Identification of a New *Arabidopsis* Disease Resistance Locus, *RPS4*, and Cloning of the Corresponding Avirulence Gene, *avrRps4*, from *Pseudomonas syringae* pv. *pisi*

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A molecular genetic approach was used to study the interaction between *Arabidopsis* and an avirulence gene from *Pseudomonas syringae* pv. *pisi*. *P. syringae* pv. *pisi* strain 151 induces a hypersensitive response (HR) when inoculated on the *Arabidopsis* accession Po-1. A genomic cosmid library was constructed from DNA from *P. syringae* pv. *pisi* strain 151 and a cosmid was identified that causes the normally virulent *P. syringae* pv. *tomato* strain DC3000 to induce an HR on Po-1. The cosmid was subcloned and a 1.2-kb DNA fragment conferring avirulence activity was sequenced. The single significant open reading frame within the 1.2-kb fragment was designated *avrRps4*. An *avrRps4*-specific probe hybridizes to DNA from all *P. syringae* pv. *pisi* strains tested. *P. syringae* strains carrying *avrRps4* induces an HR on specific accessions of both *Arabidopsis* and soybean. *Arabidopsis* accession Ws-0 is resistant to DC3000(*avrRps4*), whereas accession RLD is susceptible. Resistance segregates as a single dominant locus in a genetic cross between Ws-0 and RLD. This disease resistance locus, *RPS4*, was mapped to chromosome 5 between the molecular markers sAT2105 and KG-8.

The interaction between plants and pathogens is governed by the genetics of both organisms. Natural variation within plant or pathogen species can be used to identify some of the genetic factors that control whether the interaction results in resistance or plant disease. Historically, the gene-for-gene relationship has been used to explain the outcome of a pathogen infection (Flor 1971). This relationship predicts that if the pathogen carries an avirulence gene which is "recognized" by a specific resistance gene in the plant, a plant resistance response is induced. If either the avirulence gene or the resistance gene is absent, then the pathogen causes disease on the plant. Many bacterial avirulence genes have been cloned and most (for exceptions see Tamaki et al. 1988;

Canteros et al. 1991; Hopkins et al. 1992; Swarup et al. 1992; Bonas et al. 1993; Defeyter et al. 1993) have sequences which lack homology to each other. A few of the cloned avirulence genes affect pathogen fitness on susceptible plants (Ritter and Dangl 1995; reviewed by Dangl 1994). Recently, plant disease resistance genes have been isolated in several laboratories (Martin et al. 1993; Bent et al. 1994; Jones et al. 1994; Mindrinos et al. 1994; Whitham et al. 1994); however, the molecular signaling mechanisms by which pathogens induce resistance on plant hosts is not well understood.

Pseudomonas syringae pv. *pisi* is the causal agent of bacterial blight of pea (*Pisum sativum*). Seven races of *P. syringae* pv. *pisi* have been identified, and five avirulence genes have been postulated to control the race structure on pea (Taylor et al. 1989). The avirulence genes defining *P. syringae* pv. *pisi* race 2 (*avrRpm1/avrPpiA1*) and *P. syringae* pv. *pisi* race 3 (*avrPpi3*) have been cloned and characterized (Vivian et al. 1989; Bavage et al. 1991). Pathogens, however, can also contain avirulence genes that are recognized by nonhost plants as well as by host plants. For instance, the avirulence gene *avrRpm1/avrPpiA1* specifies disease reactions on certain lines of soybean and *Arabidopsis* as well as on pea (Debener et al. 1991; Dangl et al. 1992). Below, we describe the cloning of an avirulence gene from *P. syringae* pv. *pisi* that interacts with disease resistance loci from both *Arabidopsis* and soybean.

RESULTS

Identification and cloning of *avrRps4*.

To identify new avirulence specificities, a variety of *P. syringae* strains representing several different pathovars were assayed for their ability to induce a hypersensitive response (HR) when inoculated into *Arabidopsis* accession Po-1. Accession Po-1 was chosen for this study because it is not resistant to *P. syringae* pv. *tomato* strain DC3000 carrying the avirulence genes *avrRpt2*, *avrB*, or *avrRpm1* (M. Hinsch, unpublished observation). Thus, bacterial strains that were able to induce a resistance response (such as an HR) in Po-1 were likely to harbor avirulence genes other than those listed above. Leaves of Po-1 were pipette-infiltrated with bacterial suspensions and monitored for the appearance of an HR. *P.*

Table 1. Bacterial strains, vectors, and plasmid constructions used in this study

| Strain, vector, or plasmid	Relevant characteristics	Source or reference
Pseudomonas syringae strains		
pv. *tomato* DC3000	Pathogenic on *Arabidopsis*	Cuppels 1986
pv. *maculicola* 4326	Pathogenic on *Arabidopsis*	J. Dangl, Max-Delbrueck lab, MPG
pv. *coronafaciens* 345		T. Denny, U. of Georgia
pv. *phaseolicola* 3121		N. Panopoulos, UC Berkeley
pv *glycinea* Race 4 strain		
A29-2		N. Keen, UC Riverside
pv. *glycinea* Race 5		N. Keen, UC Riverside
pv. *pisi* 151	Rif^r, source of *avrRps4*	T. Denny, U. of Georgia
pv. *pisi* 299A	Race 1 type strain, Rif^r	John Taylor, Hort. Res. Intl., U.K.
pv. *pisi* 202	Race 2 type strain, Rif^r	John Taylor, Hort. Res. Intl., U.K
pv. *pisi* 870A	Race 3 type strain, Rif^r	John Taylor, Hort. Res. Intl., U.K
pv. *pisi* 895A	Race 4 type strain, Rif^r	John Taylor, Hort. Res. Intl., U.K
pv. *pisi* 974B	Race 5 type strain, Rif^r	John Taylor, Hort. Res. Intl., U.K
pv. *pisi* 1704B	Race 6 type strain, Rif^r	John Taylor, Hort. Res. Intl., U.K
pv. *pisi* 2491A	Race 7 type strain, Rif^r	John Taylor, Hort. Res. Intl., U.K
E. coli DH5α	F$^-$, *recA1*	Bethesda Research Laboratories
E. coli DH5αF$^-$	F$^-$, *recA1*	Gibco/BRL
Vectors		
pLAFR3	pLAFR1 containing *Hae*II fragment of pUC8, Tc^r,Tra$^-$Mob$^+$, RK2 replicon	Staskawicz et al. 1987
pLAFR6	pLAFR3 deleted for Plac with *trp* terminators flanking the polylinker	Huynh et al. 1989
pUC119	ColE1 replicon	Vieira and Messing 1987
pVSP61	Km^r, pVS1 replicon	W. Tucker, DNA Plant Technology, Inc.
Plasmids		
pRK2013	Km^r, Tra$^+$Mob$^+$, ColE1 replicon	Figurski and Helinski 1979
pBG11	pLAFR3 with ~19 kb of *P. syringae* pv. *pisi* strain 151 DNA carrying *avrRps4*	This study
pK2	*Bgl*II–*Hind*III 3-kb DNA fragment cloned into pVSP61, carries *avrRps4*	This study
pMH2	pUC119 with 3-kb region carrying *avrRps4*	This study
pMH3	pUC119 with 3-kb region in the opposite orientation of pMH2 carrying *avrRps4*	This study
p2-3a, p2-4k	Created from deletion series of pMH2, deleted 372 and 652 nucleotides from *Bgl*II site, respectively	This study
p316-1, p319-2	Created from deletion series of pMH3, deleted from the *Hind*III end to nucleotide 1239 and 1087, respectively, relative to the *Bgl*II site	This study
pL6-2-3a, pL6-2-4k, pL6-316-1a, pL6-319-2	*Eco*RI–*Hind*III fragments of p2-3a, p2-4k, p316-1, and p319-2 cloned into pLAFR6	This study
pV316-1A	*Eco*RI–*Hind*III fragments of p316-1 cloned into pVSP61, carries *avrRps4*	This study
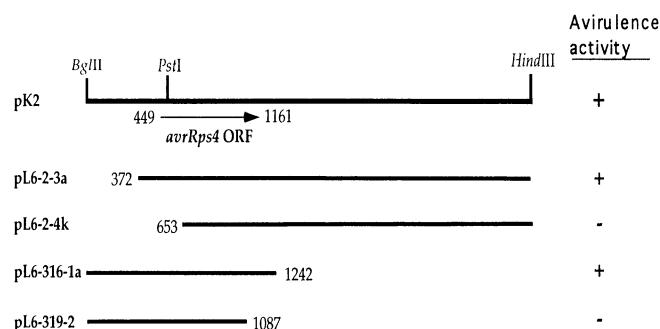
Fig. 1. Deletion analysis of *avrRps4*. A 3-kb *Bgl*II-*Hind*III fragment was cloned from a pBG11 subclone into pVSP61 (designated pK2). The *Hind*III site is derived from the polylinker of pVSP61. A set of nested deletions were made from the 3-kb fragment and the resulting fragments were cloned into pLAFR6, conjugated into *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *glycinea* race 4 and tested for their ability to induce an HR (+) in *Arabidopsis* Po-1 and soybean Harosoy, respectively. Numbers indicate nucleotide position relative to the *Bgl*II site. The *Hind*III site is approximately 3 kb from the *Bgl*II site. The open reading frame of *avrRps4* is indicated by an arrow.

syringae pv. *pisi* strain 151 induced an HR in Po-1 and therefore was likely to carry a novel avirulence gene recognized by Po-1.

To isolate the avirulence gene, a genomic library was constructed from *Sau*3AI partially digested *P. syringae* pv.

pisi strain 151 DNA which was cloned into the *Bam*HI site of the wide host range cosmid pLAFR3 (Table 1). The clones were individually conjugated from *E. coli* DH5α into DC3000 and each exconjugant was pipette-infiltrated into Po-1. Two exconjugants, out of 300 tested, induced an HR in Po-1. Cosmids isolated from the exconjugants had identical restriction patterns and the cosmid clone, pBG11, was chosen for further study. Fragments of 3 to 5 kb from *Sau*3AI partially digested pBG11 DNA were ligated into the wide host range plasmid pVSP61 (Table 1). Recombinant plasmids were moved from *E. coli* DH5α to DC3000 by conjugation. Six out of 100 of the exconjugants induced an HR when pipette-infiltrated into Po-1. A 3-kb *Bgl*II–*Hind*III fragment containing DNA common to all six clones was cloned into pVSP61 (designated pK2; Table 1 and Fig. 1) and the resulting plasmid conferred avirulence activity. The 3-kb fragment was introduced into pUC119 in both orientations, designated pMH2 and pMH3 (Table 1), and a set of nested deletions was created. The resulting fragments were cloned into pVSP61 and pLAFR6, conjugated into DC3000 and inoculated into Po-1 to test for avirulence. The smallest fragment sufficient for avirulence in Po-1, designated pV316-1A when cloned into pVSP61, is 1.2 kb (Fig. 1 and Table 1).

avrRps4 specifies disease resistance in soybean cultivars.

Because avirulence genes that specify disease reactions in

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1    GTCGATCTGAAGCAAGGCGACCAGGTACGTTTGCAAGACCTGGGCACCCAGCCTGTCGTG    60
61   GTGCAGGTCATCGAAGAGGACGGCACTGTCACGGACAAAACGGTCAACCGCCGCGAATGG   120
121  TCTGCGCAGCCGACCGCGCCTGAGCGGGAAGTGGCTCAGACCACGCCCAAGGGGCAGGCG   180
181  GCAGCAGCAGGCACACCGGAGCTGTCATCGCCTGATGAAGAAGACGGCATGAGCATGGAC   240
241  TGAGCCGTCTGGGTAACCACCTCTTTGCCTGGAGGTGGTTCCCAGAAACTATTCGTATAA   300
301  GGGGGACGGTACTGATAGCACGCTTAATGCCACGCTACATGTGGGCGAATTTCAACAGAT   360
                        p2-3a
361  TGCCAGAATATTTCCCCGAAGATTAGGAACTGTCGGCAAGTGCGAAGCCACTAAAATTTC   420
421  CGTCCTTTCCATAAGGGTAAAAATGAGTAACGCATGCCTGCTCATGCGCATTAAATTCTT   480
481  TGACAAAGAGGCTTCCCCATGACTCGAATTTCAACCAGTTCAGTAAATTCCAGCTTCAGT   540
                       M  T  R  I  S  T  S  S  V  N  S  S  F  S
541  TACTCGGCCCCTGCAGAGGAAGCGCAAAACCGCGTTTCCTCAGCCCCCACCAACTCCTCT   600
      Y  S  A  P  A  E  E  A  Q  N  R  V  S  S  A  P  T  N  S  S
                                                     p2-4k
601  CCACCTGGCACCACAACCGCTGTCGCCCAGGCGTCAGAGGGGCAAAAAAGCCGGGGGCG   660
      P  P  G  T  T  T  A  V  A  Q  A  S  E  G  Q  K  K  P  G  A
661  GTATTAAGCATGCATGCACAGCGACTGCGCCAATTGTGTGAGAGTTCATCTCACCAGTAC   720
      V  L  S  M  H  A  Q  R  L  R  Q  L  C  E  S  S  S  H  Q  Y
721  CGGCAGAACGCGCTATTAGCTAAATCTTTTGATGCTCAACGCCTAGACATTAACACTCAA   780
      R  Q  N  A  L  L  A  K  S  F  D  A  Q  R  L  D  I  N  T  Q
781  GCGGGGCCCTTCCAATAGCCCTCACCTGAACGCTCTCAACAAGCTCCAACAACGAGATTTC   840
      A  G  P  S  N  S  P  H  L  N  A  L  N  K  L  Q  Q  R  D  F
841  AAACCTGCGGCTGGCGGGCTAGAAATCCCATTTACACCCAAATCCCTATTGGGTGGAGGT   900
      K  P  A  A  G  L  E  I  P  F  T  P  K  S  L  L  G  G
901  AAACGAGTCTATCAAATTGGCTCATCGTCACGCGACGTACAAGTCTGCCCACGTGGAGCT   960
      K  R  V  Y  Q  I  G  S  S  R  D  V  Q  V  C  P  R  G  A
961  GGTGCTGCATTGAGGCAAGAAATCGAAGATAAGCAACTCATGGTTAACAATCTCACGGAC  1020
      G  A  A  L  R  Q  E  I  E  D  K  Q  L  M  V  N  N  L  T  D
1021 GAGCTTCAAGACGCTATAGATGAGGCCAACCCAGCCGAAATAGCCAATACTTCACAACAG  1080
      E  L  Q  D  A  I  D  E  A  N  P  A  E  I  A  N  T  S  Q  Q
             p319-2
1081 CTGCGCCATGCACGGGCTGACCTTGCTGACCTACAGAGACGCTTTGCGGTACTCAGAAAC  1140
      L  R  H  A  R  A  D  L  A  D  L  Q  R  R  F  A  V  L  R  N
1141 GAAGACCGCAGAATCAACCAATAACCTTAAACACATCATAGCCCCTGCCTCAAGCGAATA  1200
      E  D  R  R  I  N  Q
                          p316-1
1201 TGTAAGCCCAAGGCAAGTTGCATTTTAAATATCCTCGGGTAG  1242
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Fig. 2. Nucleotide sequence of DNA fragment conferring *avrRps4* activity and the putative amino acid sequence of *avrRps4*. The *hrp* box sequence is underlined and the potential ribosome binding site is boxed. The endpoints of deletions are marked with arrows.

Arabidopsis often specify disease reactions in other plant species (Whalen et al. 1991; Dangl et al. 1992; Wanner et al. 1993), we tested tomato and soybean lines for specific resistance to strains carrying *avrRps4*. Plants were pipette-inoculated with bacterial suspensions and were monitored for the appearance of disease symptoms or the HR. Inoculation of a number of tomato lines (Rio Grande 76R, Rio Grande 76S, UC822, Rehorot, VFNT, and Celebrity) revealed no detectable resistance to DC3000(pV316-1A). In contrast, soybean displayed natural variation of resistance to *P. syringae* pv. *glycinea* race 4 harboring pV316-1A. Soybean cultivars Harosoy and Hardee are both susceptible to *P. syringae* pv. *glycinea* race 4 (strain A29-2). When *P. syringae* pv. *glycinea* race 4(pV316-1A) was inoculated into Harosoy, a brown, necrotic HR appeared in 2 to 3 days. *P. syringae* pv. *glycinea* race4(pV316-1A) inoculated into Hardee produced water-soaking typical of the susceptible interaction.

Nucleotide sequence analysis of *avrRps4*.

The nucleotide sequence of the 1.2-kb DNA fragment carried in pV316-1A (Fig. 2) contains one large open reading frame (ORF) of 663 bp encoding a putative protein product of 221 amino acids with a predicted molecular mass of 24 kDa. The avirulence activity of the nested deletions of pMH2 and pMH3, cloned into pLAFR6, is consistent with the ORF conferring avirulence (Fig. 1). Deletions into either end of the
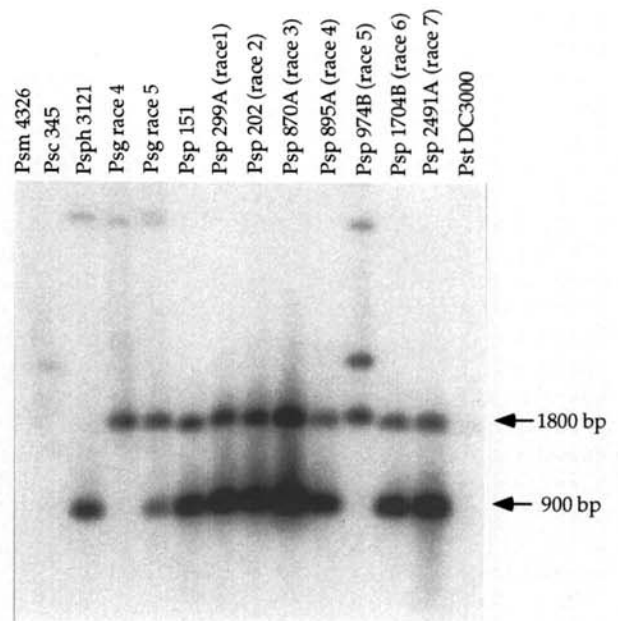

Fig. 3. DNA blot of total DNA isolated from different *Pseudomonas syringae* strains and hybridized to an *avrRps4* ORF probe. DNA was digested with *Hinc*II restriction enzyme and separated on a 0.75% agarose gel. The following abbreviations are used: *P. syringae* pv. *maculicola* (Psm), *P. syringae* pv. *coronafaciens* (Psc), *P. syringae* pv. *phaseolicola* (Psph), *P. syringae* pv. *glycinea* (Psg), *P. syringae* pv. *pisi* (Psp), *P. syringae* pv. *tomato* (Pst).

ORF resulted in loss of avirulence activity. The gene encoding this ORF is designated *avrRps4*. A purine-rich region that resembles a ribosome binding site (Shine and Dalgarno 1974) is 8 bp upstream of the predicted ORF. The region 85 bp upstream of the ORF shows strong homology to the *hrp* box, a promoter sequence found upstream of several avirulence genes in *P. syringae* pathovars (Innes et al. 1993; Salmeron and Staskawicz 1993; Mansfield et al. 1994; Lorang and Keen 1995). Based on the Kyte–Doolittle algorithm for predicting protein hydropathy (Kyte and Doolittle 1982), the amino acid sequence of *avrRps4* is hydrophilic and has no transmembrane domains. Searches using standard sequence comparison algorithms (blastp, Altschul et al. 1990; sbase, Pongor et al. 1992; blocks, Henikoff and Henikoff 1991) revealed no significant homology between the *avrRps4* sequence and other genes from several databases (Genpept, Swiss-Protein, EMBL).

Distribution of *avrRps4* in *P. syringae* strains.

A probe consisting of the ORF of *avrRps4* was hybridized to *Hinc*II-digested total DNA from a number of *P. syringae* strains (Fig. 3). The probe hybridizes to representatives of all seven reported races of *P. syringae* pv. *pisi* (Taylor et al. 1989) as well as to at least two races of *P. syringae* pv. *glycinea* and one race of *P. syringae* pv. *phaseolicola*. Consistent with the nucleotide sequence from *P. syringae* pv. *pisi* strain 151, the *avrRps4* probe hybridizes to a 900- and a 1,800-bp DNA fragment in all *P. syringae* pv. *pisi* strains tested, with the exception of *P. syringae* pv. *pisi* strain 974B. The probe does not hybridize to DNA from DC3000. The *avrRps4* probe hybridizes to a native plasmid (data not shown) of *P. syringae* pv. *pisi* 151. suggesting that *avrRps4* is plasmid borne.

Identification of natural variation of disease resistance in *Arabidopsis*.

Different accessions of *Arabidopsis* were tested for natural variation of disease resistance to DC3000(*avrRps4*). Of the 19 accessions tested (Be-0, Bla-2, Bro-A, Bs-1, Chi-1a, Col-0, Gr-1, Hs-0, La-*er*, Nd-1, Mt-0, Oy-0, Po-1, RLD, Sei-0, Sf-2, Uk-4, Ws-0, and Vi-0), RLD is the only accession susceptible to DC3000(*avrRps4*). Accession Ws-0 is resistant to DC3000(*avrRps4*) (Fig. 4) and was chosen as the resistant parent for the genetic studies described below. Growth of DC3000(*avrRps4*) within RLD and Ws-0 was monitored to confirm the disease phenotypes (Fig. 5). Four days after vacuum infiltration into RLD, DC3000(*avrRps4*) attained population levels equal to DC3000(pVSP61). In contrast, 4 days after vacuum infiltration into Ws-0, DC3000(*avrRps4*) population levels were 100 to 1,000-fold less than DC3000(pVSP61), indicating that Ws-0 was specifically resistant to DC3000 carrying *avrRps4*.

Genetic analysis of *RPS4*.

The resistance of *Arabidopsis* to DC3000(*avrRps4*) was analyzed genetically. Accessions RLD and Ws-0 were crossed and F_1 plants were resistant to DC3000(*avrRps4*) in pipette-inoculation disease assays. An assay involving the immersion of plants in bacterial suspension was used to determine the disease phenotype of the F_2 progeny. Resistance to DC3000(*avrRps4*) segregated as a single dominant locus in the F_2 progeny (686 resistant: 213 susceptible, $\chi^2 = 0.853$ for a 3:1 segregation, $P = 0.355$). The locus was designated *RPS4*.

As a first step towards cloning *RPS4*, the locus was genetically mapped by isolating DNA from F_2 and pooled F_3 plants of known disease phenotype and screening with a small number of cleaved amplified polymorphic sequences (CAPS, Konieczny and Ausubel 1993) markers that are polymorphic between Ws-0 and RLD. The CAPS marker DFR mapped approximately 5.5 cM (6 recombinant plants out of 54 plants tested) from *RPS4*. RFLP markers were then used to determine a more precise location of *RPS4*. The RFLP marker sAT2105 mapped 3.7 cM (4 recombinants/54 plants)

from *RPS4*. All four recombinants identified by sAT2105 were also identified by DFR, indicating that sAT2105 maps to the same side of *RPS4* as DFR. The RFLP marker KG-8 mapped 10.2 cM (11 recombinants/54 plants) from *RPS4*. As 10 of the 11 recombinants identified by KG-8 are different from those identified by DFR, KG-8 must be on the other side of *RPS4*.

DISCUSSION

To further our knowledge of plant disease resistance, a search was initiated to identify new bacterial avirulence genes that correspond to previously unidentified disease resistance loci in *Arabidopsis thaliana*. To identify novel avirulence specificities, we screened a number of bacterial strains for their ability to induce a resistance response on the *Arabidopsis* accession Po-1. One strain, *P. syringae* pv. *pisi* strain 151, induced an HR when inoculated into Po-1 leaves. An avirulence gene, *avrRps4*, was cloned from *P. syringae* pv. *pisi* strain 151 and the nucleotide sequence of the gene was determined. The ORF of *avrRps4* encodes a protein with a predicted molecular mass of 24 kDa. Like most bacterial avirulence genes that have been sequenced (Dangl 1994), the *avrRps4* protein is hydrophilic. The amino acid sequence of *avrRps4* does not reveal any clues about the molecular mechanism of *avrRps4* avirulence. There is an avirulence gene promoter sequence (*hrp* box) approximately 100 bp upstream of the ORF. The promoter sequence is found upstream of many avirulence and *hrp* genes from *P. syringae* (Innes et al. 1993; Salmeron and Staskawicz 1993; Mansfield et al. 1994; Lorang and Keen 1995) and has recently been implicated as the *cis*-acting sequence that is recognized by the *hrpL* transcriptional activation protein (Xiao and Hutcheson 1994).

In general, no single avirulence gene is found within all strains of a pathovar. Usually, the distribution of avirulence genes divide the strains within a pathovar into distinct races (for instance *avrRpm1/avrPpiA2* defines race 2 of *P. syringae* pv. *pisi*; Vivian et al. 1989). It is very interesting to find that the distribution of *avrRps4* (from the strains tested) is different from most bacterial avirulence genes in that all strains tested from within the *pisi* pathovar contain *avrRps4*-hybridizing sequences (Fig. 3). The *avrRps4* allele of six of
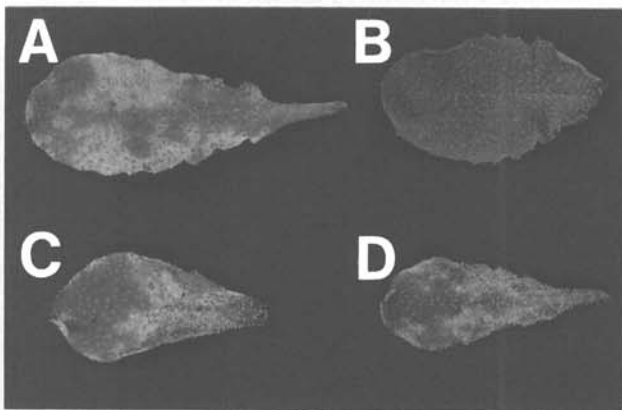

Fig. 4. Typical disease symptoms 5 days after innoculation by immersion in bacteria resuspended to an OD$_{600}$ of 0.2 in 10 mM MgCl$_2$ with 0.025% Silwet L-77: **A,** Ws-0 innoculated with DC3000(pVSP61); **B,** Ws-0 innoculated with DC3000(*avrRps4*); **C,** RLD innoculated with DC3000(pVSP61); **D,** RLD innoculated with DC3000(*avrRps4*). Leaves were removed immediately prior to photograph.
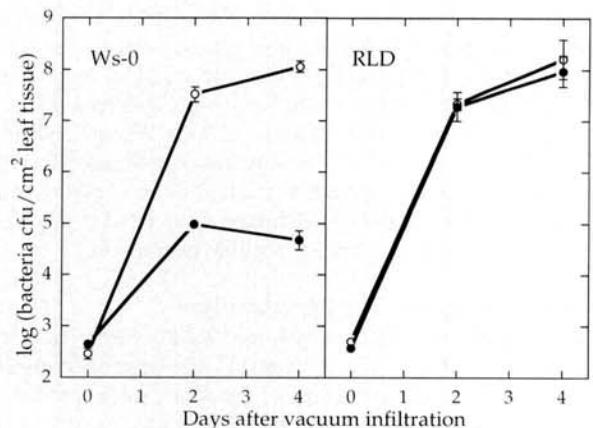

Fig. 5. Typical time course of growth of Pst DC3000(*avrRps4*) in *Arabidopsis*. Population levels of DC3000 with empty vector pVSP61 are indicated with open symbols (O). Population levels of DC3000 with *avrRps4* on plasmid pK2 are indicated with closed symbols (●).

the *P. syringae* pv. *pisi* races (race 5, strain 974B being the exception) have identical *Hinc*II and *Eco*RI restriction patterns suggesting that *avrRps4* may be conserved within many *P. syringae* pv. *pisi* strains and that *avrRps4* avirulence activity may be widespread within the *pisi* pathovar. This is not the first example of an avirulence gene that is found in all strains of a pathovar; the *Xanthomonas campestris* pv. *vesicatoria* gene, *avrBs2*, is conserved within many pathovars of *X. campestris* (Kearney and Staskawicz 1990). Based on the presence of *avrRps4*-homologous sequences in the type strains of all identified races of *P. syringae* pv. *pisi*, it seems unlikely that *avrRps4* corresponds to one of the avirulence genes defining races within the *pisi* pathovar (Taylor et al. 1989).

Like several *Pseudomonas syringae*-derived avirulence genes that specify resistance on *Arabidopsis* and soybean (Whalen et al. 1991; Wanner et al. 1993), bacteria carrying *avrRps4* induce resistance responses in certain soybean cultivars in addition to *Arabidopsis*. Soybean cultivar Harosoy displays resistance to *P. syringae* pv. *glycinea* race 4 carrying *avrRps4*, but soybean cultivar Hardee is susceptible. It will be interesting to determine what genetic factors control resistance to *P. syringae* pv. *glycinea* race 4(*avrRps4*) in soybean. Since soybean cultivar Harosoy and *Arabidopsis* accession Ws-0 are resistant to bacteria carrying *avrRps4*, both plants may have similar functional resistance genes or resistance mechanisms. It should be noted that although the *avrRps4* probe hybridizes to *P. syringae* pv. *glycinea* race 4 DNA (Fig. 3), *P. syringae* pv. *glycinea* race 4 does not display *avrRps4* activity. The *P. syringae* pv. *glycinea* race 4 allele is polymorphic relative to the active *avrRps4* allele and probably contains mutations resulting in an inactive allele.

Natural variation of disease resistance in *Arabidopsis* to a variety of pathogens has been observed and, in most cases, single loci appear to control resistance (Debener et al. 1991; Whalen et al. 1991; Parker et al. 1993; Tör et al. 1994). We have identified and genetically mapped a new disease resistance locus in *Arabidopsis* using an avirulence gene isolated from a strain of *P. syringae* pv. *pisi*. The resistance locus, *RPS4*, was identified in a cross between *Arabidopsis* accessions Ws-0 and RLD and maps to a ~15-cM interval on chromosome 5. This work is the basis for a map-based approach to the cloning and characterization of *RPS4*. Recent isolation of several plant disease resistance genes (Martin et al. 1993; Bent et al. 1994; Jones et al. 1994; Mindrinos et al. 1994; Whitham et al. 1994) has revealed some clues to the nature of plant disease resistance (reviewed by Staskawicz et al. 1995). In particular, two *Arabidopsis* genes which confer resistance to specific *P. syringae* strains have been cloned (*RPS2*; Bent et al. 1994 and *RPM1*; Grant et al. 1995) and one other has been identified and genetically mapped (*RPS5*; Simonich and Innes 1995). Comparison of their structure with that of *RPS4* should help identify specificity determinants and clarify the molecular mechanisms of plant disease resistance.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids.

Bacterial strains, vectors, and plasmid constructions are described in Table 1. *Pseudomonas syringae* strains were typically grown at 30°C on King's Medium B (King et al. 1954). Nutrient yeast growth agar (Daniels et al. 1984) was used for experiments to determine in planta bacterial multiplication. *E. coli* strains were grown at 37°C on Luria Bertani medium (Ausubel et al. 1987). Bacto Agar (Difco) at 1.5% (w/v) was added to the media for plate cultures. The plasmid pRK2013 was used (Table 1) to mobilize broad host-range vectors from *E. coli* into *P. syringae*. Antibiotics (Sigma) were used for selection at the following concentrations (in μg/ml): tetracycline (Tc), 10; rifampicin (Rif), 100; ampicillin (Ap), 50; kanamycin (Km), 25; cyclohexamide 50 μg/ml.

Recombinant techniques.

Standard techniques for DNA subcloning, plasmid preparations and gel electrophoresis were used (Ausubel et al. 1987). Standard procedures for probe preparation and DNA gel blot hybridizations were followed (Sambrook et al. 1989). The genomic cosmid library was prepared in pLAFR3 as described (Swanson et al. 1988), except *Sau*3AI partially digested DNA was size-fractionated by agarose gel electrophoresis and was eluted with an analytical electroeluter (BRL, Bethesda, MD). Plant DNA was isolated as described (Kunkel et al. 1993). Southern hybridizations were done with Hybond-N (Amersham) nylon membranes following the manufacturer's instructions. PCR primers used to make the probe corresponding to the ORF of *avrRps4* add *Nde*I and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively, of the ORF. The sequence of the 5′ primer and 3′ primer are: GCGCATATGACTCGAATTTCAACCAG and GCGGGA-TCCTTATTGGTTGATTCTG, respectively. PCR reactions were performed using a thermal cycler (model PHC2; Techne, Inc., Princeton, NJ). Conditions for amplification using pMH3 DNA as a template were as follows: 35 cycles of 30 s at 94°C, 30 s at 56°C and 3 min at 72°C. The PCR reaction contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM MgCl$_2$, 0.001% gelatin, 3% DMSO, 100 μM each of the 4 dNTPs, 200 μM primer, 50 ng of template DNA and 1 U of Taq polymerase in a 100 μl volume with 60 μl of mineral oil.

Exonuclease III deletion analysis.

To create deletions in the 3-kb *Bgl*II–*Hin*dIII fragment with *avrRps4* activity, the termini were filled in with Klenow enzyme and the resulting blunt-ended product was cloned into the *Hinc*II site of pUC119. Both orientations, designated pMH2 and pMH3, were isolated. Both pMH2 and pMH3 were digested with *Bam*HI and *Kpn*I followed by Exonuclease III from the Erase-A-Base kit of Promega (Madison, WI) to produce a series of nested deletions in the 3-kb fragment. After filling in the termini using Klenow enzyme, the DNA was fractionated by electrophoresis, eluted (GeneClean, Bio 101, La Jolla, CA), and ligated. DNA fragments resulting from the Exonuclease III deletions of pMH2 and pMH3 were cloned into the polylinker of pVSP61 at the *Hin*dIII–*Eco*RI sites. The fragments from pMH3 were cloned so that the *avrRps4* ORF was in the same orientation as the *lacZ* promoter of pVSP61. The fragments from pMH2 was cloned such that the *avrRps4* ORF was in the opposite orientation from the *lacZ* promoter. These plasmids were transformed into *E. coli* DH5α cells and then mobilized into DC3000 and *P. syringae* pv. *glycinea* Race 4 by triparental mating (Figurski and Helinski 1979).

Nucleotide sequencing.

DH5α F′ cells were transformed with the nested deletions of pMH2 and pMH3 and then infected with the helper phage M13K07 to allow for production of single-stranded DNA. Single-stranded DNA templates were prepared as described (Vieira and Messing 1987). Dideoxy chain termination sequencing with the Sequenase 2.0 kit from U.S. Biochemical Corp. (Cleveland, OH) was used to determine the sequence of both strands of the 1.2-kb fragment with avrRps4 activity.

Growth of plants, plant inoculations, in planta bacterial growth curves, and genetic analysis.

Arabidopsis thaliana accessions were obtained from the *Arabidopsis* Information Service seed bank, with the exception of RLD, which was kindly provided by Eric Holub (Hort. Res. Int., U.K.). *Arabidopsis* accessions were grown as described previously (Kunkel et al. 1993). Hypersensitive reactions were assayed by pipette infiltrating bacteria resuspended in 10 mM $MgCl_2$ to an OD_{600} of 0.02 (~2 × 10^7 CFU/ml). Leaves were scored for tissue collapse ~24 h after inoculation. Pipette infiltration with bacteria resuspended to an OD_{600} of 0.001 (~10^6 CFU/ml) in 10 mM $MgCl_2$ was used to assay disease by monitoring leaves for water-soaked chlorotic necrosis 5 days after inoculation (Kunkel et al. 1993). F_2 and F_3 disease reactions were scored after inoculation by immersion in bacterial suspensions containing a surfactant (Kunkel et al. 1993). In planta bacterial growth was measured as described (Whalen et al. 1991) except the concentration of the bacteria in the infiltration media was 5× 10^4 CFU/ml. Four tissue samples (in triplicate for each time point) were combined for a total of 0.5 cm^2 of leaf tissue, ground in 10 mM $MgCl_2$ and plated in serial dilution on NYG agar containing kanamycin, rifampicin, and cyclohexamide (Whalen et al. 1991). The in planta bacterial growth experiment was replicated 3 times. Crosses were performed by hand emasculation of flowers before anther dehiscence and brushing donor pollen over the stigma. Genetic markers sAT2105 (Pang et al. 1988) and KG-8 were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University. Genetic distances in centiMorgans (cM) were calculated from recombination frequencies using the Kosambi function (Kosambi 1944).

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