Recognition of the Avirulence Gene *avrB* from *Pseudomonas syringae* pv. *glycinea* by *Arabidopsis thaliana*

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The response of *Arabidopsis thaliana* land race Columbia to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* 4326 harboring cloned avirulence genes *avrB* and *avrC* from *P. syringae* pv. *glycinea* and *avrA* and *avrD* from *P. syringae* pv. *tomato* was examined. Only *avrB* was recognized by Columbia, as evidenced by attenuation of disease symptoms, slower bacterial multiplication *in planta*, and differentially greater induction of mRNA for several defense-related genes. This contrasts with two *A. thaliana* land races where *P. s.* pv. *maculicola* strains containing *avrB* were not recognized. These plants showed typical disease symptoms, and bacterial multiplication *in planta* was not reduced in response to *P. s.* pv. *maculicola* containing *avrB*. In addition, there was no differential induction of defense-related mRNAs in these susceptible land races after infiltration with bacteria containing or lacking *avrB*. These results extend previous observations that avirulence genes from pathogens of one host plant can be recognized by "nonhost" plants and provide the genetic framework for analysis of the plant-specified response to the bacterial *avrB* gene product in *A. thaliana*.

Additional keywords: disease resistance, hypersensitive response, lipoxygenase, peroxidase, phenylalanine ammonia-lyase.

Plants are capable of recognizing a variety of microorganisms as potential pathogens and mounting an effective resistance response. Changes in both plant and bacterial gene expression result from the interaction of phytopathogenic bacteria with their host plants, indicating that both partners produce and perceive signals. Groups of bacterial genes have been identified that are involved in both pathogenesis and induction of plant hypersensitive responses (Gabriel *et al.* 1990; Keen 1990; Tamaki *et al.* 1988; Willis *et al.* 1991), and many plant genes that are activated during resistance responses have been described (Ausubel *et al.* 1992; Bowles 1990; Graham *et al.* 1991). However, little is known about the signals produced by either partner, or the signal transduction pathway resulting in plant defense gene expression.

Pathogen genes that are critical in determining whether a bacterial strain will be virulent or avirulent on a specific host have been identified. These individual avirulence genes interact with corresponding resistance genes in host plants, resulting in the induction of the hypersensitive response (Gabriel and Rolfe 1990; Keen 1990, 1992). Such "gene-for-gene" interactions have been identified for many plant-bacterial combinations, and several bacterial avirulence genes have been cloned (De Feyter *et al.* 1991; Gabriel *et al.* 1986; Hitchin *et al.* 1989; Kobayashi *et al.* 1989; Kobayashi *et al.* 1990; Staskawicz *et al.* 1984; Staskawicz *et al.* 1987; Tamaki *et al.* 1988; Vivian *et al.* 1989). Detailed molecular analyses of these cloned avirulence genes have provided few clues as to how these genes activate hypersensitive responses in plants. In addition, no plant resistance gene corresponding to a bacterial avirulence gene has yet been cloned and characterized.

Several laboratories are using the infection of *Arabidopsis thaliana* with phytopathogenic *Pseudomonas syringae* pathovars to study the interaction of bacterial avirulence genes with plant resistance genes (Davis *et al.* 1991; Debener *et al.* 1991; Dong *et al.* 1991; Whalen *et al.* 1991). The advantages offered by *A. thaliana* as an experimental system and a summary of the establishment of *Arabidopsis*-pathogen systems have recently been reviewed (Dangl 1993; Davis 1992). Several examples of gene-for-gene interactions have been described in *A. thaliana*. Two groups simultaneously identified the bacterial *avrRpt2* gene from *P. s.* pv. *tomato* JL1065 (MM1065) (Dong *et al.* 1991; Whalen *et al.* 1991), which is recognized by some, though not all, land races of *A. thaliana*. Genetic analyses of crosses between resistant and susceptible land races indicate that this resistance is due to a single dominant locus (Innes *et al.* 1993) designated *Rps2*. In addition, a mutant of *A. thaliana* that no longer recognizes *avrRpt2* has been identified in land race Columbia. This mutation has been mapped to chromosome 4 (Kunkel *et al.* 1992). Another avirulence gene, *avrRpm1* (*avrPmaA1*), recognized by some land races of *A. thaliana*, has been isolated from *P. s.* pv. *maculicola* m2, and the corresponding resistance locus

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has been localized to an approximately 200-kb segment of chromosome 3 (Dangl *et al.* 1992b; Debener *et al.* 1991).

There are now several examples of avirulence (*avr*) genes originally identified in bacterial pathovars of one plant species that can act to attenuate virulence in "nonhost" plants (Whalen *et al.* 1988; Kobayashi *et al.* 1989; Whalen *et al.* 1991). We decided therefore to see whether avirulence genes identified and cloned from *Pseudomonas* strains that normally infect tomato and soybean could also function as avirulence genes in the crucifer pathogen *P. s.* pv. *maculicola* 4326, which produces leaf spot in *A. thaliana* (Davis *et al.* 1991; Dong *et al.* 1991; Whalen *et al.* 1991).

We show here that one of the four bacterial avirulence genes tested (*avrB*) was specifically recognized by *A. thaliana* land race Columbia. This was evidenced by attenuation of disease symptoms typically produced by infection with *P. s.* pv. *maculicola*, slower bacterial multiplication *in planta*, and differentially more rapid induction of mRNAs for several defense-associated genes. We also were able to contrast the characteristics of the recognition of the *avrB* gene in land race Columbia with the lack of recognition of this avirulence gene in two other *A. thaliana* land races. Land races Mt-0 and Bla-2 showed typical disease symptoms, and bacterial multiplication *in planta* was not reduced when they were inoculated with *P. s.* pv. *maculicola* containing *avrB*. Moreover, there was no difference in expression of defense-associated mRNAs after infiltration of *P. s.* pv. *maculicola* with or without *avrB* into these two land races.

RESULTS

Disease symptoms and bacterial multiplication of *P. s.* pv. *maculicola* containing *avrB* in *A. thaliana* land race Columbia.

Since several previously identified avirulence genes are known to function in more than one bacterial species, we wanted to determine whether avirulence genes identified and cloned from *Pseudomonas* strains that normally infect tomato and soybean could also function as avirulence genes in the crucifer pathogen *P. s.* pv. *maculicola* 4326. Hand-infiltration of Columbia leaves with *P. s.* pv. *maculicola* harboring the cloned avirulence gene *avrB7* from *P. s.* pv. *glycinea* induced mild chlorosis and drying of leaf tissue typical of a hypersensitive response within 24–48 hr. These symptoms were distinct from the water-soaked necrotic lesions with chlorotic margins produced in land race Columbia leaves in response to *P. s.* pv. *maculicola*. The distinction in symptoms produced by *P. s.* pv. *maculicola* with or without *avrB* was more striking when plants were inoculated by dipping them into a solution of bacteria containing surfactant. As shown in Figure 1, virtually no disease symptoms were observed in plants dipped in *P. s.* pv. *maculicola* with *avrB*, while plants dipped in *P. s.* pv. *maculicola* lacking *avrB* developed necrosis and chlorosis at many converging spots on the leaves, probably corresponding to bacterial entry at individual stomata. Necrosis spread from individual spots throughout the leaf over 3–4 days until bacterial multiplication ceased.

To determine whether the severity of disease symptoms corresponded with bacterial cell numbers in the leaves, we examined the growth rates of *P. s.* pv. *maculicola* with and without *avrB* in *Arabidopsis* land race Columbia. Both bacterial strains multiply two to three logs in the first 24 hr when leaves are hand-infiltrated at an initial bacterial density of 0.001 OD_{600} (Fig. 2, IIA). *P. s.* pv. *maculicola* lacking *avrB* multiply about 5 logs over 4 days when inoculated at this low dose, while *P. s.* pv. *maculicola* with *avrB* multiply about 3 logs. Most of the difference in multiplication rates between the two bacterial strains occurs after the first 24 hr.

Dipping plants into bacterial suspensions at a density of 0.2 OD_{600} produces a more dramatic difference in growth rates between *P. s.* pv. *maculicola* strains (Fig. 2, IA). *P. s.* pv. *maculicola* lacking *avrB* multiplied in Columbia leaves 3 to 4 logs over 4 days. Multiplication was limited to about 1 log in Columbia treated with *P. s.* pv. *maculicola* with *avrB*, with most of the growth occurring in the first 24 hr. The *avrB* gene thus converts a normally virulent *Pseudomonas* strain to avirulence on *A. thaliana* land race Columbia, and attenuation of virulence is correlated with a substantial reduction in symptom development and bacterial multiplication.

Disease symptoms of *A. thaliana* land race Columbia infected with *P. s.* pv. *maculicola* strains containing *avrA*, *avrC*, and *avrD* genes.

Dipping of *A. thaliana* (land race Columbia) plants into a suspension of *P. s.* pv. *maculicola* strains harboring either *avrC22* (from *P. syringae* pv. *glycinea*), or *avrA20* or *avrD33* (from *P. syringae* pv. *tomato*) produced many small, water-soaked necrotic lesions surrounded by chlorotic areas within 3 days (Fig. 1). Necrotic lesions continued to spread within a leaf for 4–5 days, after which there was no further spread of diseased tissue. These symptoms were indistinguishable in severity and timing from those typically produced upon infiltration with either *P. s.* pv. *maculicola* or *P. s.* pv. *maculicola* harboring the pDSK519 vector alone. In addition, we observed no difference in growth rates of *P. s.* pv. *maculicola* transconjugants containing *avrA*, *avrC*, and *avrD* and *P. s.* pv. *maculicola in planta* (data not shown). We therefore concluded that these three avirulence genes are not recognized in land race Columbia.

Disease symptoms and bacterial multiplication of *P. s.* pv. *maculicola* containing *avrB* in *A. thaliana* land races Mt-0 and Bla-2.

Concurrent with our studies, a preliminary report confirmed that *avrB* was recognized in land race Columbia and that *P. s.* pv. *tomato* DC3000 containing *avrB* was not recognized by two *Arabidopsis* land races, Mt-0 and Bla-2 (Innes *et al.* 1993). We tested these two land races to determine whether they recognized *P. s.* pv. *maculicola* harboring *avrB*. We found that *avrB* harbored by *P. s.* pv. *maculicola* is also not recognized by these land races when plants are either hand-infiltrated or

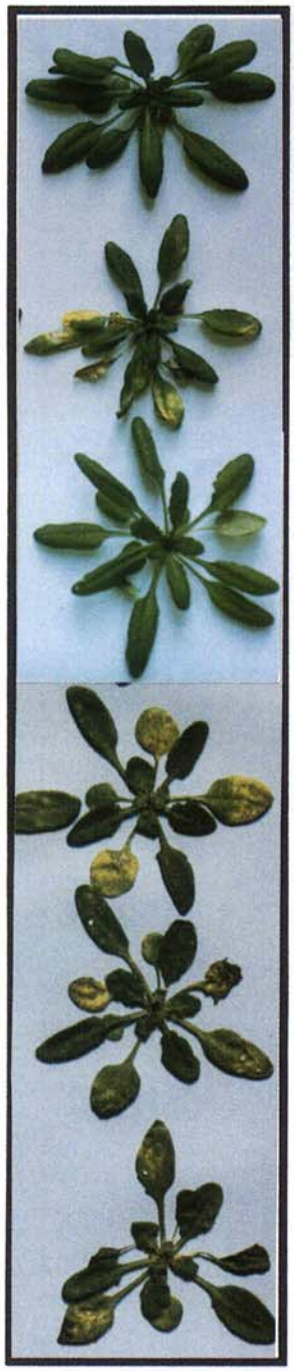

Fig. 1. Symptoms produced in *Arabidopsis thaliana* land race Columbia by *Pseudomonas syringae* pv. *maculicola* 4326 harboring bacterial avirulence genes. **A,** Plant dipped in surfactant/$MgCl_2$ solution without bacteria, and showing no symptoms. **B,** Dipped in *P. s.* pv. *maculicola* 4326 and showing typical chlorosis and water-soaked lesions 4 days later. **C,** Dipped in *P. s.* pv. *maculicola* 4326/pDSK519*avrB*, and showing no disease symptoms 4 days later. **D–F,** were dipped in *P. s.* pv. *maculicola* 4326/pDSK519 *avrA, avrC,* and *avrD,* respectively, and show typical disease symptoms 4 days later.

dipped into bacterial suspensions, as evidenced by a similar degree and similar timing of disease symptom development. There is also no difference in rates of *P. s.* pv. *maculicola* multiplication with or without *avrB* in these two land races, as shown in Figure 2, rows B and C. These results confirm that Mt-0 and Bla-2 are completely susceptible to *P. s.* pv. *maculicola* 4326 containing *avrB* and thus could provide useful comparative data in further characterization of the *avrB*-induced defense response.

Accumulation of mRNAs from defense-associated genes in response to infiltration with *P. s.* pv. *maculicola* with and without *avrB*.

A close correlation has been demonstrated between the recognition of avirulence genes by *A. thaliana* and the activation of several defense-associated genes (Dong *et al.* 1991; Keith *et al.* 1991; Melan *et al.* 1993; Ausubel *et al.* 1992). We used RNA blot analysis to examine the induction kinetics of several defense-associated genes in leaves inoculated with *P. s.* pv. *maculicola* with and without *avrB*. Representative results of Northern blots hybridized with different *A. thaliana* phenylalanine-ammmonia lyase (PAL) genes and with lipoxygenase (*LOX1*) are shown in Figures 3–5.

Increased levels of both *PAL1* and *PAL2* are detected within 1 hr after infiltration of land race Columbia with *P. s.* pv. *maculicola* with *avrB* (Figs. 3A, 4A, and data not shown), and maximal levels of *PAL1* and *PAL2* mRNAs are reached between 4 and 6 hr after infiltration. Infiltration of Columbia with *P. s.* pv. *maculicola* lacking *avrB* resulted in little or no increase in *PAL1* and *PAL2* mRNA levels.

In contrast, in response to infiltration with either bacterial strain, there is no induction of *PAL1* or *PAL2* mRNA levels in the two land races in which *avrB* is not recognized (Figs. 3B,C and 4B,C). The approximately twofold increase in *PAL1* mRNA in Bla-2 infiltrated with *P. s.* pv. *maculicola* without *avrB* as compared to infiltration with *avrB* (Fig. 3C) was not observed in other independently derived sets of RNAs.

We tested another defense-associated gene, lipoxygenase, which was previously shown to be induced by *P. s.* pv. *maculicola* harboring the *avrRpt2* gene (Melan *et al.* 1993). Lipoxygenase message was undetectable in untreated *A. thaliana* leaves of all three land races. In comparison to the negligible basal levels, there was substantial accumulation of lipoxygenase mRNA in Mt-0 and Bla-2 infiltrated with either *P. s.* pv. *maculicola* strain (Fig. 5B,C); this result is understated in Figure 5B and C, since the same scale was used in plotting accumulation in all three land races. Initial levels of lipoxygenase mRNA were also low in land race Columbia (Fig. 5A), but both *P. s.* pv. *maculicola* strains induced higher accumulation than was seen in either Mt-0 or Bla-2. A differentially earlier response to *avrB* was clearly evident in land race Columbia; lipoxygenase mRNA accumulated more rapidly in Columbia infiltrated with *P. s.* pv. *maculicola* containing *avrB*, reaching maximal levels around 18 hr after infiltration. Similar levels of lipoxygenase message were not seen until 30 hr after infiltration with *P. s.* pv. *maculicola* lacking *avrB*.

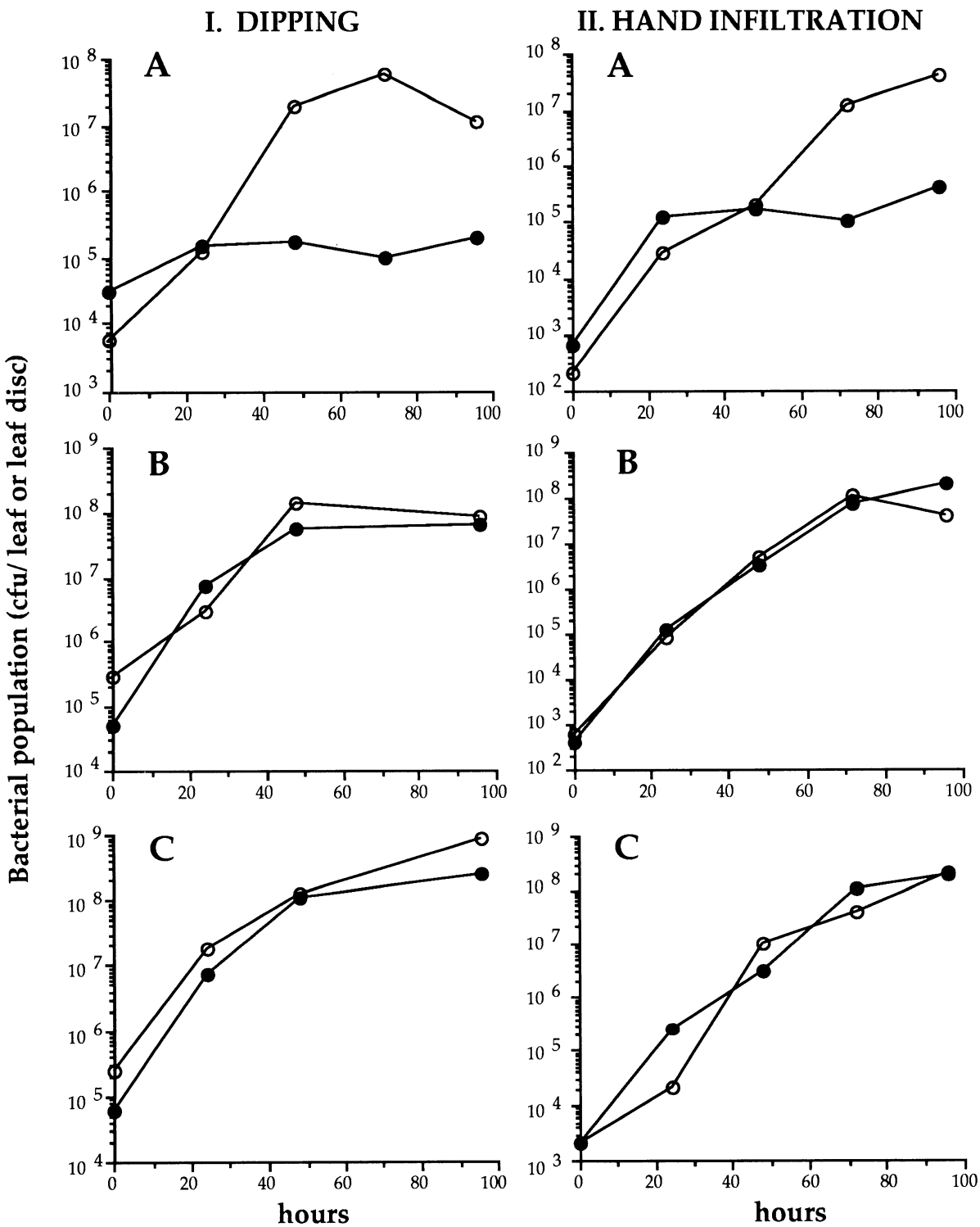

Fig. 2. Multiplication of *Pseudomonas syringae* pv. *maculicola* 4326 containing *avrB* in *Arabidopsis thaliana* land races Columbia (Col-0), Mt-0, and Bla-2. Plants were treated with *P. s.* pv. *maculicola* 4326 with (filled circles) or without (open circles) the *avrB* gene. At 24-hr intervals, four whole leaves (I) or three 0.5-cm-diameter leaf disks (II) were removed from each plant, ground together in 10 mM $MgCl_2$, and serial dilutions were plated on selective media to follow bacterial multiplication. Results are shown as colony-forming units per leaf (I) or per leaf disk (II), and are representative of two (dipped plants; I) or three (hand infiltrations, II) independent experiments. **A,** Land race Col-0. **B,** Land race Mt-0. **C,** Land race Bla-2.

DISCUSSION

We have shown that an avirulence gene previously isolated from the soybean pathogen *P. s.* pv. *glycinea* can serve to attenuate virulence in *A. thaliana* land race Columbia when introduced into the crucifer pathogen *P. s.* pv. *maculicola* 4326. Attenuation of virulence was observed as a gross reduction in disease symptoms, a significant reduction of bacterial multiplication *in planta*, and the increased or earlier expression of defense-related genes. These results extend previous observations that avirulence genes isolated from pathogens of one host plant can function to attenuate virulence in different host plants. The results also emphasize an underlying conservation of the mechanism(s) that allow the plant to recognize an avirulence gene and activate a set of defense responses.

A number of gene products have been implicated in plant defense against the spread of pathogens. Studies have shown a differential increase in either enzyme activities and/or mRNA levels during incompatible or hypersensitive defense responses in many plant species

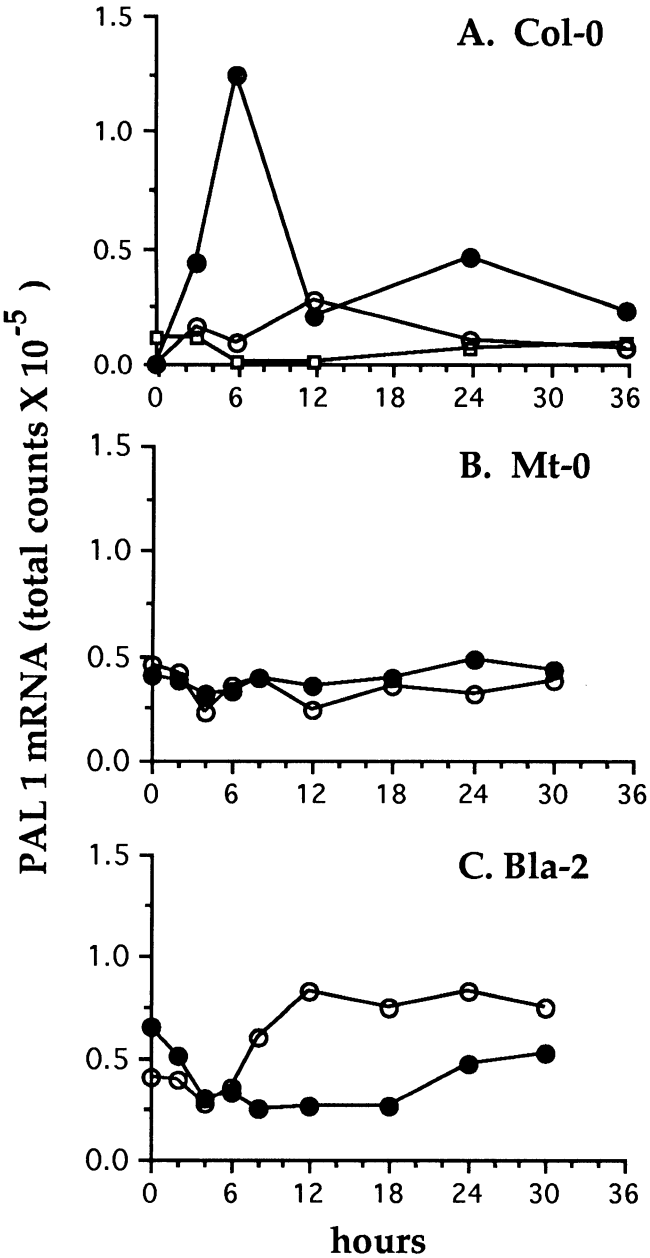

Fig. 3. *PAL1* mRNA accumulation in *Arabidopsis thaliana* land races Columbia (Col-0), Mt-0, and Bla-2. Northern blots of total RNA (10 µg/lane) from leaves harvested at the indicated number of hours after hand-infiltration with *Pseudomonas syringae* pv. *maculicola* 4326 with (filled circles) or without (open circles) the *avrB* gene were hybridized to a gene-specific probe for *PAL1*. The hybridization signal in each lane was quantified using a PhosphorImager, and the data are presented as cumulative counts from blots that were hybridized and imaged together. Data shown are representative of at least three independent experiments. **A**, Land race Col-0. **B**, Land race Mt-0. **C**, Land race Bla-2.

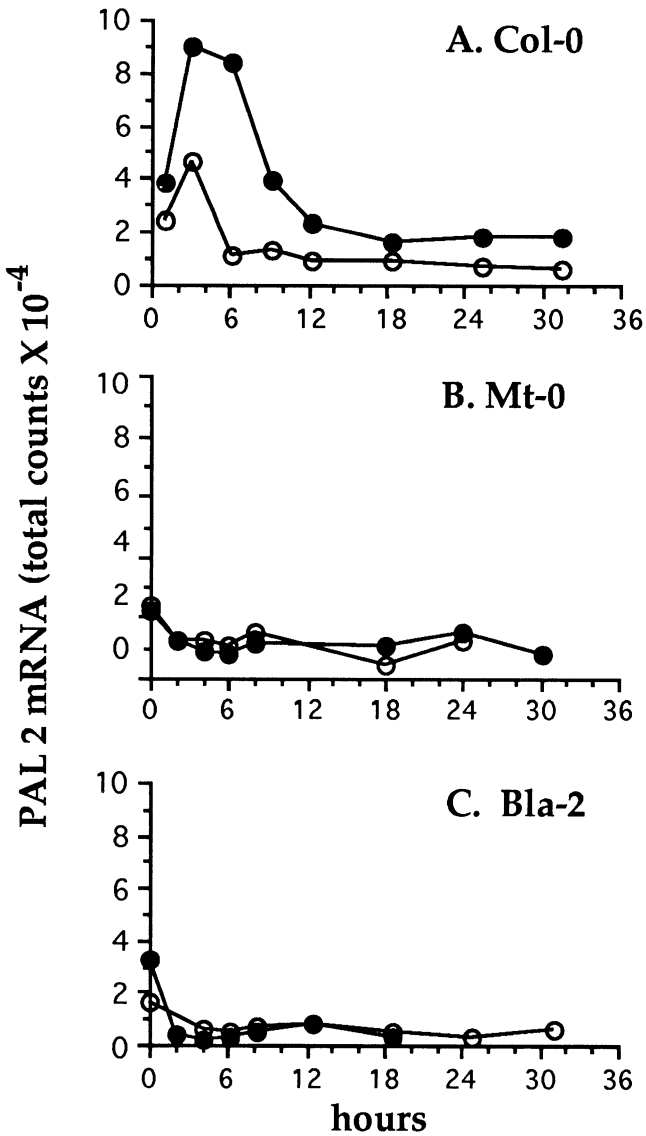

Fig. 4. *PAL2* mRNA accumulation in *Arabidopsis thaliana* land races Columbia (Col-0), Mt-0, and Bla-2. Data were obtained by hybridizing and imaging Northern blots as in Figure 3, except using a *PAL2* gene-specific probe. Filled circles, plants infiltrated with *P. s.* pv. *maculicola* 4326 containing *avrB*; open circles, plants infiltrated with *P. s.* pv. *maculicola* 4326 lacking *avrB*. **A**, Land race Col-0. **B**, Land race Mt-0. **C**, Land race Bla-2.

(reviewed in Lamb *et al.* 1989). Defense-associated genes include enzymes in the phenylpropanoid pathway resulting in lignin and phytoalexin biosynthesis. Common to both lignin and phytoalexin synthesis are PAL and 4-coumarate-CoA ligase. Cinnamyl-alcohol dehydrogenase is specific to the biosynthesis of lignins, and chalcone synthase (CHS) and chalcone isomerase are specific to the synthesis of flavonoids. Additional defense-associated gene products include cell wall components such as hydroxyproline-rich glycoproteins (HRGPs), hydrolases such as chitinase and β-1,3 glucanases, and enzymes involved in neutralizing the effects of free radicals formed during tissue damage and cell death, such as peroxidases. Lipoxygenase mRNA levels have also been shown to increase during a hypersensitive response in *Arabidopsis* (Melan *et al.* 1993). Lipoxygenase may be involved in generating fatty acid hydroperoxide precursors to signal molecules such as jasmonates, which have been shown to induce expression of many defense-associated genes (Creelman *et al.* 1992; Melan *et al.* 1993). Lipoxygenase has also recently been implicated in production of bactericidal lipid-breakdown products (Croft *et al.* 1993).

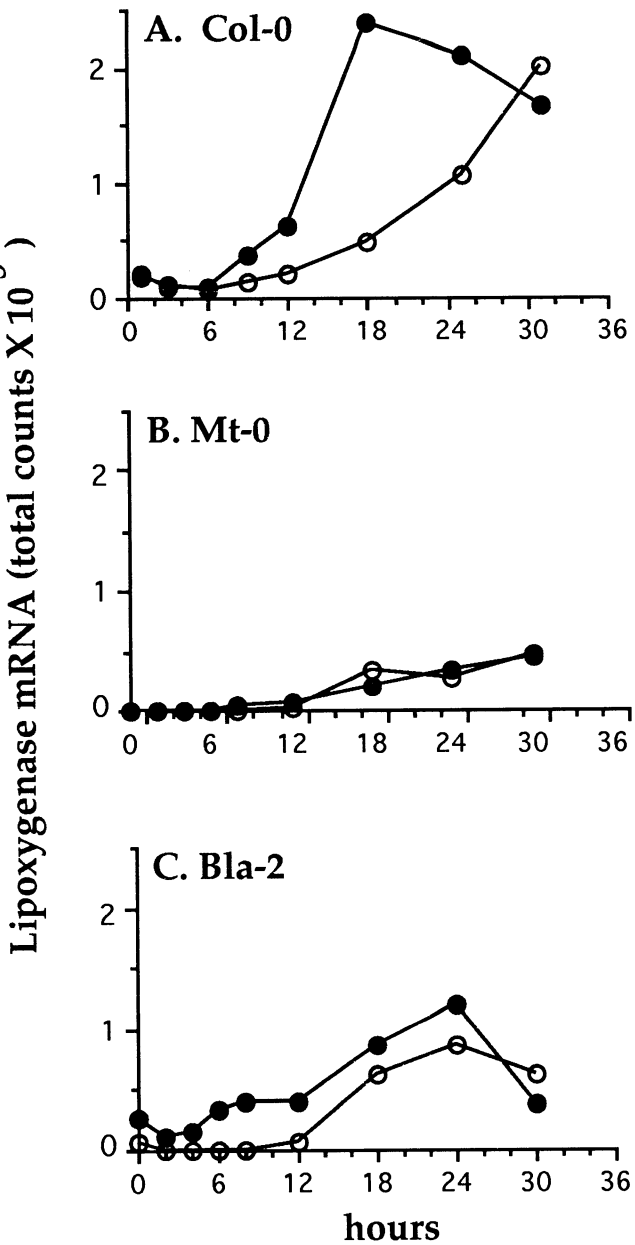

Fig. 5. Lipoxygenase mRNA accumulation in *Arabidopsis thaliana* land races Columbia (Col-0), Mt-0, and Bla-2. Data were obtained by hybridizing and imaging northern blots as in Figure 3, except with a probe for lipoxygenase. Filled circles, plants infiltrated with *P. s.* pv. *maculicola* 4326 containing *avrB*; open circles, plants infiltrated with *P. s.* pv. *maculicola* 4326 lacking *avrB*. **A,** Land race Col-0. **B,** Land race Mt-0. **C,** Land race Bla-2.

Although the importance of any of these genes in plant defense has not been conclusively demonstrated, similar patterns of defense-related gene expression in hypersensitive resistance responses have been observed in many plant species, including *Arabidopsis*. Messenger RNAs for defense-associated genes that have been shown to be induced more rapidly or to higher levels by avirulent bacteria than by virulent bacteria in *Arabidopsis* include PAL (Davis *et al.* 1991; Dong *et al.* 1991), DAHP synthase I (Keith *et al.* 1991), lipoxygenase (Melan *et al.* 1993), glutathione-S-transferase (Ausubel *et al.* 1992), and several genes of unknown function (Ausubel *et al.* 1992; Kiedrowski *et al.* 1992). The induction kinetics of one gene of unknown function, ELI3, specifically correlate with *Rpm1*-linked resistance (Kiedrowski *et al.* 1992).

We examined the pattern of expression of several defense-associated mRNAs in response to the bacterial *avrB* gene. We chose to focus on two genes in particular; we examined PAL expression because of the significance of products of phenylpropanoid metabolism in lignification, and we examined expression of a lipoxygenase because of the potential importance of lipoxygenases in producing signal molecules. PAL expression is typically transiently induced within 1–2 hr and reaches maximal expression within 6 hr during hypersensitive resistance responses. Lipoxygenase mRNA accumulation begins several hours after initial bacterial infection, peaking earlier in hypersensitive resistance responses than in susceptible responses. Both PAL and lipoxygenase are among the defense-related genes whose expression is enhanced in response to *P. s.* pv. *maculicola* 4326 containing the bacterial avirulence gene *avrRpt2* (Ausubel *et al.* 1992; Dong *et al.* 1991; Melan *et al.* 1993).

We found that there was a strict correlation between recognition of *avrB* and enhanced expression of two distinct PAL genes, *PAL1* and *PAL2*, and of the lipoxygenase gene *LOX1*, implicating these gene products in the defense response that resulted from *avrB* recognition. We were able to use gene-specific probes to determine that expression of the two *Arabidopsis* PAL genes is induced with similar kinetics and to similar extents in response to *avrB*, suggesting that products of the phenylpropanoid pathway are important in this defense response. This result is similar to the induction of total PAL mRNA in response to *avrRpt2* (Dong *et al.* 1991). Although we did not examine the expression of any other genes whose products function in the lignin biosynthesis branch of the phenylpropanoid pathway, both autofluorescence and

phloroglucinol staining indicate that increased amounts of phenolics and lignins are produced in response to *avrB* (unpublished data). A further corroboration of the involvement of lignin biosynthesis in response to *avrB* is the enhanced accumulation of a peroxidase message (ELI 11; Trezzini *et al.* 1993) in Columbia infiltrated with *P. s.* pv. *maculicola* containing *avrB* compared to Columbia infiltrated with *P. s.* pv. *maculicola* lacking *avrB* (unpublished data). There was no difference in the pattern of accumulation of peroxidase when the two land races that do not recognize *avrB* were infiltrated with either *P. s.* pv. *maculicola* strain (data not shown), extending the correlation between higher levels of defense-associated genes and *avrB* recognition. As expected, expression of CHS was not enhanced in conjunction with *avrB* recognition (unpublished data). This result is similar to the observed response to *avrRpt2* (Dong *et al.* 1991). Because flavonoids do not function as phytoalexins in *Arabidopsis* (Tsuji *et al.* 1992), it is not surprising that a gene in this branch of the phenylpropanoid pathway is unresponsive to the bacterial signal presumably perceived by other defense-associated genes.

We found that lipoxygenase mRNA accumulation was accelerated by several hours in the presence of *avrB*. This result is also similar to the results observed in response to *avrRpt2* (Melan *et al.* 1993). Although the *Arabidopsis* resistance gene to *avrB* and the *Rps2* locus are genetically separate, it appears that both early and late defense-associated gene responses to both are similar. These observations support the hypothesis that the defense responses induced by bacterial avirulence genes are the same, but that the initial recognition step(s) differ.

The inhibition in multiplication of *P. s.* pv. *maculicola* containing *avrB* relative to multiplication of *P. s.* pv. *maculicola* lacking *avrB* is likely to be the cause of the difference in the degree and distribution of symptoms observed on leaves after infection. It is not known what mediates the inhibition of multiplication of *P. s.* pv. *maculicola* containing *avrB* in plant leaves. Analysis of the *in planta* bacterial growth of the two strains (Fig. 2) shows that the increase in bacterial numbers is similar during the first 24 hr, but then slows dramatically for the strain containing *avrB*. There is thus ample time for plant "defense" products to be synthesized and excreted into the mesophyll intercellular spaces, thereby inhibiting bacterial growth. This observation suggests that it should be possible to identify secreted plant products that inhibit bacterial growth in culture and could be responsible for the inhibition of growth *in planta*. Volatile lipid breakdown products of the lipoxygenase pathway which have antibacterial activity have recently been described during a hypersensitive resistance response in bean (Croft *et al.* 1993). Similar products may play a role in inhibition of growth of *P. s.* pv. *maculicola* containing *avrB* in plant leaves, since maximum levels of *LOX1* mRNA expression are reached between 18 and 24 hr after infiltration, the time when the growth rate of *P. s.* pv. *maculicola* containing *avrB* begins to lag behind that of *P. s.* pv. *maculicola* lacking *avrB*.

The recognition of *avrB* by *A. thaliana* land race Columbia suggests that *A. thaliana* contains a resistance gene conditioning the response to *avrB*, and functionally homologous to the genetically defined soybean *Rpg1* gene (Keen *et al.* 1991; Staskawicz *et al.* 1987). Functionally homologous resistance genes to *A. thaliana Rpm1*, which recognizes bacterial *avrRpm1* (*avrPmaA1*) (Dangl *et al.* 1992b; Debener *et al.* 1991), are also found in other plant species, including bean and pea (Dangl *et al.* 1992a). The *P. s.* pv. *tomato avrRpt2* gene is recognized by some cultivars of soybean (Whalen *et al.* 1991). These results extend initial findings that *A. thaliana* can recognize specific bacterial avirulence genes and adds to the growing body of evidence that true gene-for-gene interactions can be studied in this organism.

Genetic variation in the resistance genes among plant cultivars is widespread and appears to be the rule in *Arabidopsis* as in other plants. *A. thaliana* land races that do not recognize *avrRpt2* and *avrRpm1* have been identified (Debener *et al.* 1991; Whalen *et al.* 1991). The *avrB* resistance gene is either absent or nonfunctional in two *A. thaliana* land races. At least one *A. thaliana* mutant that fails to recognize *avrRpt2* has been identified (Ausubel *et al.* 1992; Kunkel *et al.* 1992), and mutants in recognition of *avrB* are currently being sought. The variability in recognition of *avrB* among land races of *A. thaliana* provides a framework for a genetic analysis of the plant-specified response to the bacterial *avrB* gene product and for map-based cloning of the *Arabidopsis* resistance gene corresponding to *avrB*. It seems likely that *Arabidopsis* land races carrying resistance genes corresponding to the bacterial *avrA, avrC*, and *avrD* genes may exist. We are currently screening land races to look for these resistances.

In summary, the *P. s.* pv *glycinea avrB* gene (Tamaki *et al.* 1988) is recognized in the *Arabidopsis* land race Columbia, and the presence of this single bacterial gene results in attenuation of disease symptoms, inhibition of bacterial multiplication, and induction of defense-associated gene expression. Cloning of the *Arabidopsis* resistance gene corresponding to *avrB* as well as of additional resistance genes will enhance our understanding of how pathogens are recognized by plants, initiating the series of events that culminate in a resistance response.

MATERIALS AND METHODS

Plant material.

A. thaliana was planted from seed in prewetted Metromix 200 (Hummert Seed Co., St. Louis, MO). Covered flats of newly planted seeds were kept at 4° C for 2 days and then transferred to a 20° C controlled-environment room with a 12-hr photoperiod (50–150 μm mol m^{-2} sec^{-1}). After 2 wk, the clear plastic covers were removed. The 4- to 5-wk-old plants used in experiments had fully expanded rosette leaves, but had not yet bolted.

Bacterial strains and avirulence genes.

The bacterial strains and plasmids used in this study are shown in Table 1. Clones of four avirulence genes

inserted into the broad host range plasmid vector pDSK519 (Keen *et al.* 1988) are under the control of the plasmid *lac* promoter; avirulence genes were obtained from Noel Keen (Keen and Buzzell 1991). The *P. s.* pv. *maculicola* strains used in these experiments were *P. s.* pv. *maculicola* ES4326 (Dong *et al.* 1991), which is resistant to streptomycin (Sm), and *P. s.* pv. *maculicola* KD4326 (this study), which is resistant to rifampin (Rif). Plasmids containing individual avirulence genes were introduced into *P. s.* pv. *maculicola* ES4326 and *P. s.* pv. *maculicola* KD4326 by triparental mating (Ditta *et al.* 1980), using pRK2013 (Figurski *et al.* 1979) as the helper plasmid. Both pRK2013 and pDSK519-derived plasmids were maintained in *E. coli* DH5α (Bethesda Research Laboratories) for use in triparental matings. *Pseudomonas* strains containing avirulence gene plasmids were routinely grown in King's B medium, containing either 0.15% glucose or 0.5% glycerol as carbon source (King *et al.* 1954), with 50 g/L kanamycin (Km) and either 50 g/L Sm or Rif. The presence of endogenous plasmids and the introduced pDSK519 derivatives in transconjugants was confirmed by gel electrophoresis of mini-prepped plasmid DNA (Sambrook *et al.* 1989).

Bacterial treatments of plants and growth curves.

Plants were inoculated with overnight cultures of bacteria (1.6–2.2 OD_{600}) collected by centrifugation and resuspended in 10 mM $MgCl_2$ at the desired dilution using one of two methods. In the first method, bacteria were resuspended to 0.001 OD_{600} (1×10^6 cfu/ml), and hand-infiltrated into the undersides of leaves of 4- to 5-wk-old plants, as previously described (Dong *et al.* 1991). Alternatively, 4- to 5-wk-old plants growing through nylon screen in small pots were dipped into a suspension of bacteria at 0.2 OD (2×10^8 cfu/ml) containing 0.02% of the surfactant Silwet L77 (Whalen *et al.* 1991). To monitor bacterial multiplication *in planta*, four whole leaves for the dipping experiments or three 0.5-cm-diameter leaf disks for the hand-infiltration experiments were removed from each plant at 24-hr intervals. These tissues were homogenized in 10 mM $MgCl_2$, serial dilutions were plated on selective KB media, and plates were incubated at 30° C for 2 days. Growth of Km/Rif or Km/Sm-resistant bacteria from infiltrated plant leaves after at least 5 days demonstrated that the plasmids were stably maintained during the time course of our experiments.

RNA preparation and hybridization.

Eight to 10 leaves were harvested from five to 10 different plants receiving the same treatment and frozen in liquid nitrogen. Total RNA was prepared as described (Davis *et al.* 1991), and 10 μg-aliquots of RNA were separated by electrophoresis on 1.1% agarose-formaldehyde gels as described. RNA concentrations were determined by OD_{260} and confirmed by intensity of ethidium bromide staining of rRNA bands. The RNA was transferred to Hybond N (Amersham Corp.) in 20× SSC (1× SSC os 0.15 M NaCl plus 0.015 M sodium citrate) (Sambrook *et al.* 1989) overnight and was fixed onto the filters using a Stratagene Stratalinker on auto-crosslink (12 μjoules). Northern blots were prehybridized (1–2 hr) and hybridized in 5× SSC/1% SDS/1 μg ml^{-1} denatured fish sperm DNA at 65° C. Probes were prepared using a random primer labeling kit (BRL) to specific activities of 5×10^8 to 2×10^9 cpm/μg. Labeled DNA was separated from unincorporated ^{32}P-nucleotides using a 1-ml Sephadex G50 spin column (Sambrook *et al.* 1989), denatured, and added to hybridization solutions at approximately 10^7 cpm ml^{-1}. Filters were hybridized for 20–24 hr, and washed in two changes of 2× SSC/0.8% SDS for 10–15 min at 65° C. Filters to be rehybridized to a second probe were stripped by pouring boiling-hot 0.02× SSC/0.5% SDS over the filters. Damp filters were exposed to Kodak XAR5 film at −80° C with a Dupont Cronex intensifying screen, and/or imaged using a

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains/plasmids	Description	References or source
E. coli DH5α	Cloning host	Bethesda Research Laboratories
P. syringae pv. *maculicola*		
ES4326	Spontaneous Sm^r derivative	Dong *et al.* 1991
KD4326	Spontaneous Rif^r derivative	This study
Plasmids		
pRK2013	Helper plasmid for conjugations Km^r, tra$^+$, Mob$^+$, ColE1 replicon	Figurski and Helinski 1979
pDSK519	Broad host-range cloning vector with *lacZ* promoter, Km^r	Keen *et al.* 1988
pAVRA20	3.2-kb fragment containing the *P. s.* pv. *tomato avrA* gene cloned into pDSK519 downstream of the *lac* promoter	Keen and Buzzell 1991
pAVRB7	1.3-kb fragment containing the *P. s.* pv. *glycinea avrB* gene cloned into pDSK519 downstream of the *lac* promoter	Keen and Buzzell 1991
pAVRC22	1.2-kb fragment containing the *P. s.* pv. *glycinea avrC* gene cloned into pDSK519 downstream of the *lac* promoter	Keen and Buzzell 1991
pAVRD33	1.2-kb fragment containing the *P. s.* pv. *tomato avrD* gene cloned into pDSK519 downstream of the *lac* promoter	Keen *et al.* 1990
pPAL1-4	157-bp *Bgl*II/*Bam*H1 fragment including 5′ untranslated and exon 1 sequences of *A. thaliana PAL1*, cloned into the *Bam*H1 site of Bluescript SK$^+$	This study
pPAL2-2	323-bp *Bgl*II/*Nhe*I fragment including 5′ untranslated and exon 1 sequences of *A. thaliana PAL2*, cloned into *Bam*H1/*Xba*I-digested Bluescript SK$^+$	This study
pLOX1-1	*A. thaliana* lipoxygenase cDNA clone	Melan *et al.* 1993

Molecular Dynamics PhosphorImager. Quantitative data were obtained from PhosphorImager scans using the Molecular Dynamics ImageQuant software to sum total pixel values in equal-sized areas placed over individual hybridizing bands. The data from a single probe used in generating Figures 3–5 were obtained from blots that were hybridized and imaged together, and thus are directly comparable.

Probes for defense-associated genes.

All probes were made from gel-purified plasmid insert fragments. The *PAL1* probe used was a *Pvu*II fragment of *PAL1*-4, which is the 157-bp *Bgl*II/*Bam*H1 fragment of *Arabidopsis PAL1* cloned into the *Bam*H1 site of Bluescript SK+ and is specific for *PAL1*. The *PAL2* probes used were made from either *Pvu*II or *Sst*I/*Hin*dIII fragments of *PAL2*-2, which is the 323-bp *Bgl*II/*Nhe*I fragment of *Arabidopsis PAL2* cloned into *Bam*H1/*Xba*I-digested Bluescript SK+, and is specific for *PAL2*. Details concerning these PAL gene-specific probes will be published elsewhere. The lipoxygenase probe was a 1.4-kb *Eco*R1 fragment of *Arabidopsis* Lox1-1 (Melan *et al.* 1993). The chalcone synthase probe was the 3.9-kb *Hin*dIII insert fragment of *Arabidopsis* CHS3.9 (Feinbaum *et al.* 1988). The peroxidase probe was a 1.5-kb *Eco*RV fragment of ELI11 (Trezzini *et al.* 1993).

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