MPMI Vol. 8, No. 5, 1995, pp. 700–708.

Molecular Characterization of the *Pseudomonas syringae* pv. *pisi* Plasmid-Borne Avirulence Gene *avrPpiB* Which Matches the *R3* Resistance Locus in Pea

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Received 21 January 1995. Accepted 13 June 1995.

An avirulence gene (designated *avrPpiB*) from race 3 of *Pseudomonas syringae* pv. *pisi* was cloned and sequenced. The gene corresponded to a single open reading frame of 831 nt identified by transposon mutagenesis and subcloning. This ORF encodes a predicted hydrophilic protein of 276 amino acids (MW 31,300). It effects the expression of a resistance mechanism governed by a single genetic locus in pea. Cosegregation of resistance at the *R3* locus of pea was observed towards race 3 and a transconjugant carrying the cloned *avrPpiB* gene according to the predicted 3:1 ratio of resistant:susceptible F_2 progeny from a cross between Jade (*R3 R3*) and Kelvedon Wonder (*rr*) cultivars. DNA hybridization studies showed *avrPpiB* to be plasmid-borne in race 3 and suggested the presence of other alleles on one of the endogenous plasmids of races 1 and 7. Disruption of the *avrPpiB* allele of race 1 and its complementation confirmed its behavior towards pea cultivars expressing the *R3* locus. Homologs of *avrPpiB* were detected in *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *maculicola*, and *P. syringae* pv. *tomato*. The presence of *avrPpiB* homologs in *P. syringae* pv. *phaseolicola* does not match any gene-for-gene pattern of interaction with bean cultivars.

Un gène d'avirulence (nommé *avrPpiB*) provenant de la race 3 de *Pseudomonas syringae* pathovar *pisi* a été cloné et séquencé. Ce gène correspond à un cadre de lecture ouvert (ORF) de 831 nt identifié par sous-clonage et mutagénèse par transposition. Cet ORF code pour une protéine hydrophilique de 276 acides aminés (poids moléculaire 31,300). Elle est impliquée dans l'expression d'un mécanisme de résistance gouverné par un seul locus génétique chez le pois. Selon un ratio 3:1 de résistants:susceptibles vis à vis de la race 3 et un transconjugant arborant un gène *avrPpiB* cloné pour une génération F_2 provenant d'un croisement entre les cultivars Jade (*R3 R3*) et Kelvedon Wonder (*rr*), la co-ségrégation de la résistance du locus *R3* du pois a été établie. Des études d'hybridation ont montré qu'*avrPpiB* est porté par un plasmide chez la race 3 et que des allèles se retrouvent sur un des plasmides endogènes des races 1 et 7. La dislocation du gène *avrPpiB* de la race 1 et sa complémentation ont confirmé son implication au niveau de l'expression du locus *R3* des cultivars de pois. Des gènes homologues d'*avrPpiB* ont été détectés chez *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *maculicola* and *P. syringae* pv. *tomato*. La présence d'homologues chez *P. syringae* pv. *phaseolicola* ne correspond à aucun patron connu d'interaction gène-pour-gène avec le haricot.

Additional keywords: harp box, hypersensitive response, *Phaseolus*, plant resistance locus, *Pisum sativum*, promoter region, water soaking.

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The EMBL accession number of *avrPpiB1.R3* DNA sequence is X84843.

Pseudomonas syringae pv. *pisi* (*P. syringae* pv. *pisi*) are plant pathogenic bacteria infecting pea (*Pisum sativum*). Differential interactions between isolates of the pathovar and cultivars of pea suggested the occurrence of physiologic races. Seven races of *P. syringae* pv. *pisi* are currently recognized (Bevan et al. 1995). The pattern of interactions may be interpreted in terms of a gene-for-gene model, involving six matching genes for avirulence (*avr*) in the pathogen and resistance (*R*) in the host. This model predicts the outcome of the interactions being either compatible where disease symptoms, typified by water soaking, develop or incompatible where a host defense reaction termed the hypersensitive response (Klement 1963) prevents development of the disease.

The interaction between pea and *P. syringae* pv. *pisi* is well defined both in terms of the host genetics and the pathogen races (Bevan et al. 1995). However, the absence of detailed molecular analysis about the genes involved hinders our understanding of the function, origin, and evolution of the genetic determinants involved. Only one of the matching gene pair, *avrPpiA–R2*, has been characterized (Vivian et al. 1989; Dangl et al. 1992). These data suggested the involvement of a

well-conserved pair of genes. The *R2* gene has functional homologs in other plants such as *Phaseolus* and *Arabidopsis*, and a functional *avrPpiA* homolog was detected in *P. syringae* pv. *maculicola* (Dangl et al. 1992). In this paper, we present the molecular analysis of the avirulence gene from race 3 (strain 870A) matching the *R3* resistance locus of pea cultivars. The bacterial genetic locus encoding this gene, named A3 in the scheme of Taylor et al. (1989), had previously been cloned by Bavage et al. (1991). Five cosmids from a gene library of race 3 (870A) restored the A3 avirulence phenotype of PF24, a derivative of the race 1 strain 299A that had lost one of its endogenous plasmids involved in the expression of the A3 phenotype (Malik et al. 1987; Bavage et al. 1991). The genetic determinant of A3 was located on a 6.5-kb fragment (see pAV233 of Bavage et al. 1991). Here, the molecular characterization of the gene essential for the expression of the A3 phenotype and its distribution among *P. syringae* pv. *pisi* races and other *P. syringae* pathovars is presented. The broad distribution of this gene suggests a positive selection for this activity which could be involved in the ecological fitness, e. g., competitiveness and pathogenicity of these bacteria. In this paper, we will refer to the A3 phenotype as corresponding to the gene *avrPpiB* and the race 3 allele matching the *R3* locus for resistance as *avrPpiB1.R3* as recommended by Vivian and Mansfield (1993).

RESULTS AND DISCUSSION

An avirulence gene is involved in the induction of an active defense mechanism in plants. This line of defense is initiated by the activity of a single plant resistance locus. In ecological terms, it is quite surprising that phytopathogens code for genes that confer a negative advantage over the other isolates of the phyllosphere. However, some avirulence genes have been shown to be involved in pathogenicity (Kearney and Staskawicz 1990; Lorang et al. 1994; Yang et al. 1994) and thus they might confer a relative advantage during the infection process over strains without such avirulence genes. Nevertheless, on cultivars with the right resistance locus the phytopathogen will be recognized and rapidly countered. The phytopathogen with the avirulence gene will thus be outcompeted by the microorganisms that do not possess the avirulence gene. Therefore, a strong selection for the gain or loss of properties that will increase its competitiveness will occur. Certain avirulence genes have been lost and gained naturally by *P. syringae* pv. *pisi* isolates and those changes might have been driven by some kind of ecological pressure. Here, we describe one of the avirulence genes (*avrPpiB*) of *P. syringae* pv. *pisi* which has been involved in race evolution. Characterization of this gene and the study of its distribution are the first steps towards our understanding of the mechanisms behind race evolution.

Mapping of *avrPpiB1.R3*.

The pLAFR3 subclone, pAV233 was mapped and further cosmid subclones were prepared, i. e., pAV228, pAV229, and pAV403 (Fig. 1). These were tested for virulence towards the pea differential cultivars. None of these subclones conferred the A3 phenotype associated with pAV233 (Table 1). Tn*3*HoKmgus insertions into pAV233 mapped a genetic determinant involved in the expression of the A3 phenotype in the region of the 890-bp *Eco*RI fragment of pAV403 (Fig. 1; Table 1). Tn*3*HoKmgus insertions #5, 6, and 16 disrupted the A3 activity of pAV233 cloned in *P. syringae* pv. *pisi* race

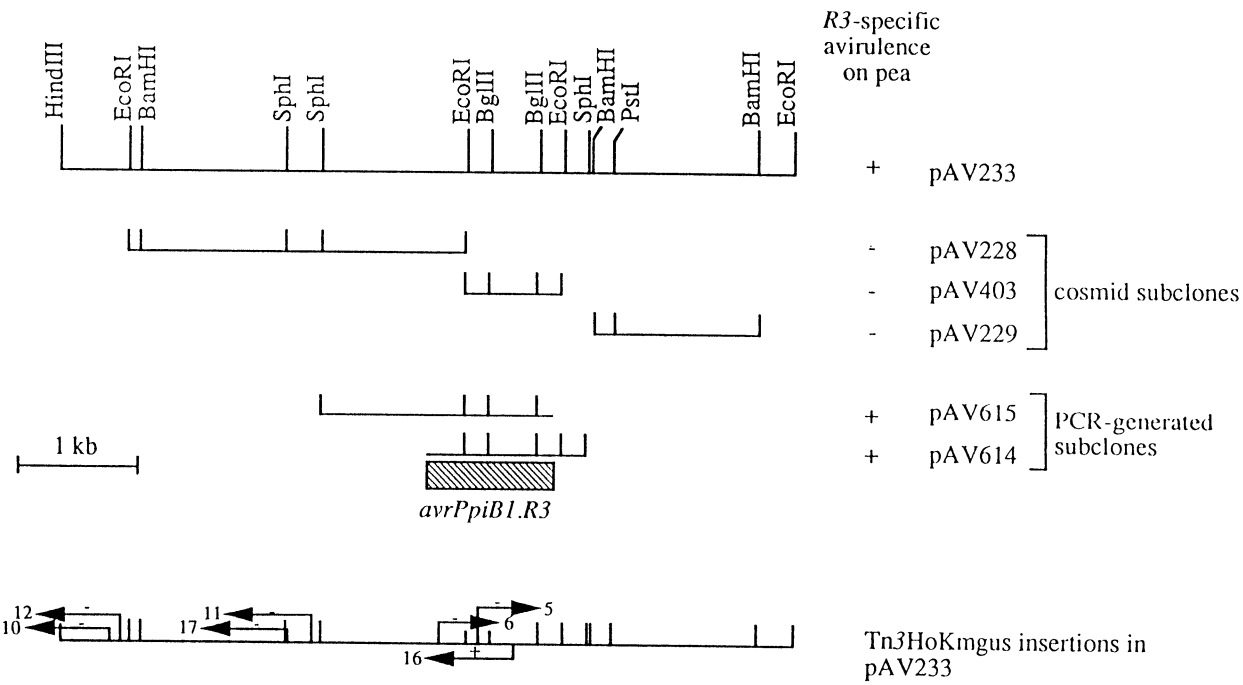

Fig. 1. Restriction endonuclease maps of the DNA fragments studied for the characterization of *avrPpiB1.R3*. The avirulence phenotype of the constructs is given. Details about the cloning vectors used are given in Table 3. The position of *avrPpiB1.R3* is shown (shaded box). The position of the various Tn*3*HoKmgus insertions in pAV233 is shown (arrows). The + and – signs above the arrows indicate the presence or absence, respectively, of Gus activity of these insertions.

4-like PT10 strain which is a race 4 strain obtained from a race 6 culture (1704B) after several genetic manipulations. The position of these Tn*3*HoKmgus insertions (Fig. 1) and the activity of the promoter-less *gus* cassette of the mutants indicated an active promoter located in the 430-bp *Bgl*II fragment of pAV233 on the opposite strand of the insert (according to Fig. 1).

Since an essential part of the A3 region overlapped the *Eco*RI fragment (890 bp) of pAV403, this fragment was subcloned in pUC19 and sequenced in both orientations. Sequence analysis showed the presence of a harp box (Innes et al. 1993) and a second *cis*-acting element (Shen and Keen 1993) known to be required for transcription of several *avr* genes (Fig. 2). Approximately 530 bp downstream from the *Eco*RI site were sequenced in both orientations to complete the sequencing of this potential *avr* gene. This revealed the presence of only one open reading frame (ORF) that could be inactivated by the Tn*3*HoKmgus insertions #5, 6, and 16 (Fig. 3A). The ORF is 831 nt long and comprises 40% G+C. A putative ribosome binding site was identified. The most similar σ^{54} promoter (Kustu et al. 1989) sequence found overlapped the start codon of the ORF (Fig. 3A). The σ^{54} RNA polymerase was suggested to be involved in the expression of some avirulence genes, e. g., *avrD* (Shen and Keen 1993). The deduced protein is 276 amino acids long (Fig. 3B) and is mainly hydrophilic with some hydrophobic residues (according to Kyte and Doolittle 1982) (Fig. 4). Its predicted molecular weight is 31,300. Searches for similarities at the DNA and amino acids levels between this sequence and other sequences were performed using the Pearson and Lipman (1988) algorithm. No convincing homology was observed with any sequences available on GenBank/EMBL and SWISSPROT. Best scores were mainly with short regions of sequences (18 to 43 amino acids) that share 20 to 35% identity in unrelated proteins. The gene was designated *avrPpiB*.

To confirm the involvement of the putative ORF in the A3 phenotype, the PCR-generated subclones pAV614 and pAV615 were conjugated into *P. syringae* pv. *pisi* strain PT10 and tested on pea cultivars (Fig. 1; Table 1). The results demonstrated that the minimal region required for the A3 phenotype was localized in the 968 bp overlapping pAV615 and pAV614 and corresponding to *avrPpiB* (Fig. 1). These constructs showed that the 107-bp region upstream the start codon of *avrPpiB1.R3* was sufficient for its expression.

Disruption of *avrPpiB*.

The pAV233 Tn*3*HoKmgus derivatives #5 and #16 (Table 1) were used in the attempt to disrupt *avrPpiB1.R3* and its homolog (*avrPpiB2.R3*) in the race 1 strain 299A. Electroporation was used to introduce the constructs into the strains. Selecting for Km^r, electro-transformants (Km^r, Tc^r) were obtained for the strain 299A but repeated attempts to electrotransform 870A failed. The pAV233 Tn*3*HoKmgus derivatives were then cured from the 299A electro-transformants while selecting for a recombination event between *avrPpiB2* and the disrupted *avrPpiB1* derivative. Kanamycin-resistant, tetracycline-sensitive recombinants were recovered and tested for their virulence toward pea differential cultivars. Ten 299A recombinants with the Tn*3*HoKmgus #5 insertion in *avrPpiB1*, and 10 with the Tn*3*HoKmgus #16 insertion were tested for each disruption experiment and all showed the absence of A3 activity. Table 1 shows the results for two representative strains, PF260 and PF261. The Tn*3*L probe (see Materials and Methods) hybridized signal with the largest plasmid in all recombinant strains tested. Introduction of pAV615 into strains PF260 and PF261 restored the A3 phenotype. The A1 phenotype of 299A was maintained in PF260 and PF261 (Table 1). This confirms the involvement of *avrPpiB* in the expression of resistance on pea cultivars with the *R3* locus.

Table 1. Virulence towards pea cultivars of *Pseudomonas syringae* pv. *pisi* strain 299A and its derivatives PF260 and PF261 with or without pAV615, and of *P. syringae* pv. *pisi* strain PT10 or PF304 harboring plasmid derivatives of pAV277 and Tn*3*HoKmgus mutants of pAV233

		Pea cultivars[a]						
Bacterial strain	**Relevant plasmid**	**KW[b] *R*[c] genes...none**	**Puget 3**	**Jade 3**	**Belinda 3**	**Vinco 1, 2, 3, 5**	**Partridge 3,4**	**Fortune 2,3,4**
299A		+	ND	–	–	–	ND	ND
PF260		+	ND	+	+	–	ND	ND
	pAV615	+	ND	–	–	–	ND	ND
PF261		+	ND	+	+	–	ND	ND
	pAV615	+	ND	–	–	–	ND	ND
PF304		+	+	ND	+	ND	ND	ND
	pAV228/pAV229/ pAV403	+	+	ND	ND	ND	ND	ND
	pAV233	+	–	ND	–	–[d]	ND	ND
PT10		+	+	+	+	+	–	–
	pAV614	+	ND	–	–	–	ND	ND
	pAV615	+	ND	–	–	–	–	–
	pLAFR3	+	ND	+	+	+	–	–
	Mutants:							
	pAV233-5/-6	+	+	+	+	+	ND	ND
	pAV233-10/-11/-12	+	–	–	–	–	ND	ND
	pAV233-16	+	+/-	+	+	+	–	–
	pAV233-17	+	–	–	–	–	ND	ND

[a] +: disease; -: hypersensitive response (HR); +/-: variable response, i. e., some plants showed disease and some the HR; ND: not done.
[b] Kelvedon Wonder.
[c] Postulated resistance genotype (Bevan et al. 1995).
[d] Tested in PT10 instead of PF304.

Genetic analysis of the *R3* locus.

Sequential inoculation of *P. syringae* pv. *pisi* race 3 strain 870A, race 4-like strain PT10, and PT10 harboring pAV615 on 78 F_2 progeny plants from a cross between cv. Kelvedon Wonder (*rr*) and Jade (*R3 R3*) was performed to study the segregation of the *R3* locus. Twenty-one plants were susceptible (diseased) and 57 plants developed a hypersensitive response to both strain 870A and strain PT10 with pAV615. All plants were susceptible to PT10 alone. Therefore, the *R3* locus cosegregated experimentally towards both 870A and PT10 harboring pAV615 at a ratio of 2.7:1. Chi-squared analysis of these data agrees with a predicted 3:1 ratio (χ^2= 0.154; P = 0.5–0.8). Cosegregation of resistance to race 3 and to race 4 harboring *avrPpiB* showed that *avrPpiB* is involved in the induction of a specific resistance governed by a single genetic locus in the plant.

Identification of *avrPpiB* homologs.

An internal *avrPpiB* PCR-generated fragment of 623 bp extending from position 498 to 1120 (according to nucleotide numbering of Fig. 3A) was used to probe *Eco*RI-digested genomic DNA from the seven wild-type races of *P. syringae* pv. *pisi*, listed in Table 2. Positive hybridization signals were obtained for isolates of races 1, 3, and 7. Hybridization signals corresponding to DNA fragments of about 3.3 and 1.9 kb were observed for isolates of races 1 (299A) and 7 (1691), and of about 2.8 and 1.0 kb for a race 3 isolate (870A). Hybridization of the endogenous plasmids of these isolates with the same probe showed *avrPpiB* to be plasmid-borne (Fig. 5). For the race 1 isolate (299A), the hybridization signal matched the position of the largest plasmid (pAV212–approximately 66 kb); for the race 3 isolate (870A), the hybridization signal matched the position of the smallest plasmid (pAV232—approximately 40 kb); for the race 7 isolate (1691), the hybridization signal matched with the position of the largest plasmid (pAV381—approximately 66 kb). This confirms the gene-for-gene interpretation of interactions between the physiologic races of *P. syringae* pv. *pisi* and the pea differentials (Bevan et al. 1994), and also indicates an absence of nonfunctional alleles in isolates of races 2, 4, 5, and 6. The identical RFLP hybridization patterns and the similarity in size for the plasmids harboring *avrPpiB* in isolates of races 1 and 7 suggest that these plasmids are closely related.

PCR was used to screen for the presence of the internal 623 bp fragment described above (position 498 to 1120 of Fig. 3A) in several isolates (listed in Table 2) of *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *maculicola*, *P. syringae* pv. *tomato*, *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *syringae*, *P. viridiflava,* and *P. putida*. Positive signals were obtained for *P. syringae* pv. *phaseolicola* isolates 1281A (race 1), 1375A (race 5), and 1299A (race 6), for *P. syringae pv. maculicola* isolate 1853, and for *P. syringae* pv. *tomato* 19. The relatedness between these PCR fragments and the *avrPpiB* alleles of *P. syringae* pv. *pisi* isolates 299A and 870A was confirmed by the analysis of restriction fragments length polymorphisms (RFLP) (Fig. 6). The PCR-RFLP patterns observed were identical suggesting conservation of

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avrPpiB1.R3       GGGAACCACATCAT.GGGTAAAAGCCACGAA
avrPpiA1.R2       GGGAAC.TCATTTTCTTTTAAAA.CCACACA
avrRpt2           GGGAACCCATTCATTGTTTGGAA.CCACCAA
avrA              TGAAACCGAAACGGCGTTGCTTG.CCACACA
avrB              TGGAACCTAATTCAGGGTAAATG.CCACACA
avrC              TGGAACCGTTCTGCAACTCG.TG.CCACTAA
avrDg             TGGAACCAAATCC.GTCCCAAAGGCCACACA
avrDt             TGGAACCAAATCC.GTCCCAAAGGCCACACA
avrPto            TGGAACCGA.TCCGCTCCCTATGACCACTCA
avrPphB1.R3       TGGAACCGAAT.GGGTCAGC.TGGACACTTA
avrPmaA1.RPM1     GGGAAC.TCATTTTCTTTTAAAA.CCACACA

consensus         tGGAACcNaatcNNNNNNNNaaaggcCACaca
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Fig. 2. Comparison of the promoter regions of *avrPpiB1.R3* and other *avr* genes. The boxed regions show highly conserved bases which are, respectively, part of a sequence similar to the consensus of the harp box (Innes et al. 1993) and of a *cis*-acting element necessary for transcription (Shen and Keen 1993). Sources of sequences are: *avrPpiA1.R2* and *avrPmaA1.RPM1*—Dangl et al. (1992); *avrRpt2*—Innes et al. (1993); *avrA*—Napoli and Staskawicz (1987); *avrB* and *avrC*—Tamaki et al. (1988); *avrD*$_\alpha$—Kobayashi et al. (1990a); *avrD*$_t$—Kobayashi et al. (1990b); *avrPto*—Salmeron et al. (1993); *avrPphB1.R3*—Jenner et al. (1991).

(A)

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1     GAATTCCGGT GCAGCGATGG ACTTTGGATC GTATCGATCT GCCCAGTTCG
51    GTCATATTGC CAGGCATCCC GGTCAGGTCT AAAAACGCTT CGTCGATAGA
101   CGCCACTTGA ACGCGAGGAA CCATGGATTC TATGATGGTC ATCATGCGAA
151   GGCTTAAGTC GCCATTTATG TGGAATGGAA AATCAACCCC TTACGTCCTA
201   ACAAAAATTC TCTACGTGAG AGCCCTTCAA GTAGATCTCC CGCCAATTTG
251   GGAACCACAT CATGGGTAAA AGCCACGAAG AGCTAAGTTG TTAGAAGCAA
300   CGTCTGGAGG CATACATGCA CGCAAATCCT TTAAGCTCTT TCAACAGAGC
351   TCAACATGGC AATCTGACTA ATGTAGAGGC CAGCCAAGTT AAATCGGCAG
401   GAACCTCTTC CACCACTAAT ATAGACAGTA AAAACATTGA AGAACATGTT
451   GCAGACAGAC TCAGTGATTT AGGCAGACCT GATGGTGGAT GGTTTTTCGA
501   GAAGTCACTT GCCACCTTGA AAAATTTAAA TCTTGAGCAG TTAGCCGGAA
551   TCCATGATGT ACTAAAATTA ACAGATGGCG TAAAGAACAT TGTCTCTTTT
601   GGAGCTCGGG AAGGAGGCTT CGAGTTGGCA ATGCAGTTTC GTCATGATTT
651   ATACAGATCT CAACATCCGG ATGAAAACTC GCCGCACGAT GCCGCAACTC
701   ATTATCTTGA TGCAATCAGC CTGCAATCAA ACAAATTTAC AAAACTTGAA
751   AAACTACAAC ATGTAGATGT ATTTAAAATG CAAAACCCGT TTTGGGATGT
801   CGGGTACAAA AACGGAATTG CGCACGCAAA AAAAATGGCA TTCTTCATAA
851   CGCCAGAGTG GCTGGGTTCT GATTTCTGTA AACAGGAATT CCAGTGGCTT
901   AGCGAAACAA AAAACAAAGA CATAAAATCT GCATTTGTGA TCTTTAAAGA
951   TGTAGACTTA AAAAGCAAAA ATATGACAAG TATCTTCAAT TTTGCAGACT
1001  TCCATAAATC ACGCGTCATG ATGGCAAGCA CACCTCCCGA ATCGGGATTG
1051  AATAATGTAA AAATCGAAAA TAGCGTTGAC CTGAATTTCA AGAGGTTATT
1101  AACTGACCGT GAGTCATGGG AACTAAATAA TTTCCTAGGC GACTAAAAAA
1151  CTCAAATCAG AGTGCCCACG ATTATCGGTG TTTTTTAGCC TCGATTTGAC
1201  TGGGCGTTCT GCCATCCAGC GTCGGCACAT CATCGAGCTT GCGTATAAGC
1251  CCATCCAATT TTTCCAGGTC ACCTTCAACA ATAGCCCGCT CATCGGCCGT
1301  CAGGGCACGG CCTCCAGATA ATGTCCGATG GACGCTTTAC TTTCCAGAGC
1351  CTGCGCTCTG GCACTGGCTT TGGGAATATT AAAATCGCAG CCAGCACACG
1401  CCATTCGATG AGGGCAA
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(B)

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1     MHANPLSSFN RAQHGNLTNV EASQVKSAGT SSTTNIDSKN IEEHVADRLS
51    DLGRPDGGWF FEKSLGTLKN LNLEQLAGIH DVLKLTDGVK NIVSFGAREG
101   GFELAMQFRH DLYRSQHPDE NSPHDAATHY LDAISLQSNK FTKLEKLQHV
151   DVFKMQNPFV DVGYKNGIAH AKKMAFFITP EWLGSDFCKQ EFQWLSETKN
201   KDIKSAFVIF KDVDLKSKNM TSIFNFADFH KSRVMMASTP PESGLNNVKI
251   ENSVDLNFKR LLTDRESWEL NNFLGD
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Fig. 3. A, DNA sequence of *avrPpiB1.R3*. Asterisks show the position of the start and stop codons. The putative ribosome binding site of the gene is underlined (thick line) and the harp box and the *cis*-acting element sequence are boxed. A putative σ^{54} promoter sequence is underlined twice. The numbered horizontal arrows indicate the position and direction of the primers used for PCR amplification (see Materials and Methods). The positions of Tn*3*HoKmgus insertions that inactivated *avrPpiB.R3* were determined by DNA sequencing (see Materials and Methods) and are indicated by the numbered vertical arrows. **B,** Predicted amino acid sequence of *avrPpiB1.R3*. The putative peptide is 276 amino acids long including the methionine of the start codon and is hydrophilic.

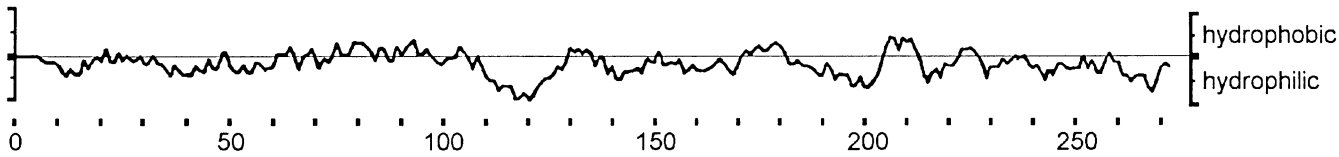

Fig. 4. Hydrophobicity plot for the predicted amino acids of the AvrPpiB protein presented in Figure 3B computed according to Kyte and Doolittle (1982). The analysis was performed using a window of nine residues.

Table 2. Bacterial strains used in this study

Strain	Relevant characteristics	Reference
E. coli		
DH5α	*sup*E44, Δ*lac*U169 (ϕ80*lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1	Hanahan 1983
HB101	*sup*E44, *hsd*S20 (r_B^- m_B^-), *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20, *xyl-mtl*-1	Boyer and Roulland-Dussoix 1969
C2110	Nal^r, *pol*A$^-$	Bonas et al. 1989
39R861	Host to four plasmids used as size markers (98, 42, 23.9, 4.6 MDa)	Threlfall et al. 1986
P. syringae pv. *pisi* (pea pathogens)		
299A	Wild-type race 1	Taylor et al. 1989
203	Wild-type race 2	Taylor et al. 1989
870A	Wild-type race 3	Taylor et al. 1989
895A	Wild-type race 4	Taylor et al. 1989
974B	Wild-type race 5	Taylor et al. 1989
1704B	Wild-type race 6	Taylor et al. 1989
1691	Wild-type race 7	Taylor et al. 1989
PF24	Avirulence mutant of 299A	Malik et al. 1987
PF260	299A derivative with *avrPpiB2.R3*:: Tn*3*HoKmgus	This work
PF261	299A derivative with *avrPpiB2.R3*:: Tn*3*HoKmgus	This work
PF304	Rif^r derivative of PF24	Malik et al. 1987
PT10	Race 4-like Rif^r derivative of 1704B	Moulton et al. 1993
P. sringae pv. *phaseolicola* (bean pathogens)		
1281A	Wild-type race 1	J. D. Taylor[a]
882	Wild-type race 2	M. L. Schuster[b]
1301A	Wild-type race 3	J. H. C. Davis[b]
1302A	Wild-type race 4	J. H. C. Davis
1375A	Wild-type race 5	D. J. Allen[b]
1299A	Wild-type race 6	J. H. C. Davis
1449B	Wild-type race 7	H. Assefa[b]
2656A	Wild-type race 8	J. D. Taylor
2709A	Wild-type race 9	J. D. Taylor
P. syringae pv. *maculicola*		
65B	Mustard isolate	J. D. Taylor
1822D	Cauliflower re-isolate of 65B	J. D. Taylor
1853A	Cauliflower isolate	J. D. Taylor
1846A	Brassica pathogen	J. D. Taylor
P. syringae pv. *morsprunorum* (cherry pathogens)		
C28	Wild-type race 1	C. M. E. Garrett[c]
C330	Wild-type race 2 - group 1	C. M. E. Garrett
C333	Wild-type race 2 - group 2	C. M. E. Garrett
E71	*trp*-1, *met*-5 C28 derivative	A. Vivian
E714	*trp*-1, *met*-5 C28 derivative	A. Vivian
P. syringae pv. *syringae*		
PS 51	Broad host range	Bender and Cooksey 1986
PS 61	Broad host range	Bender and Cooksey 1986
239A	Pea cv. Octava isolate	J. D. Taylor
245A	Pea cv. Octava isolate	J. D. Taylor
250A	Pea cv. Octava isolate	J. D. Taylor
1212	Pea isolate	G. Berry[b]
P. syringae pv. *tomato* 19 (tomato pathogen)		R. A. Lelliot[b]
P. viridiflava 803 (broad host range)		I. J. Oxtoby[b]
P. putida PaW340 (soil)		Pickup et al. 1983

[a] Horticulture Research International, Wellesbourne, UK.
[b] Provided by J. D. Taylor of Horticulture Research International, Wellesbourne, UK.
[c] Horticulture Research International, East Malling, Kent, UK

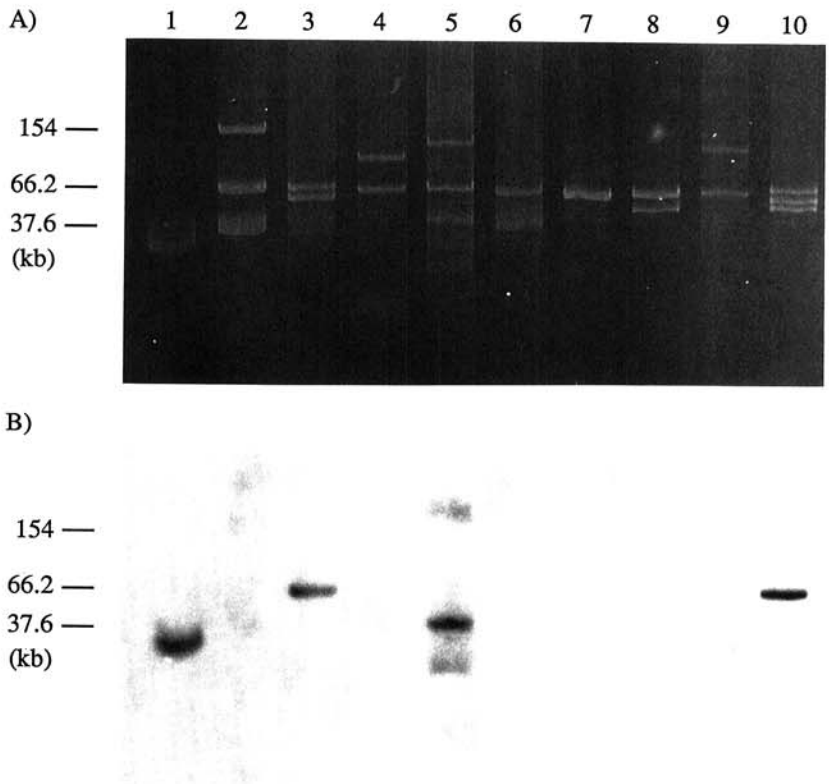

Fig. 5. DNA blot analysis of *Pseudomonas syringae* pv. *pisi* endogenous plasmids probed with *avrPpiB1.R3*. **A,** Plasmid profiles of *P. syringae* pv. *pisi* races, and, **B,** DNA hybridization of *P. syringae* pv. *pisi* endogenous plasmids with a PCR-amplified DNA probe for *avrPpiB1.R3* (see Materials and Methods). Lanes: (1) pAV615, (2) *E. coli* 39R861, and (3) *P. syringae* pv. *pisi* strain 299A: race 1, (4) 203: race 2, (5) 870A: race 3, (6) 895A: race 4, (7) PT10: race 4-like, (8) 974B: race 5, (9) 1704B: race 6, and (10) 1691: race 7. The plasmids of *E. coli* 39R861 (Table 2) were used as size markers and were converted from megadaltons to kilobase pairs using the conversion factor 1 MDa= 1.575 kb (Sambrook et al. 1989).

gene-structure. Homologs of *avrPpiA*, the other *P. syringae* pv. *pisi* avirulence gene characterized, also were shown to be conserved between pathovars (Dangl et al. 1992). The PCR screening of *avrPpiB* was further supported by DNA hybridization analysis. An internal *avrPpiB* PCR-generated fragment extending from position 668 to 878 (according to nucleotide numbering of Fig. 3A) was used to probe *Eco*RI-digested genomic DNA from the isolates that conferred the positive PCR signal analysed in Figure 6. The presence of *avrPpiB* homologs in these isolates was confirmed and variability was now detected between the RFLP patterns (Fig. 7). Genetic divergence was observed between most isolates with only a conserved pattern observed between *P. syringae* pv. *phaseolicola* isolates 1281A and 1375A. Hybridization signals corresponding to DNA fragments of about 23 and 2.0 kb were observed for *P. syringae* pv. *maculicola* isolate 1853, of about 23 kb for *P. syringae* pv. *tomato* isolate 19, of about 1.1 kb for *P. syringae* pv. *phaseolicola* isolates 1281A and 1375A, and of about 2.0 kb for *P. syringae* pv. *phaseolicola* isolate 1299A.

Homologs of *avrPpiB* are thus present in at least three other pathovars of *P. syringae*. According to the gene-for-gene interactions between *P. syringae* pv. *phaseolicola* and bean cultivars (Mansfield et al. 1994), the presence of *avrPpiB* does not match any of the described avirulence loci: race 6 does not show any avirulence activity; race 1 (A1) and race 5 (A1, A2, A4) have the A1 locus in common but *avrPpiB* was not amplified by PCR for the race 7 isolate which also shows an A1 phenotype. Assuming that these *avrPpiB* homologs are functional, beans would not appear to possess functional homologs of the *R3* locus. However, Fillingham et al. (1992) reported a susceptible response

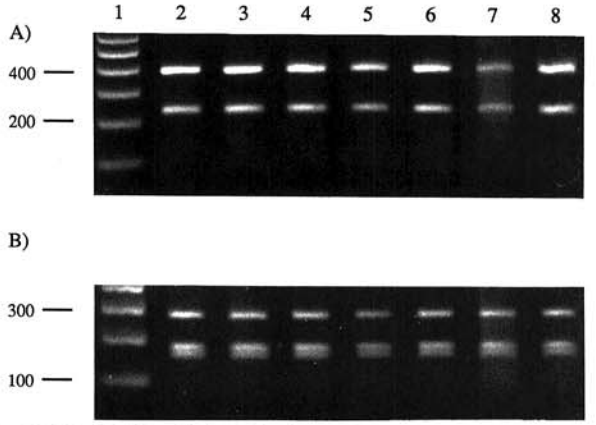

Fig. 6. PCR–RFLP analysis of the internal *avrPpiB* fragment from position 498 to 1120 of the gene sequence (Fig. 3A) amplified from liquid cultures of various *Pseudomonas* spp. isolates. **A,** *Eco*RI and, **B,** *Sau*3AI RFLP patterns of the PCR products after electrophoresis through a 2% (w. v^{-1}) agarose gel. Lanes: (1) 100-bp DNA ladder, (2) *Pseudomonas syringae* pv. *pisi* 299A (race 1), (3) *P. syringae* pv. *pisi* 870A (race 3), (4) *P. syringae* pv. *phaseolicola* 1281A (race 1), (5) *P. syringae* pv. *phaseolicola* 1375A (race 5), (6) *P. syringae* pv. *phaseolicola* 1299A (race 6), (7) *P. syringae* pv. *maculicola* 1853, and (8) *P. syringae* pv. *tomato* 19.

associated with some tissue browning within the water-soaked lesion when *P. syringae* pv. *phaseolicola* races 5 and 6 (race 1 was not tested) were inoculated on the bean cultivar Seafarer. It is conceivable from this observation that some kind of resistance was induced by the *avrPpiB* homologs. However, this resistance would be atypical and quite different from the one related to the *RPM1* (named *R2* in pea) resistance locus (requiring *avrPpiA* for its expression) which was observed in bean, pea, and *Arabidopsis* (Fillingham et al. 1992; Dangl et al. 1992). This difference could be explained if the appearance of the matching *R3* and *avrPpiB* activities were a relatively recent event in evolutionary terms with the possible emergence of different matching loci in the other hosts. Since *avrPpiB* is plasmid-borne and could be transferred laterally in the phyllosphere, this would support the notion of recent acquisition of this information by some of the other pathovars. It is possible that the acquisition of *avrPpiB* could have improved the competitiveness and/or pathogenicity of these isolates. Consequently, some hosts would have selected mechanisms to recognize this activity and counter the phytopathogen expressing it. We are currently studying this hypothesis.

MATERIALS AND METHODS

Strains and plasmids.

Bacterial strains used are listed in Table 2 and plasmids in Table 3. *P. syringae* pv. *pisi* isolates were grown at 25°C on

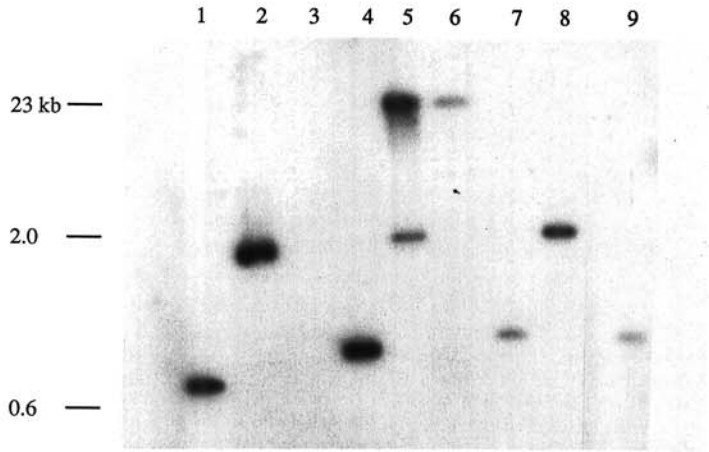

Fig. 7. DNA blot analysis of *avrPpiB* homologs from various *Pseudomonas syringae* pathovars. An internal *avrPpiB* PCR-generated fragment extending from position 668 to 878 (according to nucleotide numbering of Fig. 3A) was used to probe *Eco*RI-digested genomic DNA and pAV615. Lanes: (1) pAV615, (2) *P. syringae* pv. *pisi* 299A (race 1), (3) *P. syringae* pv. *pisi* 202 (race 2), (4) *P. syringae* pv. *pisi* 870A (race 3), (5) *P. syringae* pv. *maculicola* 1853, (6) *P. syringae* pv. *tomato* 19, (7) *P. syringae* pv. *phaseolicola* 1281A (race1), (8) *P. syringae* pv. *phaseolicola* 1299A (race 6) and (9) *P. syringae* pv. *phaseolicola* 1375A (race 5). Scale is in kilobase pairs.

Table 3. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pLAFR3	Tra$^-$ Mob$^+$ Tc^r, RK2 replicon	Staskawicz et al. 1987
pRK2013	Tra$^+$ Km^r, ColE1 replicon	Figurski and Helinski 1979
pUC18/19	Ap^r, *lacZ*	Norrander et al. 1983
pBluescript (KS-)	Ap^r, *lacZ*	Short et al. 1988
pHoKmgus	Ap^r, Km^r, *tnpA*$^-$, promoter-less *gus*	Bonas et al. 1989
pSShe	Cm^r, *tnpA*	Bonas et al. 1989
pAV212	Approx. 66- kb endogenous plasmid of *P. syringae* pv. *pisi* strain 299A	This work
pAV228	2.8 kb *Eco*RI fragment of pAV233 in pLAFR3	This work
pAV229	1.4 kb *Bam*HI fragment of pAV233 in pLAFR3	This work
pAV232	Approx. 40 kb endogenous plasmid of *P. syringae* pv. *pisi* strain 870A	This work
pAV233	6.1 kb *Hin*dIII/*Eco*RI fragment of pAV277 in pLAFR3	Bavage et al. 1991
pAV233-5/-6/-16	Tn*3*HoKmgus insertions inside *avrPpiB1.R3* in pAV233	This work
pAV233-10/-11/-12/-17	Tn*3*HoKmgus insertions outside *avrPpiB1.R3* in pAV233	This work
pAV277	*P. syringae* pv. *pisi* race 3 strain 870A pLAFR3 library clone harboring the A3 region	Bavage et al. 1991
pAV328	2.8-kb *Eco*RI fragment of pAV233 in pUC19	This work
pAV329	1.4-kb *Bam*HI fragment of pAV233 in pUC19	This work
pAV330	0.9-kb *Eco*RI fragment of pAV233 in pUC19	This work
pAV366/367	*Sph*I fragment of pAV404 in pUC19 (in both orientations)	This work
pAV381	Approx. 66-kb endogenous plasmid of *P. syringae* pv. *pisi* strain 1691	This work
pAV403	0.9-kb *Eco*RI fragment of pAV233 in pLAFR3	This work
pAV404	4.7-kb *Hin*dIII/*Pst*I fragment of pAV233 in KS-	This work
pAV612	Amplified *avrPpiB1*.R3 fragment of pAV366 in pUC18	This work
pAV613	Amplified *avrPpiB1*.R3 fragment of pAV367 in pUC18	This work
pAV614	1.4-kb *Hin*dIII fragment of pAV612 in pLAFR3	This work
pAV615	1.9-kb *Hin*dIII/*Eco*RI fragment of pAV613 in pLAFR3	This work

King's B agar (King et al. 1954) for 2 days or in Luria-Bertani (LB) broth (Miller 1972) for 24 h at 25°C. *Escherichia coli* strains were grown overnight on LB agar or LB broth at 37°C. Antibiotics were obtained from Sigma Co. (Poole, Dorset, UK) and used at the following concentrations (μg ml^{-1}): ampicillin, 100; chloramphenicol, 50; kanamycin, 10; nalidixic acid, 50; rifampicin,100; tetracycline, 10.

Plasmids from *E. coli* were extracted according to Jones and Schofield (1990) or using the Qiagen kit (Hybaid Ltd., Teddington, Middlesex, UK). Genomic DNA of *P. syringae* pv. *pisi* was obtained by the method of Denny et al. (1988). Plasmids of *P. syringae* pv. *pisi* were extracted as described by Moulton et al. (1993). Electrophoresis and visualization of DNA profiles were performed according to Sambrook et al. (1989).

Plant growth conditions and inoculations.

Growth conditions for pea plants have been described previously (Vivian et al. 1989). Strains were tested for their virulence on pea differential cultivars (listed in Table 1) by the stem inoculation method (Moulton et al. 1993). Plant responses were scored 7 days after inoculation. At least 10 plants were inoculated for each trial.

DNA cloning, transposon mutagenesis and plasmid curing.

Restriction enzymes were obtained from Gibco Life Technologies Ltd. (Paisley, Scotland) and used according to manufacturer's instructions. Ligations were performed using the T4 DNA ligase method (Sambrook et al. 1989). $CaCl_2$-mediated DNA transformations of *E. coli* were performed (Hanahan 1983; Sambrook et al. 1989). Conjugations between *E. coli* and either PF304 or PT10 strains of *P. syringae* pv. *pisi* were done according to the triparental mating method (Dangl et al. 1992) using the helper plasmid pRK2013 (Figurski and Helinski 1979). Electro-transformation of *P. syringae* pv. *pisi* was done according to Keen et al. (1993).

Mutagenesis with Tn*3*HoKmgus (Bonas et al. 1989) was as described by Dangl et al. (1992). The β-glucuronidase activity of Tn*3*HoKmgus cosmid (pLAFR3) mutants was assayed after conjugation into *P. syringae* pv. *pisi* strain PT10. The PT10 exconjugants were grown on a minimal salt medium ([g/liter] 5, NH_4Cl; 1, NH_4NO_3; 2, Na_2SO_4; 3, K_2HPO_4; 1, KH_2PO_4; and 0.1, $MgSO_4.7H_2O$; pH 7.2) supplemented with 0.5% (w/v^{-1}) glucose and 25 μg ml^{-1} cyclohexylammonium 5-bromo-4-chloro-3-indoyl β-D-glucuronic acid (Sigma Co., Poole, UK). Gus activity under these growth conditions gives blue colonies. Curing of pLAFR3 derivatives cloned into *P. syringae* pv. *pisi* was done according to Moulton et al. (1993).

DNA sequencing.

DNA sequencing was done following the procedure of Jones and Schofield (1990) and was performed after the subcloning of the restriction fragments of interest into pUC18/19 (Pharmacia, St. Albans, Hertfordshire, UK) and pBluescript (Short et al. 1988). Initial DNA sequencing of pUC18/19 derivatives was done using the M13 universal and M13 reverse universal primers. Initial DNA sequencing of pBluescript derivatives was done using the T3 and T7 primers. The other sequencing primers were then defined according to the sequence of the insert. All primers were obtained from Pharmacia (UK). About 20 primers were required to complete the sequencing in both orientations and some of them are shown in Figure 3A.

PCR DNA sequencing was performed according to the manufacturer's instructions (Pharmacia, UK) to position some of the Tn*3*HoKmgus inserts. The Tn*3*L primer (5′-AAAGAGGCGTCAGAGGC-′3) going from position 50 to 66 according to nucleotide numbering of Tn*3* (Heffron and McCarthy 1979) was used.

PCR amplification and DNA hybridizations.

Preparation of PCR and visualization of the PCR products were done according to Simonet et al. (1991) except that the *Pfu* DNA polymerase was used (Lundberg et al. 1991). PCR cycles (35 in total) were conducted on an automated thermocycler (Hybaid, UK) set at the following parameters: 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C.

PCR was used to clone the minimal region involved in the expression of *avrPpiB1.R3*. The PCR amplifications were performed using pAV367 and pAV366 (Table 3) with the M13 universal reverse primer and, respectively, a primer defined upstream (primer #1) (pAV613) or downstream (primer #16) (pAV612) *avrPpiB1.R3* ORF (Fig. 3A). These PCR fragments were blunt-ended using the Klenow fragment of polymerase I according to the instructions of the manufacturer (Pharmacia, UK) and ligated into dephosphorylated pUC18 which was digested by *Sma*I (obtained from Pharmacia, UK). RFLP study and DNA sequencing of the inserts confirmed cloning of the genetic determinant. PCR primers #8 and #15 (Fig. 3A) were used to amplify an internal portion of *avrPpiB1.R3* which was then used as a DNA probe.

Radioactive labeling of the PCR-based DNA probe was done using the Megaprime kit (Amersham, UK) and labeling of oligonucleotides was done using the T4 polynucleotide kinase (Gibco, UK). Southern blots and hybridization conditions were as described by Dangl et al. (1992).

DNA sequence analysis.

DNA sequence analysis were done using the University of Wisconsin GCG computer program (Devereux et al. 1984) through the SERC Seqnet service (Daresbury, UK).

ACKNOWLEDGMENTS

We would like to thank M.-C. Grosjean for her helpful comments on the manuscript. B. C. thanks the NSERC of Canada for the receipt of a postdoctoral fellowship. This work was supported by grants from the Gatsby Foundation and AFRC (UK). It was carried out under MAFF licence number PHF 678/81 (91) issued under the Plant Health (GB) Order 1987.

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