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Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar phaseolicola

(plant disease resistance/hypersensitive reaction/signal transduction)

ROBERT W. JACKSON*, EVANGELOS ATHANASSOPOULOS†‡, GEORGE TSIAMIS†‡, JOHN W. MANSFIELD†§, ANE SESMA¶, DAWN L. ARNOLD*, MARJORIE J. GIBBON*, JESUS MURILLO¶, JOHN D. TAYLOR‖, AND ALAN VIVIAN*

*Department of Biological and Biomedical Sciences, University of the West of England, Coldharbor Lane, Bristol BS16 1QY, United Kingdom; †Department of Biological Sciences, Wye College, Wye, Ashford, Kent TN25 5AH, United Kingdom; ¶Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Pública de Navarra, 31006 Pamplona, Spain; and ‖Horticulture Research International, Wellesbourne, Warwick CV35 9EF, United Kingdom

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ABSTRACT **The 154-kb plasmid was cured from race 7 strain 1449B of the phytopathogen *Pseudomonas syringae* pv. phaseolicola (*Pph*). Cured strains lost virulence toward bean, causing the hypersensitive reaction in previously susceptible cultivars. Restoration of virulence was achieved by complementation with cosmid clones spanning a 30-kb region of the plasmid that contained previously identified avirulence (*avr*) genes *avrD*, *avrPphC*, and *avrPphF*. Single transposon insertions at multiple sites (including one located in *avrPphF*) abolished restoration of virulence by genomic clones. Sequencing 11 kb of the complementing region identified three potential virulence (*vir*) genes that were predicted to encode hydrophilic proteins and shared the *hrp*-box promoter motif indicating regulation by HrpL. One gene achieved partial restoration of virulence when cloned on its own and therefore was designated *virPphA* as the first (*A*) gene from *Pph* to be identified for virulence function. In soybean, *virPphA* acted as an *avr* gene controlling expression of a rapid cultivar-specific hypersensitive reaction. Sequencing also revealed the presence of homologs of the insertion sequence IS*100* from *Yersinia* and transposase Tn*501* from *P. aeruginosa*. The proximity of several *avr* and *vir* genes together with mobile elements, as well as G+C content significantly lower than that expected for *P. syringae*, indicates that we have located a plasmid-borne pathogenicity island equivalent to those found in mammalian pathogens.**

Varietal resistance to halo-blight disease of bean (*Phaseolus vulgaris* L.) caused by *Pseudomonas syringae* pv. phaseolicola (*Pph*) is determined by gene-for-gene interactions involving five resistance (*R*) genes in the host and five matching avirulence (*avr*) genes in the pathogen. Depending on the presence or absence of functional *avr* genes, nine races of *Pph* have been distinguished (1, 2). The *avr* genes matching *R1*, *R2*, and *R3* have been cloned and sequenced. Their full designations are *avrPphF.R1*, *avrPphE.R2*, and *avrPphB.R3*; the terminal *R* gene designation will not be used here (3–5). Both *avrPphE* and *avrPphB* are chromosomal, whereas *avrPphF* is located on a large plasmid in those races that cause the hypersensitive reaction (HR) in cultivars of bean with the matching *R1* gene. The HR is a resistance response recognized by the rapid death of plant cells at inoculation sites and the restriction of microbial colonization (2). Additional *avr* genes located on the plasmid in *Pph* determine ability to elicit the HR in nonhost plants, *avrPphC* and a homolog of *avrD* (soybean interactors), and *avrPphD* that interacts with pea (6–8).

Certain *avr* genes, although recognized by their ability to activate plant defenses (the HR), also may have a role in pathogenicity in the absence of the interacting *R* gene in the host plant. In some cases there is a clear, qualitative effect on pathogenicity of mutations in *avr* genes, as with *avrBs2* in certain races of *Xanthomonas campestris* pv. vesicatoria in pepper and *avrRpm1* in *P. syringae* pv. maculicola in *Arabidopsis* (9, 10). However, the effect of *avr* gene mutations often is incomplete; for example, the *avrE* locus has a quantitative role in virulence in *P. syringae* pv. tomato strain PT23 but is dispensable in strain DC3000 (11). If the *avr* gene products do have a role in pathogenicity they may contribute to a redundancy of pathogenicity factors so that loss of a single *avr* gene product may not lead to total loss of ability to cause disease.

It has been proposed that gene-for-gene interactions are superimposed on an established basic, species-specific parasitism (12). Genes controlling such fundamental aspects of infection are located on well-characterized pathogenicity islands (PAIs) in animal pathogens (13, 14). There is increasing support for the hypothesis that bacteria pathogenic to animals have evolved from their nonpathogenic ancestors after acquisition of PAIs that typically contain large fragments of DNA that differ in G+C content from the rest of the genome (13). PAIs have been located on plasmids and also mapped to the chromosome. Examples of the former include the pCD1 plasmid in *Yersinia pestis* and of the latter, the islands SPI-1 and SPI-2 of *Salmonella typhimurium* (15, 16). In *Salmonella*, SPI-1 and SPI-2 both contain components of type III secretion systems (17, 18). This specialized protein delivery system is also the main component of the common *hrp* cluster found in plant pathogenic bacteria, which also may be considered a PAI. Mutations in *hrp* genes cause loss of the ability to elicit the HR in resistant plants (whether host or nonhost) and to cause disease symptoms (i.e., to be pathogenic) in susceptible cultivars. In *Ralstonia solanacearum*, *hrp* genes are located on a megaplasmid whereas they are chromosomal in *Erwinia*, *Pseudomonas*, and *Xanthomonas* (for recent reviews see refs. 19–21).

The proteins encoded by *avr* genes appear to be delivered via the type III secretion system into plant cells, where they act as elicitors of the HR. Evidence for this has been obtained by the transient expression of *avr* genes in plants, production of the Avr protein leading to activation of a rapid HR in an *R* gene-specific manner; examples include *avrPphB* and *avrPphE* (22). In animal pathogens, the type III secretion system is well

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Abbreviations: *avr*, avirulence gene; *vir*, virulence gene; HR, hypersensitive reaction; PAI, pathogenicity island; *Pph*, *Pseudomonas syringae* pv. phaseolicola; *R*, resistance gene; *dsp*, disease-specific gene.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF141883).

‡E.A. and G.T. contributed equally to this work.
§To whom reprint requests should be addressed. E-mail: j.mansfield@wye.ac.uk.

recognized as a route for export of pathogenicity factors such as Yops in *Yersinia*, invasion plasmid antigens in *Shigella*, Sips and Sops in *Salmonella*, and Esps in enteropathogenic *Escherichia coli* (23–25). Circumstantial evidence from the existence of the type III system and activity of Avr proteins inside plant cells suggests that similar pathogenicity determinants or virulence factors, as encoded by PAIs in pathogens of animals, also should be present in phytopathogenic bacteria (19).

In addition to pathogenicity factors thought to be secreted through the type III *hrp*-dependent system and to establish basic parasitism, degradative enzymes, extracellular polysaccharides, and low molecular weight toxins are produced by many plant pathogens (26). Genes encoding the pathogenicity determinants that are *hrp* independent often are clustered, but they are not generally recognized as PAIs. An example is the cluster of genes involved in the production of phaseolotoxin by *Pph*. Secretion of phaseolotoxin causes the yellow haloes that develop around lesions in bean leaves (27, 28).

Races of *Pph* harbor plasmids ranging in size from 25 to 160 kb (29). The large native plasmid (designated pAV511 in race 7 strain 1449B) is known to carry several *avr* genes. To examine the function of pAV511 we attempted to cure the plasmid by expression of its origin of replication in trans. The successfully cured strains were found to be compromised in virulence toward bean. Here we report the identification of a region on pAV511 that controls virulence, which we describe as a PAI based on the presence of several virulence genes (*vir*) and association with the transposases IS*100* and Tn*501*. We also characterize *virPphA* as the first (*A*) gene cloned for virulence functions from *Pph*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Sources of races of *Pph* are listed in ref. 1. The *E. coli* strain DH5α was used as host for clones in vectors pBluescript, pLAFR3, or pBBR1MCS-2 (2, 30).

DNA Extraction and Manipulation. Total DNA was extracted by using a Puregene DNA isolation kit (Gentra systems, distributed by Flowgen, Lichfield, Staffs., U.K.). Plasmid DNA was extracted either by alkaline lysis miniprep (31) or with a midiprep extraction kit (Qiagen, Chatsworth, CA). Restriction endonucleases, ribonuclease A, and buffers (GIBCO/BRL) were used according to manufacturer's instructions. Whole plasmids were extracted according to the method of Moulton *et al.* (32) and separated in 0.5% molecular biology agarose (Appligene, Strasbourg, France) midi gels for 4–5 h at 100 V, or for preparation of probe DNA in 0.5% ultra-pure low-melting point agarose (GIBCO/BRL). A gene library was constructed in the cosmid vector pLAFR3 (2).

Hybridization. Separated DNA was transferred to Hybond N^+ nylon membrane (ICN) by vacuum blotter (Appligene). Colony blotting was as described by Sambrook *et al.* (33), using BIOTRANS nylon colony blot. Probe DNA usually was purified by using a QIAEX II gel extraction kit (Qiagen), but pAV511 was excised from low-melting point gels. Restricted/amplified DNA was radiolabeled by using a High Prime radiolabeling kit (Boeringher Mannheim), and pAV511 was radiolabeled (1 h) in agarose by using Ready-to-Go beads (Amersham Pharmacia). Blots were hybridized (65°C, 16 h) with the labeled probes in hybridization solution (33), and then washed to high stringency (34).

Plasmid Transfer Procedures and Curing. Plasmid constructs were transferred from *E. coli* to rifampicin-resistant recipient strains of *Pseudomonas* by using a replica plate triparental mating procedure (35). Electroporations were carried out with a Gene Pulser (Bio-Rad) (36). Genomic clones were mutagenized by transposon insertion using Tn*3 gus* (37), and marker exchange mutants were obtained as described (in ref. 2).

Plasmid pPPY51 (which carries the replication gene from pAV505, a *ca*. 140-kb plasmid from race 4 strain 1302A; ref. 38) was electroporated into cells of 1449B to cure the homologous plasmid pAV511. Single-colony electroporants selected on King's B agar plates (with 100 μg/ml ampicillin) were immediately subcultured to King's B + ampicillin broth and grown overnight at 25°C. A loop full of the broth was further streaked on King's B + ampicillin plates, and the plasmid profile of strains from single colonies was examined. Plasmid pPPY51 was cured from *Pseudomonas* cells by using a cold shock method, 7 days at 4°C (39).

PCR and DNA Sequencing. Standard PCRs were performed with SuperTaq DNA polymerase and buffer (HT Biotechnologies, Cambridge, U.K.) by using either a DNA Thermal Cycler 480 or Gene Amp 2400 (Perkin–Elmer). Automated DNA sequencing was performed with an ABI 310 sequencer (Perkin–Elmer), and sequences were analyzed with a DNASTAR program. Database comparisons were made via the BLAST algorithm (40).

Pathogenicity Tests. Bacteria were tested for pathogenicity and avirulence in pods and leaves of bean cultivars (41). For assessment of the growth of bacterial populations in leaves, inoculations with suspensions of 5×10^8 colony-forming units/ml were made

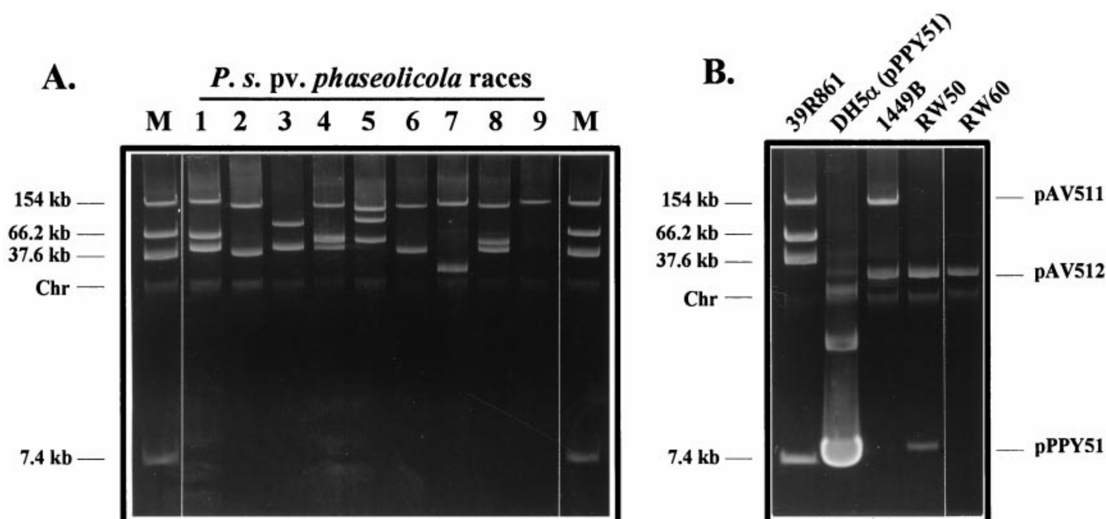

FIG. 1. Curing the 154-kb plasmid from *Pph* race 7 strain 1449B. (*A*) Plasmid profiles from races of *Pph*; M gives profile from *E. coli* 39R861 that contains plasmids of known size (44). (*B*) The 154-kb plasmid pAV511 was cured from 1449B by using the *rep* gene cloned in pPPY51. Both RW50 and RW60 have been cured of pAV511, but RW50 retains pPPY51. The smaller plasmid in 1449B, RW50, and RW60 is designated pAV512. Chr, chromosomal DNA.

into unifoliate leaves, and tissue samples were taken with a 0.6-cm diameter borer (42). Tissues were homogenized in 10 mM $MgCl_2$ and dilutions were plated on selective medium. Pathogenicity tests in *Phaseolus lunatus* L., *Pisum sativum* L., and *Nicotiana tabacum* L. were as described (1, 2, 43), and on soybean (*Glycine max* L.) plants were inoculated by using a 1-ml syringe (without needle) to infiltrate bacterial suspensions of 5×10^7 colony-forming units/ml into the underside of fully expanded primary leaves.

RESULTS

Strains of Race 7 Cured of the 154-kb Plasmid Have Reduced Virulence. Plasmid profiles from races of *Pph* are illustrated in Fig. 1*A*. The 154-kb plasmid pAV511 was cured from race 7 strain 1449B by the introduction of pPPY51, which carries a similar replication gene. Examination of plasmid profiles in several electroporants indicated loss of pAV511 (Fig. 1*B*). To confirm that the plasmid had been cured, and not forced into a recombination event with the chromosome, total and plasmid DNA were extracted and probed with the plasmid-borne *avrPphF* gene and the *rep* region on pPPY51. No hybridization was observed with either of the plasmid probes, and strains such as RW60 were used as plasmid-cured derivatives in further experiments.

The cured strains were strikingly altered in virulence as summarized in Table 1. The appearance of inoculation sites in pods of differential cultivars is illustrated in Fig. 2*A*. In cvs. Canadian Wonder and Tendergreen, race 7 is normally fully virulent, causing water-soaked lesions, but the cured strains caused brown HR-like lesions. In cv. Red Mexican, loss of plasmid-borne *avrPphF* was expected to result in a shift to virulence but instead RW60 caused restricted orange/brown lesions phenotypically different from the wild-type HR (Fig. 2*B*). In contrast to the altered responses observed in other cultivars, in A43 loss of the plasmid had no effect on appearance of the HR governed by the chromosomal *avrPphE* and *R2* gene interaction. The same pattern of loss of virulence in cured strains was apparent in pathogenicity tests on bean leaves and also on the alternative host *P. lunatus* (lima bean) cv. King of the Garden. However in nonhost tobacco and pea (as in bean cv. A43) an HR similar to that caused by the wild type was induced.

To determine whether the HR-like reaction caused after loss of pAV511 was *hrp* dependent, insertion mutants in *hrpF* (Tn*3 gus*; ref. 2) and *hrpA* (nonpolar mutation; G.T., unpublished work) were created in race 7, and the mutants then were cured of pAV511 as previously described. The *hrp* mutants of race 7 or the cured strain all failed to cause the HR or water soaking in any bean cultivar or nonhost tested. Delivery of signals leading to the formation of lesions by RW60 therefore depended on the presence of the *hrp* secretion system.

Cloning Virulence Factors. Strains lacking pAV511 were able to produce proteases, extracellular polysaccharides, and phaseolotoxin to the same levels as the wild-type race 7 (data not shown). Production of these potential pathogenicity factors therefore was not compromised by loss of the 154-kb plasmid. To clone the putative *vir* genes from pAV511, a genomic library of race 7 was constructed by using the broad host range cosmid pLAFR3. Clones harboring plasmid DNA were identified by probing colony blots with pAV511. The 48 clones selected then were mobilized by triparental mating into strain RW60, and transconjugants were tested for their ability to restore virulence on bean pods. Nine cosmid clones modified the plant's response to RW60, and they fell into two groups based on the phenotypes conferred. One group, typified by pAV521, was able to restore water-soaking ability to RW60 in cv. Tendergreen, and to a lesser extent cv. Canadian

Table 1. Reactions of strains on bean and soybean

	Bean cultivar			Soybean cultivar	
Strain	Canadian Wonder	Tender-green	Red Mexican	Osumi	Choska
P.s. pv. glycinea	HR	HR	HR	S	S
Pph race 7	S	S	HR*	HR	S−
RW60	HR	HR	HR†	N	N
RW60 (pAV518)	S−	S−	S−	HR	N
RW60 (pAV533)	S−	S−	S−	HR	N
Race 7::*virPphA* Tn*3gus*	HRd	HRd	HR*	S−	S−

S, fully susceptible water-soaked lesion; S−, slower development of lesions than S; HRd, delayed HR after initial water-soaking; N, null reaction, no cell browning.
*HR characteristic of the *avrPphF/R1* interaction.
†HR in pods more orange in color than *.

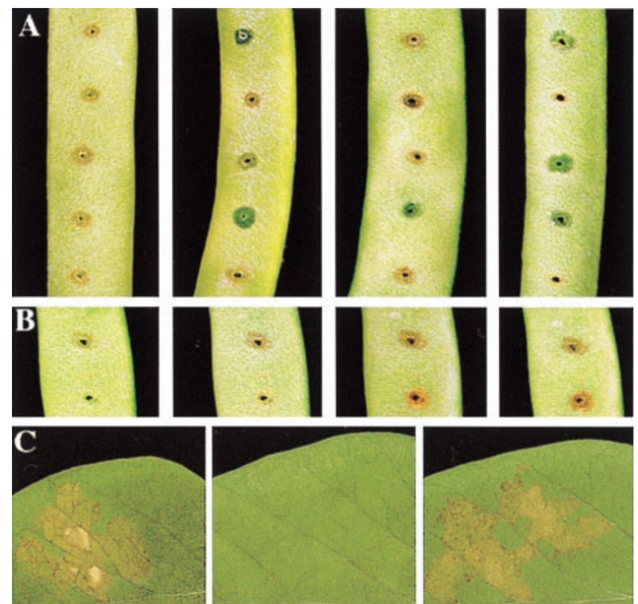

FIG. 2. Reaction phenotypes in bean pods and soybean leaves inoculated with *Pph*. (*A*) Pods of bean cultivars (from left to right): A43, Canadian Wonder, Red Mexican, and Tendergreen 3 days after stab-inoculation with (top to bottom site): *Pph* race 7 strain 1449B, cured strain RW60, complemented strains RW60 (pAV521) and RW60 (pAV518), and RW60 (pLAFR3) vector control. Note that pAV521 carries *avrPphF*, the *avr* gene matching resistance gene *R1* in Red Mexican. (*B*) Time course of HR development in pods of cv. Red Mexican photographed 1, 2, 3, and 5 days after inoculation with 1449B (*Upper*) and RW60 (*Lower*); note the more orange lesion caused by the plasmid-cured strain. (*C*) Leaves of soybean cv. Osumi 10 days after inoculation with (left to right) 1449B, RW60, and RW60 (pAV518).

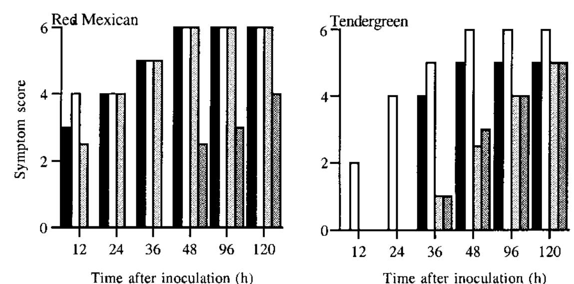

FIG. 3. Development of the HR and susceptible tissue collapse in leaves of cvs. Red Mexican and Tendergreen inoculated with suspensions of 2×10^8 cells/ml of race 7 strain 1449B (black bars), cured strain RW60 (white bars), complemented strains RW60 (pAV521) (light gray bars) and RW60 (pAV518) (dark gray bars). Symptom scores assigned to infiltrated tissue were: 0, no reaction; 1, partial glazing; 2, 100% glazing over the inoculation site; 3, up to 50% tissue collapse; 4, between 50 and 100% collapse, 5, 100% collapse; 6, 100% collapse and browning, as observed during the HR. Data are the mean scores from sites in two leaves. Note that pAV521 carries *avrPphF*, the *avr* gene matching resistance gene *R1* in Red Mexican.

Table 2. Numbers of bacteria ($\times 10^{-5}$) recovered from inoculation sites in leaves 4 days after inoculation

Cultivar	Bacterial strain*			
	Race 7	RW60 pLAFR3	RW60 pAV518	RW60 pAV521
Canadian Wonder	92.0 ± 16.0	*6 ± 0.7*	43.1 ± 16.0	15.1 ± 5.0
Red Mexican	*0.14*	*1.23 ± 0.3*	12.5 ± 1.3	*0.08*
Tendergreen	139.5 ± 0.4	*0.27 ± 0.1*	33.6 ± 0.5	38.5 ± 2.0

Bacterial numbers are given per 0.6-cm diameter disc of leaf as means from three replicates ± SEM unless <0.001.
*Data from interactions giving the HR are italicized (see Table 1).

Wonder, and also restored the *avrPphF/R1* phenotype of the HR in cv. Red Mexican, suggesting that *avrPphF* was present in these clones. The second group, typified by pAV518, conferred the ability to cause water-soaked lesions in cvs. Canadian Wonder, Tendergreen, and Red Mexican. In pods, the initial development of lesions caused by complemented RW60 in cvs. Canadian Wonder and Tendergreen was as rapid as observed with wild-type isolates of *Pph*, as shown in Fig. 2*A*. However, in contrast to fully virulent strains, after 5 days the water-soaked lesions caused by transconjugants of RW60 often developed some browning, leading to the S-classification used in Table 1. In leaves, RW60 caused a rapid HR in each cultivar and complementation (in the absence of *avrPphF*) was indicated by the slow development of susceptible symptoms, as quantified for cvs. Red Mexican and Tendergreen in Fig. 3. Restoration of virulence in transconjugants of RW60 harboring pAV518 and pAV521 also was demonstrated by increases in bacterial populations at inoculation sites (Table 2).

Mapping, Transposon Mutagenesis, and Subcloning. Restriction mapping and hybridization experiments showed that all clones that restored virulence contained part of a region of about 30 kb, which was covered by pAV518 and pAV521. PCR and hybridization analysis revealed the presence of *avr* genes *avrD* and *avrPphC* as well as *avrPphF* within this region (Fig. 4). The two genomic clones were mutagenized with Tn*3 gus* to localize genes responsible for the partial restoration of virulence; more than 200 separate insertions were tested in each cosmid. Transposons located to several different sites within pAV518 abolished the restoration of virulence by the clone. In pAV521, only two insertions were similarly effective and sequencing located these in *avrPphF* and just downstream of the gene as indicated in Fig. 4.

Fragments carrying the regions implicated in the restoration of virulence in pAV518 and pAV521, which were bounded by convenient restriction sites, were subcloned into pLAFR3 or pBBR1MCS-2 for expression in *Pph*. A 1.8-kb *Bam*HI fragment (designated pAV525), harboring a functional *avrPphF* gene, restored some water-soaking ability to RW60 on cv. Tendergreen. From pAV518, a 6.7-kb *Eco*RI fragment (designated pAV530) successfully restored pathogenicity to RW60 on cvs. Canadian Wonder, Tendergreen, and Red Mexican. The combination of insertion mutagenesis and subcloning therefore located determinants of virulence to specific regions in pAV518 and pAV521. Although some subclones significantly restored virulence, none was as fully effective as the genomic clones. For example, in pods of cv. Tendergreen RW60 (pAV530) caused water-soaked lesions but they developed slightly more slowly than those caused by RW60 (pAV518). The potential *vir* genes located by Tn*3 gus* mutagenesis appeared to have additive effects.

Sequencing *vir* Genes in pAV518. The contiguous 0.9-, 3.4-, and 6.7-kb *Eco*RI fragments from the right of pAV518 contained the site of several Tn*3 gus* insertions that abolished the restoration of pathogenicity by the genomic clone (Figs. 2 and 3). The 6.7-kb *Eco*RI fragment cloned as pAV530 possessed ability to restore virulence to RW60. Sequencing the 11-kb region of DNA revealed the presence of four possible ORFs, each of which was preceded by an upstream *hrp* box motif (indicating potential regulation by HrpL; ref. 45) as summarized in Table 3.

Transposon insertions that compromised virulence were located to sites within ORF1 and ORF4 and the putative promoter region of ORF3 (Fig. 5). The ORFs were subcloned to analyze their function. The smallest region achieving consistent restoration of water-soaking ability was a 1.8-kb *Nsi*I–*Ssp*I fragment containing only ORF1, cloned as pAV536 (Fig. 5). The gene located as ORF1 therefore was designated *virPphA* as the first (*A*) gene for virulence cloned from *Pph*. Although clones containing ORF4 alone did not restore water-soaking ability, they did cause a clear delay in the onset of the HR normally observed in pods inoculated with RW60 (data not shown). No such effect was observed with ORFs 2 or 3 (Fig. 5). For details on subclones see Table 4, which is published as supplemental data on the PNAS web site, www.pnas.org.

The proteins predicted to be encoded by *virPphA* and the other potential *vir* genes represented by ORF3 and ORF4 are hydrophilic and lack similarity to other proteins in databases. As shown in Fig. 5, repeated and inverted sequences of 1,053 bp with predicted similarity to the IS*100* transposase ORF from *Y. pestis* (64.8% amino acid identity; ref. 47) were located at either side of ORF4, flanking a region that also contained a homolog of the Tn*501* transposase from *Pseudomonas aeruginosa* (62.5% amino acid identity; ref. 48). The G+C content of the sequenced region of pAV511 was 54%, significantly lower than the overall figures of 59–61% reported for pathovars of *P. syringae* (46). Individual ORFs, except ORF2, also had low G+C percentages (Table 3).

***virPphA* Acts as an *avr* Gene in Soybean.** Race 7 of *Pph* typically causes a rapid HR in soybean leaves but in certain cultivars such as Bragg or Choska it was found to be weakly virulent, causing symptoms similar to those produced by the recognized soybean pathogen *P. syringae* pv. glycinea. The plasmid-cured strain RW60 caused a null response on all cultivars of soybean tested. Induction of the HR was restored by the genomic clone pAV518 and subclones containing *virPphA* (Table 1). Only the transposon insertions in pAV518 that were located in *virPphA* prevented effects on soybean. Therefore, *virPphA* appeared to act as a typical *avr* gene, controlling expression of a rapid HR in soybean. Phenotypes observed are illustrated in Fig. 2*C*. The significance of *virPphA* in controlling host range was confirmed by the finding that *virPphA* mutants of strain 1449B, created by marker exchange of transposons L20 or J37 (Fig. 5), were able to colonize soybean. By contrast the 1449B::*virPphA* Tn*3 gus* strains lost virulence to pods of bean cvs. Canadian Wonder and Tender-

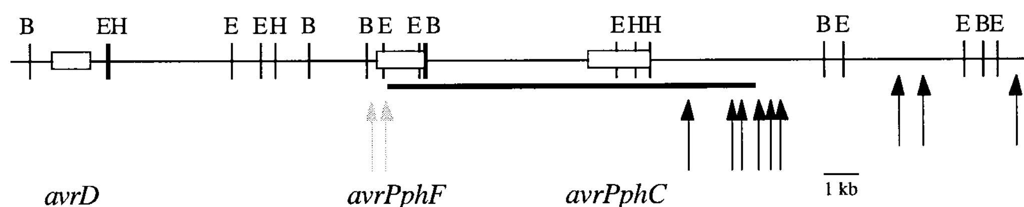

FIG. 4. Characterized region of *Pph* plasmid pAV511 found to contain the PAI. The DNA common to genomic clones pAV518 and pAV521 is marked by the underlying line. The positions of single Tn*3 gus* insertions that abolished restoration of virulence by pAV521 (gray arrows) and pAV518 (black arrows) are marked. Note that insertions that did not affect virulence were not mapped.

Table 3. Features of the four ORFs other than transposases located in the PAI

	Putative promoter region*	Position relative to ORF†	Shine-Dalgarno sequence and start codon‡	G+C%§	Predicted protein size
ORF1¶	**GGAACC**-15N-**CCAA**	−63	**GGAGAG**TCTAT<u>ATG</u>	54.01	59.6kDa
ORF2	AGAAGC-15N-**CCAC**	−94	**GCGCGAG**CTG<u>GTG</u>	59.39	22.9kDa
ORF3	**GGAACT**-15N-**CCAC**	−31	**GAGGA**TATGCG<u>GTG</u>	51.94	10.3kDa
ORF4	**GGAACT**-15N-**CCAC**	−59	**GGAGA**AAATCAGCAT<u>ATG</u>	53.09	35.4kDa

*Nucleotides in bold indicate those sequences that are conserved according to the "*hrp* box" consensus (45).
†Position relative to translation start is given relative to the position of the 3′ C in the putative HrpL promoter sequence.
‡Nucleotides in bold indicate putative Shine-Dalgarno sequences, start codons are underlined.
§The genome of *P.syringae* pvs. is reported to be 59–61% G+C (46).
¶Designated *virPphA*.

green, producing a delayed HR phenotype after initial water soaking (Table 1).

DISCUSSION

Loss of the largest plasmid (pAV511) from *Pph* caused striking changes in virulence to bean. In particular, the cured strains of race 7, such as RW60, caused the HR in previously fully susceptible cvs. Canadian Wonder and Tendergreen (Fig. 2*A*). Elicitation of the HR by RW60 depended on the presence of a functional type III secretion system, which is believed to deliver Avr proteins that have been identified because of their interaction with matching *R* genes in the plant. Removal of the plasmid-encoded virulence factors therefore has revealed the potential presence of a second tier of "masked" *avr* genes. An intriguing question is how the *vir* genes may act to block phenotypic expression of *avr* genes, which function after loss of the pAV511 plasmid.

Possible routes to virulence are outlined in Fig. 6, in which *avr* genes functioning despite the presence of Vir factors are designated α and those revealed after plasmid loss β. Three modes of action of Vir factors are suggested: (*i*) β *avr* gene suppression, (*ii*) blocking β Avr protein transfer, and (*iii*) interference with signal transduction leading to the HR and other defense responses. In view of the ability of *virPphA* to act as an α *avr* gene in soybean and the presence of the HrpL promoter motif as found upstream of other *avr* genes, it seems probable that Vir proteins, like Avr proteins, function in the plant cell. Route 3 shown in Fig. 6 therefore is the most likely pathway to virulence. Characterization of the β *avr* genes will allow this possibility to be fully explored. Interference between *avr* gene products outside the bacterium already has been proposed to explain the epistasis of *avrRpt2* over *avrRpm1* in the *P. syringae/Arabidopsis* interaction (49).

Several features of the region controlling virulence characterized on pAV511 indicate that we have identified a PAI as defined by Hacker *et al.* (14). The region contains several *vir* genes; it is associated with transposases and has G+C content significantly lower than the genome overall (46). The presence of transposases with similarity to IS*100* from the virulence plasmid of *Yersinia pestis* (48, 50) and Tn*501* from *P. aeruginosa* (51) is particularly significant. The region containing IS*100* homologs at its left and right borders, enclosing both the Tn*501* transposase and the putative *vir* locus, ORF4, has the potential to act as a transposon in *Pph*. The association of *avr* genes with mobile elements of DNA either in the form of IS or Tn elements, or by location on plasmids, recently was highlighted by Kim *et al.* (52). They point out that three of four *avr* genes from *P. syringae* previously shown to have some Vir function, *avrA*, *avrB*, *avrE*, and *avrRpm1*, are positioned close to sequences related to transposable elements, in the latter case including Tn*501*. In the 154-kb plasmid of *Pph* (pAV511) the cluster of *avr* and *vir* genes would appear to have the potential to be highly mobile.

The experiments with soybean indicate a clear link between *avr* and *vir* gene function. We have identified *virPphA* as a gene that acts as a virulence determinant in one plant (bean) but an activator of resistance (HR) in another species (soybean). Clearly the nomenclature of genes with such dual functions is rather confusing! At present we propose to name genes on the basis of their first characterized function, therefore *vir* is applied to *virPphA*, which does not appear to act as an *avr* gene in bean. Several *avr* genes have activities that cross plant species barriers; for example, *avrPphB* and *avrPpiA* match *R* genes in bean, pea, soybean, and *Arabidopsis*, but in each plant they control the HR and not susceptibility (3, 8, 19). There are similarities between the properties of *virPphA* and the disease-specific (*dsp*) locus in *Erwinia amylovora* that comprises genes designated *dspE* and *dspF* (53) or *dspA* and *dspB* (54). The *dsp* genes are absolutely required for pathogenicity but not for

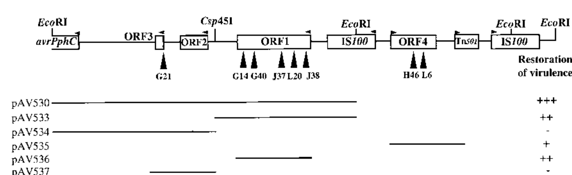

FIG. 5. Location of ORFs for ORF1 (designated *virPphA*), ORF2, ORF3, and ORF4 within the 11 kb of DNA sequenced. The directions of transcription are indicated by horizontal arrowheads. Sites of Tn*3 gus* insertions abolishing restoration of virulence by pAV518 are indicated by vertical arrows. Sequences found to have similarity to IS*100* and Tn*501* are indicated. Clone pAV530 containing the 6.7-kb *Eco*RI fragment restored virulence almost as effectively (+++) as the genomic clone pAV518 (scored as ++++); pAV533 and pAV536 were slightly less effective. Clone pAV535, containing ORF4, consistently delayed the onset of the HR caused by RW60, a reaction scored as +.

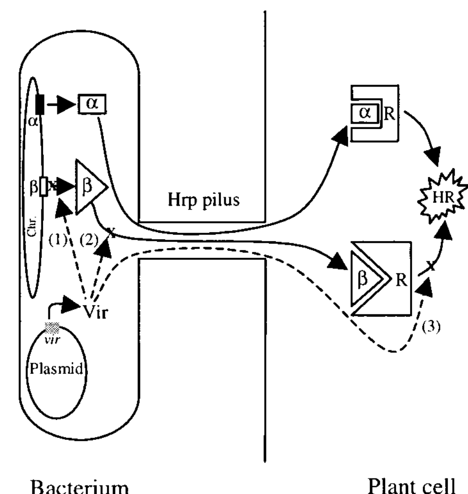

FIG. 6. Possible mechanisms by which virulence (Vir) factors block the HR in bean caused by *avr* gene activity, which is masked in wild-type *Pph*. The masked *avr* genes are designated β. Routes to virulence are via 1) suppression of β *avr* gene expression; 2) blocking delivery of the encoded β *avr* protein, or 3) interference with generation of the HR after recognition. By contrast, α *avr* genes, such as *avrPphE*, are not affected.

elicitation of the HR. Mutation in the *dsp* locus causes *E. amylovora* to behave as a *hrp* mutant in pear and reduce the severity of tissue collapse during the HR in tobacco. The *dspEF* locus is homologous to the avirulence locus *avrE* in *P. syringae* pv. tomato, which determines host range, its presence leading to a strong HR in certain soybean cultivars (11).

An intriguing aspect of the complementation of plasmid-cured strains is the quantitative nature of virulence restoration achieved. Thus, genomic clones such as pAV518 apparently containing several *vir* genes were more effective than subclones or *virPphA* alone. It is envisaged that there is redundancy and multifactorial control of virulence and that Vir factors may interact with different targets, each of which contributes to the establishment of the resistance response. Such a quantitative interaction indicates the presence of targets within the plant that may have additive effects on the activation of the HR leading to resistance. Previous analysis of HR induction in response to Avr proteins has indicated a single recognition interaction, such as AvrPto binding to the resistance gene product Pto, and a subsequent signaling cascade leading to the HR. Variation in response has been attributed to affinity of binding between the Avr protein and its target (22), or regulation via modification of the signal transduction pathway and the so-called recognition rheostat (55, 56).

Our results reinforce the emerging similarities between plant and animal pathogens both in terms of the presence of virulence factors and their location on pathogenicity islands. In addition to the common type III secretion system there may be functional similarities in the use of virulence factors to subvert defense responses. For example, the *Shigella* invasion plasmid antigen B (IpaB) recently has been found to control apoptosis in macrophages (57). The HR is thought to be a form of programmed cell death (PCD) in plants with some similarities to apoptosis, including the involvement of caspases in its execution (58, 59). *Shigella*-induced apoptosis depends on the binding of IpaB to caspase-1 (57). Similar targets in PCD pathways in plants may allow subversion of the HR and lead to disease development as a result of the activity of Vir factors such as those encoded by *virPphA*.

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1. Taylor, J. D., Teverson, D. M., Allen, D. J. & Pastor-Corrales, M. A. (1996) *Plant Pathol.* **45,** 469–478.
2. Mansfield, J., Jenner, C., Hockenhull, R., Bennett, M. A. & Stewart, R. (1994) *Mol. Plant-Microbe Interact.* **7,** 726–739.
3. Jenner, C., Hitchin, E., Mansfield, J., Walters, K., Betteridge, P., Teverson, D. & Taylor, J. (1991) *Mol. Plant-Microbe Interact.* **4,** 553–562.
4. Mansfield, J., Tsiamis, G., Puri, N., Bennett, M., Jenner, C., Stevens, C., Teverson, D., Lyons, N. & Taylor, J. (1997) in *Pseudomonas syringae Pathovars and Related Pathogens*, eds. Rudolph, K., Burr, T. J., Mansfield, J. W., Stead, D., Vivian, A. & von Kietzell, J. (Kluwer, Dordrecht, The Netherlands), pp. 385–391.
5. Vivian, A. & Mansfield, J. W. (1994) *Mol. Plant-Microbe Interact.* **6,** 9–10.
6. Kobayashi, D., Tamaki, S. J. & Keen, N. T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 157–161.
7. Yucel, I., Slaymaker, D., Boyd, C., Murillo, J., Buzzell, R. I. & Keen, N. T. (1994) *Mol. Plant-Microbe Interact.* **7,** 677–679.
8. Wood, J. R., Vivian, A., Jenner, C., Mansfield, J. W. & Taylor, J. D. (1994) *Mol. Plant-Microbe Interact.* **7,** 534–537.
9. Kearney, B. & Staskawicz, B. J. (1990) *Nature (London)* **346,** 385–386.
10. Swords, K. M. M., Dahlbeck, D., Kearney, B., Roy, M. & Staskawicz, B. J. (1996) *J. Bacteriol.* **178,** 4661–4669.
11. Lorang, J. M., Shen, H., Kobayashi, D., Cooksey, D. & Keen, N. T. (1994) *Mol. Plant-Microbe Interact.* **7,** 508–515.
12. Heath, M. C. (1981) *Phytopathology* **71,** 1121–1123.
13. Groisman, E. A. & Ochman, H. (1996) *Cell* **87,** 791–794.
14. Hacker, J., Blum-Oehler, G., Mühldorfer, I. & Tschäpe, H. (1997) *Mol. Microbiol.* **23,** 1089–1097.
15. Hu, P., Elliott, J., McCready, P., Skowronski, E., Garnes, J., Kobayashi, A., Brubaker, R. R. & Garcia, E. (1998) *J. Bacteriol.* **180,** 5192–5202.
16. Cornelis, G. R. & Wolf-Watz, H. (1997) *Mol. Microbiol.* **23,** 861–867.
17. Ochman, H., Soncini, F. C., Solomon, F. & Groisman, E. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7800–7804.
18. Shea, J. E., Hensel, M., Gleeson, C. & Holden, D. W. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2593–2597.
19. Alfano, J. R. & Collmer, A. (1997) *J. Bacteriol.* **179,** 5655–5662.
20. Bonas, U. (1994) in *Bacterial Pathogenesis of Plants and Animals: Molecular and Cellular Mechanisms*, ed. Dangl, J. L. (Springer, Berlin), pp. 79–98.
21. Van Gijsegem, F., Genin, S. & Boucher, C. (1993) *Trends Microbiol.* **1,** 175–180.
22. Stevens, C., Bennett, M. A., Athanassopoulos, E., Tsiamis, G., Taylor, J. D. & Mansfield, J. W. (1998) *Mol. Microbiol.* **29,** 165–177.
23. Menard, R., Prevost, M. C., Gounon, P., Sansonetti, P. & Dehio, C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1254–1258.
24. Conazo, C. M. & Galan, J. E. (1997) *Mol. Microbiol.* **24,** 747–756.
25. Knutton, S., Rosenshine, I., Pallan. M. J., Nisan, I., Neves, B. C., Bain, C., Wolff, C., Dougan, G. & Frankel, G. (1998) *EMBO J.* **17,** 2166–2176.
26. Van Gijsegem, F. (1997) *Eur. J. Plant Pathol.* **103,** 291–301.
27. Mitchell, R. E. (1976) *Nature (London)* **260,** 75–76.
28. Zhang, Y. X. & Patil, S. S. (1997) *Mol. Plant-Microbe Interact.* **10,** 947–960.
29. Jackson, R. W. (1997) Ph.D. thesis (University of the West of England, Bristol, United Kingdom).
30. Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop II, R. M. & Peterson K. M. (1995) *Gene* **166,** 175–176.
31. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7,** 1513–1523.
32. Moulton, P. J., Vivian, A., Hunter, P. J. & Taylor, J. D. (1993) *J. Gen. Microbiol.* **139,** 3149–3155.
33. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
34. Arnold, D. L., Athey-Pollard, A., Gibbon, M. J., Taylor, J. D. & Vivian, A. (1996) *Physiol. Mol. Plant Pathol.* **49,** 233–245.
35. Vivian, A., Atherton, G. T., Bevan, J. R., Crute, I. R., Mur, L. A. J. & Taylor, J. D. (1989) *Physiol. Mol. Plant Pathol.* **34,** 335–344.
36. Keen, N. T., Shen, H. & Cooksey, D. A. (1992) in *Molecular Plant Pathology: A Practical Approach*, eds. Gurr, S. J., McPherson, M. J. & Bowles, D. J. (IRL, Oxford), pp. 45–50.
37. Bonas, U., Stall, R. E. & Staskawicz, B. (1989) *Mol. Gen. Genet.* **218,** 127–136.
38. Gibbon, M. J., Sesma, A., Canal, A., Wood, J. R., Hidalgo, E., Brown, J., Vivian, A. & Murillo, J. (1999) *Microbiology* **145,** 325–334.
39. Malik, A. N. (1985) Ph.D. thesis (Thames Polytechnic, London).
40. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
41. Harper, S., Zewdie, N., Brown, I. R. & Mansfield, J. W. (1987) *Physiol. Mol. Plant Pathol.* **31,** 153–172.
42. Hitchin, F. E., Jenner, C. E., Harper, S., Mansfield, J. W., Barber, C. E. & Daniels, M. J. (1989) *Physiol. Mol. Plant Pathol.* **34,** 309–322.
43. Malik, A. N., Vivian, A. & Taylor, J. D. (1987) *J. Gen. Microbiol.* **133,** 2393–2399.
44. Threlfall, E. J., Rowe, B., Ferguson, J. L. & Ward, L. R. (1986) *J. Hyg.* **97,** 419–426.
45. Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R. & Staskawicz, B. J. (1993) *J. Bacteriol.* **175,** 4859–4869.
46. DeLey, J. (1968) *Annu. Rev. Phytopathol.* **6,** 63–90.
47. Lindler, L. E., Plano, G. V., Burland, V., Mayhew, G. F. & Blattner, F. R. (1998) *Infect. Immun.* **66,** 5731–5742.
48. Brown, N. L., Winnie, J. N., Fritzinger, D. & Pridmore, R. D. (1985) *Nucleic Acids Res.* **13,** 5657–5669.
49. Ritter, C. & Dangl, J. L. (1996) *Plant Cell* **8,** 251–257.
50. McDonough, K. A. & Hare, J. M. (1997) *J. Bacteriol.* **179,** 2081–2085.
51. Podladchikova, O. N., Dikhanov, G. G., Rakin, A. V. & Heesemann, J. (1994) *FEMS Microbiol. Lett.* **121,** 269–274.
52. Kim, J. F., Charkowski, A. O., Alfano, J. R., Collmer, A. & Beer, S. V. (1998) *Mol. Plant-Microbe Interact.* **11,** 1247–1252.
53. Bogdanove, A. J., Kim, J. F., Wei, Z., Kolchinsky, P., Charkowski, A. O., Conlin, A. K., Collmer, A. & Beer, S. V. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 1325–1330.
54. Gaudriault, S., Malandrin, L., Paulin, J.-P. & Barny, M.-A. (1997) *Mol. Microbiol.* **26,** 1057–1069.
55. Mansfield, J., Bennett, M., Bestwick, C. & Woods-Tör, A. (1997) in *The Gene-for-Gene Relationship in Host-Parasite Interactions*, eds. Crute, I. R., Burden, J. J. & Holub, E. B. (CAB International, London), pp. 265–292.
56. Jones, A. M. & Dangl, J. L. (1996) *Trends Plant Sci.* **1,** 114–119.
57. Hilbi H., Moss, J. E., Hersh, D., Chen, Y., Arondel, J., Banerjee, S., Flavell, R. A., Yuan, J., Sansonetti, P. J. & Zychlinsky, A. (1998) *J. Biol. Chem.* **273,** 32895–32900.
58. Richberg, H. H., Aviv, D. M. & Dangl, J. L. (1998) *Curr. Opin. Plant Biol.* **1,** 480–485.
59. du Pozo, O. & Lam, E. (1998) *Curr. Biol.* **8,** 1133–1136.