Recognition of the Avirulence Gene \textit{avrB} from \textit{Pseudomonas syringae pv. glycinea} by \textit{Arabidopsis thaliana}

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The response of \textit{Arabidopsis thaliana} land race Columbia to the bacterial pathogen \textit{Pseudomonas syringae pv. maculicola} 4326 harboring cloned avirulence genes \textit{avrB} and \textit{avrC} from \textit{P. syringae pv. glycinea} and \textit{avrA} and \textit{avrD} from \textit{P. syringae pv. tomato} was examined. Only \textit{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 \textit{A. thaliana} land races where \textit{P. s. pv. maculicola} strains containing \textit{avrB} were not recognized. These plants showed typical disease symptoms, and bacterial multiplication in planta was not reduced in response to \textit{P. s. pv. maculicola} containing \textit{avrB}. In addition, there was no differential induction of defense-related mRNAs in these susceptible land races after infiltration with bacteria containing or lacking \textit{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 \textit{avrB} gene product in \textit{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 \textit{et al.} 1990; Keen 1990; Tamaki \textit{et al.} 1988; Willis \textit{et al.} 1991), and many plant genes that are activated during resistance responses have been described (Ausubel \textit{et al.} 1992; Bowles 1990; Graham \textit{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 \textit{et al.} 1991; Gabriel \textit{et al.} 1986; Hitchin \textit{et al.} 1989; Kobayashi \textit{et al.} 1989; Kobayashi \textit{et al.} 1990; Staskawicz \textit{et al.} 1984; Staskawicz \textit{et al.} 1987; Tamaki \textit{et al.} 1988; Vivian \textit{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 \textit{Arabidopsis thaliana} with phytopathogenic \textit{Pseudomonas syringae} pathovars to study the interaction of bacterial avirulence genes with plant resistance genes (Davis \textit{et al.} 1991; Debener \textit{et al.} 1991; Dong \textit{et al.} 1991; Whalen \textit{et al.} 1991). The advantages offered by \textit{A. thaliana} as an experimental system and a summary of the establishment of \textit{Arabidopsis}-pathogen systems have recently been reviewed (Dangl 1993; Davis 1992). Several examples of gene-for-gene interactions have been described in \textit{A. thaliana}. Two groups simultaneously identified the bacterial \textit{avrRpt2} gene from \textit{P. s. pv. tomato} IL1065 (MM1065) (Dong \textit{et al.} 1991; Whalen \textit{et al.} 1991), which is recognized by some, though not all, land races of \textit{A. thaliana}. Genetic analyses of crosses between resistant and susceptible land races indicate that this resistance is due to a single dominant locus (Innes \textit{et al.} 1993) designated \textit{Rps2}. In addition, a mutant of \textit{A. thaliana} that no longer recognizes \textit{avrRpt2} has been identified in land race Columbia. This mutation has been mapped to chromosome 4 (Kunkel \textit{et al.} 1992). Another avirulence gene, \textit{avrRpm1} (\textit{avrPmaA1}), recognized by some land races of \textit{A. thaliana}, has been isolated from \textit{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 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, IA). 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. tomaton) 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
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-ammonia lyase (PAL) genes and with lipoygenase (LOXI) 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, lipoygenase, which was previously shown to be induced by P. s. pv. maculicola harboring the avrRpt2 gene (Melan et al. 1993). Lipoygenase 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 lipoygenase 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 lipoygenase 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; lipoygenase 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 lipoygenase message were not seen until 30 hr after infiltration with P. s. pv. maculicola lacking avrB.

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/MgCl₂ 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/pDSK519avrB, and showing no disease symptoms 4 days later. D–F, were dipped in P. s. pv. maculicola 4326/pDSK519avrA, avrC, and avrD, respectively, and show typical disease symptoms 4 days later.
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 MgCl₂, 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 (HRPGs), 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. Lipoxigenase mRNA levels have also been shown to increase during a hypersensitive response in Arabidopsis (Melen et al. 1993). Lipoxigenase 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; Melen et al. 1993). Lipoxigenase has also recently been implicated in production of bactericidal lipid-breakdown products (Croft et al. 1993).

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), lipoxigenase (Melen 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, EL3, 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 lipoxigenase because of the potential importance of lipoxigenases 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. Lipoxigenase mRNA accumulation begins several hours after initial bacterial infection, peaking earlier in hypersensitive resistance responses than in susceptible responses. Both PAL and lipoxigenase 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; Melen 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 lipoxigenase 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

![Graphs A, B, C showing lipoxigenase 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 lipoxigenase. 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.](image-url)
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; Trezzi 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 (Tsuij 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 (Mellan 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 soybean Rpgl 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-specific 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 μmol 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 mating. *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 OD600) collected by centrifugation and resuspended in 10 mM MgCl2 at the desired dilution using one of two methods. In the first method, bacteria were resuspended to 0.001 OD600 (1 x 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 x 10^6 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 MgCl2, 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 OD260 and confirmed by intensity of ethidium bromide staining of RNA bands. The RNA was transferred to Hybond N (Amersham Corp.) in 20x SSC (1x SSC is 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 h) and hybridized in 5x SSC/1% SDS/10 μg/ml denatured fish sperm DNA at 65°C. Probes were prepared using a random primer labeling kit (BRL) to specific activities of 5 x 10^6 to 2 x 10^7 cpm/μg. Labeled DNA was separated from unincorporated 32P-nucleotides using a 1-ml Sephadex G50 spin column (Sambrook et al. 1989), denatured, and added to hybridization solutions at approximately 107 cpm ml-1. Filters were hybridized for 20–24 h, and washed in two changes of 2x SSC/0.8% SDS for 10–15 min at 65°C. Filters were rehybridized to a second probe were stripped by pouring boiling-hot 0.02x 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**

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<th>Bacterial strains/plasmids</th>
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<td><strong>E. coli DH5a</strong></td>
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<td><strong>P. syringae pv. maculicola</strong></td>
<td>Spontaneous Sm' derivative</td>
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<td>Spontaneous Rif' derivative</td>
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<td>KD4326</td>
<td>Helper plasmid for conjugations Km', tra+, Mob+, ColEl1 replicon</td>
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<td><strong>Plasmids</strong></td>
<td></td>
<td>Keen et al. 1988</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Broad host-range cloning vector with lacZ promoter, Km'</td>
<td>Keen and Buzzell 1991</td>
</tr>
<tr>
<td>pDSK519</td>
<td>3.2-kb fragment containing the P. s. pv. tomato avrA gene cloned into pDSK519 downstream of the lac promoter</td>
<td>Keen and Buzzell 1991</td>
</tr>
<tr>
<td>pAVRB7</td>
<td>1.3-kb fragment containing the P. s. pv. glycinea avrB gene cloned into pDSK519 downstream of the lac promoter</td>
<td>Keen and Buzzell 1991</td>
</tr>
<tr>
<td>pAVRC22</td>
<td>1.2-kb fragment containing the P. s. pv. glycinea avrC gene cloned into pDSK519 downstream of the lac promoter</td>
<td>Keen and Buzzell 1991</td>
</tr>
<tr>
<td>pAVRD33</td>
<td>1.2-kb fragment containing the P. s. pv. tomato avrD gene cloned into pDSK519 downstream of the lac promoter</td>
<td>Keen and Buzzell 1991</td>
</tr>
<tr>
<td>pPAL1-4</td>
<td>157-bp BglII BamHI fragment including 5' untranslated and exon 1 sequences of A. thaliana PAL1, cloned into the BamHI site of Bluescript SK*</td>
<td>This study</td>
</tr>
<tr>
<td>pPAL2-2</td>
<td>323-bp BglII/Nhel fragment including 5' untranslated and exon 1 sequences of A. thaliana PAL2, cloned into BamHI/Xbal-digested Bluescript SK*</td>
<td>This study</td>
</tr>
<tr>
<td>pLOX1-1</td>
<td>A. thaliana lipoxygenase cDNA clone</td>
<td>Melan et al. 1993</td>
</tr>
</tbody>
</table>
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 PvuII fragment of PAL1-4, which is the 157-bp BglII/BamHI fragment of Arabidopsis PAL1 cloned into the BamHI site of Bluescript SK+ and is specific for PAL1. The PAL2 probes used were made from either PvuII or SstI/HindIII fragments of PAL2-2, which is the 323-bp BglII/NheI fragment of Arabidopsis PAL2 cloned into BamHI/Xbal-digested Bluescript SK+, and is specific for PAL2. Details concerning these PAL gene-specific probes will be published elsewhere. The lipoxigenase probe was a 1.4-kb EcoRI fragment of Arabidopsis Lox1-1 (Mellan et al. 1993). The chalcone synthase probe was the 3.9-kb HindIII insert fragment of Arabidopsis CHS3.9 (Feinbaum et al. 1988). The peroxidase probe was a 1.5-kb EcoRV fragment of ELI11 (Trezzini et al. 1993).

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LITERATURE CITED


