

Identification of *Pseudomonas syringae* Pathogens of *Arabidopsis* and a Bacterial Locus Determining Avirulence on Both *Arabidopsis* and Soybean

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To develop a model system for molecular genetic analysis of plant-pathogen interactions, we studied the interaction between *Arabidopsis thaliana* and the bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*). *Pst* strains were found to be virulent or avirulent on specific *Arabidopsis* ecotypes, and single ecotypes were resistant to some *Pst* strains and susceptible to others. In many plant-pathogen interactions, disease resistance is controlled by the simultaneous presence of single plant resistance genes and single pathogen avirulence genes. Therefore, we tested whether avirulence genes in *Pst* controlled induction of resistance in *Arabidopsis*. Cosmids that determine avirulence were isolated from *Pst* genomic libraries, and the *Pst* avirulence locus *avrRpt2* was defined. This allowed us to construct pathogens that differed only by the presence or absence of a single putative avirulence gene. We found that *Arabidopsis* ecotype Col-0 was susceptible to *Pst* strain DC3000 but resistant to the same strain carrying *avrRpt2*, suggesting that a single locus in Col-0 determines resistance. As a first step toward genetically mapping the postulated resistance locus, an ecotype susceptible to infection by DC3000 carrying *avrRpt2* was identified. The *avrRpt2* locus from *Pst* was also moved into virulent strains of the soybean pathogen *P. syringae* pv *glycinea* to test whether this locus could determine avirulence on soybean. The resulting strains induced a resistant response in a cultivar-specific manner, suggesting that similar resistance mechanisms may function in *Arabidopsis* and soybean.

INTRODUCTION

Genetic analyses of plant-pathogen interactions have demonstrated that disease resistance is often determined by single genes in the two interacting organisms (Flor, 1971). These studies suggest that expression of host resistance involves specific recognition of the pathogen. Single pathogen genes called avirulence genes are required for this recognition, and detection of avirulent pathogens is determined by plant genes termed resistance genes. Thus, for each avirulence gene in the pathogen, there is a corresponding resistance gene in a resistant plant, and resistance is observed only when both genes are present. These genetic relationships have been recorded for a large number of plant-pathogen interactions (Ellingboe, 1981; Keen and Staskawicz, 1988). The identification of a single avirulence gene predicts the presence of a corresponding plant resistance gene, and avirulence genes have been used to identify resistance genes (Whalen et al., 1988; Keen and Buzzel, 1990).

It is not known how the end products of resistance gene

and avirulence gene expression interact or how this interaction leads to disease resistance. Several bacterial avirulence genes have been cloned and characterized (Staskawicz et al., 1984; Gabriel et al., 1986; Napoli and Staskawicz, 1987; Staskawicz et al., 1987; Bonas et al., 1988; Ronald and Staskawicz, 1988; Swanson et al., 1988; Whalen, et al., 1988; Hitchin et al., 1989; Kobayashi et al., 1989; Vivian et al., 1989; Kelemu and Leach, 1990; Min-savage et al., 1990). The function of most avirulence genes remains unclear, but recent evidence suggests that one of these genes has a role in extracellular elicitor production (Keen et al., 1990). No resistance genes have been isolated, nor has the function of any been determined. Progress in cloning a resistance gene has been impeded by the lack of an identified protein or function, and cloning based on genetic map position is difficult in most crop plants. The small genome and well-developed molecular genetics of *Arabidopsis thaliana* (Meyerowitz, 1989) should expedite cloning of both disease resistance genes and genes required for transduction of the recognition event into a physiological response. Therefore, we are developing the interaction between *Arabidopsis* and *Pseudomonas syringae* pv *tomato* (*Pst*) as a model plant-pathogen system (Whalen and Staskawicz, 1990). The development of Ara-

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bidopsis as a model system for study of plant-bacterial interactions is also being pursued by Fred Ausubel and colleagues at Harvard University (Dong et al., 1991), and our laboratories are cooperating in this effort.

Arabidopsis has become well established as a model for genetic and molecular studies of flowering plants (Somerville, 1989). However, little is known about its interactions with pathogens. Recent investigations have shown that *Arabidopsis* is a host for several groups of pathogens, including viruses, bacteria, and fungi (Susnova and Polak, 1975; Koch and Slusarenko, 1990; Schott et al., 1990; Simpson and Johnson, 1990). No disease resistance genes have been defined in *Arabidopsis* and no avirulence genes that cause a resistant response in *Arabidopsis* have been identified. In this report, we characterize the interaction between *Arabidopsis* and *P. syringae* pathovars *tomato* and *maculicola*. Four cosmid clones from avirulent *Pst* strains are described that convert virulent strains of *Pst* to avirulence on *Arabidopsis* ecotype Col-0. As a first step in characterizing putative Col-0 resistance genes, an *Arabidopsis* ecotype that lacks resistance to *Pst* carrying the cloned avirulence locus *avrRpt2* has been identified. Finally, evidence is presented that bacteria carrying the avirulence locus *avrRpt2* are recognized by certain soybean cultivars, suggesting that the same or a functionally equivalent resistance gene is present in both *Arabidopsis* and soybean.

RESULTS

Identification of Bacterial Pathogens of *Arabidopsis*

Eighteen *Arabidopsis* ecotypes of diverse geographical origin were inoculated with four strains of *P. syringae* pv *maculicola* (*Psm*), a pathogen of tomato and crucifers. Table 1 summarizes these results. Although the intensity of the response of *Arabidopsis* ecotypes to the four *Psm* strains varied, *Psm* was virulent and induced disease symptoms on all 18 *Arabidopsis* ecotypes. *Psm* strain 4326 generally induced the strongest response, a grey-brown lesion with marginal chlorosis. On a few ecotypes, 4326 produced strong, spreading chlorosis. *Psm* strains 2744 and 4981 were generally less virulent than 4326 and induced either a grey-brown lesion with light chlorosis at the margin or chlorosis spreading out from the inoculation site and no lesion. *Psm* strain 795 was the least virulent on all ecotypes, inducing light chlorosis. On the four *Brassica* species tested, the relative intensity of the response to a given *Psm* strain was the same as that seen on *Arabidopsis* (Table 1). All four *Psm* strains induced equivalent disease symptoms on tomato (Table 1).

Because we did not observe variation in the response of *Arabidopsis* ecotypes to the four *Psm* strains (i.e., none of the 18 different ecotypes tested appeared to be resistant to any of the four *Psm* strains), 30 geographically

Table 1. Virulence of *P. syringae* pv *maculicola* and *P. syringae* pv *tomato* on Three Host Genera

<i>P. syringae</i>	<i>Brassica</i>		
	spp ^a	tomato ^a	<i>Arabidopsis</i> ^b
<i>pv maculicola</i>			
4326	+++	++	++ +
795	+	++	+
2744	++	++	++
4981	+++	++	++
<i>pv tomato</i>			
DC3000	+++	+++	+++ ^{c,d}
1065	±	+++	—
T1	+	+++	—
3435	NT	+++	+++ ^d
3455	NT	+++	+++ ^d
Others ^e	NT	++/+++	— ^f

+++ , highly virulent; ++ , moderately virulent; + , less virulent; ± , less virulent to avirulent; — , avirulent; NT , not tested.

^a Tested on three *Brassica oleracea* (cabbage, cv Tastic; cauliflower, cv Snowball; broccoli cv Emperor) and one *B. campestris* (turnip, cv Just Right), and on tomato cv Peto76.

^b Tested on the following ecotypes: Aa-0, Ag-0, Co-4, Col-0, Cvi-0, En-2, Greenville, Hau-0, Kas-1, Kindalville, Ll-0, Mh-0, Ms-0, Mv-0, Nd-0, No-0, Sei-0, Turk Lake.

^c Also tested on the following *Arabidopsis* ecotypes: Ba-1, Bla-4, Br-0, Cvi-0, Ge-1, Gs-0, Gu-0, Hs-0, Kä-0, Po-1, Sh-0, Stw-0.

^d Virulent on most ecotypes; see Table 2 for exceptions.

^e Includes other *P. syringae* pv, *tomato* strains tested: 16-1B, 19-2, 19-3, 156, 688, 864, 1065, 2846, 3358, 5109, 9501, 3647, 4498, BM-G13, JL1002, JL1006, JL1015, JL1043, JL1065, JL1102, JL1118, JL1124, PT-7, and PT-8.

^f Inoculated on ecotype Col-0 only.

diverse *P. syringae* pv *tomato* strains were tested. Initially, we confirmed the virulence of all 30 strains on tomato (Table 1). Variation in the response of *Arabidopsis* ecotype Col-0 to different *Pst* strains was evident (Table 1). *Pst* strains DC3000, 2844, 3455, and 3435 induced strong disease symptoms that were similar to those induced by *Psm* strain 4326, producing a grey-brown lesion with chlorosis spreading out from the lesion. Figure 1A shows the response of ecotype Col-0 to strain DC3000. Ecotype Col-0 was resistant to the other 26 *Pst* strains. In typical resistant reactions, no leaf infection symptoms were evident (Figure 1B), although mild chlorosis or necrosis was occasionally observed. *Pst* strains DC3000, JL1065, and T1 were used in further studies; these strains induced symptoms on the four *Brassica* species ranging from severe (DC3000) to mild (JL1065).

Bacterial growth in plant leaves was monitored to determine whether the lack of symptom development correlated with restriction of pathogen multiplication. Entire rosettes of ecotype Col-0 were vacuum infiltrated with suspensions of a single *Pst* strain and leaf samples were taken at 2-day intervals. The virulent strain DC3000 multiplied approximately 5 orders of magnitude over 6 days to reach final

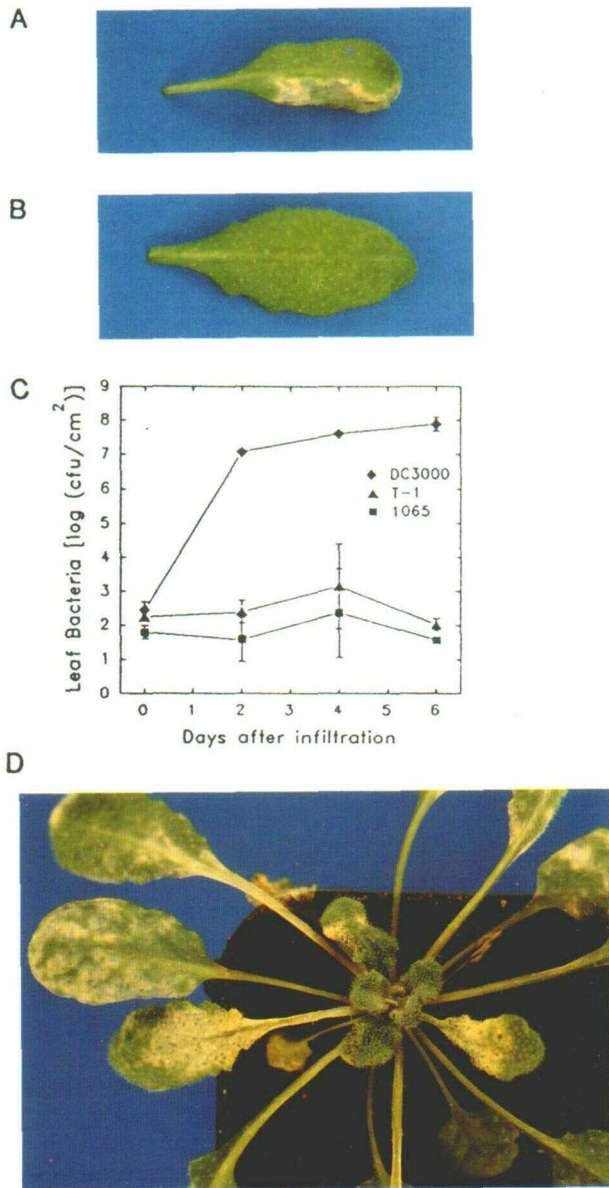


Figure 1. Resistant and Susceptible Phenotypes of *Arabidopsis* Infected with *P. syringae* pv *tomato*.

(A) *Arabidopsis* ecotype Col-0 leaf 5 days after inoculation with *Pst* strain DC3000. Leaf was inoculated on one side by pressure infiltration of a 1×10^6 cfu/mL suspension of bacteria.
 (B) *Arabidopsis* ecotype Col-0 leaf 5 days after inoculation with *Pst* strain JL1065. Inoculation protocol was identical to that for (A).
 (C) Growth of *Pst* strains DC3000, JL1065, and T1 within *Arabidopsis* ecotype Col-0 leaves. Plants were inoculated by vacuum infiltration of a 1×10^5 cfu/mL bacterial suspension. Data points represent mean \log_{10} (cfu/cm²) \pm sample SD.
 (D) *Arabidopsis* ecotype Col-0 5 days after inoculation with *Pst* strain DC3000. Leaves were inoculated by dipping them into a 2×10^8 cfu/mL bacterial suspension in 0.01% L-77, 10 mM MgCl₂.

leaf concentrations of 10^7 to 10^8 colony-forming units (cfu)/cm² of leaf, whereas growth of strains JL1065 and T1 was extremely limited (Figure 1C). Growth of the crucifer pathogen *Psm* 4326 in ecotype Col-0 was essentially the same as *Pst* DC3000 (data not shown).

Development of Bacterial Inoculation Procedures

Several growth regimes and inoculation methods were tested to identify conditions that increased the reproducibility of disease phenotypes. Plants grown under 8-hr days proved best for inoculation; these plants produced multiple large leaves before onset of bolting. Juvenile (small circular) leaves and plants that had visibly initiated flowering shoots gave variable results. For hand inoculations, dose-response studies indicated that virulent bacteria caused no symptoms when infiltrated in suspensions below 10^5 cfu/mL, whereas avirulent bacteria often caused chlorosis and necrosis at concentrations above 5×10^7 cfu/mL (data not shown). Therefore, we used 1×10^6 cfu/mL as our standard concentration; phenotypes were scored 5 days after inoculation.

A new method for inoculation of large numbers of plants was developed that utilized the surfactant Silwet L-77 (Union Carbide). L-77 is a silicon-based copolymer that depresses surface tension sufficiently to allow aqueous droplets to spread evenly over the leaf surface and to penetrate stomatal openings. Dipping or spraying plants with 2×10^8 cfu/mL suspensions of *Pst* in 10 mM MgCl₂/0.01% L-77 resulted in the development of disease phenotypes on *Arabidopsis* that were quite similar to those observed in naturally occurring field infections of tomato (Figure 1D). For all ecotype/strain pairings tested with L-77, development of resistant or susceptible reactions correlated with the phenotypes predicted from traditional hand or vacuum infiltrations. Toxicity of L-77 was visually apparent at concentrations above 0.1%. L-77 has been tested with similar results for inoculations of tomato and pepper with *Pst* and *Xanthomonas campestris* pv *vesicatoria*, respectively (F. Carland, D. Dahlbeck, and B. Staskawicz, unpublished results).

Natural Variation in Avirulence:Resistance Relationships

During the initial examination of the response of *Arabidopsis* to wild-type isolates of *Pst*, variation was found in both the virulence of a given strain on different ecotypes and the resistance of a given ecotype to different strains. Table 2 summarizes these results. *Pst* strains DC3000, 3435, and 3455 were virulent on most *Arabidopsis* ecotypes, but for each strain at least one resistant ecotype was identified. Ecotype Sf-2 was resistant to all 30 *Pst* strains tested (Tables 1 and 2).

Table 2. Natural Variation in Avirulence of *P. syringae* pv *tomato* and Resistance of *Arabidopsis* Ecotypes

<i>P. syringae</i> pv <i>tomato</i>	<i>Arabidopsis</i>				
	Col-0	Uk-4	Be-0	Bus-0	Sf-2
DC3000	+ 4.6 ± 0.1	- 2.4 ± 0.2	+ 3.7 ± 0.4	- 2.2 ± 0.6	- 1.1 ± 0.1
3435	+ 3.7 ± 0.3	± 2.7 ± 0.3	NT	+ 3.8 ± 0.6	- 1.2 ± 0.2
3455	+ 3.6 ± 0.4	+ 4.3 ± 0.2	- 1.5 ± 0.4	- 2.0 ± 0.6	- 1.0 ± 0.0

Disease ratings scored on a scale of 1 to 5. Numbers are mean ± 1 SE of the mean; average sample size 14 (range 6 to 39). >3.5 = + (virulent); <2.5 = - (avirulent); 2.5 to 3.5 = ± (intermediate); NT, not tested.

Isolation of Avirulence Loci from *P. syringae* pv *tomato* Genomic Libraries

To ascertain whether the resistant reaction of *Arabidopsis* to *Pst* strains JL1065 and T1 was determined by avirulence genes, genomic libraries of DNA from JL1065 and T1 were constructed in the wide host range cosmid vector pLAFR3 and were conjugated into the virulent strain *Pst* DC3000.

Transconjugants were inoculated onto *Arabidopsis* ecotype Col-0 and reactions were scored 4 days to 5 days thereafter. Table 3 lists the four cosmid clones that converted the normally virulent DC3000 to avirulence on ecotype Col-0. These cosmids had no apparent effect on the virulence of DC3000 on tomato (see below). Cosmid p4-24 was isolated in a screen of 1170 cosmids from *Pst* strain JL1065, and pT1371, pT1381, and pT1390 were

Table 3. Virulence of *P. syringae* pv *tomato* Strains on *Arabidopsis* Ecotypes

<i>P. syringae</i> pv <i>tomato</i>	<i>Arabidopsis</i>				
	Col-0	Po-1	Hs-0	Sei-0	Gs-0
DC3000	+ 4.6 ± 0.1	+ 4.7 ± 0.1	+ 4.7 ± 0.1	+ 3.8 ± 1.0	+ 4.7 ± 0.2
1065	- 1.0 ± 0.0	- 1.0 ± 0.0	- 1.0 ± 0.0	NT	NT
T1	- 1.0 ± 0.0	NT	NT	NT	NT
DC3000(pT1371)	± 3.0 ± 0.2	+ 4.1 ± 0.2	± 3.4 ± 0.3	+ 4.5 ± 0.3	+ 3.9 ± 0.3
DC3000(pT1381)	- 2.0 ± 0.2	- 2.4 ± 0.4	- 2.4 ± 0.4	± 3.0 ± 1.0	- 1.4 ± 0.4
DC3000(pT1390)	- 1.6 ± 0.1	+ 4.4 ± 0.2	± 3.2 ± 0.3	± 3.0 ± 0.9	- 2.0 ± 0.5
DC3000(p4-24)	- 1.7 ± 0.2	+ 4.8 ± 0.2	± 3.4 ± 0.4	- 2.3 ± 0.7	- 2.3 ± 0.8
DC3000(pLH12)	- 1.8 ± 0.2	+ 4.9 ± 0.1	± 2.8 ± 0.6	NT	NT
DC3000(pLH12 Ω)	+ 4.7 ± 0.1	+ 4.8 ± 0.2	+ 4.8 ± 0.1	NT	NT

Disease ratings scored on a scale of 1 to 5. Numbers are mean ± 1 SE of the mean; average sample size 20 (range 4 to 82). >3.5 = + (virulent); <2.5 = - (avirulent); 2.5 to 3.5 = ± (intermediate); NT, not tested.

derived from a screen of 350 cosmids from strain T1. The strength of avirulence conferred by these loci differed; cosmid p4-24 was the most consistent and complete in reducing disease symptoms on Col-0. This cosmid was chosen for further characterization. Introduction of this cosmid into the virulent strains *Pst* 3435 and 3455 and *Psm* 4326 also resulted in conversion to avirulence on ecotype Col-0 (data not shown). Avirulence activity was localized to a 1.4-kb DNA fragment, and we have designated this locus *avrRpt2*. Construct pLH12 carries this 1.4-kb region in pLAFR3. An insertional disruption of *avrRpt2* was constructed by introduction of an Ω fragment (Prentki and Krisch, 1984). The resulting plasmid pLH12 Ω does not convert DC3000 to avirulence on *Arabidopsis* ecotype Col-0 (Table 3), indicating that a single operon and possibly a single gene encodes the avirulence activity. In the course of these studies, we exchanged avirulence locus clones with Fred Ausubel's laboratory and found by restriction enzyme analysis and DNA gel blot hybridization that their clone pMMXR1 from *Pst* strain JL1065 carried *avrRpt2*. Clone pMMXR1 also converts *Pst* strain DC3000 and *Psm* strain 4326 to avirulence on ecotype Col-0 (Dong et al., 1991).

Several other cosmid clones that reduced virulence of *P. syringae* on *Arabidopsis* Col-0 were identified during our initial screen of genomic libraries. However, these cosmids conferred phenotypes (such as reduced growth in vitro or within a susceptible *Pst* host, tomato cv Peto76) that indicated a nonspecific reduction in virulence. Four cosmid clones were isolated from a library of *Pst* JL1065 based on their conversion of *Psm* 4326 to avirulence on *Arabidopsis* Col-0, but these did not convert *Pst* DC3000 to avirulence on Col-0. These clones had homology with the *hrp* cluster from *Pst* JL1065 (data not shown), a group of loci required for pathogenicity on susceptible hosts and elicitation of the hypersensitive response on resistant hosts (Lindgren et al., 1988). The nonspecific avirulence caused by these four clones was possibly due to a general reduction in virulence caused by interference between heterologous *hrp* regions (C. Boucher, D. Dahlbeck, and B. Staskawicz, manuscript in preparation). A fifth cosmid clone, p10656.21 (preliminarily reported in Whalen and Staskawicz, 1990), did convert *Pst* strain DC3000 to avirulence on Col-0 and did not hybridize to the *Pst* *hrp* region. However, further characterization revealed that this clone did not behave as a classical avirulence gene. Clone p10656.21 (and subclones thereof) reduced the in vitro growth rate of *Pst* strain DC3000 compared with strain DC3000 (pLAFR3). Furthermore, p10656.21 reduced growth of *Pst* DC3000 by 1 order of magnitude in Cvi-0, an *Arabidopsis* ecotype classified as susceptible to DC3000 (p10656.21) based on visual inspection (data not shown). Similar reductions in in vitro growth and growth in plants were conferred by another candidate avirulence locus (cosmid pT1460) isolated from *Pst* strain T1 (data not shown).

Identification of *Arabidopsis* Ecotypes with Differing Responses to *P. syringae* pv *tomato* Strains Carrying Cloned Avirulence Loci

Arabidopsis ecotype Col-0 potentially carries resistance genes that determine specific recognition of bacteria carrying the avirulence loci reported in Table 3. To test this hypothesis genetically, it was necessary to identify *Arabidopsis* ecotypes or mutants susceptible to *Pst* DC3000 carrying a given avirulence locus. More than 30 ecotypes were screened for susceptibility; results from a subset of these inoculations are reported in Table 3. The majority of ecotypes were resistant to DC3000 carrying any of the four avirulence loci. However, ecotypes Po-1, Sei-0, Hs-0, and Gs-0 were less resistant than Col-0 upon inoculation with DC3000 carrying specific avirulence loci (Table 3). Ecotype Po-1 is of special interest because it was susceptible to infection by DC3000 carrying *avrRpt2*.

Induction of a Hypersensitive Response in *Arabidopsis* by *Pst* Strains Expressing *avrRpt2*

In many plant-pathogen interactions, the resistant reaction is characterized by localized death of host cells in the region of infection [the hypersensitive response (HR); Klement, 1982]. No HR was observed when we inoculated *Arabidopsis* with virulent or avirulent strains of *Pst* at bacterial concentrations of 1×10^6 cfu/mL. In inoculations of pepper with *X. campestris* pv *vesicatoria* or soybean with *P. syringae* pv *glycinea*, resistant plants only show an HR when avirulent bacteria are inoculated at a density above approximately 10^7 cfu/mL (D. Dahlbeck and B. Staskawicz, unpublished results). To determine whether *Pst* strains expressing the putative avirulence gene *avrRpt2* induced an HR on *Arabidopsis*, we inoculated strains JL1065, DC3000(pLH12), and DC3000(pLH12 Ω) into the resistant ecotype Col-0 at cell concentrations of 10^7 and 10^8 cfu/mL. At 10^7 cfu/mL, strain DC3000(pLH12) induced a collapse of host tissue in the inoculated region within 16 hr of inoculation, whereas no tissue collapse was observed with strain DC3000(pLH12 Ω) until 48 hr after inoculation. Strain JL1065 induced only a partial collapse, even at 48 hr, at this level of inoculum. At 10^8 cfu/mL, both JL1065 and DC3000(pLH12) induced a complete collapse of the inoculated region within 16 hr of inoculation. However, at 10^8 cfu/mL, the virulent strain DC3000(pLH12 Ω) induced a complete collapse within 24 hr. On the susceptible ecotype Po-1, the phenotypes induced by strains DC3000(pLH12) and DC3000(pLH12 Ω) were indistinguishable; neither strain induced tissue collapse until 48 hr after inoculation when an inoculum of 10^7 cfu/mL was used. Strain JL1065 induced no symptoms on ecotype Po-1, even when inoculated at 10^8 cfu/mL.

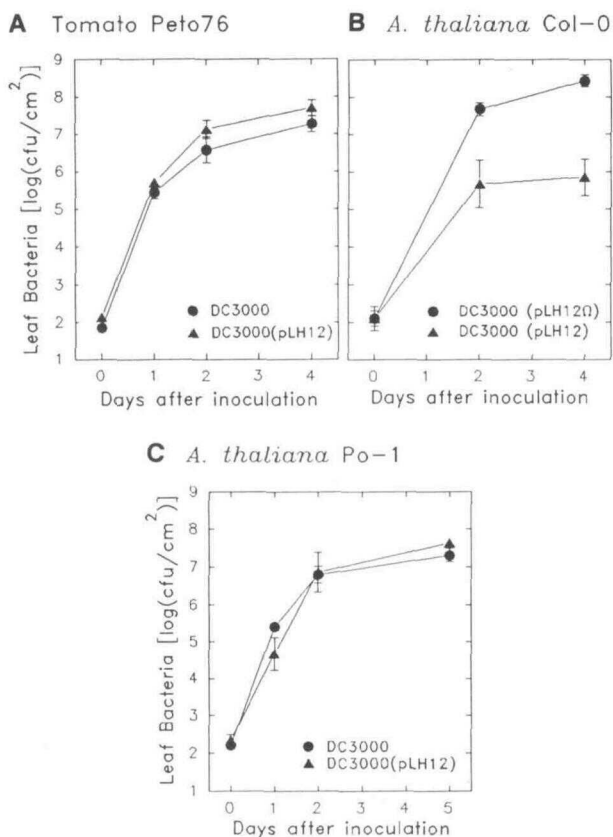


Figure 2. Growth of *Pst* Strain DC3000 with and without Avirulence Locus *avrRpt2* in Plant Leaves.

Data points represent mean log₁₀ (cfu/cm²) \pm sample SD.

(A) Tomato cultivar Peto76 inoculated with *Pst* strain DC3000 or DC3000(pLH12).

(B) *Arabidopsis* ecotype Col-0 inoculated with *Pst* strain DC3000(pLH12 Ω) or DC3000(pLH12).

(C) *Arabidopsis* ecotype Po-1 inoculated with *Pst* strain DC3000 or DC3000(pLH12).

Effect of *avrRpt2* on Bacterial Growth in Plants

To ascertain whether the phenotypes reported in Table 3 reflected the level of bacterial growth inside the plant leaves, the effect of *avrRpt2* on growth of *Pst* strain DC3000 was determined. Tomato cv Peto76 and *Arabidopsis* ecotypes Col-0 and Po-1 were vacuum infiltrated with bacterial suspensions, and bacterial growth within leaves was monitored over time. Figure 2 summarizes the results. In both tomato and *Arabidopsis*, DC3000 populations increased by approximately 10⁵ over the first 3 days before reaching a plateau at approximately 10⁷ cfu/cm². The equivalent growth in tomato of DC3000 with and without pLH12 (Figure 2A) suggested that *avrRpt2* has no significant effect on the general virulence of this strain. Addition of pLH12 reduced growth of DC3000 in *Arabidopsis* ecotype Col-0 leaves by 10²-fold to 10³-fold,

whereas DC3000 carrying a disrupted *avrRpt2* (pLH12 Ω) behaved similarly to wild type (Figure 2B). The susceptibility of ecotype Po-1 to DC3000(pLH12) was also confirmed (Figure 2C). Equivalent growth of DC3000 with and without *avrRpt2* in ecotype Po-1 again indicated that this locus does not affect virulence in general.

Observation of Infected Leaves Using Scanning Electron Microscopy

The correlation between macroscopic phenotypes of infected leaves and bacterial growth was further documented with scanning electron microscopy. *Arabidopsis* ecotype Col-0 leaves were hand inoculated on one side of

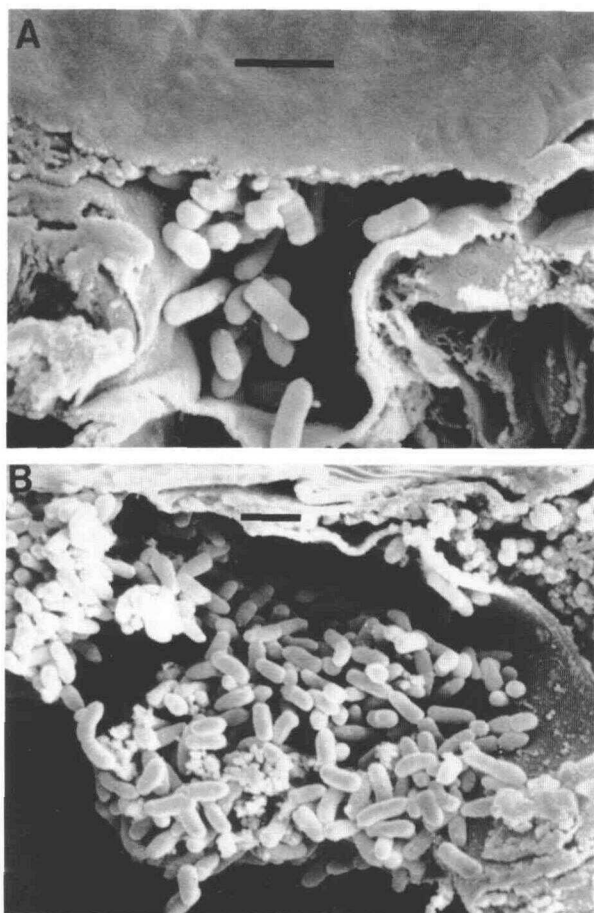


Figure 3. Scanning Electron Micrographs of *Arabidopsis* Ecotype Col-0 Leaves 4 Days after Inoculation with *Pst* Strain DC3000 with or without *avrRpt2*.

Leaves were fixed and then sectioned transversely. Bar = 2 μ m.

(A) Inoculated with DC3000(pLH12).

(B) Inoculated with DC3000(pLH12 Ω).

the midvein with suspensions of DC3000(pLH12) or DC3000(pLH12 Ω). After inoculation, samples were taken immediately and after 2 days and 4 days. Although no bacteria were evident in samples taken on day 0, sections taken on day 2 contained clusters of one to five bacterial cells at a frequency of approximately one cluster per every 100 visible plant cells. These clusters seemed to occur only in the extracellular spaces of leaf mesophyll, and no obvious differences in frequency or morphology were observed between inoculations with virulent or avirulent bacteria. Figure 3A shows leaves that were sampled 4 days after inoculation with the avirulent strain DC3000(pLH12). Clusters were again infrequently observed and, when present, tended to contain a maximum of 10 to 20 bacterial cells. In contrast, leaves sampled 4 days after inoculation with the virulent strain DC3000(pLH12 Ω) displayed more frequent bacterial clusters (approximately one per 20 visible plant cells) and these clusters contained up to many hundreds of bacterial cells (Figure 3B). No bacteria were observed in limited examination of uninoculated tissue on the opposite side of the midvein. Col-0 leaves inoculated with wild-type *Pst* strain DC3000 gave results essentially similar to those obtained with DC3000(pLH12 Ω), whereas no bacteria were observed in leaves inoculated with the avirulent strain JL1065.

Avirulence Locus *avrRpt2* from *P. syringae* pv *tomato* also Determines Resistance of Soybean to *P. syringae* pv *glycinea*

Previous work has shown that a single avirulence gene can function in multiple pathovars (Whalen et al., 1988; Kobayashi et al., 1989). For example, avirulence gene *avrD* from *P. syringae* pv *tomato* converts the soybean pathogen *P. syringae* pv *glycinea* (*Psg*) from virulence to avirulence on specific cultivars of soybean (Kobayashi et al., 1990), and soybean resistance to bacteria carrying *avrD* segregates as a single dominant gene (Keen and Buzzel, 1990). We introduced *avrRpt2* into the virulent *Psg* strain A29-2 to test whether this putative avirulence gene could convert *Psg* to avirulence on specific soybean cultivars. Inoculations were done at high bacterial cell densities (10^8 cfu/mL) to assay for induction of an HR. An HR is typically produced by soybean plants during a resistant response (Staskawicz et al., 1984). On soybean cultivars Centennial, Flambeau, and Harosoy, a light but distinct HR became visible 30 hr after inoculation with *Psg* strain A29-2(pLH12). On cultivars Acme and Norchief, no HR was detected, and water-soaking phenotypes typical of a susceptible response appeared approximately 72 hr after inoculation. Inoculation with wild-type *Psg* strain A29-2 produced water-soaking symptoms on all cultivars. Bacterial growth curves performed on cultivars Acme and Centennial confirmed that visible phenotypes correlated with reduction in bacterial cell growth. On cultivar Centennial, growth of a

strain carrying pLH12 was reduced approximately 1.5 orders of magnitude after 5 days relative to growth of the strain carrying pLH12 Ω (data not shown).

DISCUSSION

Success in elucidating the molecular basis of disease resistance in plants will depend on the concurrent use of genetics, biochemistry, and molecular biology to study a given plant-pathogen interaction. Biochemical and molecular responses to pathogen invasion have been well studied (Lamb et al., 1989; Dixon and Harrison, 1990), but causal roles in resistance have been difficult to demonstrate without genetic analysis based on defined loci and mutations. Genetic studies of many plant-pathogen interactions have demonstrated that resistance depends on the simultaneous presence of corresponding resistance and avirulence genes (Ellingboe, 1982). Studies of the molecular mechanisms underlying these interactions have been hampered, however, by difficulties in molecular genetic analysis of the plant species for which resistance genes have been described. Many pathogen species have also proven to be intractable to molecular genetic analysis. We have focused on interactions between *Arabidopsis* and bacterial pathogens to circumvent many of these problems. *Arabidopsis* is proving to be extremely useful for molecular genetic analysis (Somerville, 1989). Bacterial pathogens are also highly suitable for such analysis, and several genes controlling bacterial pathogenicity and avirulence already have been characterized (Keen and Staskawicz, 1988).

To develop a plant-pathogen system involving *Arabidopsis*, we first identified a bacterial pathogen species, *Pst*, that was virulent on *Arabidopsis*. We then identified strains within this species that were avirulent on at least one *Arabidopsis* ecotype. Responses of *Arabidopsis* to *Pst* were classified as resistant or susceptible based on visual examination, scanning electron microscopy, and bacterial growth *in vivo* (Figures 1 to 3 and Tables 1 to 3). In all cases, a susceptible response assessed visually and by scanning electron microscopy correlated with a high level of bacterial growth *in vivo*, whereas the resistant response was associated with a reduced level of growth. These observations demonstrate that the interaction between *Arabidopsis* and *Pst* is similar to other well-studied plant-pathogen interactions.

Our identification of *Arabidopsis* ecotypes that differ in their response to wild-type *Pst* strains complements other work on bacterial pathogens and *Arabidopsis*. Simpson and Johnson (1990) identified *Arabidopsis* ecotypes that showed differential responses to a single *X. campestris* pv *campestris* (*Xcc*) strain and *Xcc* strains that were virulent and avirulent on a single *Arabidopsis* ecotype. These results open the way for isolation of avirulence and resist-

ance genes that could potentially control this interaction. Biochemical responses of *Arabidopsis* to bacterial pathogens were investigated by Davis and Ausubel (1989), who characterized elicitor-induced defense responses in suspension-cultured cells of *Arabidopsis*. They reported that cells treated with the pectin degrading enzyme α -1,4-endopolygalacturonic acid lyase from *Erwinia carotovora* pv *carotovora* accumulated substantial levels of several enzymes involved in phenylpropanoid biosynthesis. Phenylpropanoids have been implicated in defense responses in several plant-pathogen systems (Hahlbrock and Scheel, 1989). Recently, it has been found that the avirulent *Pst* strain JL1065 induces transcript accumulation of the phenylalanine ammonia lyase (PAL) gene when inoculated into *Arabidopsis* (Dong et al., 1991). PAL is the first enzyme in the phenylpropanoid pathway. Moreover, Dong et al. (1991) report that when the avirulence locus *avrRpt2*, described above, is transferred to the virulent *Psm* strain 4326, the resulting strain induces the PAL gene to greater levels than strain 4326 without *avrRpt2*.

The interaction of *Pst* with *Arabidopsis* is similar to that of *Pst* with tomato. In natural infections, *Pst* generally enters the intercellular spaces of tomato leaves through stomata or wounds and forms a necrotic lesion with marginal chlorosis (Bashan et al., 1981). These lesions are similar in appearance to those produced upon inoculation of *Arabidopsis* with *Pst* suspended in L-77 (Figure 1D).

The data presented in Table 2 indicate that there is heterogeneity in the occurrence of avirulence and resistance determinants in *Pst* and *Arabidopsis*. One ecotype was susceptible to all three *Pst* strains (Col-0), whereas another was resistant to all three (Sf-2). Differences in infection phenotypes on Uk-4, Bus-0, and Sf-2 suggest that there are at least three distinct resistance genes and/or mechanisms of resistance in these ecotypes. Ecotype Col-0 either lacks all three of these genes or mechanisms or is blocked in some other required step, yet retains resistance to *Pst* strains JL1065 and T1 (Table 1). In addition, resistance could be controlled by separate mechanisms in any two ecotypes that are both resistant to the same *Pst* strain.

To ensure that resistance of *Arabidopsis* to *P. syringae* pv *tomato* was conditioned by a single resistance/avirulence gene pair, we constructed avirulent pathogens by the addition of cloned avirulence loci to otherwise virulent strains. A major step toward developing this model system was, thus, the identification of natural variation for avirulence among *Pst* strains. This allowed us to clone and partially characterize several putative avirulence genes from the avirulent *Pst* strains JL1065 and T1. Criteria for identification of avirulence genes included induction of resistance on specific host genotypes with no reduction in virulence on susceptible genotypes. The induction of resistant as opposed to susceptible plant reactions by strains differing only in the expression of *avrRpt2* can, therefore, be attributed to the presence of a single avirulence locus.

We are currently using a combination of sequence and deletion analysis to determine whether *avrRpt2* is a single gene.

In many plant-pathogen interactions, the resistant reaction is characterized by localized death of host cells in the region of infection, which has been called the hypersensitive response (Klement, 1982). When *Arabidopsis* was inoculated with *Pst* at densities above 1×10^7 cfu/mL, both avirulent and virulent strains produced collapse of plant cells that developed into a necrotic lesion surrounded by chlorosis (data not shown). However, this collapse occurred earlier in inoculations with avirulent strains, suggesting that the avirulent strains are inducing a hypersensitive response. Thus, at a bacterial concentration of 1×10^7 cfu/mL, inoculation with *Pst* strain DC3000(pLH12) induced a hypersensitive response within 16 hr, whereas no collapse was observed within 48 hr in leaves inoculated with DC3000(pLH12 Ω). Interestingly, the wild-type avirulent strain JL1065 did not induce collapse within 16 hr unless inoculated at a 10-fold higher cell concentration. One possible explanation for this difference is that strain JL1065 fails to multiply inside *Arabidopsis* leaves, whereas strain DC3000(pLH12) initially multiplies quite rapidly (Figures 1C and 2B). Thus, by 12 hr after inoculation of a 1×10^7 cfu/mL suspension of DC3000(pLH12), population levels would be expected to be as great or greater than those of strain JL1065 inoculated at 10^6 cfu/mL. Inoculation densities of 1×10^6 cfu/mL were of limited diagnostic utility, however, because the timing of symptom appearance was very similar between virulent and avirulent strains. At densities below 5×10^6 cfu/mL, avirulent *Pst* strains produce no visible reaction on *Arabidopsis* (Figure 1B). The resistant response at low densities may involve dispersed, microscopic HRs that do not coalesce to form visible necrotic lesions.

We observed that *Pst* DC3000 carrying *avrRpt2* grew to higher levels in *Arabidopsis* Col-0 leaves than JL1065, the wild-type *Pst* strain from which *avrRpt2* was derived (Figures 1C and 2B). This difference in virulence on Col-0 was also reflected in our scoring of visual phenotypes (Table 3) because *Pst* DC3000 carrying *avrRpt2* occasionally induced patches of chlorosis on inoculated leaves, whereas *Pst* JL1065 did not. Additional avirulence genes that are specific for other Col-0 resistance genes may be present in JL1065 and together may induce a stronger resistance reaction on Col-0. Alternatively, *avrRpt2* constructs used in this study may not include DNA sequences required for full expression, or strain JL1065 may lack functions required for pathogenesis on *Arabidopsis*.

The identification of *avrRpt2* suggests, in accordance with the gene-for-gene paradigm, that *Arabidopsis* ecotype Col-0 carries a single resistance gene specific for *avrRpt2* (Flor, 1971; Whalen et al., 1988; Keen and Buzzel, 1990). The isogenic pathogen strains allowed us to identify *Arabidopsis* ecotype Po-1, which putatively lacks a corresponding resistance gene. Po-1 would not have been

identified using strain JL1065 (the source of *avrRpt2*) because this strain was avirulent on Po-1. With the identification of an ecotype susceptible to *Pst* strains carrying *avrRpt2*, we are in a position to evaluate the genetic basis of resistance corresponding to *avrRpt2*. We have crossed the resistant ecotype Col-0 to susceptible ecotype Po-1 and will follow segregation in F2 populations. If a single locus for resistance is identified, it can be mapped relative to the extensive collection of *Arabidopsis* restriction fragment length polymorphism markers as a first step toward cloning the gene by chromosomal walking (Chang et al., 1988; Nam et al., 1989). We are also pursuing identification of mutagenized plants deleted for the locus, which would allow subsequent cloning by genomic subtraction methods (Straus and Ausubel, 1990; Wieland et al., 1990). The surfactant L-77 is likely to find its greatest use in mutant screens such as this because it eliminates the need for time-consuming hand inoculation of individual plant leaves.

We have found that some, but not all, soybean cultivars give a resistant response upon inoculation with *Psg* carrying the *avrRpt2* locus from *Pst*. Because the interaction conditioned by avirulence genes and resistance genes is specific, we expect that resistant soybean cultivars such as Centennial may have a resistance gene functionally equivalent to the gene in *Arabidopsis* ecotype Col-0. The implied similarity of resistance mechanisms between *Arabidopsis* and soybean suggests that *Arabidopsis* resistance genes can be used to isolate similar loci from soybean. More generally, it may be possible to expand the resistance of crop plants by transformation using *Arabidopsis* resistance genes. These results highlight the potential diversity of contributions that *Arabidopsis* research can make to the field of plant pathology.

METHODS

Bacterial Strains, Media, and Plasmids

Psm strains 4326, 795, 2744, and 4981 and *Pst* strains 3435, 3455, 864, 2844, 2846, 3358, 3647, 4498, 5109, and 9501 were obtained from the New Zealand culture collection. *Pst* strains DC3000, T1, and BM-G13 were kindly provided by D. Cuppels and *Pst* strains JL1065, JL1002, JL1006, JL1015, JL1043, JL1102, JL1118, and JL1124 were kindly provided by J. Lindeman. *Pst* strains PT-7, PT-8, and 688 were kindly provided by M. Schroth. *Psg* strain A29-2 (Race 4) was obtained from N. Keen. *Escherichia coli* DH5 α (Bethesda Research Laboratories) was the recipient in the construction of the *Pst* JL1065 and T1 cosmid libraries and for subclones. *Pst* and *Psm* strains were cultured at 30°C on King's Medium B (King et al., 1954). *E. coli* strains were grown at 37°C on Luria-Bertani medium (Maniatis et al., 1982). Bacto agar (Difco) at 1.5% (w/v) was added to media for plate cultures. Antibiotics (Sigma) were used for selection at the following concentrations: tetracycline, 10 to 20 mg/L; rifampicin, 100 mg/L; spectinomycin (Sp), 20 mg/L; streptomycin (Sm), 30 mg/L; cyclohexamide, 50 mg/L. The broad host range vector pLAFR3

(Staskawicz et al., 1987) was used for the cosmid library and for subclones. The omega (Ω) fragment encoding Sp' (Prentki and Krisch, 1984) was used for site-directed mutagenesis. The helper plasmid pRK2013 was used in triparental matings (Figurski and Helinski, 1979) to mobilize cosmids from *E. coli* into *Psm*, *Pst*, and *Psg*.

Growth of Plants, Plant Inoculations, and in Vivo Growth Curves

Arabidopsis thaliana ecotypes were obtained from the Arabidopsis Information Service seed bank. *Arabidopsis* ecotypes were grown from seed in growth chambers under an 8-hr photoperiod at 24°C. *Arabidopsis* seeds were sown in 2-inch-square plastic pots in soil consisting of a 3:3:1 mix of UC-mix (potting soil):peat:sand. Seedlings were fertilized once every 2 weeks with 0.7 g/L Ra-Pid-Gro Plant Food 23-19-17 (Ortho Consumer Products, San Francisco, CA). Leaves of 6-week-old to 9-week-old seedlings that had not initiated flowering shoots and did not exhibit any signs of purple pigmentation were inoculated and left attached to the plant. Only leaves that had just fully expanded were used. Turnip (*Brassica campestris* cv Just Right), cabbage (*B. oleracea* cv Tastie), cauliflower (*B. oleracea* cv Snowball Y), broccoli (*B. oleracea* cv Emperor), tomato (*Lycopersicon esculentum* cv Peto76, J. Watterson, Petoseed Company, Saticoy, CA), pepper (*Capsicum annuum*), and soybean (*Glycine max* cvs Acme, Centennial, Flambeau, Harosoy, and Norchief) were grown from seed in greenhouses in clay pots with UC-mix potting soil. *Brassica*, tomato, pepper, and soybean plants were incubated in growth chambers with 12-hr photoperiods at 24°C 1 day before inoculation.

Reactions of *Arabidopsis* and *Brassica* plants to inoculation with *P. syringae* strains and transconjugants were determined by infiltrating approximately 10 μ L of a bacterial suspension (10^6 cfu/mL in 10 mM MgCl₂) into intact leaves as described (Swanson et al., 1988). Plants were returned to growth chambers and disease phenotypes were scored 5 days after inoculation. For the results reported in Table 1, disease phenotypes were qualitatively assessed. For Tables 2 and 3, disease levels were rated from 1 (no visible necrosis or chlorosis) to 5 (complete necrosis of the infiltrated region with chlorotic margins). A minimum of four inoculations were done for each strain-plant combination, and the scores were averaged. Average scores above 3.5 are listed as virulent (+), 2.5 to 3.5 as intermediate (\pm), and less than 2.5 as avirulent. Tomato plants were inoculated with bacterial suspensions of both 10^6 cfu/mL and 10^5 cfu/mL. Soybean plants were inoculated with bacterial suspensions of 5×10^8 cfu/mL.

For large-scale inoculations, we used the surfactant Silwet L-77 (Union Carbide). L-77 was added to bacterial suspensions (2×10^8 cfu/mL) at a concentration of 0.01% (v/v). Leaves of whole *Arabidopsis* plants were dipped in the above suspension or sprayed with a hand-pump spray bottle. Symptoms were scored at 3 days to 5 days after application.

To determine levels of bacterial growth in the leaves of *Arabidopsis*, leaves of six plants per strain were vacuum infiltrated with bacterial suspensions of 10^5 cfu/mL. Bacterial populations in leaves were sampled by taking four leaf discs (two per plant) using a No. 1 cork borer (0.4 cm diameter), macerating the discs in 10 mM MgCl₂, and plating appropriate dilutions on fresh King's B agar containing rifampicin and cyclohexamide. Population sizes were examined on the days indicated; three replicates were taken for each sampling.

Recombinant DNA Techniques

Standard techniques for DNA subcloning, plasmid preparations, and agarose gel electrophoresis of DNA fragments were used (Ausubel et al., 1987). Genomic cosmid libraries of *Pst* strains JL1065 and T1 were prepared in the vector pLAFR3 as described (Swanson et al., 1988). Avirulence locus *avrRpt2* was subcloned from cosmid p4-24 by partial digestion with *Sau3A*, gel purification of 3-kb to 5-kb fragments, and ligation into the *Bam*HI site of pLAFR3. Active constructs pABL18 and pABL30, containing 3.6-kb and 3.7-kb inserts, respectively, were identified by conjugation into *Pst* strain DC3000 and testing on *Arabidopsis* ecotype Col-0. The inserts in these two clones overlapped by 2.2 kb. A 1.4-kb *Hind*III fragment that was contained within this overlap was then isolated from pABL30 and cloned into pLAFR3 to yield pLH12. pLH12 avirulence activity was disrupted by insertion of an Ω fragment (*Sp*/*Sm*^r) (Prentki and Krisch, 1984) into the *Sac*I site located 0.7 kb from either end of the insert.

Electron Microscopy

Arabidopsis leaves were hand inoculated on one side of the midvein with a 10^6 cfu/mL suspension of bacteria. Entire leaves were removed from plants 20 min, 2 days, and 4 days after inoculation and immediately placed in ice-cold 2% glutaraldehyde/0.1 M cacodylate buffer. After refrigeration for at least 24 hr, samples were step dehydrated in ethanol and critical point dried. Leaves were then hand sectioned in the transverse plane, mounted, sputter coated, and examined by scanning electron microscopy.

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