min after addition of camlaexin. Values are mean and standard deviation of four independent replicates. Similar results were obtained in two independent experiments.

1985; Spratt 1977; Tsai and Frasch 1982). However, no differences between the parent and any of the mutants were discovered (data not shown).

P. syringae mutants partially resistant to camalexin are not more virulent.

P. syringae mutants that were fully resistant to camalexin could be used to demonstrate whether camalexin plays an important defense role; if camalexin tempered the virulence of *P. syringae*, camalexin-resistant mutants would be more virulent than wild type. However, Figure 6 shows that two mutants, #6 and #9, grew to the same density as the parent *Psm* ES4326 in *Arabidopsis* leaves. This result is difficult to interpret, as 500 μg of camalexin per ml kills at least 99.9% of the cells in a culture of mutants #6 or #9. Mutant #9 also grew to the same density as wild-type *Psm* ES4326 in the *pad1* mutant (data not shown).

Camalexin resistance may result from multiple mutations.

A concerted effort to clone the gene(s) responsible for the camalexin resistance phenotype of mutant #9 was unsuccessful. Because we did not know whether the resistance phenotypes were dominant or recessive, two different strategic approaches were used in the cloning experiments. First, assuming that resistance was dominant, we conjugated a genomic cosmid library constructed from mutant #9 DNA into wild-type *Psm* ES4326 and the transconjugants were subjected to the same camalexin resistance selection as the original mutagenized culture of *Psm* ES4326 described in Materials and Methods. However, no resistant cells were obtained. Second, assuming resistance was recessive, we conjugated a genomic cosmid library constructed from *Psm* ES4326 DNA into mutant #9. In this second strategy, because we were looking for loss of the resistant phenotype and because we could not use a camalexin killing assay on agar (due both to the limited solubility and availability of camalexin), we made the assumption that the same mutation was the cause of both the camalexin- and tetracycline-resistant phenotypes. We reasoned that using tetracycline resistance as a marker for camalexin resistance was valid because all six putative camalexin-resistant mutants were also more resistant to tetracycline. Individual transconjugant clones from the second conjugation were screened for any that were no longer able to grow on King's medium B plates containing 0.5 mg of tetracycline per ml. However, none were found.

Both of the libraries used in the attempt to identify the gene(s) responsible for the camalexin-resistant phenotype of mutant #9 were more than 99.5% representative and approximately 1,150 transconjugants were screened, more than enough to assure 99% probability of identifying the desired clone. Because of the very low rate of appearance of camalexin mutants, one likely explanation of our failure to identify a cosmid clone that would genetically complement the camalexin resistance phenotype is that the resistant phenotype of mutant #9 is due to mutations at at least two unlinked loci.

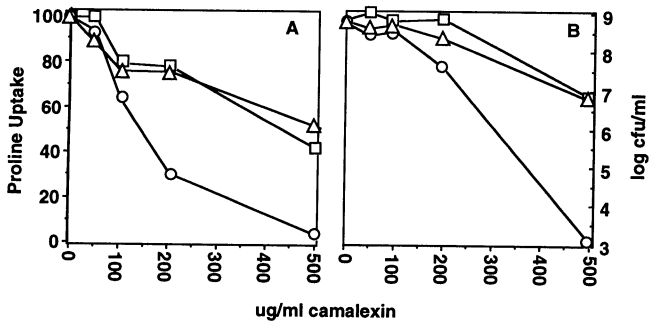

Fig. 5. Effects of camalexin on viability and proline uptake of *Pseudomonas syringae* pv. *maculicola* and mutant derivatives partially resistant to camalexin. Symbols: circles, *P. syringae* pv. *maculicola* strain ES4326; triangles, mutant #6; squares, mutant #9. For proline uptake, counts per minute indicate ^{14}C-proline content of chloramphenicol-treated *Psm* ES4326. ^{14}C-proline and various concentrations of camalexin in dimethyl sulfoxide (DMSO), final concentration 5% (vol/vol), were added to chloramphenicol-treated cells. After 90 s, cells were collected onto a filter, washed, and counted. For viability, various concentrations of camalexin in DMSO, final concentration 5% (vol/vol), were added to *Psm* ES4326 in King's medium B. Colonies were plated on King's medium B plates containing streptomycin after a 30-min incubation. Similar results were obtained in two independent experiments.

Table 2. Cross resistance of *Pseudomonas syringae* pv. *maculicola* strain ES4326 camalexin-resistant mutants to tetracycline and nalidixic acid

Strain	Tetracycline (μg/ml)	Nalidixic acid (μg/ml)
Mutant #9	0.5[a]	30[a]
Mutant #7	0.4	30
Mutant #6	0.4	30
Mutant #8	0.4	15
Mutant #4	0.2	5
Mutant #1	0.2	5
Psm ES4326	0.1	5

[a] Values represent the highest concentration of antibiotic on which the strains formed colonies on plates.

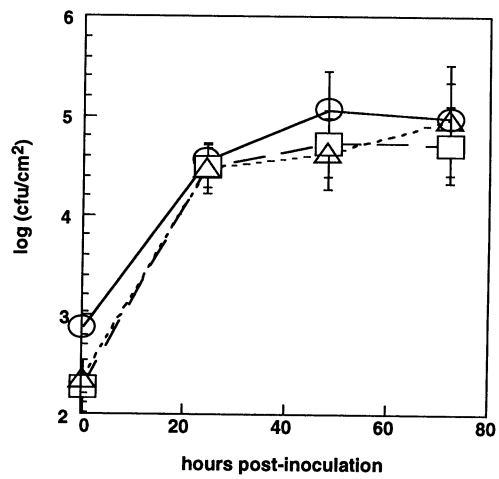

Fig. 6. Growth of *Pseudomonas syringae* pv. *maculicola* and mutant derivatives partially resistant to camalexin in *Arabidopsis* leaves. Wild-type Col-0 plants were infected with *P. syringae* pv. *maculicola* strain ES4326, mutant #6, or mutant #9, at a dose of 300 CFU/cm^2 leaf area. At indicated times, samples were cut from infected leaves and bacterial densities determined. Each point represents mean and standard deviation of six replicate samples. Symbols: circles, *Psm* ES4326; squares, mutant #6; triangles, mutant #9. Similar results were obtained with *Psm* ES4326 and mutant #9 in an independent experiment; growth of mutant #6 was only quantitated once.

Camalexin toxicity to *Arabidopsis*.

Because other phytoalexins have been shown to be toxic to host cells, we tested whether camalexin would kill *Arabidopsis* suspension culture cells. At 100 or 500 μg/ml, camalexin caused significant death of suspension culture cells as measured by Evans blue dye retention (Fig. 7). Camalexin killing of *Arabidopsis* tissue culture cells prompted us to investigate whether camalexin-mediated cell death is primarily responsible for plant-cell death observed during a virulent *P. syringae* infection. The previous isolation of *Arabidopsis pad* mutants facilitated this analysis. We reasoned that if camalexin caused significant plant cell death, there should be less cell death in a *pad* mutant than in a wild-type plant following infection with *Psm* ES4326. We therefore infected *pad1*, *pad3,* and wild-type *Arabidopsis* plants with *Psm* ES4326 and monitored cell death by the Evans blue dye retention method. Plants were infected with *Psm* ES4326 at approximately 3×10^3 CFU/cm^2. After 2 days, cell death and bacterial growth were quantitated. Plant cell death as measured by the Evans blue dye method was approximately the same in the wild-type and the *pad1* and *pad3* mutant plants (Table 3). One complicating factor in this experiment, however, was that *pad1* is more susceptible than wild-type *Arabidopsis* to *P. syringae* infection. Therefore, as a control, we also infected another *Arabidopsis* mutant, *eds*6-1, which is also more susceptible to *P. syringae* infection (about the same as *pad1*), but which synthesizes wild-type levels of camalexin (Glazebrook et al. 1996). Again, plant cell death was approximately the same in this mutant and in wild type and *pad1* and *pad3*. However, in this particular experiment, the initial bacterial inoculum was too high for growth differences between *pad1* and *eds*6-1 and wild type to be observable. These data indicate that camalexin is not a major cause of plant cell death in intact, infected tissue.

DISCUSSION

In this paper, we have described a variety of studies concerning the mode of action of the *Arabidopsis* phytoalexin in the compatible interaction between *Arabidopsis* ecotype Columbia and the virulent bacterial pathogen *P. syringae* pv. *maculicola* strain ES4326. In general, the role of phytoalexins, if any, in the defense response to virulent bacterial pathogens is poorly understood. In our particular model system, because *Psm* ES4326 elicits high levels of camalexin biosynthesis and because camalexin is toxic to *Psm* ES4326 in vitro, we were particularly interested in determining whether a successful pathogen must circumvent the toxic effects of a host's phytoalexin.

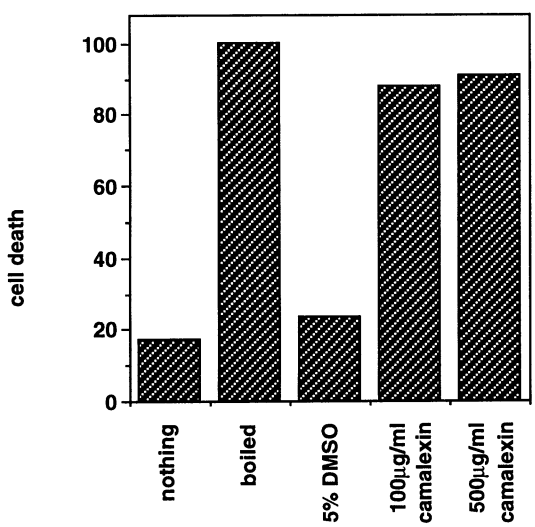

Fig. 7. Camalexin toxicity to *Arabidopsis* suspension culture cells. After the indicated treatment, suspension culture cells were stained with Evans blue to determine cell death. Similar results were obtained in an independent experiment.

We first demonstrated that camalexin is a broad spectrum toxicant, toxic toward bacterial, fungal, and plant cells. There was quite a bit of variation in the sensitivity of the tested microorganisms toward camalexin; however, this variability did not relate to whether the microorganism was phytopathogenic. For example, the *Arabidopsis* pathogen *Psm* ES4326 and the nonpathogen *E. coli* exhibited similar sensitivities to camalexin. Similarly, *F. oxysporum* and *S. cerevisae* were equally sensitive to camalexin, even though *F. oxysporum* is phytopathogenic and *S. cerevisiae* is not. The only bacterial strain that was highly sensitive to camalexin was *B. subtilis,* a gram-positive bacterium. However, although it has been previously observed that gram-positive bacteria are generally more sensitive than gram-negative bacteria to phytoalexins and antibiotics (Smith and Banks 1986), we observed that the gram-positive species *L. monocytogenes* (which is not a plant pathogen) exhibited the same sensitivity to camalexin as *Psm* ES4326 and *E. coli.*

We next investigated the mechanism by which camalexin kills *P. syringae.* This was of interest because the mode of toxicity of sulfur-containing phytoalexins had not previously been reported. We found that camalexin, like other phytoalexins, rapidly disrupts the integrity of the inner membrane. Within minutes, camalexin blocks proline uptake, macromolecules leak out, and viability drops six orders of magnitude. At the same time, most of the camalexin in solution becomes associated with the bacterial cells, rather than remaining free in solution. This allows the few bacterial cells that were not killed initially to continue dividing and to repopulate the culture. Camalexin is a highly lipophilic molecule and would be expected to associate with other lipophilic compounds such as membrane phospholipids. Consistent with the hypothesis that camalexin is a nonspecific membrane antibiotic, neither *P. syringae* nor *E. coli* appear to be able to mutate readily, if at all, to become camalexin resistant.

We were not able to select for *Psm* ES4326 mutants that were completely resistant to camalexin; indeed, to our knowl-

Table 3. Cell death, pathogen growth, and camalexin levels in wild-type *Arabidopsis* and in various mutants

Arabidopsis strain	Cell death (A_{592})[a]	Bacterial density (log CFU/cm^2)[a]	Camalexin levels (% of wild type)[b]
Col (wild type)	1.69 ± 0.49	6.4 ± 0.4	100
pad1-1	0.79 ± 0.27	6.5 ± 0.5	30
pad3-1	1.32 ± 0.21	6.9 ± 0.6	≤1
eds6-1	0.78 ± 0.41	7.2 ± 0.4	100

[a] Values are the amount of Evans blue dye retained, an indication of cell death, and the density of *Pseudomonas syringae* pv. *maculicola* strain ES4326 3 days after infection of 3×10^3 CFU/cm^2 leaf area. Similar results were obtained in an independent experiment.

[b] Values are the average of several previous experiments and were not determined in this same experiment.

edge, there are no other reports of pathogen mutants resistant to a host phytoalexin. This makes sense from an evolutionary perspective since it is clearly to the advantage of a host if its pathogens cannot mutate to become resistant to the antimicrobial compounds that it produces. On the other hand, we did find mutants that were partially resistant to camalexin. The frequency at which we obtained these mutants, approximately 1 in 10^9 in a heavily mutagenized culture, is consistent with the idea that mutations at more than one locus are required. If these loci are unlinked, it would explain why we were unable to identify a cosmid clone that would complement the mutant phenotype of the mutants.

Two independent *P. syringae* mutants that were partially resistant to camalexin showed no enhanced ability to grow in *Arabidopsis* leaves relative to the parent strain. Because 99.9% of the cells of the partially resistant mutants are killed by camalexin (in vitro), the failure of the mutants to grow better in *Arabidopsis* leaves was the expected result even if camalexin plays a major role in limiting virulent *P. syringae* infections, as we previously hypothesized on the basis that *P. syringae* exhibits enhanced growth in some camalexin-deficient mutants (Glazebrook and Ausubel 1994). The fact that partially resistant camalexin mutants do not have a growth advantage in planta would also help explain why partially resistant mutants would not be selected under natural conditions. Indeed, we found that *Psm* ES4326, a Brassica pathogen, exhibited no more resistance to camalexin than *E. coli*. On the other hand, it is possible that camalexin does not play a major role in the *Arabidopsis* defense response, in which case it is not surprising that the partially resistant camalexin mutants showed no growth advantage compared with wild-type *P. syringae*.

Another possible explanation for the observation that the partially resistant camalexin mutants did not show enhanced virulence is that in acquiring resistance to camalexin, they concomitantly became generally less fit due to a cell membrane alteration. Thus, a growth advantage they might have acquired by becoming partially resistant to camalexin was negated by a nonspecific growth disadvantage. If this is the case, a resistant mutant would be expected to grow less well than *Psm* ES4326 in a *pad* mutant plant. However, mutant #9 grew just as well as wild-type *Psm* ES4326 in the *pad1* mutant and in wild-type *Arabidopsis* and had no obvious growth defects in vitro.

The cross-resistant phenotypes of the mutants to tetracycline and nalidixic acid implies changes in the outer membrane, because the gram-negative outer membrane is a permeability barrier for many antibiotics, including tetracycline and nalidixic acid (Nikaido 1994). However, we were unable to detect changes in outer membrane proteins or lipopolysaccharides from any of the mutants.

Because *Psm* ES4326 appeared to be as sensitive to camalexin as a variety of nonphytopathogenic bacteria, we were interested in determining whether *Psm* ES4326 could potentially circumvent the toxic effects of camalexin by either degrading it or becoming tolerant to it. However, we found no evidence that *Psm* ES4326 could either degrade or acquire tolerance to camalexin following growth in sublethal camalexin concentration or in intercellular fluid extracted from infected *Arabidopsis* leaves. The inability of *Psm* ES4326 to metabolize camalexin contrasts with the ability of other pathogens to degrade the relevant phytoalexin as a necessary (but not sufficient) prerequisite, for virulence (Denny et al. 1987; Denny and VanEtten 1981; Denny and VanEtten 1983a; Denny and VanEtten 1983b; Macfoy and Smith 1979; Macfoy and Smith 1986; Miao and VanEtten 1992; Tegtmeier and VanEtten 1982). On the other hand, mutants of *Nectria haematococca* that have lost the capacity to degrade the pea phytoalexin, pisatin, are only slightly compromised for virulence (H. VanEtten, personal communication) (VanEtten et al. 1994).

Camalexin levels induced by *Psm* ES4326 in *Arabidopsis* leaves reach 1 to 2 $\mu g/cm^2$ and, with the thickness of an *Arabidopsis* leaf estimated to be 0.01 cm, camalexin concentrations are approximately 100 to 200 $\mu g/cm^3$. This should be a level sufficient to kill *Arabidopsis* cells, based on the toxicity we measured of camalexin to *Arabidopsis* tissue culture cells. It was therefore of interest to determine whether camalexin plays a role in *Arabidopsis* cell death observed during a compatible interaction with *Psm* ES4326. Previous studies of the role of phytoalexins in mediating host cell death have been limited to incompatible interactions, in which it is been established that phytoalexins do not appear to mediate hypersensitive cell death. Our results, indicating that there is no more host cell death during a virulent *P. syringae* infection in an *Arabidopsis pad1* mutant than in a wild-type plant, suggest that camalexin also does not play a significant role in non-hypersensitive-mediated plant cell death.

There are several possible mechanisms by which a plant could protect itself from the toxic effects of its own phytoalexin, including sequestration of phytoalexins away from host tissues, as in the case of the sorghum–*Colletotrichum graminicola* interaction in which phytoalexin-filled vacuoles are targeted to sites of pathogen penetration (Snyder and Nicholson 1990). It is also possible that a host resistance mechanism is coordinately induced with phytoalexin synthesis. Our observation that *Psm* ES4326 is not sensitive to camalexin in intercellular fluid extracted from infected *Arabidopsis* leaves suggests that camalexin may be sequestered in an inactive form. Similar observations have been reported previously; for example, polar lipid extracts from pea have been demonstrated to decrease the sensitivity of *Aphanomyces euteiches* to the pea phytoalexin pisatin (Sweigard and VanEtten 1987).

In conclusion, a potential role for camalexin in the *Arabidopsis* defense response to a virulent pathogen cannot be readily ascribed to any distinguishing feature of the mode of camalexin toxicity to either bacterial pathogens or to plant host cells. While it is not possible to readily measure the camalexin concentrations to which *Psm* ES4326 is exposed in planta, our estimates of in planta camalexin concentrations are below the threshold toxicity level determined in vitro. Moreover, camalexin exhibits decreased toxicity in vitro when mixed with *Arabidopsis* intercellular fluids, and *Psm* ES4326 mutants with greater tolerance to camalexin do not appear to be more virulent. Taken together, these results suggest that camalexin does not play a major role in limiting *Psm* ES4326 growth in *Arabidopsis*.

MATERIALS AND METHODS

Strains, media, and growth conditions.

P. syringae pv. *maculicola* strain ES4326 (*Psm* ES4326) (Dong et al. 1991), *P. syringae* pv. *phaseolicola* strain 3121

(*Psp* 3121) (Rahme et al. 1992), *Xanthomonas campestris* pv. *campestris* strain BP109 (*Xcc* BP109) (Weiss et al. 1994), *Saccharomyces cerevisiae* strain EGY48 (Gyuris et al. 1993), and *Bacillus subtilis* strain PY79 (Youngman et al. 1984) have been described. *Listeria monocytogenes* 10403S was obtained from Andy Camilli (Tufts University, Boston). *P. syringae* was grown at 28°C either in King's medium B (10 mg of proteose peptone per ml; 1.5 mg of K_2HPO_4 per ml; 15 mg of glycerol per ml; 0.4 mg $MgSO_4$ per ml) (King et al. 1954) supplemented with appropriate antibiotics (100 μg of streptomycin per ml for *Psm* ES4326 or 25 μg of rifampicin per ml for *Psp* 3121) or in M9 minimal salts medium (Gibco BRL, Bethesda, MD) supplemented with 0.4% glycerol. *Xcc* BP109 was grown at 28°C in Luria-Bertani (LB) medium (Ausubel et al. 1996) supplemented with 25 μg of rifampicin per ml. *S. cerevisiae* was grown at 28°C in yeast extract, peptone, dextrose (YPD) medium (Ausubel et al. 1996). *Escherichia coli* MM294 and *B. subtilis* PY79 were grown at 37°C in LB without antibiotics. *Fusarium oxysporum* #5 (Mauch-Mani and Slusarenko 1994) was grown under long-wave UV light (365 nm) at room temperature on potato dextrose agar (Difco, Detroit, MI). *L. monocytogenes* 10403S was grown at 37°C in brain-heart infusion (Difco). *Arabidopsis thaliana* ecotype Columbia ("*Arabidopsis*" for simplicity) was grown in Metromix 2000 (W. R. Grace) soil, either in a climate-controlled greenhouse (20 ± 2°C, relative humidity 60 ± 30%), on a 16-h light, 8-h dark cycle, or in a Conviron growth chamber (20 ± 2°C, relative humidity 90%) on a 12-h light/dark cycle under 125 $\mu E\ s^{-1}\ m^{-2}$ fluorescent illumination. *Arabidopsis pad* mutant plants have been described previously (Glazebrook and Ausubel 1994). The *Arabidopsis eds*6-1 mutant was isolated as more sensitive to *Psm* ES4326 (Glazebrook et al. 1996). Plants grown in the growth chamber were used for experiments involving determination of bacterial growth. Other experiments were conducted on plants grown in the greenhouse. *Arabidopsis* cell suspension cultures were obtained from Beth Laughner and Robert Ferl (University of Florida, Gainesville) and cultivated as described (Ferl and Laughner 1989).

Determination of camalexin toxicity.

Synthetic camalexin was kindly provided by William A. Ayer (University of Alberta, Edmonton). A camalexin stock was made in DMSO at 10 mg/ml; this was diluted to final concentrations as indicated for individual experiments and DMSO concentrations were adjusted to 5% (vol/vol) in all samples. For determination of toxicity to bacteria and *S. cerevisiae*, 5 to 500 μg of camalexin per ml was added to logarithmically growing cells at a density of approximately 1×10^8 CFU/ml. After 5 min to 2 h, cells were diluted for plating on appropriate media. The effect of camalexin on the final bacterial density of a saturated culture was determined by inoculating the appropriate growth media containing 25 to 500 μg of camalexin per ml and 5% DMSO with approximately 1×10^6 CFU/ml. After incubation at the appropriate temperature for 24 h, the OD_{600} of each culture was measured as an indication of bacterial density. Toxicity toward *F. oxysporum* was determined by placing 100 μg of camalexin on small disks of sterile filter paper. The disks were then placed on potato dextrose agar plates 3 cm away from an agar plug removed from a saturated, 2-week-old plate of *F. oxysporum*. The fungus grew out from the inoculation plug until it reached inhibitory camalexin concentrations diffusing from the disk of filter paper. Camalexin was extracted and quantitated from agar plugs taken from a known distance from the disks and a minimum inhibitory concentration of camalexin was estimated from the zone of clearing and these camalexin measurements. Intercellular fluid was isolated from *Arabidopsis* leaves by vacuum infiltration of water, followed by centrifugation at 6,000 rpm (IEC Clinical Centrifuge, International Equipment Co., Needham Heights, MA) to remove intercellular fluid. Cell death in *Arabidopsis* suspension cell cultures, grown as described (Ferl and Laughner 1989), after 30 min of camalexin treatment or 10 min in a boiling water bath, was determined by quantitation of Evans blue dye retention, as previously described for soybean cell suspension cultures (Levine et al. 1994). Cell death in *Arabidopsis* leaves was measured in an adaptation of the above method. Three days after infection with *Psm* ES4326 at approximately 3×10^3 CFU/cm^2, leaf disks were vacuum infiltrated with Evans blue dye. After a 30-min incubation in the dye, the disks were washed with water and then incubated in 50% methanol and 1% sodium dodecyl sulfate (SDS) at 50°C for 30 min. The methanol/SDS solution was quantitated at OD_{600}.

Determination of membrane integrity.

Measurement of proline uptake of chloramphenicol-treated *Psm* ES4326 was accomplished by a combination of previously described methods (Fraimow et al. 1991; Galvez et al. 1991; Ruhr and Sahl 1985; Schuller et al. 1989). In brief, cells were treated with 100 μg of chloramphenicol per ml for at least 3 min. ^{14}C-proline (205.5 mCi/mmol) and camalexin and/or DMSO was added at various times and after various incubation times; cells were filtered onto 0.22-μm-pore filters (Millipore, Bedford, MA) and washed with 2×5 ml of 0.1 M LiCl. Filters were allowed to dry and then counted in 5-ml of UniverSol (ICN, Costa Mesa, CA) in a Beckman LS7800 scintillation counter. For ion leakage measurements, cells were washed three times with distilled water and then treated with toluene and camalexin and/or DMSO. After 60 min, the cells were pelleted and the conductivity of the supernatant was measured with a Radiometer CDM3 conductivity meter. Serine deaminase activity was assayed as described (Isenberg and Newman 1974) except that OD_{440} was measured. Protein leakage was assayed as described (Jackson and DeMoss 1965), except that cells were grown overnight in the presence of ^{3}H-leucine (155 Ci/mmol) and treated with toluene and camalexin and/or DMSO. After 60 min, the cells were pelleted, the supernatant was precipitated with trichloroacetic acid (TCA), and the precipitate was collected onto filters and counted.

Mutant selection and bacterial genetics.

Psm ES4326 was mutagenized with 20 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) per ml for 60 min following standard procedures (Miller 1972). After mutagenesis, cells were divided into 100 aliquots, each containing approximately 1×10^7 cells, and allowed to grow overnight in nonselective media. No more than 5×10^6 cells from each of these cultures were inoculated into King's medium B containing 500 μg of camalexin per ml. Cultures that were turbid after 2 to 3 days were diluted 1:100 into fresh media containing 500 μg of camalexin per ml. After three such rounds of

selection, turbid cultures were streaked on nonselective medium and individual colonies were tested for camalexin resistance by inoculation into 500 μg of camalexin per ml as described above. Cross resistance of putative camalexin-resistant mutants to selected antibiotics was determined by plating between 100 and 1,000 cells per 9 cm petri plate on King's medium B containing 0 to 1 μg of nalidixic acid per ml or 0 to 30 μg of tetracycline per ml; colonies were allowed to form over 48 h.

Cosmid libraries were constructed by standard procedures (Ausubel et al. 1996). Briefly, bacterial DNA was isolated, partially digested with the restriction enzyme *Sau*3AI, and fractionated on a sucrose gradient. Fragments between 10 and 30 kb were ligated into the vector pJS1 (Rahme et al. 1995; Roberts et al. 1990) cut at a unique *Bam*HI site. Ligations were electroporated into *E. coli* DH5α and cosmids were selected by plating on ampicillin. The libraries were conjugated into *P. syringae* by standard techniques (Ausubel et al. 1996) with plasmid pRK600 to provide transfer functions (Finan et al. 1986).

Determination of bacterial growth in planta.

Leaves were infected by forcing a suspension of bacterial cells in 10 mM $MgSO_4$ solution through the stomata with a 1-ml syringe without a needle. At various times after inoculation, samples were cut from infected leaves with a #2 cork borer. Each sample consisted of 1 or 2 disks, ground in 500 μl of 10 mM $MgSO_4$ with a plastic pestle. Samples were diluted into 10 mM $MgSO_4$ and plated on King's medium B.

Camalexin determination.

Camalexin was extracted in hot methanol and quantitated as described previously (Glazebrook and Ausubel 1994).

ACKNOWLEDGMENTS

We thank W. A. Ayer for providing synthetic camalexin without which this work would not have been possible, B. Laughner and R. Ferl for the *Arabidopsis* suspension cell culture, and D. Fraenkel for helpful discussions. This work was supported by research grants from the National Institutes of Health (GM48707) and the United States Department of Agriculture (94-37303-0464).

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