Evidence That the *Pseudomonas syringae* pv. *syringae* *hrp*-Linked *hrmA* Gene Encodes an Avr-Like Protein That Acts in an *hrp*-Dependent Manner Within Tobacco Cells

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A 25-kb DNA region, previously cloned from *Pseudomonas syringae* pv. *syringae* 61 in cosmid pHIR11, enables nonpathogenic bacteria such as *Pseudomonas fluorescens* and *Escherichia coli* to elicit the hypersensitive response (HR) in tobacco (*Nicotiana tabacum*). *hrmA* is located within this region, adjacent to a conserved cluster of *hrp* genes, and is essential for nonpathogens to elicit the HR. DNA sequence analysis suggested that *hrmA* was the second of two genes in an operon and was preceded by an open reading frame (ORF), ORF1, which is predicted to encode a 10.9-kDa protein. DNA gel blot analysis revealed that sequences hybridizing with a DNA fragment internal to *hrmA* were absent from *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *tabaci* 11528, and *P. syringae* pv. *glycinea* race 4 U1, but present in *P. syringae* pv. *tomato* DC3000. A 2.4-kb *Bam*HI-*Avr*II fragment carrying *hrmA*, ORF1, and native regulatory sequences was subcloned into broad-host-range vector pDSK519 and electroporated into *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tabaci* 11528. The presence of the *hrmA* locus had no apparent effect on the ability of *P. syringae* pv. *syringae* B728a to cause brown spot of bean, but it caused *P. syringae* pv. *tabaci* 11528 to elicit the defense-associated HR rather than disease in *N. tabacum* cvs. Xanthi N and Xanthi NC and *N. clevelandii*. Furthermore, *N. debeyii*, *N. glutinosa*, *N. rustica*, and *N. tabacum* cvs. Petit Havana and Samsun responded with the HR to *P. fluorescens*(pHIR11). In contrast, *N. benthamiana*-*P. syringae* pv. *tabaci* interactions were unaffected by the presence of *HrmA*, and *P. fluorescens*(pHIR11) did not elicit the HR in *N. benthamiana*. The *hrmA* ORF was subcloned into pFLAG-CTC, which expressed *HrmA* with a C-terminal FLAG synthetic epitope fusion. *Escherichia coli* MC4100 cells carrying the functional *hrp* cluster and the *hrmA*-FLAG derivative secreted the HrpZ harpin, but not *HrmA*-FLAG, to the medium, as indicated by immunoblot analysis with M2 anti-FLAG and polyclonal anti-HrpZ antibodies. The *hrmA* ORF was also subcloned into plant expression vector pFF19 and then biolistically

delivered, along with pFF19G (expressing β -glucuronidase), into suspension-cultured tobacco cells. Histochemical staining 24 h later revealed substantial β -glucuronidase activity in cells receiving pFF19G and pFF19 but not in those receiving pFF19G and pFF19-*HrmA*. Thus, internal production of *HrmA* was deleterious to tobacco cells.

Host-specific, gram-negative, phytopathogenic bacteria typically elicit the hypersensitive response (HR) in plants with which they are incompatible. The HR is a rapid, localized necrosis that results from the apparent programmed death of plant cells in contact with incompatible pathogens and is associated with active plant defense (Klement 1982; He et al. 1993; Dietrich et al. 1994; Greenberg et al. 1994). The ability to elicit the HR or be pathogenic is controlled by *hrp* genes, which are probably universal among phytopathogenic bacteria in the genera *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia* (Bonas 1994). *hrp* genes are now thought to be primarily involved in protein secretion, with nine of them encoding highly conserved components of a type III protein secretion system that is also present in animal-pathogenic *Yersinia*, *Shigella*, and *Salmonella* spp. These nine genes have been redesignated as *hrc* (hypersensitive response and conserved) (Bogdanove et al. 1996). The *hrp/hrc* genes are clustered, and the 25-kb cluster from *P. syringae* pv. *syringae* 61, carried on cosmid pHIR11, enables nonpathogens like *P. fluorescens* and *Escherichia coli* to elicit the HR (Huang et al. 1988). Thus, pHIR11 encodes a minimal set of proteins required for HR elicitation.

The HrpZ harpin is an elicitor of the HR and is encoded by pHIR11 and all strains of *P. syringae* investigated (He et al. 1993; Preston et al. 1995). HrpZ, like the previously identified *Erwinia amylovora* HrpN harpin (Wei et al. 1992), is a glycine-rich, cysteine-lacking, heat-stable protein that is secreted in culture in an *hrp*-dependent manner and can elicit an HR-like necrosis in the leaves of tobacco and several other plants (He et al. 1993). The biochemical activity and biological function of HrpZ is uncertain. HrpZ has no apparent role in the host specificity of *P. syringae* (Preston et al. 1995), and deletion of *hrpZ* from the *P. syringae* pv. *syringae* genome only slightly reduces the ability of the bacterium to elicit the HR (Alfano et al. 1996). More puzzling are the observations

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that deletion of *hrpZ* from pHIR11 produces a variable HR deficiency in nonpathogens carrying the cosmid and that mutations in the pHIR11 *hrmA* gene abolish HR elicitation activity without affecting HrpZ synthesis or secretion (Alfano et al. 1996). *hrmA* (hypersensitive response modulator) is adjacent to the *hrp/hrc* cluster in a region that appears variable in *P. syringae* and contains the *avrPphE* gene in *P. syringae* pv. *phaseolicola* (Heu and Hutcheson 1993; Mansfield et al. 1994). *P. fluorescens*(pHIR11) *hrmA* mutants do not elicit the HR, whereas *P. syringae* pv. *syringae* 61 *hrmA* mutants elicit a reduced HR but appear unaffected in pathogenicity (Huang et al. 1991). The *hrmA* coding region encodes a predicted 41-kDa protein with no homology to known proteins, and which is regulated by the HrpL alternative sigma factor (Heu and Hutcheson 1993; Xiao and Hutcheson 1994). The location of *hrmA*, the limited distribution of *hrmA* in *P. syringae* pathovars, the regulation by HrpL, and the phenotype of *hrmA* mutations suggested that the gene encodes an Avr (avirulence)-like protein that has a primary role in elicitation of the HR in tobacco leaves by nonpathogenic bacteria carrying pHIR11 (Alfano et al. 1996).

Bacterial *avr* genes are defined by their ability to interact with cognate plant resistance (*R*) genes in a gene-for-gene manner to trigger a genotype-specific HR (Staskawicz et al. 1984; Keen 1990). *avr* genes are known to control the cultivar-specific interactions of races within several pathovars of *P. syringae*, and they may control the host specificity of *P. syringae* at the pathovar-host species level as well (Kobayshi et al. 1989). They are typically identified by screening a broad-host-range cosmid library of donor-race DNA in a recipient race that is normally compatible on tester plant cultivars that are incompatible with the donor. Conversion of the interaction to incompatibility (avirulence) indicates the presence of an *avr* gene.

Our hypothesis that HrmA is functioning as an Avr protein

led us to test the ability of *P. fluorescens*(pHIR11) to deliver known Avr proteins, such as AvrB. This ultimately produced evidence that AvrB must function inside plant cells following delivery by the pHIR11-encoded Hrp system (Gopalan et al. 1996). The ability of the type III protein secretion systems of *Yersinia*, *Shigella*, and *Salmonella* spp. to deliver virulence proteins directly into animal cells had been demonstrated previously and may be a general property of the system in both animal and plant pathogens (Rosqvist et al. 1994, 1995). Now there is evidence that three additional bacterial Avr proteins function inside plant cells (Leister et al. 1996; Scofield et al. 1996; Tang et al. 1996; Van den Ackerveken et al. 1996). Furthermore, pHIR11 has been shown to be able to deliver the signals from several *avr* genes that were heterologously expressed in *P. fluorescens* or *E. coli* (Gopalan et al. 1996; Pirhonen et al. 1996).

In this report we use *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *tabaci*, and biolistically transformed tobacco cells to obtain further evidence that the pHIR11-encoded HrmA has Avr-like properties and that it functions within plant cells. A preliminary account of this work has been published (Collmer et al. 1996).

RESULTS

A small ORF resides between the *hrp/avr* box and *hrmA*.

An *hrp/avr* box, which has been reported to be responsive to transcriptional activation by the HrpL alternative sigma factor (Xiao and Hutcheson 1994), is located 518 bp upstream of the *hrmA* translational start (Fig. 1). Beginning 126 nucleotides downstream of the *hrp/avr* box, an open reading frame (ORF), ORF1, was found that predicted a protein with 96 amino acids, a molecular weight of 10,888, an acidic pI of 5.6, and a high leucine content of 18% (Fig. 2). A potential ribosome-binding site, AAGGG, was found 10 nucleotides upstream from the ini-

Fig. 1. Map of pHIR11 detailing location of *hrmA*, ORF1, and a previously constructed TnPhoA insertion. Subset of *hrc* genes within the *hrp* cluster denoted with shaded boxes. ORF1 and *hrmA* depicted as hatched boxes. Arrow underneath ORF1 and *hrmA* indicates direction of transcription. Black box upstream of ORF1 represents the *hrp/avr* box (not drawn to scale); lollipop within the *hrmA* sequence shows point of insertion of the TnphoA mutation carried on pCPP2071. BamHI (B) and AvrII (A) sites are shown.

tiation codon. Three other initiation codons are located 39, 45, and 93 bp upstream of the putative ORF1 start, and in the same reading frame, but none of these is preceded by a consensus ribosome-binding site. The predicted ORF1 protein lacked an N-terminal signal peptide and any likely membrane-spanning domains. A BLAST search revealed that the predicted ORF1 protein shared similarity with ExsC from *P. aeruginosa*. ORF1 was 22% identical to ExsC (Frank and Iglewski 1991), and the sequence similarity shared between the two predicted proteins was in the same region of each. The similarity was particularly high (33% identity) in a 45-amino-acid region in the N-terminal portion of both proteins.

Confirmation of the pCPP2071 *hrmA*::TnPhoA mutation.

E. coli carrying a previously isolated pHIR11-derivative, pCPP2071, which has a TnphoA insertion mapping within *hrmA*, is unable to elicit an HR on tobacco (Huang et al. 1991). In view of the discovery of ORF1, we wanted to confirm that the mutation carried on pCPP2071 was within *hrmA* rather than ORF1. A 7-kb *Bam*HI fragment containing TnphoA and adjacent *P. syringae* DNA was subcloned from pCPP2071 into pUC18. Sequencing from the end of TnphoA into flanking *P. syringae* DNA confirmed that the TnphoA insertion carried on pCPP2071 was within *hrmA* (Fig. 1). Therefore, the *hrmA* gene is required for HR elicitation by non-pathogens carrying pHIR11.

DNA sequences hybridizing with *hrmA* are not present in *P. syringae* pv. *tabaci*.

The *hrmA* locus is located on the left side of the *hrp* cluster on pHIR11 (Fig. 1). The *hrmA* ORF was amplified by polymerase chain reaction (PCR), with pCPP2324 as a template, and cloned into pFLAG-CTC. The insert was then excised by *Nde*I/*Bgl*II double digestion and used as a probe for DNA gel blot hybridization. *P. syringae* pv. *tabaci* 11528, *P. syringae* pv. *glycinea* race 4 U1, *P. syringae* pv. *tomato* DC3000, *P. sy-*

ringae pv. *syringae* B728a, and *P. syringae* pv. *syringae* 61 total DNA samples were probed under conditions of moderate stringency following *Eco*RI digestion. No significant hybridization was observed with *P. syringae* pv. *tabaci*, *P. syringae* pv. *glycinea*, or *P. syringae* pv. *syringae* B728a DNA (Fig. 3). Hybridization was observed with *P. syringae* pv. *tomato*, and the hybridizing band had a different size than that in the *Eco*RI digest of *P. syringae* pv. *syringae* 61 DNA. Thus, *hrmA* is limited in its distribution among several experimentally important model strains of *P. syringae*, and a highly similar *hrmA* homolog is absent from *P. syringae* pv. *tabaci* 11528.

hrmA converts *P. syringae* pv. *tabaci*, but not *P. syringae* pv. *syringae*, to incompatibility on its respective host.

P. syringae pv. *tabaci* 11528 causes wildfire disease of tobacco, and *P. syringae* pv. *syringae* B728a is a highly virulent agent of brown spot of bean. pCPP2349, which carries the *hrmA*⁺ *Bam*HI-AvrII fragment in the broad-host-range vector pDSK519 (Fig. 1), was constructed and electroporated into *P. syringae* pv. *tabaci* 11528 and *P. syringae* pv. *syringae* B728a. *P. syringae* pv. *tabaci*(pCPP2349) and *P. syringae* pv. *tabaci*(pDSK519) were infiltrated into tobacco (*Nicotiana tabacum* L. 'Xanthi N') leaves at five different concentrations ranging from 5×10^8 to 5×10^3 cells per ml. Leaf panels infiltrated with *P. syringae* pv. *tabaci*(pCPP2349) at the highest concentration displayed a typical hypersensitive collapse within 24 h, whereas those infiltrated with lower concentrations of the bacterium failed to produce disease symptoms even after 5 days (Fig. 4). In contrast, *P. syringae* pv. *tab-*

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TGCTGACGTTTCTGTACGCGCATGGAACCTTATCGGGAATAATTGCCACCCACCGGACA 60
AATCCACAGTGGCGAACCGATTATGAGTAGCCTTTTCTACAAAACGTTGCTTGATGACTT 120
TTCAAGATGCATGGAGATGCCCGCCTTGGCGTTTGACGATAAGGGTGCCTGCAACATGAT 180
CATCGACAAGGCATTGCTCTGACGCTGTTGCGCGACGACACGCATCAACGTTTGTGCT 240
I D K A F A L T L L R D D T H Q R L L L
GATTGGTCTGCTTGAGCCACACAGGATCTACCTTGCAGCGCCTGTTGGCTGGCGCTCT 300
I G L L E P H E D L P L Q R L L A G A L
CAACCCCTTGTGAATGCCGCCCGCCGCAATTGCTGGGATGAGCAAGCGGCTGTACCA 360
N P L V N A G P G I G W D E Q S G L Y H
CGCTTACCAAGCATCCCGCGGAAAAAGTCAGCGTGAGATGCTGAAGCTCGAAATTGC 420
A Y Q S I P R E K V S V E M L K L E I A
AGGATTGGTCAATGGATGAAGTGTGGCGAGAACCCGACGTCACCGCGCTTTCAATC 480
G L V E W M K C W R E A R T
TTCCGACGTATTTCACTACTCTTGTCTTAGCGTAAGGAGCTTTTGTGAACCTATCCAT 540
M N P I H
hrmA

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Fig. 2. DNA sequence of the ORF1 region upstream of *hrmA*. Putative *hrp/avr* box denoted with single overlines, the putative ribosome-binding sites of ORF1 and *hrmA* with double overlines. The GenBank accession number for ORF1 is U96179.

Fig. 3. DNA gel blot showing distribution of sequences hybridizing with *hrmA* among several experimentally important strains of *Pseudomonas syringae*. Total DNA from indicated strains was digested with *Eco*RI and resolved on a 0.8% agarose gel before electroblotting and hybridization at moderate stringency with a ³²P-labeled *hrmA* probe. Lane 1, *P. syringae* pv. *tabaci* 11528; lane 2, *P. syringae* pv. *glycinea* race 4 U1; lane 3, *P. syringae* pv. *tomato* DC3000; lane 4, *P. syringae* pv. *syringae* B728A; lane 5, *P. syringae* pv. *syringae* 61.

aci(pDSK519) elicited no visible response 24 h after inoculation, but at several inoculum levels it produced disease symptoms within 5 days (Fig. 4). Furthermore, the growth of *P. syringae* pv. *tabaci*(pDSK519) was substantially higher than that of *P. syringae* pv. *tabaci*(pCPP2349) at all inoculum levels (data not shown). *P. syringae* pv. *syringae*(pCPP2349) and *P. syringae* pv. *syringae*(pDSK519) were similarly infiltrated into bean (*Phaseolus vulgaris* L. 'Eagle') leaves at 5×10^8 , 5×10^6 , 5×10^5 , and 5×10^4 cells per ml. Both strains were identical in failing to elicit any visible response in the first 24 h at any concentration and in producing a slowly spreading lesion at all but the lowest concentration (data not shown). Thus, *hrmA* converts the interaction of *P. syringae* pv. *tabaci* and tobacco to incompatibility, but it has no apparent effect on the interaction of *P. syringae* pv. *syringae* B728a with bean cv. Eagle.

The reactions of *hrmA*⁺ bacteria with other *Nicotiana* spp. and *N. tabacum* cultivars were tested by infiltrating leaves with *P. fluorescens*(pHIR11) at 5×10^8 cells per ml and *P. syringae* pv. *tabaci*(pDSK519) and *P. syringae* pv. *tabaci*(pCPP2349) at both 1×10^8 and 1×10^6 cells per ml. Plant reactions were then scored at 1 and 5 days. *N. tabacum* cv. Xanthi NC and *N. clelandii* responded, as did *N. tabacum* L. cv. Xanthi N (data not shown). That is, in each of these plants, *P. fluorescens*(pHIR11) elicited an HR at 5×10^8 cells per ml, and *P. syringae* pv. *tabaci* at 1×10^6 cells per ml caused disease after several days but failed to produce disease symptoms

when carrying pCPP2349. *N. debeyi*, *N. glutinosa*, *N. rustica*, and *N. tabacum* cvs. Petit Havana and Samsun were tested only with *P. fluorescens*(pHIR11) at 5×10^8 cells per ml, but all responded with a typical HR. In contrast, *N. benthamiana* failed to respond with the HR when infiltrated with any of the bacteria carrying *hrmA*, and it produced disease symptoms when inoculated with *P. syringae* pv. *tabaci*, regardless of the presence of *hrmA*. Thus, the *R* gene proposed to interact with *hrmA* appears to be present in all cultivars of *N. tabacum* tested and in most *Nicotiana* spp.

HrmA-FLAG is not secreted in culture under conditions in which HrpZ is secreted.

To determine if HrmA, like HrpZ, can be secreted in culture by *E. coli* MC4100 cells carrying pHIR11, we constructed pCPP2352, a derivative of *hrmA* that produced full-length HrmA carrying a C-terminal fusion with the FLAG synthetic epitope (Hopp et al. 1988). *E. coli* MC4100 cells carrying both pHIR11 and pCPP2352 were grown in *hrp*-derepressing minimal medium to late-log phase, and then cells were separated from the medium by centrifugation. Proteins in the cell and supernatant fractions were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis with M2 anti-FLAG and polyclonal anti-HrpZ antibodies. HrpZ was detected in the culture supernatant, whereas HrmA-FLAG was not, despite the presence of a large cell-bound pool (Fig. 5). pCPP2324

Fig. 4. Responses of tobacco leaves at 1 and 5 days post-infiltration to different levels of inoculum of *P. syringae* pv. *tabaci* 11528 either lacking or heterologously expressing the *hrmA* region carried on pCPP2349. On both leaves, panels on the left and right sides were inoculated with vector control and *hrmA*⁺ strains, respectively. Beginning with the lowest panel on each leaf half and progressing upward, the levels of inoculum were 5×10^8 , 5×10^6 , 5×10^4 , and 5×10^3 cells per ml.

(HrmA⁺) and pCPP2352 (HrmA-FLAG⁺) restored the HR phenotype to *P. syringae* pv. *syringae* 61-2071 equally well (data not shown).

Transient expression of *hrmA* in tobacco cells leads to apparent cell death.

A site of HrmA action in the interior of tobacco cells was suggested by the observations that HrmA was not released to the medium of bacteria carrying pHIR11 and had no apparent effect when infiltrated into tobacco leaf intercellular spaces, even though it was absolutely required for HR elicitation (data not shown). To determine whether expression of *hrmA* within tobacco cells was lethal, a transient expression protocol similar to that used by Mindrinos et al. (1994) was employed. In this technique, plant cells are doubly transformed with vectors expressing β -glucuronidase (GUS) and a test gene; if the test gene is lethal, GUS activity is reduced or eliminated. The *hrmA* ORF was subcloned into the plant expression vector pFF19 and biolistically delivered, along with the GUS⁺ plasmid pFF19G, into suspension-cultured tobacco cells. After incubation for 24 h, the tobacco cells were histochemically stained for GUS activity. Filters covered with tobacco cells co-bombarded with pFF19G and pFF19 showed extensive GUS activity, whereas those co-bombarded with pFF19G and

pFF19-HrmA showed a 30-fold reduction in the number of blue spots (Fig. 6). This indicated that the expression of *hrmA* within tobacco cells was deleterious.

DISCUSSION

We have provided further evidence that *P. syringae* pv. *syringae* 61 HrmA has properties of an Avr protein interacting with an unidentified *R* gene that is present in nonhost tobacco but not in host bean. Introduction of the *hrmA* gene into *P. syringae* pv. *tabaci* 11528 and *P. syringae* pv. *syringae* B728a, two strains we have shown to naturally lack the gene, converts only the former to incompatibility with its host. Such an ability to confer avirulence is part of the original operational definition of *avr* genes (Staskawicz et al. 1984; Keen 1990). HrmA has two additional properties that are consistent with an Avr-like function, as predicted by the hypothesis that some Avr proteins act within plant cells following contact-dependent delivery via the Hrp secretion system (Alfano and Collmer 1996; Gopalan et al. 1996). First, bacterial elicitation of a HrmA-dependent HR requires a functional Hrp secretion system, even though HrmA was shown not to be secreted in culture. Second, HrmA has an apparently lethal effect on tobacco cells but only when produced in the interior of these cells. Several aspects of these observations and the *hrmA* locus are discussed below.

hrmA appears to be the second of two genes in the *hrmA* operon. The upstream ORF1 uses typically preferred *P. syringae* pv. *syringae* codons to encode a predicted 10.9-kDa protein with weak similarity to *P. aeruginosa* ExsC. ExsC is the gene product of *exsC*, which is the first gene in a three-gene operon known as the trans-regulatory locus for exoenzyme S synthesis because it is required for synthesis of the secreted ExoS protein (Frank and Iglewski 1991). Interestingly, the other two genes of this operon, *exsB* and *exsA*, have homologs involved in the regulation and secretion of the type III-secreted Yop virulence proteins of *Yersinia* spp. ExsC has an apparent role in the translation or stability of ExoS (Goranson et al. 1997). Recently, it has been shown that the amino terminal portion of ExoS is similar to the type III-secreted YopE protein of *Yersinia* (Yahr et al. 1995; Kaniga et al. 1996), and that ExoS is secreted by a type III pathway (Yahr et al. 1996). Therefore, even though the sequence similarity between the predicted proteins encoded by ORF1 and ExsC is weak, it is

Fig. 5. Immunoblot analysis of the distribution of HrpZ and HrmA-FLAG between cell-bound and culture fluid fractions of *Escherichia coli* MC4100 cells carrying pHIR11 and pCPP2352. Bacterial cells were removed from a mid-log phase culture by centrifugation, and then the presence of HrmA-FLAG and HrpZ in each fraction was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblot analysis with M2 anti-FLAG monoclonal antibodies and anti-HrpZ polyclonal antibodies. Supernatant fractions were concentrated 20-fold and cell fractions were concentrated fivefold relative to initial culture volumes before electrophoresis.

Fig. 6. Transient expression assay for deleterious effects of *hrmA* expression within tobacco cells. Tobacco suspension-cultured cells were collected on Whatman #4 filters and biolistically co-bombarded with pFF19G and either pFF19 or pFF19-HrmA, as indicated. Cells on filters were incubated for 24 h, histochemically stained for β -glucuronidase activity, and then photographed.

likely that the similarity is significant and that ORF1 codes for a protein that has some role in HrmA function. One attractive possibility that we are currently testing is that ORF1 encodes a chaperone for HrmA secretion, similar to the chaperones that are present in the type III secretion systems of animal pathogens and that are required for secretion of specific virulence proteins (Wattiau et al. 1996). By confirming that the TnPhoA insertion in pCPP2071 was in *hrmA*, we confirmed that *hrmA* is required for bacteria carrying pHIR11 to elicit the HR. Functionally nonpolar mutations are now required to determine if ORF1 is also required. We converted *P. syringae* pv. *tabaci* to avirulence by using a clone carrying the complete locus, including ORF1, *hrmA*, and upstream regulatory sequences. Consequently, it is unclear whether *hrmA* is sufficient to convert *P. syringae* pv. *tabaci* to avirulence, although the *hrmA* coding region alone is sufficient to have a deleterious effect in tobacco cells.

As previously reported (Heu and Hutcheson 1993), *hrmA* is present in relatively few strains of *P. syringae*. Here we tested for its presence specifically in several experimentally important model strains. As predicted, *hrmA* is lacking in the tobacco pathogen *P. syringae* pv. *tabaci* 11528. It is also lacking in *P. syringae* pv. *syringae* B728a, a model for the study of epiphytic fitness factors, and *P. syringae* pv. *glycinea* race 4, a model for the study of *avr*-mediated race-cultivar interactions. However, sequences hybridizing with *hrmA* were found in *P. syringae* pv. *tomato* DC3000, an extensively studied strain that is pathogenic on both *Arabidopsis* and tomato. We are now cloning the hybridizing locus from DC3000 to learn whether it is linked with an ORF1 homolog, has the same effects as the *P. syringae* pv. *syringae* 61 *hrmA* when expressed in *P. syringae* pv. *tabaci* or tobacco cells, and contributes to virulence.

Our data suggest that the *R* gene proposed to interact with *hrmA* is present in many *Nicotiana* spp. but lacking in legumes. This is consistent with previous observations that *P. syringae* pv. *syringae* 61 is a weak pathogen of legumes such as bean and pea, but is a strong elicitor of the HR in tobacco, and that nonpathogenic bacteria carrying pHIR11 elicit the HR in tobacco but not in legumes (Huang et al. 1988). We have further explored this relationship by demonstrating with bean and several *Nicotiana* spp. and *N. tabacum* cultivars that *P. fluorescens*(pHIR11) elicits the HR only in plants in which heterologous *hrmA* expression can convert a virulent *P. syringae* strain to avirulence. Thus, the reason that the *hrp* cluster carried on pHIR11 is the only one from *P. syringae* reported to enable nonpathogens to elicit the HR is likely due to two factors. The DNA cloned contains a complete set of conserved *hrp* secretion and positive regulatory genes, and a linked variable region happens to contain an *avr* gene that interacts with an *R* gene in tobacco, the plant most commonly tested for bacterial HR elicitation activity. This model is further supported by the observation that the range of plants in which bacteria carrying pHIR11 elicit the HR can be extended by heterologous expression of appropriate *avr* genes (e.g., *avrB*; Gopalan et al. 1996; Pirhonen et al. 1996).

hrmA shares some properties with *avrE* and others with *avrB*. Kobayashi et al. (1989) originally isolated *avrE* from *P. syringae* pv. *tomato* on the basis of its ability to convert *P. syringae* pv. *glycinea* race 4 (virulent on a wide range of tester soybean cultivars) to avirulence on several cultivars. Further

analysis revealed that *avrE* appears to interact with an unknown *R* gene present in all soybean cultivars tested, occurs in a complex locus, is part of the *hrp* regulon, and is linked to the *hrp* cluster (Lorang and Keen 1995). These properties are similar to those of *hrmA*. The similarities of *hrmA* and *P. syringae* pv. *glycinea* *avrB* are based on a rather different analysis of *avr* gene function. Both are required for *P. fluorescens*(pHIR11) or normally compatible *P. syringae* strains to elicit the HR in certain plants (Huang et al. 1991; Gopalan et al. 1996; Pirhonen et al. 1996). Both require conserved Hrp secretion system components, such as HrcC (Alfano et al. 1996; Gopalan et al. 1996; Pirhonen et al. 1996), for their action, yet neither is secreted in culture under conditions in which HrpZ is secreted. Neither has any apparent effect when infiltrated into the intercellular spaces of leaves carrying the cognate resistance genes, but both elicit apparent cell death when heterologously expressed in the interior of such plant cells (Gopalan et al. 1996). Demonstration of the *R* gene-dependent lethal activity of AvrB in plant cells was aided by the existence of isogenic *Arabidopsis* lines that are differential for the *RPM1* *R* gene (Gopalan et al. 1996). Unfortunately, the putative *R* gene interacting with *hrmA* appears to be present in all of the cultivars of *N. tabacum* that we tested, so we could not explore the effect of transient *hrmA* expression in cells from a cultivar that does not respond hypersensitively to bacteria carrying *hrmA*.

The *hrmA* gene was originally named because of its weak HR phenotype in *P. syringae* pv. *syringae* 61 (Huang et al. 1991). As we have described above, *hrmA* is *avr*-like, but without a demonstrable *R* gene interaction. Therefore, its classification as an *avr* gene is uncertain. Indeed, our concept of the role of Avr proteins in *P. syringae* pathogenesis is being transformed by the accumulating evidence that these factors have a primary role in virulence and that a key property of several of them is that they act within plant cells following secretion by the Hrp pathway (Alfano and Collmer 1996). Thus, rather than considering HrmA as an "Avr-like" protein, it may be more useful to think of both HrmA and many Avr proteins (e.g., AvrB, AvrPto, AvrRpt2, and AvrBs3) as members of a larger class of "Hops" (Hrp-dependent outer proteins). This designation is analogous to the Yops (*Yersinia* outer proteins) designation for the proteins secreted by the prototypical *Yersinia* *ysc*-encoded type III secretion system, but is broadened here to "Hrp-dependent" rather than "*Pseudomonas*" to reflect the current, conveniently broad usage of "Hrp" and "Avr" for all plant pathogens. As with the Yops, *P. syringae* proteins in the Hop class may be targeted to the milieu or to the interior of host cells. Thus, it appears that the pHIR11 Hrp system, while sufficient to deliver Hop proteins targeted to the host cell, has no intrinsic ability to elicit the HR in the absence of such proteins. That is, as with the Yops, the Hops that are targeted to the interior of host cells are the key effectors of the interaction.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids are listed in Table 1. *E. coli* strains were routinely grown in LM (Hanahan 1983) at 37°C, but MC4100 cultures carrying pHIR11 were grown at 30°C in King's B broth (King et al. 1954). To express the *hrp* genes in

culture, bacteria were grown in the *hrp*-derepressing medium of Huynh et al. (1989). *P. syringae* strains were grown in King's B medium at 30°C. Inoculum for virulence assays was obtained by washing cells off of overnight King's B plates with 5 mM 2-[N-morpholino]ethanesulfonic acid and harvesting them by centrifugation at $3,000 \times g$ for 10 min, followed by resuspension in the same buffer. Antibiotics were used at the following concentrations (μg/ml): ampicillin, 100; tetracycline, 20; kanamycin, 50; nalidixic acid, 20; rifampicin, 50.

Plant materials.

N. benthamiana, *N. clevelandii*, *N. debeyi*, *N. glutinosa*, *N. rustica*, *N. tabacum* cv. Xanthi N, *N. tabacum* cv. Xanthi NC, *N. tabacum* cv. Petit Havana, *N. tabacum* cv. Samsun, and bean (*Phaseolus vulgaris* cv. Eagle) were grown in a greenhouse with supplemental metal halide lamp illumination and a photoperiod of 16 h. Bacteria were infiltrated into tobacco leaves through a small dissecting-needle wound, with syringes lacking needles. To inoculate bean, leaves were similarly infiltrated, but through stomata without prior wounding. The

NT1 line of *N. tabacum* suspension-cultured cells were grown in NT1 medium (Paszy and Lurquin 1987) at 150 rpm (shaker model G2, New Brunswick Scientific, Edison, NJ) and at 24°C. Four-day-old cells, in early-log phase, were used for biolistic experiments.

DNA manipulations.

Plasmid DNA manipulations were done by standard procedures (Sambrook et al. 1989). The procedures used have been described previously for the isolation of chromosomal DNA from *P. syringae* (Silhavy et al. 1984), PCR amplification of DNA for subcloning (Alfano et al. 1996), and DNA gel blot hybridizations with Immobilon-N membrane (Millipore, Bedford, MA). Hybridization and washing were done at 50°C in $1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.2% SDS. Both strands of a 700-bp DNA region upstream of *hrmA* were sequenced with synthetic primers and the ABI 373A sequencer (Applied Biosystems, Foster City, CA) at the Cornell Biotechnology Center. DNA sequences were analyzed with the DNASTar software package (DNASTar, Madison, WI). pDSK519 and pCPP2349 were introduced into *P. syringae* strains by electroporation as described.

Plasmid constructions.

The 2.4-kb *Bam*HI-AvrII *hrmA*⁺ fragment from pHIR11 was first cloned into pBluescript II KS⁻ to produce pCPP2324. This plasmid was used as a template for DNA sequencing. The pCPP2324 insert was re-cloned as a *Bam*HI-*Sst*I fragment into pDSK519 to produce pCPP2349. To clone *hrmA* into pFLAG-CTC, the ORF was amplified from pCPP2324 by PCR by standard procedures (Innis et al. 1990) with the following primers that introduced *Nde*I and *Bgl*II restriction sites at the 5' and 3' ends of *hrmA*, respectively: 5'-ATGACATATGAACCCCTATCCATGCA-3' and 5'-ATGAAGATCTGTTTCGCGCCCTGAGCGC-3'. Similarly, the following primers, which introduced *Bam*HI and *Pst*I restriction sites at the 5' and 3' ends of the amplified *hrmA* product, respectively, were used to amplify and clone *hrmA* into pFF19: 5'-ATGAGGATCCGTGAACCCCTATCCATGCA-3' and 5'-ATG ACTGCAGTCAGTTTCGCGCCCTGAG-3'.

Immunoblot assays for HrmA and HrpZ in culture fluids.

E. coli MC4100(pCPP2352) was grown overnight in King's B broth and then diluted to an OD₆₀₀ of 0.6 in 10 ml of *hrp*-derepressing minimal medium (Huynh et al. 1989). After 18 h of further incubation, bacterial cells were separated from culture fluids by centrifugation at $3,000 \times g$ for 10 min and resuspended in 2 ml of fresh medium. Culture supernatants containing 1 mM phenylmethylsulfonyl fluoride were passed through a 0.2-μm filter and concentrated 20-fold by ultrafiltration with Centriprep concentrators (Amicon, Beverly, MA). SDS-PAGE was done by standard procedures (Sambrook et al. 1989). For immunoblot analysis, separated proteins were transferred to Immobilon-P (Millipore). HrpZ was recognized with rabbit polyclonal antibodies raised to HrpZ and then followed with goat-anti-rabbit IgG alkaline phosphate conjugate as secondary antibody. Commercially available M2 anti-FLAG monoclonal antibodies (IBI/Kodak, New Haven, CT) and goat-anti-mouse IgG alkaline phosphate conjugate were used to detect HrmA-FLAG. Both secondary antibody conjugates were purchased from Sigma (St. Louis, MO) and used at

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
DH5α	<i>hsdR17 recA1 endA1 relA1</i> Nal ^r	Life Technologies (Gaithersburg, MD)
MC4100	F- Δ (<i>lac</i>)U169 <i>araD136 relA rpsL thi</i> Sm ^r	Oliver and Beckwith 1981
<i>Pseudomonas syringae</i>		
pv. <i>syringae</i> 61	Wild type, Nal ^r	Huang et al. 1988
pv. <i>syringae</i> 61-2071	<i>hrmA::TnPhoA</i>	Huang et al. 1991
pv. <i>syringae</i> B728a	Wild type, Rif ^r	Willis et al. 1988
pv. <i>tabaci</i> 11528	Wild type, Rif ^r	American Type Culture Collection
pv. <i>tomato</i> DC3000	Wild type, Rif ^r	Cuppels 1986
pv. <i>glycinea</i> race 4 U1	Wild type	N. T. Keen
Plasmids		
pUC18	Cloning vector, Ap ^r	Yanisch-Perron et al. 1985
pBluescript II KS ⁻	Cloning vector, Ap ^r	Stratagene (La Jolla, CA)
pDSK519	IncQ vector, Km ^r	Keen et al. 1988
pCPP2324	pBluescript II KS ⁻ carrying 2.4-kb <i>Bam</i> HI-AvrII <i>hrmA</i> ⁺ fragment	This work
pCPP2349	pDSK519 carrying 2.4-kb <i>Bam</i> HI-AvrII <i>hrmA</i> ⁺ fragment	This work
pFLAG-CTC	FLAG expression vector, Ap ^r	IBI/Kodak (New Haven, CT)
pCPP2352	pFLAG-CTC carrying <i>hrmA</i> ORF in <i>Nde</i> I- <i>Bgl</i> II sites	This work
pCPP2356	pUC18 carrying <i>TnphoA</i> and flanking DNA from pCPP2071	This work
pFF19	Plant expression vector	Timmermans et al. 1990
pFF19G	pFF19 carrying <i>uidA</i>	Timmermans et al. 1990
pFF19-HrmA	pFF19 carrying <i>hrmA</i> ORF	This work

their recommended dilutions. Membrane-bound secondary antibodies were visualized by chemiluminescence with the Western Light chemiluminescent detection system (Tropix, Bedford, MA).

Biolistics.

Particle bombardment procedures were done as described (Russell et al. 1991) with some modifications. Briefly, 4-day-old tobacco suspension-cultured cells grown in NT1 medium were diluted so the fresh weight of tobacco cells was about 100 mg/ml. Four milliliters of diluted suspension-cultured cells was collected by filtration on 5.5-cm Whatman #4 filter paper. Tobacco cells on filter paper supports were transferred to NT1 plates. Biolistic transformation of suspension-cultured tobacco cells was done with the PDS-1000/He apparatus (Bio-Rad, Hercules, CA) and 1,300-lb/in² rupture disks. For each co-bombardment, 0.5 mg of tungsten particles (Sylvania GTE Products, Towanda, PA) was coated with 1.0 µg of pFF19G and 0.5 µg of either pFF19 or pFF19-HrmA. After bombardment, tobacco cells were incubated at 24°C for 24 h, then cells on filter paper supports were transferred to clean petri plates and histochemically stained for 24 h with 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as a substrate (Jefferson et al. 1987).

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