Research Note

Detection of a Gene in Pea Controlling Nonhost Resistance to *Pseudomonas syringae* pv. *phaseolicola*

John R. Wood,[1] Alan Vivian,[1] Carol Jenner,[1] John W. Mansfield,[2] and John D. Taylor[3]

[1]Department of Biological Sciences, University of the West of England, Frenchay Campus, Coldharbour Lane, Bristol, BS16 1QY, [2]Department of Biological Sciences, Wye College, Wye, Ashford, Kent, TN25 5AH, and [3]Horticulture Research International, Wellesbourne, Warwick, CV35 9EF, United Kingdom

Received 15 October 1993. Revision received 24 March 1994. Accepted 26 April 1994.

Cloned DNA from an approximately 150-kb plasmid in the bean pathogen *Pseudomonas syringae* pv. *phaseolicola* conferred avirulence on *P. syringae* pv. *pisi* toward its host, pea. Avirulence was determined by two loci, which appeared to match a single dominant resistance gene in the pea cultivar Kelvedon Wonder, the first gene for nonhost resistance to be identified in pea.

Development of the technology to transfer genes between pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* has allowed the genetic dissection of determinants of nonhost and race-specific avirulence (Staskawicz *et al.* 1984; Whalen *et al.* 1988, 1991, 1993; Kobayashi *et al.* 1989; Keen 1990; Dangl *et al.* 1992; Fillingham *et al.* 1992; Ronald *et al.* 1992). For example, the transfer of individual clones from a genomic library of *X. c.* pv. *vesicatoria* to *X. c.* pv. *phaseoli* led to the isolation of the avirulence gene *avrRxv*. The cultivar specificity found for the interaction of *avrRxv* with bean (*Phaseolus vulgaris* L.) provided the first demonstration that the gene-for-gene concept, traditionally applied to race-cultivar interactions, could be extended to interactions between pathogens and nonhost plants (Flor 1955; Crute 1985; Heath 1991; Whalen *et al.* 1988). The most detailed analysis of species specificity concerns the interactions between the tomato pathogen *P. s.* pv. *tomato* and soybean (*Glycine max* L.), a nonhost of this pathogen. Five avirulence genes from *P. s.* pv. *tomato*—*avrA, avrD, avrE, avrPto* (from strain PT23), and *avrRpt2* (from strain JL1065)—have been shown to be recognized by soybean (Kobayashi *et al.* 1989; Keen *et al.* 1990; Ronald *et al.* 1992; Lorang *et al.*, in press).

The emerging theme is that bacterial *avr* gene–resistance gene pairs cross pathogen, pathovar, and plant species boundaries (Carney and Denny 1990; Dangl *et al.* 1992; Fillingham *et al.* 1992; Swarup *et al.* 1992; Whalen *et al.* 1988, 1991, 1993). Genes for avirulence are, effectively, probes for matching resistance genes in host and nonhost plants. The *avrPpiA* gene (previously named *avrAspiI*) from *P. s.* pv. *pisi* (Vivian *et al.* 1989; Vivian and Mansfield 1993) was found to alter the virulence of *P. s.* pv. *phaseolicola* to bean and *P. s.* pv. *maculicola* to *Arabidopsis* in a cultivar- or ecotype-specific manner (Dangl *et al.* 1992). The activity of *avrPpiA* has therefore demonstrated the presence, in bean and *Arabidopsis,* of functional homologs of the *R2* gene for resistance to *P. s.* pv. *pisi* (Dangl *et al.* 1992; Fillingham *et al.* 1992). The *avrPphB* gene (previously named *avrPph3*) from *P. s.* pv. *phaseolicola* races 3 and 4 was found to confer avirulence on *P. s.* pv. *pisi* in all cultivars of pea examined (Fillingham *et al.* 1992; Vivian and Mansfield 1993). In this article we report an extension of the search for genes in *P. s.* pv. *phaseolicola* that control the expression of avirulence in the nonhost pea (*Pisum sativum* L.).

Approximately 1,000 clones from a gene library of *P. s.* pv. *phaseolicola* race 4 strain 1302A (Jenner *et al.* 1991) were mated into *P. s.* pv. *pisi* race 1 strain PF247 (Vivian *et al.* 1989) by the use of the helper plasmid pRK2013 (Figurski and Helinski 1979), and the transconjugants were tested for changes in virulence on seedlings of the pea cultivar Kelvedon Wonder. Plants were stab-inoculated below the stipule, with a sterile entomological mounting pin coated in bacterial cells taken from a 48- to 72-hr culture on agar (Malik *et al.* 1987). Seven clones (designated pPPY40 to pPPY46) were associated with the production of restricted brown lesions in

Table 1. Bacteria recovered from inoculation sites in stems of the pea cultivars Kelvedon Wonder and Martus, 3 and 8 days after inoculation with *Pseudomonas syringae* pv. *pisi*[a]

	$\log_{10}$cfu recovered per site			
	Kelvedon Wonder		Martus	
Strain	3 days	8 days	3 days	8 days
PF247, race 1	8.24 ± 0.14	8.11 ± 0.13	8.40 ± 0.08	8.45 ± 0.08
PF247 (pPPY40)	7.14 ± 0.10	6.16 ± 0.20	8.18 ± 0.05	8.38 ± 0.13
PT10, race 4	7.77 ± 0.16	8.04 ± 0.21	8.08 ± 0.13	8.53 ± 0.10
PT10 (pPPY40)	7.00 ± 0.12	6.84 ± 0.11	7.80 ± 0.10	8.09 ± 0.03

[a]Pea plants were stabbed with a sterile entomological mounting pin and inoculated by placing 10^5 cfu of the strain in 1 μl of 0.25× Ringer's solution on the wound. Each mean represents recoveries of bacteria from three plants (for PT10 strains) or five plants (for PF247 strains). Tissue was excised from 0.5 cm above to 0.5 cm below the inoculation site and was ground, with a pestle and mortar, in 1 ml of 0.25× Ringer's solution. Serial dilutions were plated on King's B agar (King *et al.* 1954) with appropriate antibiotics for selection of the strains. The two strains of *P. s.* pv. *pisi* were examined in different experiments.

Corresponding author: Alan Vivian.

Present address of John Wood: Scottish Agricultural Science Agency, East Craigs, Edinburgh, EH12 8NJ, United Kingdom.

MPMI Vol. 7, No. 4, 1994, pp. 534-537
©1994 The American Phytopathological Society

Kelvedon Wonder rather than the large, spreading, water-soaked lesions characteristic of the compatible interaction. Since no race-specific resistance genes have been detected in Kelvedon Wonder, it was clear that the determinants responsible for resistance are distinct from those involved in race-cultivar specificity in the interaction between *P. s.* pv. *pisi* and pea. Digestion of insert DNA from the selected clones and hybridization using pPPY40 as a probe indicated that five of the clones shared common insert fragments, while pPPY42 and pPPY43 did not (data not shown). Further studies focused on pPPY40, which was screened for activity on a range of pea genotypes. Only one cultivar, Martus, was fully susceptible to transconjugants containing pPPY40.

The differences in phenotype observed in *P. s.* pv. *pisi* containing pPPY40 were confirmed in cultivars Kelvedon Wonder and Martus by examination of bacterial growth at inoculation sites (Table 1). Strains of *P. s.* pv. *pisi* races 1 and 4 showed an increase from the 10^5 cfu inoculated to 10^8 cfu after 8 days on both pea cultivars. Similar increases occurred in both strains containing pPPY40 on Martus; but, on Kelvedon Wonder, after an initial increase to 10^7 cfu after 3 days, populations of transconjugants declined. Thus the presence of pPPY40 caused a 50- to 100-fold reduction in the number of colony-forming units at inoculation sites in Kelvedon Wonder, a result consistent with the formation of restricted lesions.

A sample of 222 F_2 progeny from a cross between the pea cultivars Martus and Kelvedon Wonder were tested for their reaction to *P. s.* pv. *pisi* isolate PF247 (pPPY40). The observed ratio of resistant progeny to susceptible progeny was

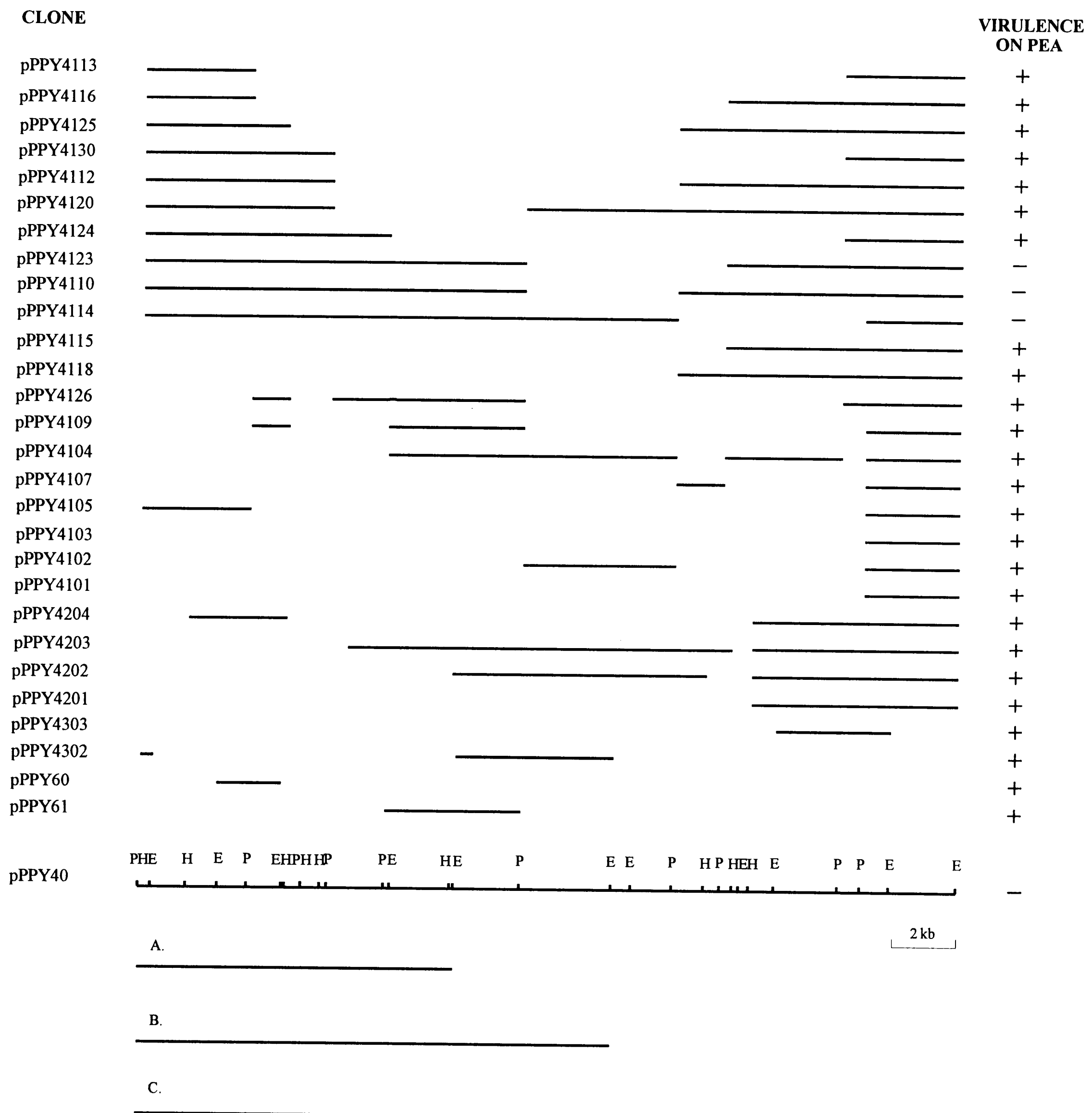


Fig. 1. Subcloning of pPPY40 and analysis of pPPY40, 41, 44, 45, and 46 to locate determinants of avirulence. Subclones containing the regions of pPPY40 indicated by the lines were mo_bilized with pRK2013 (Figurski and Helinski 1979) into *Pseudomonas syringae* pv. *pisi* strains PF247 or PT10, and the transconjugants were tested for changes in virulence toward the pea cultivar Kelvedon Wonder (+ = virulent; – = avirulent). Line A indicates the pPPY40 *Eco*RI restriction fragments also present in cosmids pPPY41 and pPPY45. Line B indicates the pPPY40 *Eco*RI restriction fragments also present in cosmids pPPY44 and pPPY46. Line C defines the region of pPPY40 deduced to be required for the avirulence phenotype. The recognition sites for *Eco*RI, *Hin*dIII, and *Pst*I are indicated by E, H, and P, respectively.

167:55. Chi-square analysis of these results, assuming they represented a 3:1 ratio, gave $\chi^2 = 0.006$, $P = 0.05–0.1$, indicating the presence of a single dominant resistance gene in Kelvedon Wonder, matching the avirulence phenotype conferred by pPPY40.

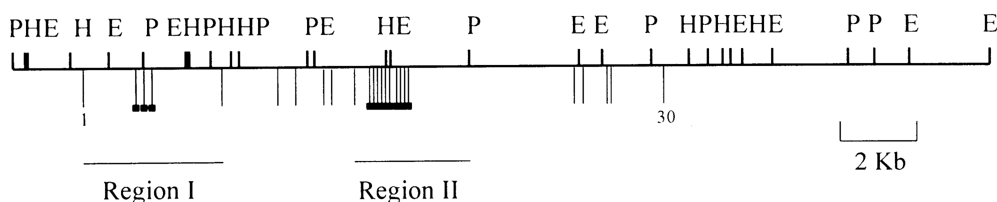
The insert of pPPY40 was mapped with three restriction enzymes and subcloned by standard molecular techniques (Ausubel *et al.* 1989; Sambrook *et al.* 1989). Although a wide range of subclones were generated, the avirulence phenotype was retained only by the clones harboring a 12.2-kb *Pst*I fragment, as indicated in Figure 1. Comparison of the restriction patterns and hybridization analysis of genomic clones pPPY41, pPPY44, pPPY45, and pPPY46 showed that they all shared this 12.2-kb *Pst*I fragment, which determined avirulence (Fig. 1).

Cosmid pPPY40 was mutagenized by the use of Tn*3*HoKmGus (Bonas *et al.* 1989). The resulting mutants were transferred into *P. s.* pv. *pisi* strains PF247 and PT10 (Moulton *et al.* 1993) and tested for loss of avirulence toward Kelvedon Wonder. Two regions of pPPY40 insert DNA were identified as being essential for the avirulence phenotype (Fig. 2A). A single insertion in either of these regions resulted in reversion to virulence in *P. s.* pv. *pisi.* The two regions (designated I and II) are defined in Figure 2 from a combination of the Tn*3*HoKmGus insertion and subcloning data. Clones in pLAFR3 generated from the two regions (pPPY60, with a 2.1-kb *Eco*RI fragment containing most of region I, and pPPY61, with a 4.3-kb *Pst*I fragment containing region II) were individually unable to confer avirulence on *P. s.* pv. *pisi* (Fig. 1). Despite numerous attempts, we were unable to transfer the transposons which caused a loss of the avirulence phenotype into strains of *P. s.* pv. *phaseolicola* by marker exchange. This suggests that the mutagenized loci may have a vital function in the bean pathogen.

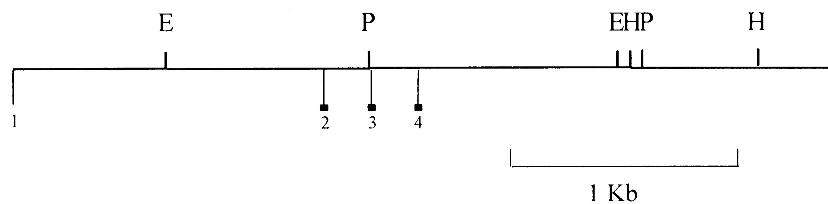
Plasmid DNA recovered from *P. s.* pv. *phaseolicola* isolate 1302A by alkaline lysis and phenol extraction (Moulton *et al.* 1993) was probed with pPPY40 and internal fragments. Results obtained confirmed that regions I and II are derived from a *P. s.* pv. *phaseolicola* plasmid of approximately 150 kb.

In conclusion, it is clear that we have isolated DNA from a *P. s.* pv. *phaseolicola* plasmid that confers on isolates of *P. s.* pv. *pisi* avirulence toward pea. Avirulence is controlled by two loci within pPPY40, which together appear to interact with a single dominant resistance gene in Kelvedon Wonder, the first gene for nonhost resistance identified in pea. Our results therefore suggest the operation of a "genes-for-gene" interaction between *P. s.* pv. *phaseolicola* and pea, a nonhost of this pathovar.

A. pPPY40



B. Region I detail



Region II detail

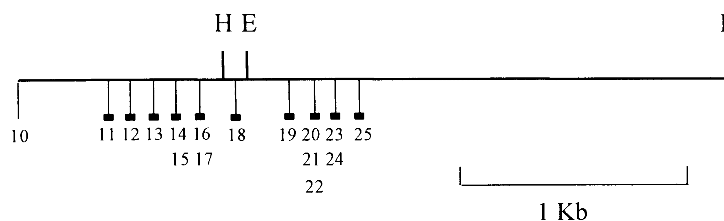


Fig. 2. Regions that determine avirulence located in pPPY40 by transposon mutagenesis. **A,** Restriction map of pPPY40 showing Tn*3*HoKmGus insertions. **B,** Enlargements of regions I and II, which are involved in the avirulence phenotype. The recognition sites for *Eco*RI, *Hin*dIII, and *Pst*I are indicated by E, H, and P, respectively. Tn*3*HoKmGus insertions are sequentially numbered 1 to 30. The vertical lines with squares represent the position of insertions that caused a loss of the avirulent phenotype conferred by pPPY40; other vertical lines represent the position of insertions that did not affect this phenotype.

ACKNOWLEDGMENTS

We wish to thank Jane Fillingham and Paul Hunter for helpful discussions and assistance with some of the plant experiments. This work was supported by the Agricultural and Food Research Council and was carried out under Ministry of Agriculture, Fisheries and Food license numbers 678/84 (72) and PHF1227B/361/23 issued under the Plant Pests (Great Britain) Order 1980.

LITERATURE CITED

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. 1989. Short Protocols in Molecular Biology. Greene Publishing Associates and Wiley Interscience, New York.

Bonas, U., Stall, R. E., and Staskawicz, B. J. 1989. Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*. Mol. Gen. Genet. 218:127-136.

Carney, B. F., and Denny, T. P. 1990. A cloned avirulence gene from *Pseudomonas solanacearum* determines incompatibility on *Nicotiana tabacum* at the host species level. J. Bacteriol. 172:4836-4843.

Crute, I. R. 1985. The genetic bases of relationships between microbial parasites and their hosts. Pages 80-142 in: Mechanisms of Resistance to Plant Disease. R. S. S. Fraser, ed. Martinus Nijhoff and W. Junk, Dordrecht, Netherlands.

Dangl, J. L., Ritter, C., Gibbon, M. J., Mur, L. A. J., Wood, J. R., Goss, S., Mansfield, J., Taylor, J. D., and Vivian, A. 1992. Functional homologs of the *Arabidopsis RPM1* disease resistance gene in bean and pea. Plant Cell 4:1359-1369.

Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on plasmid functions provided *in trans*. Proc. Natl. Acad. Sci. USA 76:1648-1652.

Fillingham, A. J., Wood, J., Bevan, J. R., Crute, I. R., Mansfield, J. W., Taylor J. D., and Vivian, A. 1992. Avirulence genes from *Pseudomonas syringae* pathovars *phaseolicola* and *pisi* confer specificity towards both host and non-host species. Physiol. Mol. Plant Pathol. 40: 1-15.

Flor, H. H. 1955. Host-parasite interactions in flax rust—Its genetics and other implications. Phytopathology 45:680-685.

Heath, M. C. 1991. The role of gene-for-gene interactions in the determination of host species specificity. Phytopathology 81:127-130.

Jenner, C., Hitchin, E., Mansfield, J., Walters, K., Betteridge, P., Teverson, D., and Taylor, J. 1991. Gene-for-gene interactions between *Pseudomonas syringae* pv. *phaseolicola* and *Phaseolus*. Mol. Plant-Microbe Interact. 4:553-562.

Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annu. Rev. Genet. 24:447-463.

Keen, N. T., Tamaki, S., Kobayashi, D., Gerhold, D., Stayton, M., Shen, H., Gold, S., Lorang, J., Thordal-Christensen, H., Dahlbeck, D., and Staskawicz, B. 1990. Bacteria expressing avirulence gene D produce a specific elicitor of the soybean hypersensitive reaction. Mol. Plant-Microbe Interact. 3:112-121.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44: 301-337.

Kobayashi, D. Y., Tamaki, S. J., and Keen, N. T. 1989. Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean. Proc. Natl. Acad. Sci. USA 86:157-161.

Lorang, J. M., Shen, H., Kobayashi, D., and Keen, N. T. 1994. The role of *Pseudomonas syringae* pv. *tomato* avirulence genes in virulence and host range. Mol. Plant-Microbe Interact. (In press.)

Malik, A. N., Vivian, A., and Taylor, J. D. 1987. Isolation and partial characterization of three classes of mutant in *Pseudomonas syringae* pv. *pisi* with altered behaviour towards their host, *Pisum sativum*. J. Gen. Microbiol. 133:2393-2399.

Moulton, P. J., Vivian, A., Hunter, P. J., and Taylor, J. D. 1993. Changes in cultivar-specificity toward pea can result from transfer of plasmid RP4 and other incompatibility group P1 replicons to *Pseudomonas syringae* pv. *pisi*. J. Gen. Microbiol. 139:3149-3155.

Ronald, P. C., Salmeron, J. M., Carland, F. M., and Staskawicz, B. J. 1992. The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J. Bacteriol. 174: 1604-1611.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Staskawicz, B. J., Dahlbeck, D., and Keen, N. T. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. USA 81:6024-6028.

Swarup, S., Yang, Y., Kingsley, M. T., and Gabriel, D. W. 1992. A *Xanthomonas citri* pathogenicity gene, *pthA,* pleiotropically encodes gratuitous avirulence on nonhosts. Mol. Plant-Microbe Interact. 5:204-213.

Vivian, A., and Mansfield, J. 1993. A proposal for a uniform genetic nomenclature for avirulence genes in phytopathogenic pseudomonads. Mol. Plant-Microbe Interact. 6:9-10.

Vivian, A., Atherton, G. T., Bevan, J. R., Crute, I. R., Mur, L. A. J., and Taylor, J. D. 1989. Isolation and characterization of cloned DNA conferring specific avirulence in *Pseudomonas syringae* pv. *pisi* to pea (*Pisum sativum*) cultivars, which possess the resistance allele, R2. Physiol. Mol. Plant Pathol. 34:335-344.

Whalen, M. C., Stall, R. E., and Staskawicz, B. J. 1988. Characterization of a gene from a tomato pathogen determining hypersensitive resistance in non-host species and genetic analysis of this resistance in bean. Proc. Natl. Acad. Sci. USA 85:6743-6747.

Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell 3:49-59.

Whalen, M. C., Wang, J. F., Carland, F. M., Heiskell, M. E., Dalhbeck, D., Minsavage, G. V., Jones, J. B., Scott, J. W., Stall, R. E., and Staskawicz, B. J. 1993. Avirulence gene *avrRxv* from *Xanthomonas campestris* pv. *vesicatoria* specifies resistance on tomato line Hawaii 7998. Mol. Plant-Microbe Interact. 6:616-627.