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Highly conserved sequences flank avirulence genes: isolation of novel avirulence genes from *Pseudomonas syringae* pv. *pisi*

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DNA sequences flanking two *avr* genes (*avrPpiA1* and *avrPpiB1*) from *Pseudomonas syringae* pv. *pisi* show a high degree of similarity. Specific primers designed from the conserved regions were used in PCR amplifications with all *P. syringae* pv. *pisi* races. As well as amplifying the expected *avrPpiA*- and *avrPpiB*-containing fragments, two additional fragments were amplified: one contained a single open reading frame (ORF1) and was found in races of genomic group II (2, 3A, 4A and 6); the second fragment contained two open reading frames (ORF2 and ORF3), separated by 658 nt, and was detected in all races. All three ORFs had G+C ratios (46·9–48 mol%) that were significantly less than that for *P. syringae* and each was preceded by a potential *hrp* box promoter. In *P. syringae* pv. *phaseolicola*, ORF1 and ORF2 each elicited a strong non-host hypersensitive reaction on bean leaves; ORF1 was designated *avrPpiG*, the product of which had strong similarity to AvrRxv, AvrBsT and YopP. ORF2 was identical to a gene, designated *avrPpiC*, previously isolated from *P. syringae* pv. *pisi* race 5. ORF3 was always found in association with *avrPpiC* and both were detected in a wide range of *P. syringae* pathovars. In contrast, *avrPpiG* was only detected in strains of *P. syringae* pv. *pisi* genomic group II and *P. syringae* pv. *coronafaciens* (ICMP 3113). In *P. syringae* pv. *pisi*, *avrPpiG* was plasmid-borne and *avrPpiC* and ORF3 were chromosomal. This conservation of flanking sequences has implications for the horizontal transfer of avirulence and virulence genes, suggesting that specific regions of the bacterial genome act as sites for their integration/excision.

Keywords: *Pseudomonas syringae*, conserved DNA sequences, *avr*, plasmid, *rulAB*

INTRODUCTION

The specificity between bacterial phytopathogens of the genera *Pseudomonas* and *Xanthomonas* has in general been shown to involve gene-for-gene matching of a pathogen avirulence (*avr*) gene with a plant resistance (*R*) gene, resulting in a localized defence reaction, termed the hypersensitive reaction (HR). The HR is a programmed cell death event that leads to inhibition of pathogen movement and nutrient acquisition, effectively terminating the infection (Keen, 1990; Mudgett & Staskawicz, 1998). Absence of matching *avr* and *R* genes results in compatibility between the pathogen and its plant host and disease is the outcome. Over 50 *avr* genes have now been isolated from a variety of different bacterial plant pathogens. Often these genes lack homology to other genes of known function held in databases (Vivian & Gibbon, 1997; Vivian & Arnold, 2000). However, a common feature of *Pseudomonas syringae* *avr* genes is that their G+C ratios are predominantly in the range of 40–52·5 mol%, well below the normal range of 59–61 mol% for the species (DeLey, 1968; Vivian & Gibbon, 1997).

Avirulence genes appear to operate both in determining cultivar specificity among races of a single pathovar in *P. syringae* and *Xanthomonas campestris* and also at the pathovar/host plant species level in non-host recognition (Kobayashi *et al.*, 1989, 1990; Fillingham *et al.*,

Abbreviations: HR, hypersensitive reaction; PAI, pathogenicity island.

The GenBank accession numbers for the sequences determined in this work are AJ277495 and AJ277496.

1992; Innes *et al*., 1993; Wood *et al*., 1994; Yucel *et al*., 1994; Simonich & Innes, 1995). The recent detection of a gene, *virPphA*, in *P. syringae* pv. *phaseolicola*, shown to be essential for full virulence towards the host plant bean, has provided some insight into the functional significance of *avr* genes. The same gene functions as an *avr* gene toward soybean, inducing a rapid cultivar-specific HR (Jackson *et al*., 1999). Recently, Tsiamis *et al*. (2000) showed that the gene *avrPphF* has multiple effector functions toward different plant hosts, supporting the notion that *avr* genes are probably virulence (*vir*) genes that are recognized by certain plants which do not act as hosts. There is also evidence that pathogenicity is redundantly encoded by *vir* genes (Yang *et al*., 1996; Jackson *et al*., 1999) and therefore the detection of potential *vir* genes is most readily accomplished through screening pathogen gene libraries for *avr* genes.

The genomic context of *avr* genes has attracted increasing attention since the earliest indications that genes *avrB* and *avrC* from *P. syringae* pv. *glycinea* were flanked by repeat DNA sequences (Staskawicz *et al*., 1987). A number of *vir*/*avr* genes have been associated with potentially mobile elements, such as insertion sequences, transposons and bacteriophage DNA, which might play a role in their horizontal transfer (Kim *et al*., 1998). It is also clear that in some cases the distribution of *avr* genes is not random and that many are organized on pathogenicity islands (PAIs), which include DNA sequences indicative of gene mobility such as transposases, flanking direct repeats, or insertion sequences (Jackson *et al*., 1999; Alfano *et al*., 2000; Kjemtrup *et al*., 2000).

In *P. syringae* pv. *pisi*, an 8·5 kb DNA fragment containing the avirulence gene *avrPpiA1* is present in the chromosome of race 2, but is not present in race 4B. Sequence analysis of the 8·5 kb fragment revealed the presence of *rulAB* genes, which encode tolerance to UV radiation and are widely distributed on plasmids of the pPT23A family in *P. syringae* pathovars (Sesma *et al*., 1998; Sundin & Murillo, 1999). They are homologues of the *umuDC* mutagenic DNA repair system identified in *Escherichia coli* (Smith & Walker, 1998). Kim & Sundin (2000) have recently demonstrated the likely significance of UV-B (290–320 nm) for the function of *rulAB* in the survival of *P. syringae* in the phyllosphere habitat. In *P. syringae* pv. *pisi* race 2, the *rulB* gene has been disrupted by a 4·5 kb length of DNA, which includes *avrPpiA1* and ORFs with similarity to bacteriophage and transposase genes (Arnold *et al*., 2000).

In this study we investigated the sequences flanking two *avr* genes, previously isolated from *P. syringae* pv. *pisi*: *avrPpiA1* from race 2 strain 203 matches the resistance gene *R2* in certain pea cultivars (Vivian *et al*., 1989; Dangl *et al*., 1992; Bevan *et al*., 1995) and *avrPpiB1* from race 3A strain 870A matches the gene *R3* (Cournoyer *et al*., 1995). Homologues of *avrPpiA1* are present in races 5 and 7, whilst homologues of *avrPpiB1* are present in races 1 and 7 (Cournoyer *et al*., 1995; Gibbon *et al*., 1997). The races of *P. syringae* pv. *pisi* can be divided into two genomic groups based on PCR amplification with two sets of specific oligonucleotide primers (Arnold *et al*., 1996). Although *avrPpiA* and *avrPpiB* occur in races from both genomic groups, we show here that there is a high level of DNA sequence conservation in the regions flanking these *avr* genes and that these sequences are repeated elsewhere in the genome flanking previously undetected *avr* genes. The presence of conserved sequences therefore provides a route to the potential detection of novel *avr*/*vir* genes.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are shown in Tables 1, 6 and 7. *P. syringae* pathovars were grown at 25 °C on King's B agar (KB) (King *et al*., 1954) for 2 d or in Luria–Bertani (LB) broth (Miller, 1972) for 16 h. *E. coli* strains were grown overnight on LB agar or LB broth at 37 °C. Antibiotics (Sigma) were used at the following concentrations (μg ml^{-1}): tetracycline, 10; ampicillin, 125; kanamycin, 25; chloramphenicol, 150; nitrofurantoin, 100.

Bacteria were mated using Eppendorf-tube matings as described by Vivian *et al*. (1989), using the helper plasmid pRK2013 (Figurski & Helinski, 1979) with selection for pseudomonads on nitrofurantoin. For marker-exchange mutagenesis, clones were mutagenized with transposon Tn*3gus* (Bonas *et al*., 1989).

DNA manipulations. DNA manipulations were performed as described by Sambrook *et al*. (1989). Total bacterial DNA was extracted from an overnight broth using a Puregene DNA isolation kit (Flowgen). Plasmid DNA was extracted using the alkaline lysis method or using a Qiagen midi-prep kit. Restriction enzymes, obtained from Gibco Life Technologies, were used according to the manufacturer's instructions. DNA fragments were ligated using T4 DNA ligase (Appligene) and transformed into *E. coli* strain DH5α. PCR products were cloned using the Original TA Cloning kit (Invitrogen). DNA sequencing was carried out by MWG-Biotech UK Ltd (Milton Keynes). The sequences obtained were analysed using programs at http://www.ncbi.nlm.nih.gov/and using the University of Wisconsin GCG package, accessed through the MRC Human Genome Mapping Project, Hinxton, UK.

PCR. The PCR reactions were as described by Arnold *et al*. (1996) using 30 cycles with an annealing temperature of 58 °C. PCR products (20 μl) were separated on 1·5% agarose gels (100 V); bands of interest were excised from the gel and the DNA purified using QIAEX II (Qiagen).

Hybridization. DNA digests and PCR products for hybridization analysis were transferred from agarose gels to nylon membranes by vacuum blotting with a commercial apparatus (Appligene). For dot-blot analysis of DNAs, each bacterial strain to be tested was treated as described by Arnold *et al*. (1996). The DNA samples were dot-blotted on a nylon membrane [Biotrans (+) nylon membrane (ICN)] using a commercial apparatus (Bio-Rad). Plasmid inserts and PCR bands, extracted from agarose gels, were labelled with deoxy[α-^{32}P]cytidine 5′-triphosphate using the random primer oligo-labelling method (Feinberg & Vogelstein, 1983) with a High Prime kit (Roche Diagnostics). Blots were hybridized (65 °C, 16 h) using the labelled probes in the hybridization solution (Sambrook *et al*., 1989). Membranes were washed to high stringency as described by Arnold *et al*. (1996).

Production of ORF-specific probes. Specific DNA fragments were produced for all three ORFs as follows: pAV624 (ORF1) was digested with *Eco*RI and *Sma*I to give an 800 bp fragment.

Table 1. Bacterial strains, vectors and plasmids

Strain, vector or plasmid	Relevant properties	Source or reference
Strains*		
P. syringae pv. *phaseolicola*		
1448A	Race 6, wild-type isolate	Fillingham *et al.* (1992)
1449B	Race 7, wild-type isolate	Taylor *et al.* (1996)
RW60	1449B cured of 154 kb plasmid pAV511	Jackson *et al.* (1999)
P. syringae pv. *syringae*		
1212R	1212, Rif^r	Gilmartin (1997)
P. syringae pv. *pisi*		
895A	Race 4A, wild-type isolate	Taylor *et al.* (1989); Arnold *et al.* (1996)
PT10	Race 4B, Rif^r	Moulton *et al.* (1993)
PT10.1	PT10 ORF3::Tn*3*HoKmGus	This study
PG8	974B *avrPpiC*::Tn*3*HoKmGus	This study
P. syringae pv. *tomato*		
DC3000	Spontaneous Rif^r derivative of NCPPB1106	Cuppels (1986)
E. coli		
DH5α	*recA lacZ*ΔM15	Hanahan (1983)
INVαF′	As DH5α except *lacZ*ΔM15 Δ(*lacZYA-argF*) *deoR*$^+$ F′	Invitrogen
Vectors		
pLAFR3	Tc^r Tra$^-$ Mob$^+$ *cos*, RK2 replicon	Staskawicz *et al.* (1987)
pCR2.1	Ap^r, *lacZ*	Invitrogen
pBBR1MCS	Cm^r	Kovach *et al.* (1994)
pBBR1MCS-2	Km^r	Kovach *et al.* (1995)
pBBR1MCS-3	Tc^r	Kovach *et al.* (1995)
Plasmids		
pRK2013	Km^r Tra$^+$, ColE1 replicon	Figurski & Helinski (1979)
pAV624	1605 bp PCR fragment (primers DA72 and DA73) from 895A in pCR2.1	This study
pAV625	2493 bp PCR fragment (primers DA72 and DA73) from 299A in pCR2.1	This study
pAV626	Insert of pAV624 released with *Kpn*I and *Xba*I in pBBR1MCS, ORF1 = *avrPpiG1*	This study
pAV627	Insert of pAV624 released with *Kpn*I and *Xba*I in pBBR1MCS-3, ORF1 = *avrPpiG1*	This study
pAV628	1000 bp PCR fragment (primers DA88 and 89) from pAV625 in pCR2.1, containing ORF2 = *avrPpiC2*	This study
pAV629	1191 bp PCR fragment (primers DA92 and 93) from pAV625 in pCR2.1, containing ORF3	This study
pAV630	Insert of pAV628 released with *Kpn*I and *Xho*I in pBBR1MCS-3, ORF2 = *avrPpiC2*	This study
pAV631	Insert of pAV629 released with *Kpn*I and *Xba*I in pBBR1MCS-3, ORF3	This study
pAV632	Insert of pAV625 released with *Eco*RI in pBBR1MCS-2, ORF2 = *avrPpiC2* and ORF3	This study

* Wild-type strains are numbered in the HRI collection series.

PCR was performed using primers DA90/91 and DA94/95 (Table 2) on pAV625 to amplify internal fragments of ORF2 (285 bp) and ORF3 (268 bp), respectively. All probes were excised from gels and purified using QIAEX II (Qiagen).

Subcloning of ORFs into broad-host-range vectors. The insert from pAV624 (ORF1) was cloned into the broad-host-range vectors pBBR1MCS and pBBR1MCS-3 (Kovach *et al.*, 1994, 1995) to produce pAV626 and pAV627, respectively. ORF2 plus flanking sequence was amplified by PCR from pAV625 with primers DA88 and DA89 (Table 2). The resulting 1000 bp fragment was cloned into pCR2.1 (pAV628), and then subcloned as a *Kpn*I/*Xho*I fragment into pBBR1MCS-3 to produce pAV630. ORF3 plus flanking sequence was amplified by PCR from pAV625 with primers DA92 and DA93 (Table 2) and the resulting 1191 bp fragment cloned into pCR2.1 (pAV629), which was subcloned as a *Kpn*I/*Xba*I fragment

Table 2. Oligonucleotide primers

Name	Sequence (5′–3′)	Sequence coordinate at 5′ end of primer	GenBank accession no.
DA72	TCGCCATTTATGTGGAATGG	2452	AJ251482
DA73	GCCCAGTCAAATCGAGGC	3641	AJ251482
DA88	TCTATAGCATGATACGCGGC	89	AJ277496
DA89	AGTGTCGTGGATCCACAAG	1089	AJ277496
DA90	TTACGCCCTATGCATCGGC	507	AJ277496
DA91	TCTGTGCTCACCTGCCGC	792	AJ277496
DA92	GCATGTCATTTGTCCAACGG	1320	AJ277496
DA93	GCCCAGTCAAATCGAGGCG	2492	AJ277496
DA94	ACGAGAGCCGGCACATTGC	1971	AJ277496
DA95	TCCTCTTTGCCAGATCCCG	2239	AJ277496

into pBBR1MCS-3 to produce pAV631. This strategy enabled each cloned ORF to be transferred by triparental mating into *P. syringae* pathovars for pathogenicity tests *in planta*: these were as described by Moulton *et al.* (1993) for pea seedlings, by Harper *et al.* (1987) for bean leaves, by Jackson *et al.* (1999) for soybean, and by Dangl *et al.* (1992) for *Arabidopsis thaliana*.

RESULTS

DNA sequences flanking *avrPpiA1* and *avrPpiB1*

The DNA sequences flanking the *P. syringae* pv. *pisi* avirulence genes *avrPpiA1* (Dangl *et al.*, 1992; Arnold *et al.*, 2000) and *avrPpiB1* (Cournoyer *et al.*, 1995) were compared using the GCG program BESTFIT. Regions of similarity both upstream and downstream of the two avirulence genes were detected (Fig. 1a). The first 190 bp upstream of the deposited *avrPpiB1* sequence had 66 % identity (with one gap) to an area upstream of *avrPpiA1* and the last 274 bp downstream of the deposited *avrPpiB1* sequence had 96 % identity with one gap to an area downstream of *avrPpiA1*.

In view of the conserved nature of the regions flanking the two different *avr* genes, we undertook further DNA analysis beyond the limit of the deposited *avrPpiB1* sequences. This involved single-stranded analysis of a total of 5656 nt, which was compared to the deposited

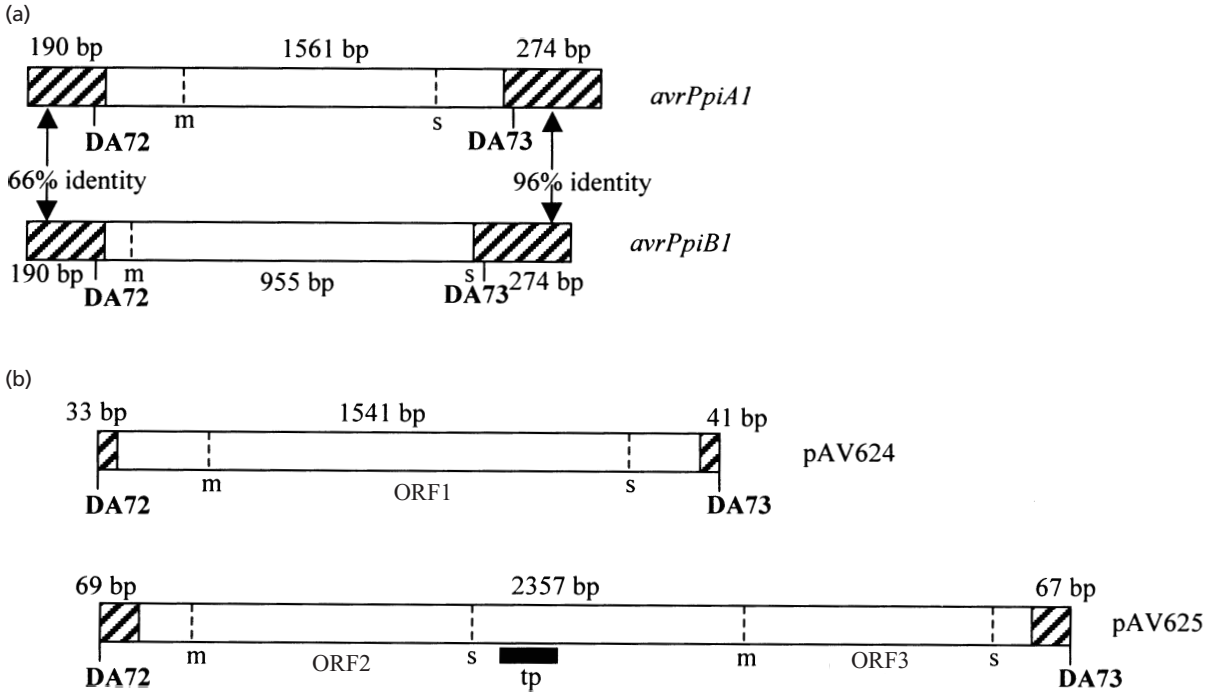

Fig. 1. Diagrammatic representation of DNA regions from *P. syringae* pv. *pisi* flanked by conserved sequences. (a) The avirulence genes *avrPpiA1* and *avrPpiB1* (GenBank accession numbers AJ251482 and X84843, respectively). (b) The inserts of clones pAV624 and pAV625, containing ORFs 1, 2 and 3, after PCR amplification. Hatched areas, area of similarity between two DNA sequences; m, methionine start codon; s, stop codon; DA72 and DA73, PCR primers; tp, homology to part of a putative transposase subunit from *P. alcaligenes.*

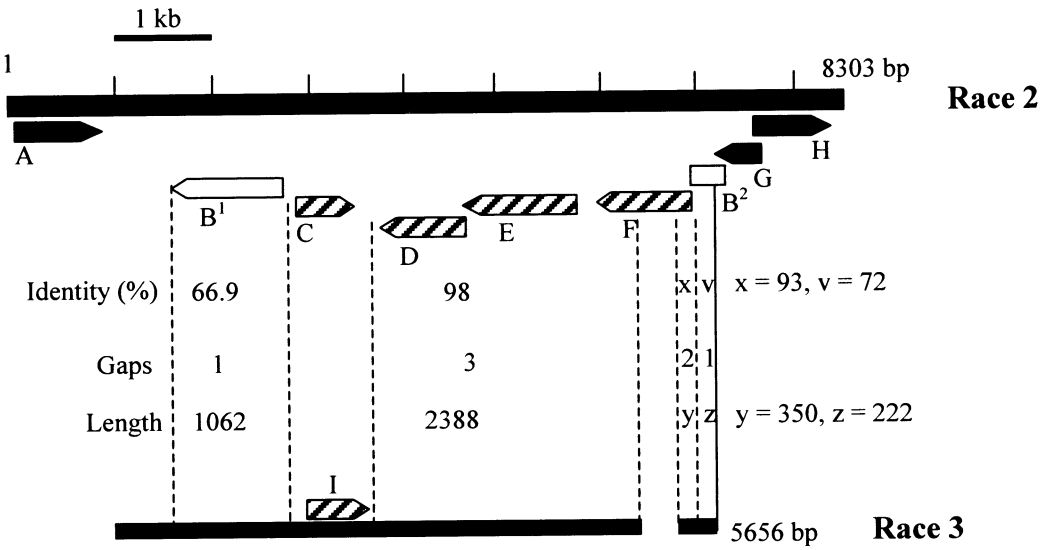

Fig. 2. Comparison of the DNA sequences flanking avirulence genes *avrPpiA1* (ORF shown as C) from *P. syringae* pv. *pisi* race 2 and *avrPpiB1* (ORF shown as I) from *P. syringae* pv. *pisi* race 3. Predicted ORFs are shown for the sequence from race 2 (AJ251482): A, *rci*; B^1 and B^2, *rulB*; D, transposase homologue; E, gene 18 from bacteriophage SPP1; F, *SSS*; G, *rulA*; H, hypothetical protein from *amyA-fliE* intergenic region (for details see Arnold *et al.*, 2000). The percentage identity of the DNA sequences determined by BESTFIT are shown.

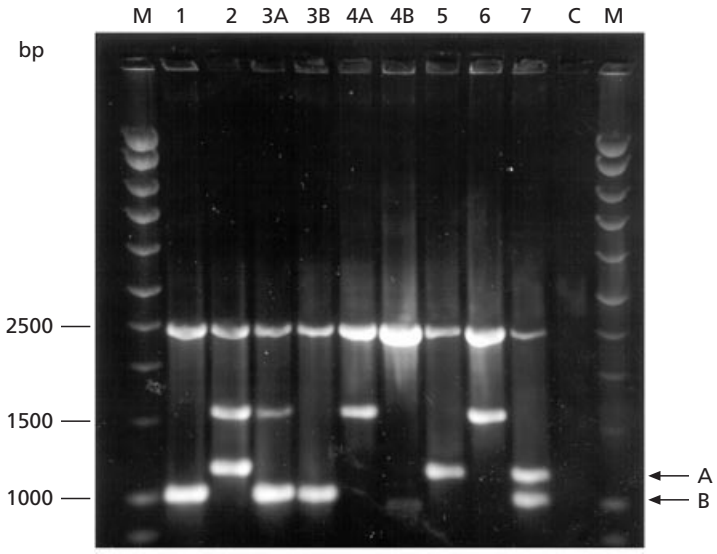

Fig. 3. PCR amplification products obtained from genomic DNA of races of *P. syringae* pv. *pisi* with primers DA72 and DA73. Lanes: M, Hyperladder I (Bioline); 1, 299A (race 1); 2, 203 (race 2); 3A, 870A (race 3A); 3B, 4585 (race 3B); 4A, 895A (race 4A); 4B, PN8 (race 4B); 5, 974B (race 5); 6, 1704B (race 6); 7, 1691 (race 7); C, no DNA. Strain numbers are those of HRI Culture Collection, Wellesbourne except for PN8. A = *avrPpiA1*; B = *avrPpiB1*.

sequence of 8·5 kb surrounding *avrPpiA1* (GenBank accession no. AJ251482) (Arnold *et al.*, 2000). Analysis revealed that the region is highly conserved, except for a 532 nt deletion in the race 3 sequence and the two *avr* genes (Fig. 2). The sequence showed that, upstream of the *avr* genes, the homology extended to the end of the *rulB* gene and downstream the homology extended to the start of *rulB*, which was also the end of the cloned insert DNA.

PCR with flanking sequence-specific primers

PCR primers, DA72 and DA73 (Table 2), were designed from highly conserved areas upstream and downstream of the *avrPpiA1* and *avrPpiB1* genes. The upstream primer (DA72) was in an inverted repeat that is immediately inside a disrupted *rulB* gene and the downstream primer (DA73) was in a transposase-like ORF. The primers were used to amplify fragments from each of the races of *P. syringae* pv. *pisi* (Fig. 3). A band of 1190 bp, corresponding to *avrPpiA1*, was amplified from races 2, 5 and 7, and a band of 1047 bp, corresponding to *avrPpiB1*, was amplified from races 1, 3A, 3B and 7. The presence of these bands was in agreement with the reported racial distribution of the *avr* genes in *P. syringae* pv. *pisi* (Cournoyer *et al.*, 1995; Gibbon *et al.*, 1997). In addition, a band of ~ 1500 bp was amplified in races 2, 3A, 4A and 6, and a band of

Table 3. Features of genes identified using conserved primers DA72 and DA73, compared to *avrPpiA1* and *avrPpiB1*

Gene	Putative promoter region*	Position relative to ORF†	Shine–Dalgarno sequence and start codon‡	G+C (mol%)§	ORF length (aa)	Predicted protein size (kDa)
avrPpiA1	**GGAAC**T-15N-**CCAC**	−37	**AAAGGG**TTTTT<u>ATG</u>	43·8	220	24·4
avrPpiB1	**GGAACC**-16N-**CCAC**	−40	**GGAGG**CATAC<u>ATG</u>	40	276	31·3
avrPpiG1‖	**GGAACC**-16N-**CCAC**	−95	**GGAGAA**CATCACG<u>ATG</u>	48	362	40·7
avrPpiC2¶	**GGAAC**T-15N-**CCAC**	−36	**AGGG**CGCTGAAAAT<u>ATG</u>	46·9	269	29·0
ORF3	**GGAACC**-16N-**CCAC**	−187	**AGGA**TAACT<u>ATG</u>	47·5	218	24·3

* Nucleotides in bold indicate those that are conserved according to the '*hrp* box' consensus sequence (Innes *et al*., 1993).

† Position of the 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* pathovars is reported to be 59–61 mol% G+C (DeLey, 1968).

‖ ORF1.

¶ ORF2.

Table 4. Protein similarities to AvrPpiG1 (ORF1 gene product) from *P. syringae* pv. *pisi* race 4A

Homologies were identified using the BLASTX algorithm.

Protein	Length	Length of homology (aa)	Identity/ positives (%)	Number of gaps	GenBank accession no.	Reference
AvrBsT	350	320	44/58	10	AF156163	Ciesiolka *et al*. (1999)
AvrRxv	373	324	38/56	15	L20423	Whalen *et al*. (1993)
ORF5	411	326	26/40	15	AF232005	Alfano *et al*. (2000)
YopP	288	264	23/40	34	AF023202	Mills *et al*. (1997)

∼ 2500 bp in all races. The 1500 bp band from race 4A and the 2500 bp band from race 1 were cloned in pCR2.1 to produce clones pAV624 and pAV625, respectively. The sequences of the cloned inserts (1605 bp and 2493 bp) were deposited at EMBL as accession numbers AJ277495 and AJ277496, respectively.

A BESTFIT analysis comparing the insert in pAV624 and the *avrPpiA1* sequence showed 97% identity with one gap over 33 bp to the region upstream and 90% identity over 41 bp to the region downstream of *avrPpiA1*. A similar analysis for the pAV625 insert revealed 83% identity with one gap over 70 bp to the region upstream and 83% identity over 66 bp to the region downstream of the gene.

Predictive analysis indicated that the insert in pAV624 contained a single ORF (1086 bp) encoding a potential protein of 362 aa (ORF1) and that pAV625 contained two ORFs (807 and 654 bp) encoding potential proteins of 269 and 218 aa (ORF2 and ORF3, respectively) with a gap of 658 bp between them (Fig. 1b). All three ORFs appeared to possess a Shine–Dalgarno ribosome-binding site just upstream of the predicted translation start and an upstream '*hrp* box' consensus sequence (Innes *et al*., 1993; Table 3).

A BLASTX search showed that the product of ORF1 was similar to two avirulence proteins (Table 4), AvrBsT (Ciesiolka *et al*., 1999) and AvrRxv (Whalen *et al*., 1993) and an effector protein, YopP (Mills *et al*., 1997). No significant homology was found to ORF3. However, ORF2 was identical to a gene, *avrPpiC*, previously isolated by function in *P. syringae* pv. *phaseolicola* from a gene library of *P. syringae* pv. *pisi* (Fillingham, 1994; Goss, 1995). A region specifying 54 aa, between the two ORFs, was found to have significant homology (53% identities; 54% positives) to the C-terminal region of a putative transposase subunit from *P. alcaligenes* (Yeo *et al*., 1998).

Investigation of phenotypes on plant hosts

The three ORFs were subcloned in appropriate broad-host-range vectors (Table 1), conjugated into *P. syringae* pv. *phaseolicola* race 6 strain 1448A (compatible on all bean cultivars) and tested on a range of bean cultivars by leaf infiltration. *P. syringae* pv. *phaseolicola* race 7 strain 1449B caused a HR typical for incompatibility towards its normal host in cultivars Red Mexican and A43, whereas *P. syringae* pv. *pisi* race 4A strain 895A (used as a control) caused a rapid non-host HR on all cultivars tested, as did 1448A (ORF1) and 1448A (ORF2) (Table 5, Fig. 4); strain 1448A (ORF3) remained compatible

Table 5. Bean reaction phenotypes associated with carriage of *avrPpiG*, *avrPpiC* and ORF3 on bean cultivars

Bacterial strains and plasmid transconjugants (see Table 1) were inoculated on bean cultivars and observed 4 d post-inoculation. 1448A harbouring any of the vectors pBBR1MCS, pBBR1MCS-2 or pBBR1MCS-3 caused disease symptoms identical to 1448A alone. HR, non-host hypersensitive reaction, observed as a very rapid tissue collapse of inoculated tissue (6–12 h) and white desiccated lesion; hr, host-cultivar-specific hypersensitive reaction, observed as a rapid tissue collapse (12 h) and a white/brown lesion; S, susceptible.

Bacterial strain (plasmid)	Bean cultivar			
	Tendergreen	Red Mexican	A43	Canadian Wonder
Ppi 895A, race 4A	HR	HR	HR	HR
Pph 1449B, race 7	S	hr	hr	S
Pph 1448A, race 6	S	S	S	S
1448A (pAV626; ORF1 = *avrPpiG1*)	HR	HR	HR	HR
1448A (pAV630; ORF2 = *avrPpiC2*)	HR	HR	HR	HR
1448A (pAV631; ORF3)	S	S	S	S
1448A (pAV632; ORF2 = *avrPpiC2* and ORF3)	HR	HR	HR	HR

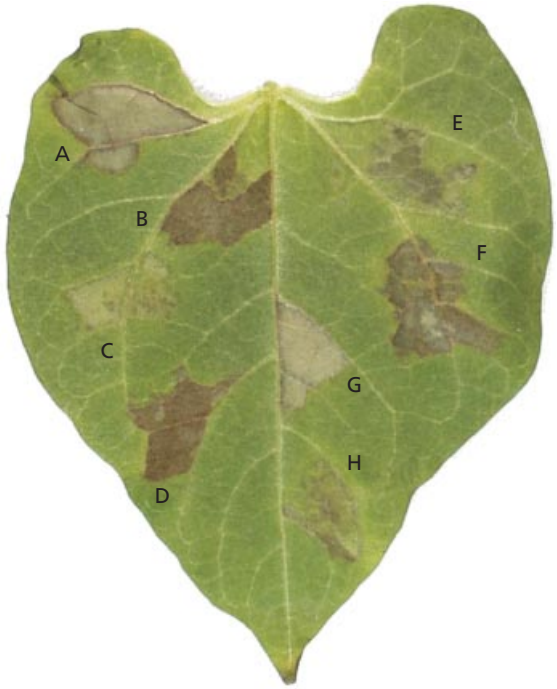

Fig. 4. Differentiation of hypersensitive reaction and susceptible reactions on a leaf of bean cultivar Red Mexican. Suspensions of bacteria (5×10^8 c.f.u. ml^{-1}) were inoculated as described in Methods and the resulting symptoms were recorded 4 d.p.i. A, *P. syringae* pv. *phaseolicola* 1448A (pAV626; ORF1 = *avrPpiG1*), non-host HR; B, 1448A (pAV630; ORF2 = *avrPpiC2*), non-host HR; C, 1448A (pAV631; ORF3), S; D, 1448A (pAV632; ORF2 = *avrPpiC2* and ORF3), non-host HR; E, 1448A, S; F, *P. syringae* pv. *phaseolicola* 1449B, host-specific HR due to *avrPphF/R1* gene interaction; G, *P. syringae* pv. *pisi* 895A, non-host HR; H, 1448A (pBBR1MCS) vector only control, S. HR = hypersensitive reaction, S = susceptible (disease).

with all cultivars. In recognition of its behaviour as an avirulence gene, ORF1 was designated *avrPpiG1* and ORF2 was designated *avrPpiC2* [to distinguish it from the race 5 allele *avrPpiC1* (Fillingham, 1994; Goss, 1995), according to the nomenclature of Vivian & Mansfield (1993)].

The three ORFs were also tested on the pea differential series (Bevan *et al*., 1995) in *P. syringae* pv. *pisi* race 4B strain PT10, *P. syringae* pv. *phaseolicola* strains 1448A and RW60 (a race 7 strain 1449B lacking a plasmid-borne PAI required for pathogenicity to bean) and *P. syringae* pv. *syringae* strain 1212R (compatible on all pea cultivars). None of the ORFs caused any change in the phenotype of these strains towards pea. The ORFs were also tested on soybean cultivars Hutcheson, Osumi, Kochi and Katai in *P. syringae* pv. *phaseolicola* strain RW60, but with no observable effect on the null phenotype. A similar lack of induction of the HR was observed when transconjugants in *P. syringae* pv. *phaseolicola* strain 1448A or *P. syringae* pv. *tomato* strain DC3000 were tested in *Arabidopsis thaliana* ecotypes Col-0, Nd-0 and Ws-3.

Marker-exchange mutagenesis and phenotypes on plant hosts

A derivative (PG8) of the *P. syringae* pv. *pisi* strain 974B a Tn*3gus* insertion in *avrPpiC1*, and a derivative (PT10.1) of the race 4B strain PT10 with a Tn*3gus* insertion in ORF3, were inoculated in the pea differential series (Bevan *et al*., 1995). No difference in symptoms between the original strains and their marker-exchanged mutants was detected. A similar result was observed when the marker-exchanged mutants and parent strains were inoculated in the leaves of four bean cultivars: no difference was detected in the symptoms observed. Repeated attempts to marker-exchange the *avrPpiG* gene in *P. syringae* pv. *pisi* group II races 2, 3A, 4A and 6 were unsuccessful.

Distribution and genomic location of *avrPpiG*, *avrPpiC* and ORF3 in *P. syringae* pv. *pisi*

ORF-specific DNA fragments for all three genes were used to probe dot-blots of a range of *P. syringae* isolates.

Table 6. Distribution of *avrPpiG*, *avrPpiC* and ORF3 among *P. syringae* isolates

Dot blots of strains were hybridized with ORF-specific probes. The following *Pseudomonas* strains gave negative results: *P. syringae* pvs *phaseolicola* (strains of all 9 races; Mansfield *et al*., 1994; Taylor *et al*., 1996), *savastanoi* (NCPPB 639), *apii* (0988-2; Hendson *et al*., 1992) and *morsprunorum* (ICMP 5795), *P. marginalis* pvs *pastinaceae* (NCPPB 806), *alfalfae* (NCPPB 2644) and *marginalis* (NCPPB 247), *P. corrugata* (NCPPB 2445), *P. cichorii* (ICMP 907), *P. caricapapayae* (NCPPB 1873), *P. tolaasii* (NCPPB 2192T), *P. agarici* (NCPPB 1999), *P. asplenii* (NCPPB 959).

P. syringae **pathovar**	**Race**	**Isolate***	***avrPpiG*†**	***avrPpiC*†**	**ORF3†**
pisi	1	HRI 299A (I)	−	+	+
pisi	2	HRI 203 (II)	+	+	+
pisi	3A	HRI 870A (II)	+	+	+
pisi	3B	HRI 4585 (I)	−	+	+
pisi	4A	HRI 895A (II)	+	+	+
pisi	4B	PN8 (I)	−	+	+
pisi	5	HRI 974B (I)	−	+	+
pisi	6	HRI 1704B (II)	+	+	+
pisi	7	HRI 1691 (I)	−	+	+
maculicola	NA	HRI 1819A	−	+	+
maculicola	NA	m4‡	−	−	−
maculicola	NA	m2‡	−	+	+
maculicola	NA	HRI 1821A	−	+	+
maculicola	NA	HRI 1814	−	+	+
glycinea	4	PG4180§	−	+	+
glycinea	NA	NCPPB 2411	−	+	+
glycinea	NA	NCPPB 3318	−	+	+
glycinea	4	49a/90‖	−	+	+
tomato	NA	HRI 19	−	+	+
tomato	NA	DC3000¶	−	+	+
syringae	NA	HRI 1212R	−	−	−
aptata	NA	HRI 2201	−	+	+
coronafaciens	NA	ICMP 3113	+	−	−
lachrymans	NA	ICMP 3988	−	+	+

NA, No information is available concerning race structure for this strain.

*HRI, Horticulture Research International, Wellesbourne, UK; ICMP, International Collection of Micro-organisms from Plants, Auckland, New Zealand; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK. The genomic group is given in parentheses (Arnold *et al*., 1996).

†−, No hybridization; +, hybridization.

‡Dangl *et al*. (1992).

§Bender *et al*. (1993).

‖Ullrich *et al*. (1993).

¶Cuppels (1986).

The results (Table 6) indicated that *avrPpiG* was present only in races 2, 3A, 4A and 6 of *P. syringae* pv. *pisi*, whereas *avrPpiC* and ORF3 were widely distributed among *P. syringae* and always present together in the same strain.

Digests of total genomic and plasmid DNAs from races of *P. syringae* pv. *pisi* were blotted and probed with radiolabelled ORF-specific probes. The results showed that *avrPpiG* homologues were plasmid-borne in races 2, 3A, 4A and 6 (data not shown). Both *avrPpiC* and ORF3 were located on the chromosome and present in all races (data not shown). Based on hybridization analysis using undigested plasmids (data not shown), *avrPpiG* homologues are located on pAV222 (85 kb) in race 2 strain 203, pAV232 (110 kb) in race 3A strain 870A, pAV241 (45 kb) in race 4A strain 895A and pAV398 (110 kb) in race 6 strain 1704B.

Search for novel genes flanked by the conserved sequences

Primers DA72 and DA73 (Table 2) were used in PCR amplifications with a further group of *P. syringae* pathovars (Table 7). A 2493 bp band was found in all the strains tested that produced a positive hybridization

Table 7. Fragments amplified by PCR from *P. syringae* pathovars with flanking sequence primers DA72 and DA73

Amplified fragments were separated on agarose gels and the sizes given are estimated from co-migration based on the following which were sequenced: 973 bp in strain 11528, 1047 bp in HRI 870A, 1190 bp in HRI 203, 1461 bp in ICMP 3113, 1605 bp in HRI 895A, 2493 bp in HRI 299A. No bands were obtained in genomospecies group 1 with pv. *syringae* NCPPB 3023 or HRI 1212, pv. *atrofaciens* NCPPB 2612, pv. *lapsa* NCPPB 2096, or pv. *papulans* NCPPB 2848 or NCPBB 3262; in group 2 with pv. *morsprunorum* ICMP 5795 or C28 (Errington & Vivian, 1981), pv. *phaseolicola* HRI 1375A, HRI 1449B or HRI 2709A, or pv. *savastanoi* NCPBB 639 or ITM 317A; in group 3 with pv. *maculicola* m4 (Dangl *et al*., 1992); in group 4 with pv. *porri* NCPBB 3364 or NCPBB 3336, or pv. *garcae* NCPBB 2708 or NCPBB 588; in group 6 with *P. viridiflava* ICMP 2848.

P. syringae **pathovar**	**Strain***	**Group†**	**Bands (bp)‡**			
			(***avrPpiB***)	(***avrPpiA***)	(***avrPpiG***)	(***avrPpiC*** **and ORF3**)
aptata	HRI 2201	1				2493
	HRI 2204	1				2493
pisi	HRI 299A (race 1)	1	1047			2493
	HRI 203 (race 2)	1		1190	1605	2493
glycinea	PG4180§	2				2493
	NCPPB 2411	2				2493
lachrymans	ICMP 3988	2	1047			2493
tabaci	11528‖	2	973			
tomato	DC3000¶	3	1047			2493
antirrhini	HRI 152E	3				2493
maculicola	HRI 1819A	3		1190		2493
	HRI 65	3		1190		2493
apii	0988-2#	3		1190		
coronafaciens	ICMP 3113	4			1461	

*HRI, Horticulture Research International, Wellesbourne, UK; ICMP, International Collection of Micro-organisms from Plants, Auckland, New Zealand; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK; ITM, Culture Collection of the Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy.

† Genomospecies groups (Gardan *et al*., 1999); groups 5, 7, 8 and 9 were not tested.

‡ Names of corresponding genes in *P. syringae* pv. *pisi* are given in parentheses.

§ Bender *et al*. (1993).

‖ J. Turner, University of East Anglia, Norwich, UK.

¶ Cuppels (1986).

Hendson *et al*. (1992).

signal with *avrPpiC2*- and ORF3-specific probes. A band corresponding to *avrPpiA* was found in *P. syringae* pv. *maculicola* strains 1819A and 65 and in *P. syringae* pv. *apii* strain 0988-2. A band corresponding to *avrPpiB* was found in *P. syringae* pv. *tomato* strain DC3000 and *P. syringae* pv. *lachrymans* strain ICMP 3988. Two novel bands were amplified: a 973 bp band in *P. syringae* pv. *tabaci* strain 11528 and a 1461 bp band in *P. syringae*. pv. *coronafaciens* strain ICMP 3113. These two bands were cloned and sequenced. The *P. syringae* pv. *tabaci* strain 11528 band did not appear to contain an ORF. The 1461 bp band from *P. syringae*. pv. *coronafaciens* strain ICMP 3113 contained a single ORF with very high DNA identity (94·5%) to *avrPpiG1*. The difference in size between the PCR fragment from *P. syringae* pv. *coronafaciens* strain ICMP 3113 and *P. syringae* pv. *pisi* strain 895A represents a gap of 144 bp in the *P. syringae*. pv. *coronafaciens* sequence between the '*hrp* box' and the ATG start codon.

DISCUSSION

Two previously identified *avr* genes, *avrPpiA* and *avrPpiB* from *P. syringae* pv. *pisi*, were shown to be flanked by conserved DNA sequences, extending at least 1061 bp and 3493 bp, upstream and downstream of the genes. This appears to indicate that the *avr* genes are embedded in a region of DNA that originated in a common ancestor. We used the conserved regions to design two PCR primers, located in regions previously identified by Arnold *et al*. (2000) as part of an 8·5 kb DNA sequence surrounding *avrPpiA1*. The primers were used to isolate two regions of DNA from the genome of *P. syringae* pv. *pisi*, containing one and two novel genes, respectively, bounded in part at least by these conserved sequences. In each case the orientation of the ORFs within the conserved sequences is maintained, implying that there could be some functional significance in the arrangement, beyond serving as possible sites for recombination.

A novel gene, *avrPpiG*, was present only in races of *P. syringae* pv. *pisi* belonging to the genomic group II of Arnold *et al.* (1996) and was also isolated from *P. syringae*. pv. *coronafaciens* strain ICMP 3113, but on a smaller PCR fragment. This gene, when present in *P. syringae* pv. *phaseolicola*, resulted in a non-host HR in all bean cultivars tested. Database searches revealed that AvrPpiG is similar to two proteins, AvrRxv and AvrBsT, both specified by *avr* genes from *X. campestris* pv. *vesicatoria*, and matching resistance in tomato (*Lycopersicon esculentum*) (Whalen *et al.*, 1993; Ciesiolka *et al.*, 1999). Another similar gene was recently found by Alfano *et al.* (2000) in an area they termed an Exchangeable Effector Locus (EEL) from *P. syringae* pv. *syringae* (B728a) flanking the *hrp/hrc* gene cluster. These genes provide direct evidence of a link to virulence determinants in animal pathogens, seen in their similarity to YopP, which is presumed to act as an effector in the induction of apoptosis in macrophages by *Yersinia enterocolitica* (Mills *et al.*, 1997). A marker-exchange mutant of *avrPpiG* could not be obtained because the group II strains of *P. syringae* pv. *pisi*, which are the only ones containing this gene, do not readily accept broad-host-range plasmids (Moulton *et al.*, 1993).

The gene *avrPpiC1* was previously identified by screening a genomic library of *P. syringae* pv. *pisi* race 5 strain 974B for *avr* genes functioning in *P. syringae* pv. *phaseolicola* race 5 strain 52 (Fillingham, 1994; Goss, 1995). The gene conferred the ability to cause a strong HR on all bean cultivars normally compatible with race 5. Insertional mutagenesis with Tn*3gus* enabled a marker-exchange mutant of strain 974B to be created, but no change in symptoms was detected on either pea or bean. A similar lack of change of phenotype was seen with a marker-exchange mutant of strain PT10 with an insertion in ORF3. The present study has shown that *avrPpiC* is linked to a second, putatively *hrp*-regulated gene, ORF3, which invariably accompanies it in the strains tested. Both genes are flanked by the conserved sequences and although *avrPpiC* clearly can function as an *avr* gene alone, both genes may be required for an (as yet undetected) virulence function. The failure to observe any changes in virulence toward pea or avirulence towards bean suggests that both virulence and non-host avirulence are redundantly encoded by these pathogens. The distribution of these genes among pathovars of *P. syringae* may reflect aspects of their precise function *in planta* and whether they involve specific roles towards a limited number of host plants or a more general and perhaps fundamental role. While *avrPpiG* appears to be restricted to a single genomic group (II) within *P. syringae* pv. *pisi*, *avrPpiC* and ORF3 are widely distributed in strains representing pathovars in the genomospecies 1 (pvs *aptata* and *pisi*), 2 (pvs *glycinea* and *lachrymans*) and 3 (pvs *maculicola* and *tomato*) as proposed by Gardan *et al.* (1999).

The flanking region primers in some cases amplified up to three bands per strain, implying duplication of the region bounded by the *rulB* gene. The finding that *avrPpiA*, *avrPpiB*, *avrPpiC* and *avrPpiG* are all located between the flanking primers suggests that this region may serve as a hotspot for the integration (and possibly the excision) of *avr* genes. The relatively wide conservation of the flanking sequence regions across members of genomospecies 1, 2, 3 and 4 imply a role in pathogen evolution over a relatively long period of time or, alternatively, efficient horizontal transfer of these regions. An important question remaining unanswered is the extent to which this genomic region offers a vehicle for rapid evolution of novel pathogen specificities. In summary, the primers described here provide a novel method for detecting potential *avr* and *vir* genes and through these may identify novel PAIs in bacterial genomes.

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