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Molecular Cloning of a *Pseudomonas syringae* pv. *syringae* Gene Cluster That Enables *Pseudomonas fluorescens* To Elicit the Hypersensitive Response in Tobacco Plants

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A cosmid clone isolated from a genomic library of *Pseudomonas syringae* pv. *syringae* 61 restored to all Tn*5* mutants of this strain studied the ability to elicit the hypersensitive response (HR) in tobacco. Cosmid pHIR11 also enabled *Escherichia coli* TB1 to elicit an HR-like reaction when high levels of inoculum (10^9 cells per ml) were infiltrated into tobacco leaves. The cosmid, which contains a 31-kilobase DNA insert, was mobilized by triparental matings into *Pseudomonas fluorescens* 55 (a nonpathogen that normally causes no plant reactions), *P. syringae* pv. *syringae* 226 (a tomato pathogen that causes the HR in tobacco), and *P. syringae* pv. *tabaci* (a tobacco pathogen that causes the HR in tomato). The plant reaction phenotypes of all of the transconjugants were altered. *P. fluorescens*(pHIR11) caused the HR in tobacco and tomato leaves and stimulated an apparent proton influx in suspension-cultured tobacco cells that was indistinguishable from the proton influx caused by incompatible pathogenic pseudomonads. *P. syringae* pv. *tabaci*(pHIR11) and *P. syringae* pv. *syringae* 226(pHIR11) elicited the HR rather than disease symptoms on their respective hosts and were no longer pathogenic. pHIR11 was mutagenized with Tn*phoA* (Tn*5* IS*50*$_L$::*phoA*). One randomly chosen mutant, pHIR11-18, no longer conferred the HR phenotype to *P. fluorescens*. The mutation was marker-exchanged into the genomes of *P. syringae* pv. *syringae* strains 61 and 226. The Tn*phoA* insertions in the two pseudomonads abolished their ability to elicit any plant reactions in all plants tested. The results indicate that a relatively small portion of the *P. syringae* genome is sufficient for the elicitation of plant reactions.

The hypersensitive response (HR) of higher plants is characterized by the rapid, localized necrosis of tissues invaded by an incompatible pathogen (a microorganism that is pathogenic only on another host) and is associated with resistance to the pathogen (11). Elicitation of the HR by phytopathogenic bacteria is readily observed by infiltrating leaves with suspensions containing $>10^6$ bacteria per ml (10). Tobacco leaf tissue collapses within 24 h after inoculation with *Pseudomonas syringae* pv. *syringae*, for example. When tobacco plants are inoculated with lower concentrations of this bacterium, no visible symptoms appear because the HR affects an insufficient number of plant cells to cause the whole tissue to collapse (26). If tobacco leaves are inoculated with various concentrations of the compatible pathogen *P. syringae* pv. *tabaci*, watersoaked lesions (disease symptoms) develop, but only after a delay of 3 or 4 days. Only in the compatible interaction do the bacteria multiply for a prolonged period. Nonpathogenic bacteria, e.g., *Pseudomonas fluorescens* and *Escherichia coli*, elicit no plant responses in these tests and fail to multiply. Elucidation of the molecular basis for the interactions of the 41 *P. syringae* pathovars (host range variants) (20) with various plants should yield insights into a universal plant defense response and mechanisms of host-parasite specificity.

Three recent observations have provided new insights into the elicitation of the HR by *P. syringae* pathovars. (i) Several genes required for elicitation of the HR have been identified by Tn*5* mutagenesis (5, 6, 16, 23), and some have been cloned (14, 19). These *hrp* genes are required for both the HR and pathogenicity and appear to be present in all *P. syringae* pathovars (13, 14, 21). (ii) Molecular cloning experiments with the soybean pathogen *P. syringae* pv. *glycinea* have demonstrated the existence of *avr* (avirulence) genes that control the host range of pathogen races on differential soybean cultivars in a gene-for-gene manner (24, 25). The observation that an *avr* gene cloned from *P. syringae* pv. *tomato* renders *P. syringae* pv. *glycinea* incompatible on several cultivars of soybean and *P. syringae* pv. *tabaci* incompatible on tobacco suggests that *avr* genes may control host range at both the cultivar and species level (D. Y. Kobayashi and N. T. Keen, Phytopathology **76**:1099, 1986). (iii) Finally, the plasmalemma K^+/H^+ exchange response (XR) of tobacco cells that occurs soon after inoculation with incompatible pseudomonads (1, 2) has been shown to be correlated genetically, as well as physiologically, with the HR: Tn*5* mutants of *P. syringae* pv. *syringae* that are unable to elicit the XR in tobacco cells are also unable to elicit the HR in tobacco leaves (3).

The biochemical basis for elicitation of plant reactions (XR, HR, and disease symptoms) by the *P. syringae* pathovars remains elusive. Hrp^- mutants must be identified in planta, and the only phenotype of cloned *hrp* genes is their

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TABLE 1. Bacteria and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
E. coli		
HB101	F' *hsdS20 (hsdR hsdM) recA13 arg-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44G thi leu*	17
TB1	Δ (*lac pro*) *thi strA ara hsdR* (r_K^- m_K^+) ϕ80d*lacZ*ΔM15	Bethesda Research Laboratories
DH5α	*endA1 hsdR17* (R_k^- M_k^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* ϕ80d*lacZ*ΔM15	Bethesda Research Laboratories
CC118	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoAv20 galE galK thi rpsE rpoB argE*(AM) *recA1*	C. Manoil (18)
P. syringae pv-*syringae*		
61	WT isolated from wheat, Nal^r	M. Sasser
B1–B7	HR^- Tn*5* mutants of strain 61	3
226	Nal^r derivative of B78 isolated from tomato	T. P. Denny
61-18	Tn*phoA* mutant of strain 61	This work
226-18	Tn*phoA* mutant of strain 226	This work
P. fluorescens 55	Nal^r	M. Sasser
P. syringae pv. *tabaci* 11528	WT pathogenic to tobacco, Nal^r	M. Sasser
Plasmids		
pLAFR3	IncP *cos*$^+$ *rlx*$^+$, tetracycline resistant	24
pRK2013	IncP Tra $RK2^+$ Δ*repRK2 repE1*$^+$, kanamycin-resistant	7
pUC19	Ampicillin resistant	27
pPR328	Chloramphenicol-resistant derivative of pUC9	22
pUC191	pUC19 containing Tn*5*-flanking genomic DNA of B7	This work
pHIR1, −3, −4, −5, −8, −10, −11, −12	pLAFR3 containing genomic DNA of *P. syringae* pv. *syringae* 61	This work
pHIR11–18	pHIR11 with Tn*phoA* insertion	This work
pHIR11H22	22-kb *Hin*dIII fragment from pHIR11 subcloned into pLAFR3	This work
pHIR11BB6	5.9-kb *Bam*HI-*Eco*RI fragment from pHIR11 subcloned into pLAFR3	This work
pHIR11BB7	2.5-kb *Eco*RI fragment from pHIR11 subcloned into pLAFR3	This work
pHIR11BB8	8.4-kb *Bam*HI-*Eco*RI fragment from pHIR11 subcloned into pLAFR3	This work

[a] WT, wild type.

ability to complement appropriate *hrp* mutations. The dimensions of the genomic requirement for bacterial elicitation of plant reactions have not been established, and identification of the actual elicitor through gene manipulation has not been possible. In this paper, we report the molecular cloning from *P. syringae* pv. *syringae* 61 of a 31-kilobase (kb) genomic DNA insert which contains all of the genes necessary for bacterial elicitation of the HR and the XR by the nonpathogen *P. fluorescens* and which enables *E. coli* to cause a weak HR.

(Preliminary accounts of this work have been published elsewhere [H.-C. Huang, R. Schuurink, T. P. Denny, C. J. Baker, M. M. Atkinson, S. W. Hutcheson, and A. Collmer, Phytopathology 77:987, 1987; H.-C. Huang, I. Yucel, A. Collmer, and S. W. Hutcheson, Phytopathology 77:1753, 1987].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* pv. *syringae* 61 was isolated from wheat in Delaware by M. Sasser (University of Delaware). Strain 61 causes the HR on tobacco and tomato and was originally chosen for this work because it was a good recipient for Tn*5* mutagenesis with pGS9 (12). *P. syringae* pv. *tabaci* 11528 and *P. fluorescens* 55 were also obtained from M. Sasser. *P. syringae* pv. *syringae* 226 is pathogenic on tomato and was isolated from tomato in Georgia. All of these pseudomonads are spontaneous nalidixic acid-resistant (Nal^r) derivatives. pLAFR3 and recombinant derivatives were propagated in *E. coli* TB1 or *E. coli* CC118. Other plasmids were carried in *E. coli* HB101.

Media and growth conditions. *Pseudomonas* strains were grown on King medium B (KB) (9) broth at 28°C. *E. coli* was cultured on Luria-Bertani (LB) medium (17) at 37°C. Media were supplemented with appropriate antibiotics at the following concentrations: kanamycin, 50 μg/ml; nalidixic acid, 50 μg/ml; tetracycline, 20 μg/ml; and ampicillin, 100 μg/ml (50 μg/ml in broth).

Growth of plants and tobacco cell cultures. Tobacco (*Nicotiana tabacum* L. var. Samsun) and tomato (*Lycopersicum esculentum* Mill. var. Rutgers) used in HR assays were grown under greenhouse conditions and then transferred to the laboratory and maintained at 28°C with 18 h of illumination per day during experiments. Tobacco cell suspension cultures were derived from *Nicotiana tabacum* var. Hicks as described by Atkinson et al. (2). Plant reaction assays were also performed on 10-day-old wheat plants (*Triticum aestivum* L. var. Saluda) grown at 28°C with 18 h of illumination.

Plant reaction assays. Bacteria were prepared by washing

colonies grown for 24 to 48 h from KB agar plates or by growing cultures in KB broth for 24 h. In both cases, bacteria were harvested by centrifugation, suspended in 10 mM sodium phosphate buffer (pH 7.2), and adjusted to appropriate cell densities. Inoculations were performed by pricking leaves with a dissecting needle and then pressing the blunt end of a tuberculin syringe against the leaf surface while supporting the leaf with a finger (3). The same technique was used for tobacco, tomato, and wheat plants. The plants were observed 18 to 24 h later for development of the HR or over a period of several days for development of disease symptoms. The XR of tobacco cells was assayed by monitoring the increase in medium pH (2). Assay mixtures containing 5×10^8 bacteria per ml, 0.175 M mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, and 0.5 mM MES (morpholineethanesulfonic acid) were adjusted to pH 6.00 ± 0.05 with NaOH. The assay mixture was incubated at 25°C with constant shaking (125 rpm), and the pH was monitored periodically.

Measuring bacterial populations in planta. Bacteria were grown on KB agar containing appropriate antibiotics overnight and suspended in 10 mM potassium phosphate, pH 7.0. Bacterial suspensions containing ca. 10^5 cells per ml were infiltrated into the intercellular space of tobacco leaves as described above. Samples of inoculated tissue were obtained by excision with a 6-mm cork borer, and the bacteria were released by grinding the tissue in a microcentrifuge tube as described by Bertoni and Mills (4). Bacterial populations were then determined by dilution plating on KB agar containing appropriate antibiotics.

Bacterial matings. Triparental matings were carried out by mixing 100 μl of recipient Nal^r pseudomonads at 10^8 cells per ml with 25 μl each of donor *E. coli* TB1(pLAFR3) clones or *E. coli* CC118(pHIR11::Tn*phoA*) (Tn*phoA* is Tn*5* IS*50*$_L$::*phoA*) and helper strain *E. coli* HB101(pRK2013) at 10^7 cells per ml (7). The mating mixture was spread on KB agar and incubated at 28°C for 16 h. The cells were then washed from the surface of the agar with 2 ml of sterile water and plated on KB medium supplemented with nalidixic acid and tetracycline. Single-colony transfers were used to purify the transconjugants.

Recombinant DNA manipulations. Restriction enzyme digestions, agarose gel electrophoresis, plasmid extractions, nick translations, Southern blot analysis, and bacterial transformations ($CaCl_2$ procedure) were performed as described by Maniatis et al. (17). Colony filter hybridization was done with Whatman 541 filter paper by the method of Maas (15). Restriction enzymes were obtained from Bethesda Research Laboratories.

Southern blot analysis was done with GeneScreen Plus (Du Pont) according to the manufacturer's instructions (except that dextran sulfate was omitted) with ^{32}P-labeled (10^6 dpm) probes. Autoradiographs were obtained by exposing the blots to Kodak XAR-5 film at −70°C.

Construction of genomic library and cloning of Tn*5* and flanking DNA from mutant *P. syringae* pv. *syringae* 61-B7. Total genomic DNA of wild-type *P. syringae* pv. *syringae* 61 was partially digested with *Sau*3A and size fractionated in a linear sucrose gradient (10 to 40%) to yield 15- to 30-kb fragments. The cosmid vector pLAFR3 (24) was digested to completion with *Bam*HI, treated with calf intestine alkaline phosphatase, and ligated to the size-selected genomic DNA fragments. The recombinant molecules were packaged in vitro and transduced into *E. coli* TB1.

Total genomic DNA of mutant B7 was digested with *Eco*RI, and fragments ca. 23 kb long were isolated by the dialysis tube electroelution method of Maniatis et al. (17). The isolated fragments were ligated into *Eco*RI-digested and calf intestine alkaline phosphatase-treated pUC19, and recombinant molecules were transformed into *E. coli* HB101. Plasmid DNA from one kanamycin-resistant transformant was used to probe the wild-type genomic library.

Construction of subclones from pHIR11. The 22-kb *Hin*dIII fragment in pHIR11 was subcloned into pLAFR3 to construct pHIR11H22 by (i) digestion of pHIR11 with *Hin*dIII, (ii) resolution of restriction fragments by electrophoresis through a 0.7% agarose gel, (iii) electroelution of the 22-kb fragment from an excised gel piece with an Elutrap (Schleicher & Schuell), (iv) ligation of the 22-kb fragment with pLAFR3 that had been digested with *Hin*dIII and treated with calf intestine alkaline phosphatase, and (v) introduction of the recombinant plasmid into *E. coli* DH5α by electroporation with a Gene Pulser (Bio-Rad) according to the manufacturer's instructions (except that 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4] was used instead of 7 mM sodium phosphate), followed by selection for tetracycline resistance. To construct a series of subclones derived from the 8.2-kb *Bam*HI-*Bgl*II fragment of pHIR11, the fragment was first subcloned into the *Bam*HI site of pPR328 essentially as described above. The pPR328 recombinant plasmid was then digested completely with *Bam*HI and partially with *Eco*RI. Restriction fragments were electroeluted from an agarose gel and separately ligated into pLAFR3 that had been digested to completion with *Bam*HI and *Eco*RI. Transformants were selected for tetracycline resistance. The subclones were designated pHIRBB6 (5.7-kb insert), pHIR11BB7 (2.5-kb insert), and pHIR11BB8 (8.2-kb insert).

Tn*phoA* mutagenesis of pHIR11. pHIR11 was transformed into *E. coli* CC118. Transformants were grown to early stationary phase in LB broth containing 10 mM $MgSO_4$, 0.2% (wt/vol) maltose, and tetracycline and then infected with λ::Tn*phoA* (18) at a multiplicity of 1, and incubated at 30°C for 15 min. The culture was diluted 1:10 into LB broth and incubated with shaking at 30°C for 6 h. Portions (0.2 ml) were plated on LB agar containing kanamycin (300 μg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate disodium salt (40 μg/ml) and incubated for 2 to 3 days at 30°C. Plasmids in mutant colonies (regardless of alkaline phosphatase phenotype) were then mobilized by triparental matings into *P. fluorescens* for plant reaction assays.

Marker exchange mutagenesis of strains 61 and 226. Mutant cosmid pHIR11-18 was mobilized from *E. coli* CC118 into *P. syringae* pv. *syringae* 61 and *P. syringae* pv. *syringae* 226 by triparental mating. Cells from single transconjugant colonies were used to inoculate 5 ml of KB broth supplemented with nalidixic acid and kanamycin. After incubation with shaking for 24 h, 0.1 ml of each culture was transferred to 5 ml of fresh medium. The bacteria were subcultured in this way for 5 days, and the final cultures were diluted and spread on KB agar containing nalidixic acid and kanamycin. Kanamycin-resistant and tetracycline-sensitive colonies in the final populations were identified by cross-picking on KB agar supplemented with appropriate antibiotics.

RESULTS

Molecular cloning of strain 61 DNA hybridizing with DNA flanking the Tn*5* insertion in an HR$^-$ XR$^-$ mutant. Tn*5* mutants of *P. syringae* pv. *syringae* 61 that were affected in HR and XR phenotypes have been isolated and physiologi-

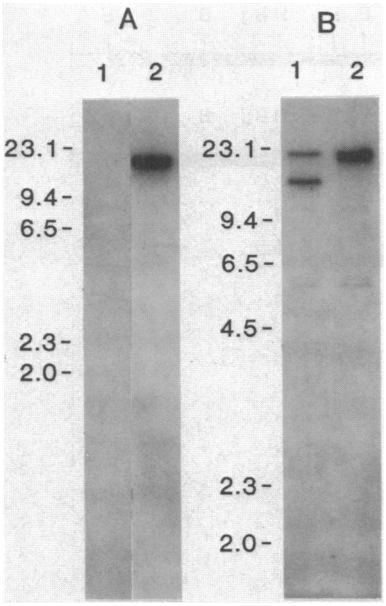

FIG. 1. Southern blot analysis of genomic DNA from *P. syringae* pv. *syringae* 61 (lanes 1) and its Tn*5* mutant B7 (lanes 2). Total genomic DNA was digested with *Eco*RI and electrophoresed through a 0.7% agarose gel. DNA fragments were transferred to GeneScreen Plus and probed with (A) ^{32}P-labeled pGS9 (which contains Tn*5*) or with (B) ^{32}P-labeled pHIR11. Sizes are shown in kilobases.

cally characterized (3). The Tn*5* insertions were analyzed by probing Southern blots of *Eco*RI-digested mutant genomic DNA with ^{32}P-labeled pGS9, the Tn*5* suicide vector. Mutant B7 contained a single Tn*5* insertion (Fig. 1) and produced no reaction in HR or XR bioassays (3). Genomic DNA from the mutant was digested with *Eco*RI and cloned into the *Eco*RI site of pUC19. Transformants containing the *Eco*RI fragment with the Tn*5* insertion were selected by their resistance to kanamycin. pUC191 was isolated from one of these transformants and labeled with ^{32}P. A library of wild-type *P. syringae* pv. *syringae* 61 DNA was constructed by ligating chromosomal DNA that had been partially digested with *Sau*3A into the *Bam*HI site of cosmid pLAFR3. Of 550 resulting clones probed by colony hybridization with labeled pUC191, 18 gave a positive signal. The cosmids in eight of these were analyzed for their ability to restore the HR phenotype to the strain 61 mutants.

Restoration of the HR phenotype in strain 61 HR$^-$ mutants by recombinant cosmids. Cosmids were mobilized from *E. coli* transductants to *P. syringae* pv. *syringae* 61 mutants by triparental matings with the helper plasmid pRK2013. Transconjugants were selected by their resistance to nalidixic acid and tetracycline and were typically obtained at a frequency of 4×10^{-5} per recipient cell. Tobacco leaves were injected with the transconjugants at a concentration of 5×10^7 cells per ml and observed 24 h later for the appearance of typical HR symptoms. The complementation patterns of the cosmids fell into three classes (Table 2). (i) The sole representative of the first class, pHIR1, was unable to restore HR activity to any of the mutants. (ii) pHIR3, pHIR4, pHIR5, pHIR8, and pHIR10 restored HR activity to three of the six mutants tested. (iii) pHIR11 and pHIR12 restored HR activity to all of the mutants. pHIR10 and pHIR11 were chosen as representatives of the latter two classes for further characterization.

TABLE 2. Restoration of HR response to Tn*5* mutants of *P. syringae* by cosmids carrying cloned wild-type *P. syringae* DNA

Mutant	Restoration[a] by cosmid pHIR-:							
	1	3	4	5	8	10	11	12
B7	−	+	+	+	+	+	+	+
B6	−	+	+	+	+	+	+	+
B5	−	+	+	+	+	+	+	+
B4	−	−	−	−	−	−	+	+
B3	−	−	−	−	−	−	+	+
B1	−	−	−	−	−	−	+	+

[a] +, HR phenotype restored; −, bacteria caused no plant reaction.

Restriction map of cosmids pHIR10 and pHIR11 and location of the B7 mutation. Mapping of the insert DNA revealed that pHIR10 contained 23 kb of *P. syringae* pv. *syringae* 61 DNA internal to the 31-kb insert present in pHIR11 (Fig. 2). The location of the B7 mutation was determined by restriction mapping of pUC191 and confirmed by probing Southern blots of *Eco*RI-digested mutant B7 genomic DNA with ^{32}P-labeled pHIR11 (Fig. 1 and 2). Only pHIR11 contained the entire 14-kb *Eco*RI fragment that was the target of the B7 mutation. The ability of pHIR11 to complement or suppress all of the Tn*5* HR$^-$ mutations in strain 61 led us to test the phenotypic expression of this cosmid in nonpathogenic bacteria.

Elicitation of the HR by nonpathogenic bacteria containing pHIR11. *E. coli* TB1 carrying pHIR11 was found to cause an HR-like reaction in tobacco, but the response occurred only when 10^9 cells per ml were injected, was spotty within the infiltrated area, and did not develop until 36 h after inoculation (Fig. 3 and Table 3). pHIR11 was introduced into *P. fluorescens* by triparental mating, and the transconjugant was tested for its ability to elicit the HR at 10^7 cells per ml in tobacco, tomato, and wheat plants. The resulting HR in tobacco leaves was indistinguishable in timing or appearance from the HR elicited by *P. syringae* pv. *syringae* 61 (Fig. 3). *P. fluorescens*(pHIR11) also caused an HR-like reaction in wheat and tomato, but only when 10^8 cells per ml were infiltrated into the leaves (Table 3). None of these bacteria lacking pHIR11, including those containing pHIR10, caused any plant reaction, even at high inoculum levels.

Conversion of *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae* 226 to incompatible pathogens on their respective hosts by pHIR11. To determine the effect of pHIR11 on the interaction of compatible pseudomonads with their hosts, the cosmid was introduced into *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae* 226, which are pathogenic on tobacco and tomato, respectively (8, 20). Both strains without the recombinant cosmid caused spreading, necrotic lesions which developed within 3 days after their respective hosts were inoculated with bacteria at a concentration of either 10^5 or 10^7 cells per ml. However, both strains behaved like incompatible pseudomonads on their normal hosts when carrying pHIR11: transconjugants infiltrated into either host produced no symptoms at 10^5 and a typical HR at 10^7 cells per ml (Table 3 and Fig. 3).

To address the possibility that an avirulent variant of *P. syringae* pv. *tabaci* was selected during bacterial conjugation, pHIR11 was cured from *P. syringae* pv. *tabaci* (pHIR11), and the cured strain was retested for virulence. The transconjugant was cured by subculturing the strain in fresh KB broth lacking antibiotics (0.1 ml into 5 ml of fresh medium) daily for 5 days. Tetracycline-sensitive colonies in the final population were identified by cross-picking on KB

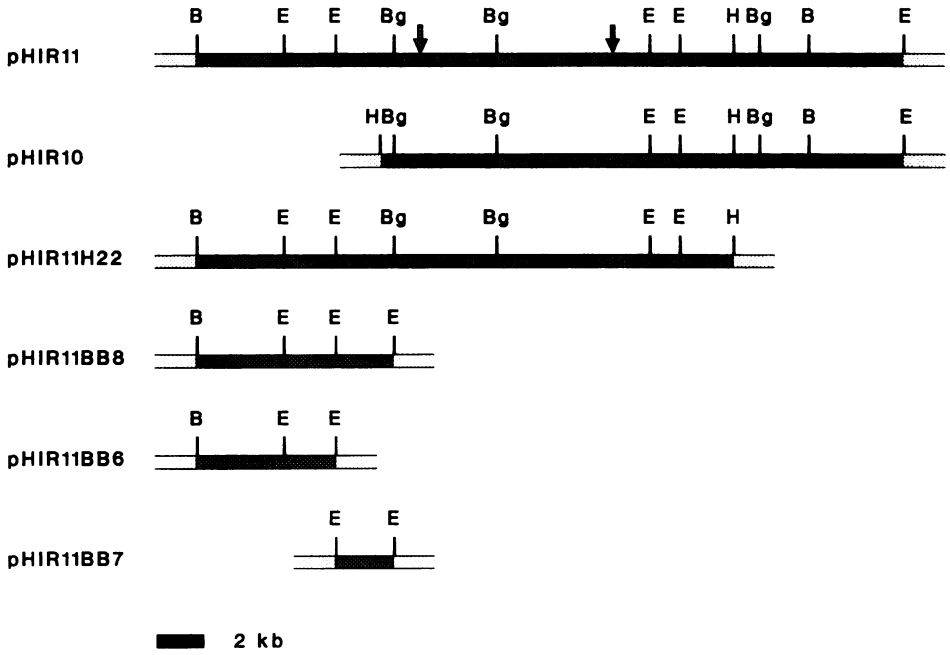

FIG. 2. Restriction map of the *P. syringae* pv. *syringae* 61 chromosomal DNA inserts in cosmids pHIR11, pHIR10, and subclones derived from pHIR11. *P. syringae* sequences are represented by the heavy shaded bars. The locations of the B7 Tn*5* insertion and the pHIR11-18 Tn*phoA* insertion are denoted by solid and cross-hatched arrows, respectively. B, E, Bg, and H, Cleavage sites for restriction endonucleases *Bam*HI, *Eco*RI, *Bgl*II, and *Hin*dII, respectively.

agar supplemented with antibiotics. Of 88 nalidixic acid-resistant colonies, 19 were tetracycline sensitive; 4 of these were infiltrated into tobacco leaves at a concentration of 10^5 cells per ml. All produced typical wildfire symptoms within 7 days after inoculation.

The incompatibility of *P. syringae* pv. *tabaci*(pHIR11) with tobacco was further documented by monitoring bacterial populations in inoculated leaf areas (Fig. 4). Populations of *P. syringae* pv. *tabaci* increased more than 1,000-fold in the 5 days following inoculation, whereas populations of *P.*

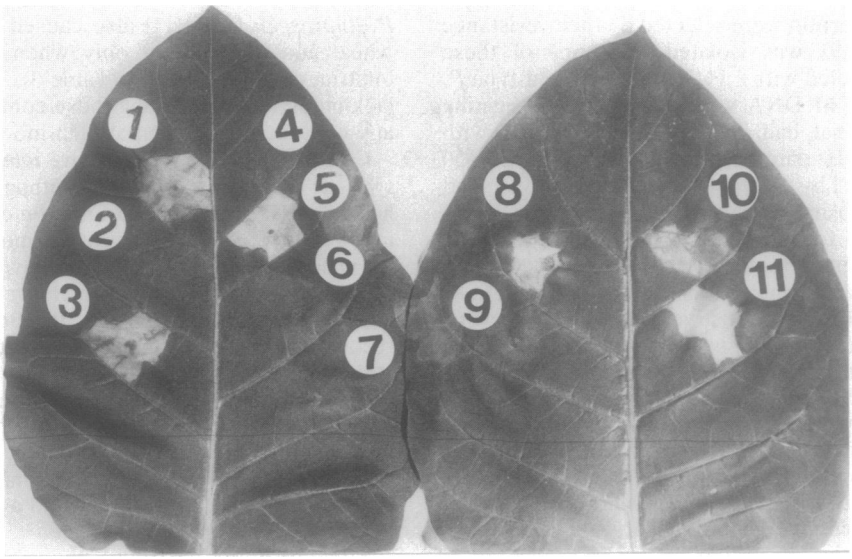

FIG. 3. Responses of tobacco leaf tissue to bacteria with altered plant reaction genotypes. Leaf panels were infiltrated with 5×10^7 (5×10^9 for *E. coli*) cells per ml, observed 24 h later, and then photographed after 4 days. Strains: 1, wild-type *P. syringae* pv. *syringae* 61; 2, Tn*5* mutant *P. syringae* pv. *syringae* 61-B7; 3, *P. syringae* pv. *syringae* 61-B7(pHIR11); 4, *P. fluorescens*; 5, *P. fluorescens*(pHIR11); 6, *P. fluorescens* carrying pHIR11-18 with Tn*phoA* insertion in cosmid; 7, *P. syringae* pv. *syringae* 61-18 with mutation in pHIR11-18 marker exchanged into the chromosome; 8, *E. coli* TB1(pHIR11); 9, *E. coli* TB1; 10, *P. syringae* pv. *tabaci*; 11, *P. syringae* pv. *tabaci*(pHIR11). Leaf tissue collapse was evident within 24 h in plants infiltrated with *P. syringae* pv. *syringae* 61 and pseudomonads carrying pHIR11 and occurred within 34 h in the tissue infiltrated with *E. coli* TB1(pHIR11). The spreading necrosis and yellowing caused by *P. syringae* pv. *tabaci* did not develop until 3 days after inoculation.

TABLE 3. Plant reactions elicited by bacteria carrying cosmid pHIR11 or a mutation introduced by marker-exchange with pHIR11::Tn*phoA*

Strain	Reaction of test plant[a] at indicated inoculum (cells/ml)			
	Tobacco		Tomato	
	10^7	10^5	10^7	10^5
P. syringae pv. *syringae* 61	HR	—	HR	—
P. syringae pv. *syringae* 226	HR	—	D	D
P. syringae pv. *tabaci*	D	D	HR	—
P. fluorescens	—	—	—	—
E. coli	—	—	—	—
P. fluorescens(pHIR11)	HR	—	—[b]	—
E. coli(pHIR11)	—[c]	—	—[d]	—
P. syringae pv. *tabaci*(pHIR11)	HR	—	HR	—
P. syringae pv. *syringae* 226(pHIR11)	HR	—	HR	—
P. fluorescens(pHIR11-18)	—	—	—	—
P. syringae pv. *syringae* 61-18	—	—	—	—
P. syringae pv. *syringae* 226-18	—	—	—	—

[a] HR, Typical HR appeared within 24 h; D, disease symptoms; —, no visible reaction.
[b] Typical HR appeared at 10^8 cells per ml.
[c] Necrosis developed in portions of infiltrated area following inoculation with 10^9 cells of *E. coli*(pHIR11) per ml but not of *E. coli* without the cosmid.
[d] No plant reaction developed at 10^9 cells per ml.

syringae pv. *tabaci*(pHIR11) only declined in the leaf tissue. The population of *P. fluorescens*, with or without pHIR11, also declined in leaf tissue.

Elicitation of the XR by bacteria containing pHIR11 and subclones derived from pHIR11. Incompatible *P. syringae* strains that elicit the HR in tobacco leaves also elicit a rapid K^+ efflux/H^+ influx (XR) in suspension-cultured tobacco cells which is readily detected as an increase in medium pH (2). To determine whether elicitation of the HR by pHIR11 involved the same physiological process, pseudomonad transconjugants that were converted to an HR^+ phenotype by pHIR11 were also tested for their ability to elicit a pH increase in the medium of suspension-cultured tobacco cells. All pseudomonads carrying pHIR11 were found to cause this response in tobacco cells (Fig. 5). Furthermore, *P. syringae* pv. *syringae* 61 elicited a more rapid pH response when containing pHIR11, although no difference was observed in the timing and appearance of the HR elicited by *P. syringae* pv. *syringae* 61 containing pHIR11 rather than the pLAFR3 vector control.

The XR bioassay was used to analyze the reactions of tobacco cells to *P. fluorescens* and *P. syringae* pv. *tabaci* transconjugants carrying plasmids with various portions of the pHIR11 insert DNA (Table 4). Whereas only pHIR11 could confer the XR phenotype to *P. fluorescens*, several of these plasmids could confer the XR phenotype to *P. syringae* pv. *tabaci*. It is particularly noteworthy that pHIR10

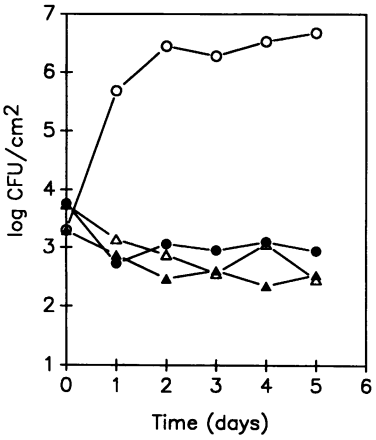

FIG. 4. Bacterial multiplication in tobacco leaves. Bacterial populations were monitored by dilution plating of disrupted tissue at the indicated times following inoculation. Values represent the mean from three plate counts for each of three independent inoculations. Symbols: open circles, *P. syringae* pv. *tabaci*; solid circles, *P. syringae* pv. *tabaci*(pHIR11); open triangles, *P. fluorescens*; solid triangles, *P. fluorescens*(pHIR11).

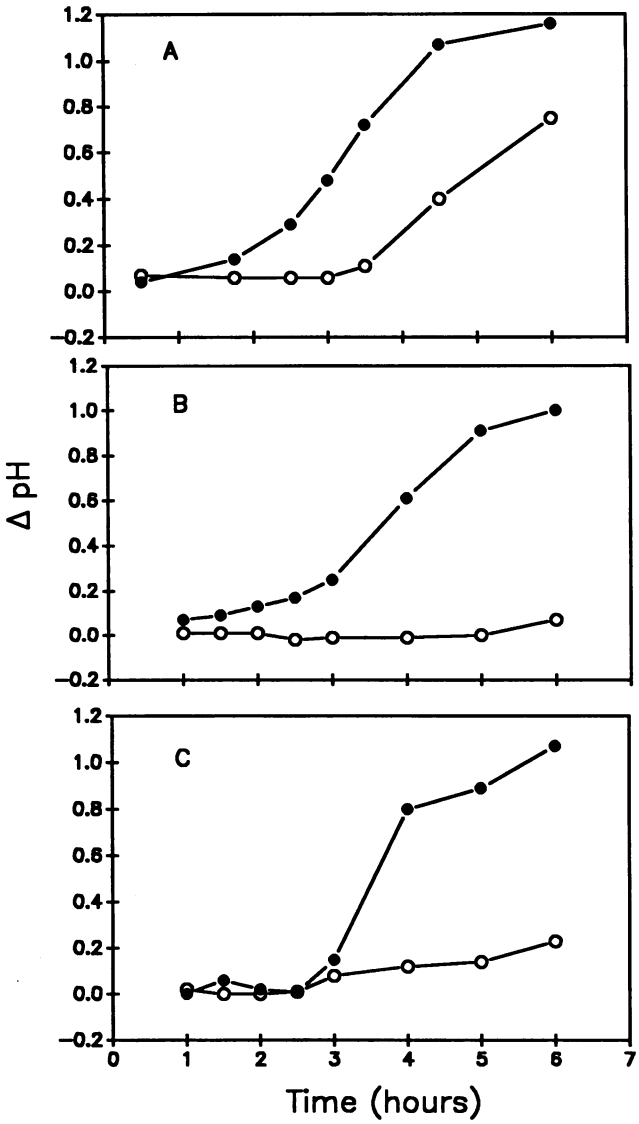

FIG. 5. Elicitation of net proton influx in suspension-cultured tobacco cells by pseudomonads carrying pHIR11. The pH of the cell culture medium was monitored following inoculation with bacteria at 10^8 cells per ml. (A) Symbols: open circles, *P. syringae* pv. *syringae* 61(pLAFR3); solid circles, *P. syringae* pv. *syringae* 61 (pHIR11). (B) Symbols: open circles, *P. syringae* pv. *tabaci* (pLAFR3); solid circles, *P. syringae* pv. *tabaci*(pHIR11). (C) Symbols: open circles, *P. fluorescens*; solid circles, *P. fluorescens* (pHIR11).

TABLE 4. Elicitation of the XR in cultured tobacco cells by *P. fluorescens* and *P. syringae* pv. *tabaci* transconjugants carrying pHIR11, pHIR10, or subclones derived from pHIR11

Plasmid	Elicitation of XR[a]	
	P. fluorescens	*P. syringae* pv. *tabaci*
pHIR11	XR	XR
pHIR10	—	XR
pHIR11H22	—	XR
pHIR11BB6	—	XR
pHIR11BB7	—	—
pHIR11BB8	—	XR

[a] XR, Alkaline pH differential of at least 0.5 units relative to uninoculated controls was observed within 6 h after inoculation of tobacco suspension cultures; —, no pH differential larger than 0.1 unit was observed.

and pHIR11BB8 (or pHIR11BB6) could confer the XR phenotype, since their DNA inserts do not overlap.

Characterization of a Tn*phoA* mutation in pHIR11 and marker exchange of the mutation into the genomes of strains 61 and 226. To further test whether pathogenic pseudomonads and *P. fluorescens*(pHIR11) elicited the HR in tobacco by the same mechanism, a mutation was introduced into pHIR11 and then marker-exchanged into the genomes of *P. syringae* pv. *syringae* strains 61 and 226. The cosmid was mutagenized with Tn*phoA*, a Tn*5* derivative which generates gene fusions resulting in target protein-alkaline phosphatase hybrids (18). Because alkaline phosphatase is active only if exported out of the cytoplasm, only colonies producing exported protein-alkaline phosphatase hybrids are blue on medium containing 5-bromo-4-chloro-3-indolylphosphate. Twenty-nine Tn*phoA*-containing (kanamycin-resistant) pHIR11 derivatives were mobilized from *E.coli* CC118 to *P. fluorescens* by triparental matings; five transconjugants were unable to elicit the HR. None of the HR$^-$ pHIR11:Tn*phoA* mutants conferred the blue colony phenotype to *E. coli* CC118 or *P. fluorescens* in KB medium. HR$^-$ mutant pHIR11-18 was randomly chosen for further study. The site of the Tn*phoA* insertion was restriction mapped (Fig. 2), and the cosmid was mobilized from *E. coli* to *P. syringae* pv. *syringae* strains 61 and 226. The mutation was marker-exchanged into the pseudomonad genomes by repeated subculturing of transconjugants in medium containing kanamycin but lacking tetracycline. When injected into tobacco leaves at 10^7 cells per ml, neither *P. fluorescens*(pHIR11-18) nor *P. syringae* pv. *syringae* 61-18 elicited any reaction (Fig. 3). *P. fluorescens*(pHIR11-18), *P. syringae* pv. *syringae* 61-18, and *P. syringae* pv. *syringae* 226-18 failed to elicit reactions in any of the plants tested (Table 3; data for wheat not shown). It is particularly significant that *P. syringae* pv. *syringae* 226-18 no longer caused disease symptoms (or any reaction) on its host plant, tomato.

Southern blot analysis of the genomic DNA from *P. syringae* pv. *syringae* strains 61 and 226, probed with pHIR11, revealed conservation of the homologous *Eco*RI restriction fragments (Fig. 6). Southern blot analysis also confirmed that the Tn*phoA* insertion in *P. syringae* pv. *syringae* 226-18 was marker-exchanged into the same *Eco*RI fragment that had been determined to be the site of the insertion pHIR11 (Fig. 7). Similar analysis of the genomic DNA of 108 strains of *P. syringae* pv. *syringae* isolated from a variety of diseased plants from all over the world revealed that 15 strains produced the same pattern of *Eco*RI fragments hybridizing with pHIR11 as did strains 61 and 226 (T. P. Denny, unpublished results).

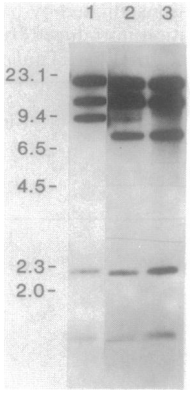

FIG. 6. Southern blot analysis of genomic DNA from *P. syringae* pv. *syringae* strains 61 and 226 probed with pHIR11. Total genomic DNA was digested with *Eco*RI, electrophoresed, and probed as in Fig. 1 with ^{32}P-labeled pHIR11 except that autoradiography of lanes 2 and 3 was enhanced by the use of a Lightning-Plus intensifying screen. Lanes: 1, pHIR11; 2, strain 61; 3, strain 226. Sizes are shown in kilobases.

DISCUSSION

We have cloned from *P. syringae* pv. *syringae* 61 a 31-kb DNA fragment containing a functional set of genes controlling bacterial elicitation of the HR and XR in tobacco. The phenotypic expression of these genes in nonpathogenic bacteria provides new tools for exploring the molecular basis of the elicitation of plant reactions by *P. syringae* pathovars. Cosmid pHIR11, which contains this gene cluster, alters the plant reaction phenotypes of bacteria in several ways. (i) It complements or suppresses all HR$^-$ and XR$^-$ mutations studied in *P. syringae* pv. *syringae* 61 and enhances the XR in the wild-type strain. (ii) It converts *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae* 226 into incompatible pathogens on their normal hosts. (iii) It causes the nonpathogen *P. fluorescens* to elicit HR and XR responses in tobacco that are typical of incompatible pathogenic pseudomonads. (iv) It also causes *E. coli* to elicit an HR-like response in tobacco leaves. These observations indicate that only a relatively small region of the genome is required for pseudomonads to elicit plant reactions.

Lindgren et al. (14) described the isolation of a 25-kb fragment of *P. syringae* pv. *phaseolicola* DNA that contains a cluster of *hrp* genes. The cluster complements seven of

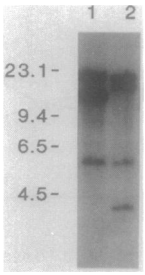

FIG. 7. Southern blot analysis of genomic DNA from *P. syringae* pv. *syringae* 226 and Tn*phoA* mutant strain 226-18. Total genomic DNA was digested with *Eco*RI, electrophoresed, and probed as in Fig. 1 with ^{32}P-labeled pHIR11. Note that the presence of two internal *Eco*RI sites in Tn*phoA* results in the appearance of two new bands in lane 2. Lanes: 1, strain 226; 2, strain 226-18. Sizes are shown in kilobases.

eight Hrp$^-$ mutations in *P. syringae* pv. *phaseolicola* and is conserved in a variety of *P. syringae* pathovars (13, 21) but does not enable *P. fluorescens* or *E. coli* to elicit the HR. We postulate that pHIR11 also contains *hrp* genes and that they are phenotypically expressed in nonpathogens because of the fortuitous cloning of a complete set of *hrp* genes or because of differences in the expression of *hrp* genes isolated from the two pathovars. Although we have not tested the homology between pHIR11 and the *P. syringae* pv. *phaseolicola hrp* gene cluster, we have shown that the pHIR11 insert DNA is conserved without restriction site polymorphism in *P. syringae* pv. *syringae* 226, a strain pathogenic on tomato, and in ca. 14% of various other pathogenic *P. syringae* pv. *syringae* strains tested. All strains tested share homology with pHIR11.

The role of *avr* genes in the elicitation of plant reactions by bacteria carrying pHIR11 remains unclear. The observations with *P. syringae* pv. *glycinea* (25), *P. syringae* pv. *phaseolicola* (14), and *P. syringae* pv. *tomato* (Kobayashi and Keen, Phytopathology **76**:1099, 1986) suggest that HR elicitation requires both *hrp* genes and an *avr* gene corresponding to a resistance gene in the test plant. Accordingly, one interpretation of our data is that pHIR11 contains both *hrp* genes and an *avr* gene. The conversion of *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae* 226 to incompatible pathogens by pHIR11 would then be explained by the presence of an *avr* gene recognized by tobacco and tomato. However, three observations suggest that the altered plant reaction phenotypes conferred to pathogenic pseudomonads by pHIR11 result from increased *hrp* copy number rather than from the presence of an *avr* gene. First, *P. syringae* pv. *syringae* 61 has an enhanced XR phenotype when carrying pHIR11. Second, pHIR11 converts *P. syringae* pv. *syringae* 226 to incompatibility even though there is no apparent restriction fragment polymorphism between the pHIR11 insert and the homologous region of the strain 226 genome, which suggests that genes controlling host range are located elsewhere. Third, more than one locus in pHIR11 is capable of conferring the XR phenotype to *P. syringae* pv. *tabaci*.

A mutation introduced into pHIR11 and subsequently marker-exchanged into the genomes of *P. syringae* pv. *syringae* strains 61 and 226 provides direct evidence that pHIR11 harbors at least one gene that confers the Hrp phenotype and further suggests that incompatible pseudomonads and *P. fluorescens*(pHIR11) elicit the HR by the same mechanism. The pHIR11-18 mutation resulted in loss of the HR phenotype in all of the bacteria tested, and it also caused loss of the pathogenicity phenotype in *P. syringae* pv. *syringae* 226 on its host, tomato. Tn*phoA* was chosen as a mutagen in anticipation of future experiments that will utilize the ability of the transposon to generate hybrid proteins and to systematically search the pHIR11 insert DNA for the presence of genes encoding exported proteins.

The evidence that bacterial elicitation of the XR and the HR occurs by a common mechanism was extended by the observation that all of the pseudomonads carrying pHIR11 elicited a strong increase in the pH of the medium of suspension-cultured tobacco cells. (It should be noted that killing of plant cells would result in disruption of the vacuole and acidification of the medium.) The failure of *E. coli*(pHIR11) to elicit a pH increase may not be significant, because much higher levels of inoculum were required for elicitation of the HR by this strain.

None of our observations suggest that pHIR11 confers pathogenicity to saprophytic pseudomonads or extends the host range of *P. syringae* pathovars. For example, *P. syringae* pv. *tabaci*(pHIR11) and *P. fluorescens*(pHIR11) caused an HR-like reaction in wheat only when artificially high levels of inoculum were used, and none of the bacteria containing pHIR11 caused any reaction other than a limited HR in either tobacco or tomato. Thus, although these genes are sufficient for the HR and may be required for pathogenicity, pathogenicity requires other genes, as previously reported by Lindgren et al. (14).

Because of its remarkable ability to confer altered plant reaction phenotypes to various bacteria and its genetic manipulability, pHIR11 provides a promising new avenue for exploring the mechanisms by which the *P. syringae* strains elicit the HR, cause disease, and define host ranges.

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