Interaction of *Xanthomonas campestris* with *Arabidopsis thaliana*: Characterization of a Gene from *X. c.* pv. *raphani* That Confers Avirulence to Most *A. thaliana* Accessions

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Infiltration of leaves of *Arabidopsis thaliana* accession Columbia with *Xanthomonas campestris* pathovar *campestris* leads to bacterial growth and disease symptoms reminiscent of those incited in *Brassica* plants inoculated under the same conditions. A search among *A. thaliana* accessions for variation in the reaction phenotype to strains of *X. campestris* pathovars *campestris, aberrans*, and *raphani* showed that there were no clear differential responses between plant accessions to the individual bacterial strains tested. *X. c.* pv. *raphani* strain 1067 was avirulent to all *A. thaliana* accessions tested. A gene was cloned from *X. c.* pv. *raphani* 1067 which, when transferred into the virulent *X. c.* pv. *campestris* strain 8004, strongly reduced symptom development and bacterial growth in *A. thaliana* Columbia plants but did not affect virulence to *Brassica* plants. The gene (denoted *avrXca*) interacted with all *A. thaliana* accessions tested except one, Kas-1, which developed disease symptoms and supported growth of the transconjugant to levels similar to those with *X. c.* pv. *campestris* 8004 alone. Sequence analysis of *avrXca* revealed a probable open reading frame encoding a protein of 66,566 Da that has no homology with other known sequences. A sequence motif conserved among *hrp* genes was identified in the 5′ noncoding region of *avrXca*, and features characteristic of a signal peptide were found in the N-terminal portion of the presumed AvrXca protein. DNA from different phytopathogenic bacteria contained sequences hybridizing with *avrXca* in related *X. campestris* pathovars but not in *Erwinia* or *Pseudomonas* strains.

Additional keywords: crucifer, disease resistance, pathogenesis.

A major goal in plant pathology is to understand the molecular basis of specificity in plant-pathogen interactions. Classical genetic studies have established gene-for-gene relationships in several host-pathogen systems in which the expression of resistance is controlled by matching pairs of loci in the pathogen and host (Flor 1971; Ellingboe 1981). These loci condition resistance in the plant and avirulence in the pathogen (Keen 1990), and their interaction, either directly or indirectly, initiates a rapid defense response that prevents successful establishment of the pathogen in the plant tissue.

Avirulence genes have been cloned from several pathogenic bacteria that infect crop plants (Staskawicz *et al.* 1984; Gabriel *et al.* 1986; Bonas *et al.* 1988; Ronald and Staskawicz 1988; Swanson *et al.* 1988; Hitchin *et al.* 1989; Vivian *et al.* 1989; Kelemu and Leach 1990; De Feyter and Gabriel 1991; Jenner *et al.* 1991). Also, single avirulence genes have been used to test the predictions of the genetic models (Minsavage *et al.* 1990) and have identified hitherto unknown corresponding resistance genes in different host cultivars (Herbers *et al.* 1992) and in nonhost plants (Kobayashi *et al.* 1989; Whalen *et al.* 1988, 1991; Fillingham *et al.* 1992). The biochemical function of avirulence gene products remains obscure, although recent work shows that an avirulence gene (*avrD*) from *Pseudomonas syringae* pathovar *tomato* acts by producing a low molecular weight extracellular elicitor, which is then specifically recognized by the plant (Keen *et al.* 1990; Keen and Buzzell 1991).

Genetically defined plant genes conditioning specific resistance to pathogens are believed to function in initial host-pathogen recognition processes, and they are likely to be constitutively expressed. Moreover, since there is no knowledge of their protein products, direct cloning strategies are precluded. An alternative and potentially powerful strategy is to use map-based cloning to isolate a gene that is defined only by its phenotype. Thus, a phenotypic mutant or natural variant can be mapped to a chromosomal location by following its segregation relative to genetic and polymorphic DNA markers. In this respect, the small cruciferous weed *Arabidopsis thaliana* (L.) Heynh. is proving to be the organism of choice for molecular genetic studies on plant development (Meyerowitz 1989; Konz *et al.* 1992).

Recent reports show that *A. thaliana* can be infected by the bacterial phytopathogens *Pseudomonas syringae* (Davis *et al.* 1991; Debener *et al.* 1991; Dong *et al.* 1991; Whalen *et al.* 1991) and *Xanthomonas campestris* (Simpson and Johnson 1990; Daniels *et al.* 1991; Tsuji *et al.* 1991; Parker *et al.*, in press). Natural variation in the reaction

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phenotype among different *A. thaliana* accession lines (ecotypes) was observed, suggesting that resistance genes may be identified genetically. Also, two avirulence genes have been isolated from avirulent strains of *P. s.* pv. *tomato* (Dong *et al.* 1991; Whalen *et al.* 1991) and from *P. s.* pv. *maculicola* (Debener *et al.* 1991) that are specifically recognized only by certain *A. thaliana* accessions. *A. thaliana* may therefore be a useful model plant for the isolation of resistance genes, using the molecular genetic tools so applicable to this organism.

In this study we found that *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot of crucifers (Williams 1980), caused disease symptoms on *A. thaliana* reminiscent of those incited on turnip (*Brassica campestris*) plants. A search was undertaken among different *A. thaliana* accessions for variation in the reaction phenotype to strains of *X. c.* pv. *campestris* and to other crucifer-infecting *X. campestris* pathovars. Most plant-pathogen combinations resulted in disease, but several "incompatible" interactions were identified. We describe the isolation of a gene from an avirulent *X. c.* pv. *raphani* strain that converts a normally pathogenic *X. c.* pv. *campestris* strain to avirulence in *A. thaliana* Columbia (Col-0) plants. The avirulence gene was recognized by all other *A. thaliana* accessions tested except one, Kas-1. In this accession, bacteria harboring the gene grew and produced disease symptoms similar to the virulent parent *X. c.* pv. *campestris* strain. The data provide the basis for a genetic analysis of a putative resistance gene in *A. thaliana* Col-0 recognizing a single defined *X. c.* pv. *raphani* avirulence gene.

RESULTS

Infection of *A. thaliana* by *X. c.* pv. *campestris*.

The host range of *X. c.* pv. *campestris* includes essentially all cultivated brassicas tested and some other crucifers (Williams 1980). In preliminary experiments, the standard *X. c.* pv. *campestris* laboratory strain 8004 was found to cause disease symptoms on *A. thaliana* Col-0 plants similar to those incited on turnip plants (*B. campestris* 'Just Right'). Several inoculation methods were tested, including dipping leaves into concentrated bacterial suspensions and wound-inoculating (Simpson and Johnson 1990). Infiltration through the underside of the leaf produced the clearest and most consistent symptom development and so was used in further experiments. Infiltration of leaves with a suspension of 10^6 colony forming units (cfu)/ml produced chlorosis in the inoculated area after 3 days, followed by necrosis after 5 to 6 days (Fig. 1). The chlorotic zone was seen to spread only to adjacent uninoculated parts of the leaf. Numbers of viable bacteria recovered from inoculated leaves increased by 100- to 1,000-fold over a period of 4 to 6 days (Fig. 2A). Bacteria were not recovered from uninoculated leaves for up to 10 days, indicating that the infection was not systemic. Also, seeds produced from

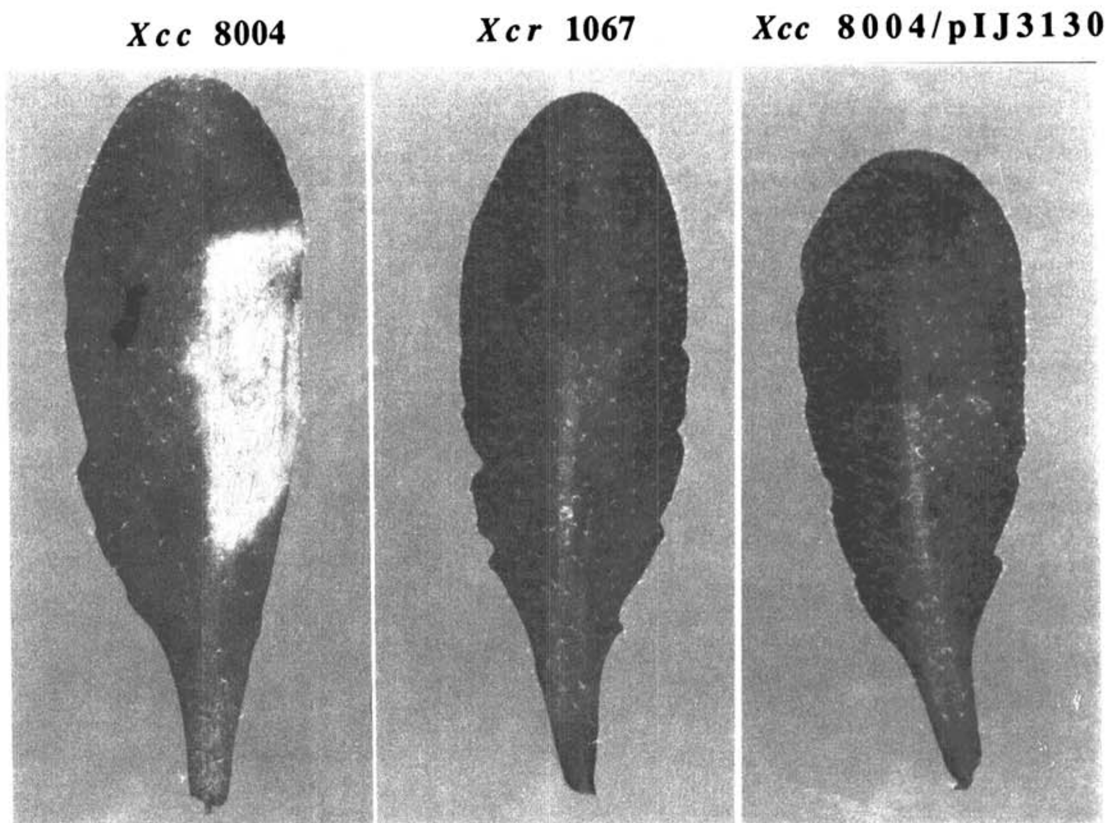

Fig. 1. Symptom expression of *Arabidopsis thaliana* Col-0 leaves 5 days after infiltration with suspensions of *Xanthomonas campestris* pv. *campestris* (Xcc) 8004, *X. c.* pv. *raphani* (Xcr) 1067, and *X. c.* pv. *campestris* 8004/pIJ3130 at 10^6 cfu/ml. Inoculation with *X. c.* pv. *campestris* 8004/pIJ3200 without insert DNA produced the same symptoms as *X. c.* pv. *campestris* 8004 alone.

infected plants were not contaminated.

Several classes of mutants of *X. c.* pv. *campestris* 8004 that are altered in pathogenicity on turnip plants were tested for their ability to cause disease on Col-0 plants. As described elsewhere (Parker *et al.*, in press), the pathogenicity of all mutants was reduced compared with the wild-type *X. c.* pv. *campestris* 8004 by the criteria of symptom development and growth in the leaf. The two most severely affected, ME-29 (Osbourn *et al.* 1990) and XchA2 (a *hrp* mutant described by Arlat *et al.* 1991), also gave no visible symptoms on turnip plants. The results indicated that the symptoms observed with *X. c.* pv. *campestris* 8004 were a consequence of pathogenesis and not a nonspecific reaction of the plant to bacterial infiltration.

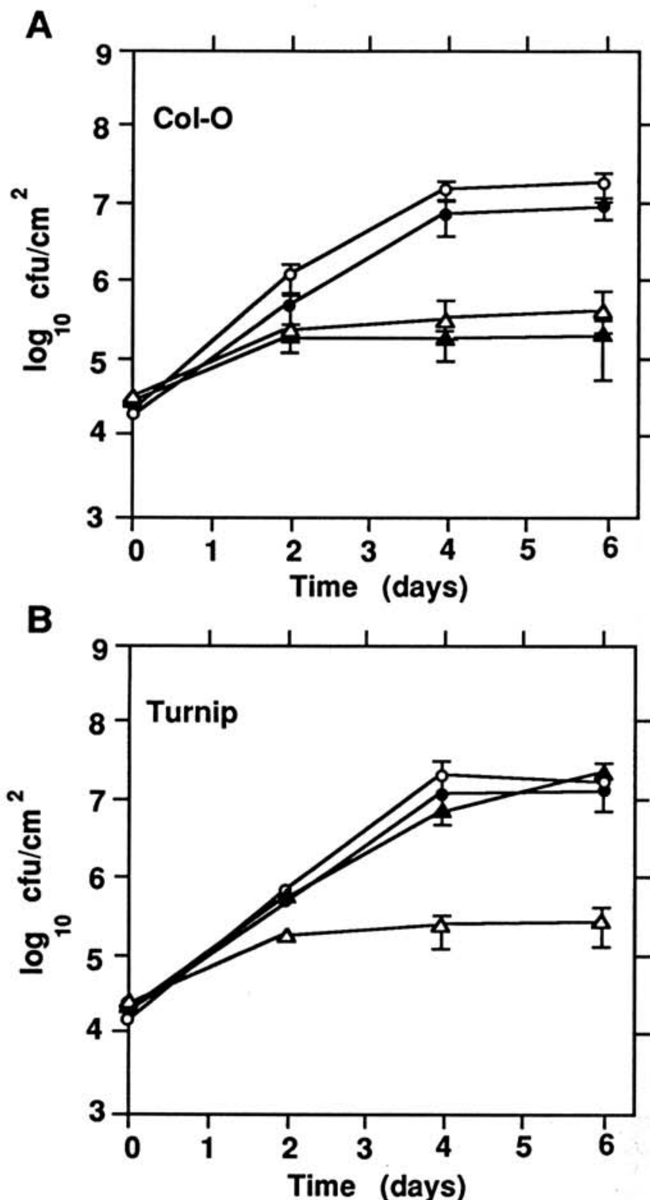

Fig. 2. Growth of *Xanthomonas campestris* pv. *campestris* 8004 (○), *X. c.* pv. *raphani* 1067 (△), *X. c.* pv. *campestris* 8004/pIJ3200 (●), and *X. c.* pv. *campestris* 8004/pIJ3130 (▲) in leaves of **A,** *Arabidopsis thaliana* Col-0 and **B,** turnip (*B. campestris* 'Just Right') after infiltration with a suspension of 10^6 cfu/ml.

Natural variation among *A. thaliana* accessions and *X. campestris* strains.

Eighteen independent wild isolates of *X. c.* pv. *campestris* and two *X. c.* pv. *aberrans* isolates were screened for natural variation in the reaction phenotype with five different *A. thaliana* accession lines (Col-0, La-*er*, Nd-0, JI-1, and 0y-0; described in Materials and Methods) using an inoculum concentration of 10^6 cfu/ml. Differences in disease symptom severity (aggressiveness) between bacterial strains were observed, but there were no clear differential responses between accessions (results not shown). Several *X. c.* pv. *raphani* strains were also tested. Strain 1946 was virulent on all ecotypes, strains 2345 and 2586 produced mild symptoms only, and strain 1067 was avirulent, as shown for Col-0 plants (Fig. 1). *X. c.* pv. *raphani* strain 1067 caused chlorosis and tissue darkening upon infiltration of turnip (cv. Just Right) plants with 10^6 cfu/ml, but symptom development was slower and less severe than with *X. c.* pv. *campestris* strain 8004 (data not shown).

Cloning an avirulence gene from *X. c.* pv. *raphani* 1067.

We postulated that the avirulence of *X. c.* pv. *raphani* 1067 results from the interaction of an avirulence gene (or genes) with resistance gene(s) in *A. thaliana.* In order to identify putative avirulence genes from *X. c.* pv. *raphani* 1067, individual clones containing 1067 genomic DNA were transferred by conjugation into the virulent strain *X. c.* pv. *campestris* 8004, and the transconjugants infiltrated into Col-0 leaves at 10^6 cfu/ml. One clone containing a 23-kb insert (denoted pIJ3130) rendered *X. c.* pv. *campestris* 8004 avirulent. Growth rate in NYG broth (described in Materials and Methods) and production of extracellular enzymes in 8004(pIJ3130) were the same as for *X. c.* pv. *campestris* 8004 (results not shown).

No symptoms were evident in Col-0 leaves inoculated with 10^6 cfu/ml of 8004(pIJ3130) compared with 8004 alone or 8004 containing the cosmid pIJ3200 without insert (Fig. 1), and growth of the transconjugant was reduced to levels comparable with *X. c.* pv. *raphani* 1067 (Fig. 2A). However, in turnip leaves 8004(pIJ3130) was as

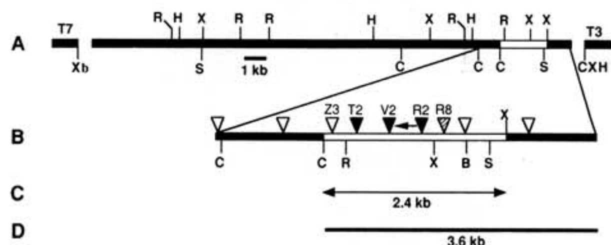

Fig. 3. Location of *Xanthomonas campestris* pv. *raphani* 1067 avirulence activity on cosmid pIJ3130 by Tn*5lac*-B20 mutagenesis. **A,** Restriction enzyme digestion map of pIJ3130. Restriction enzyme sites: R, *Eco*RI; H, *Hin*dIII; X, *Xho*I; C, *Cla*I; S, *Sst*I; Xb, *Xba*I; B, *Bam*HI. **B,** Location of Tn*5lac*-B20 insertions on an expanded view of the right end of pIJ3130. The arrow shows the direction of transcription based on β-galactosidase assays. Tn*5lac*-B20 mutants Z3, T2, V2, R2, and R8 of 8004/pIJ3130 were inoculated at a concentration of 10^6 cfu/ml onto Col-0 leaves, and disease symptoms were recorded after 3 to 5 days. ▽, Insertion with no effect on avirulence phenotype of 8004/pIJ3030; ▼, Insertion restoring virulence of 8004/pIJ3130; ▼, Insertion giving an intermediate phenotype. **C,** Portion (2.4 kb) of pIJ3130 that has been sequenced. **D,** Fragment (3.6 kb) subcloned from pIJ3130 into pIJ3200, giving pIJ3132.

virulent as *X. c.* pv. *campestris* 8004, as determined by bacterial growth in leaves (Fig. 2B) and disease symptom appearance and progression (not shown). pIJ3130 was relatively stable in bacteria *in planta*. About 8% of bacteria recovered from Col-0 or turnip leaves after 6 days had lost the tetracycline resistance marker of the cloning vector. The specificity of the avirulence effect on Col-0 plants indicated that the clone contained an avirulence gene or genes and was not, for example, a negative-acting regulatory element that depresses pathogenicity when the copy number is increased by cloning (Tang *et al.* 1990).

Restriction mapping of pIJ3130.

The restriction endonucleases *Eco*RI, *Hin*dIII, *Xho*I, *Sst*I, and *Cla*I were used to derive the map shown in Figure 3A.

Transposon mutagenesis of pIJ3130.

pIJ3130 was mutagenized with Tn*5lac*-B20 as described by Arlat *et al.* (1991). Approximately 300 mutant clones were obtained, and insertions in the 23-kb insert were located on the restriction map and their orientations deduced. When selected mutant plasmids were transferred to *X. c.* pv. *campestris* 8004, four were found to have lost the ability to confer avirulence towards *A. thaliana* Col-0. The transposon insertions were located in a cluster at the right side of pIJ3130 (Fig. 3B). Three of the four (T2, V2, and R2) caused complete loss of function, i.e., the bacteria harboring the mutant plasmids were fully virulent to Col-0. The fourth, R8, gave an intermediate phenotype. All other insertions tested had no effect on avirulence. *X. c.* pv. *campestris* 8004(pIJ3130::Tn*5lac*-V2) (avr$^-$) and 8004(pIJ3130::Tn*5lac*-Z3) (avr$^+$) were tested for

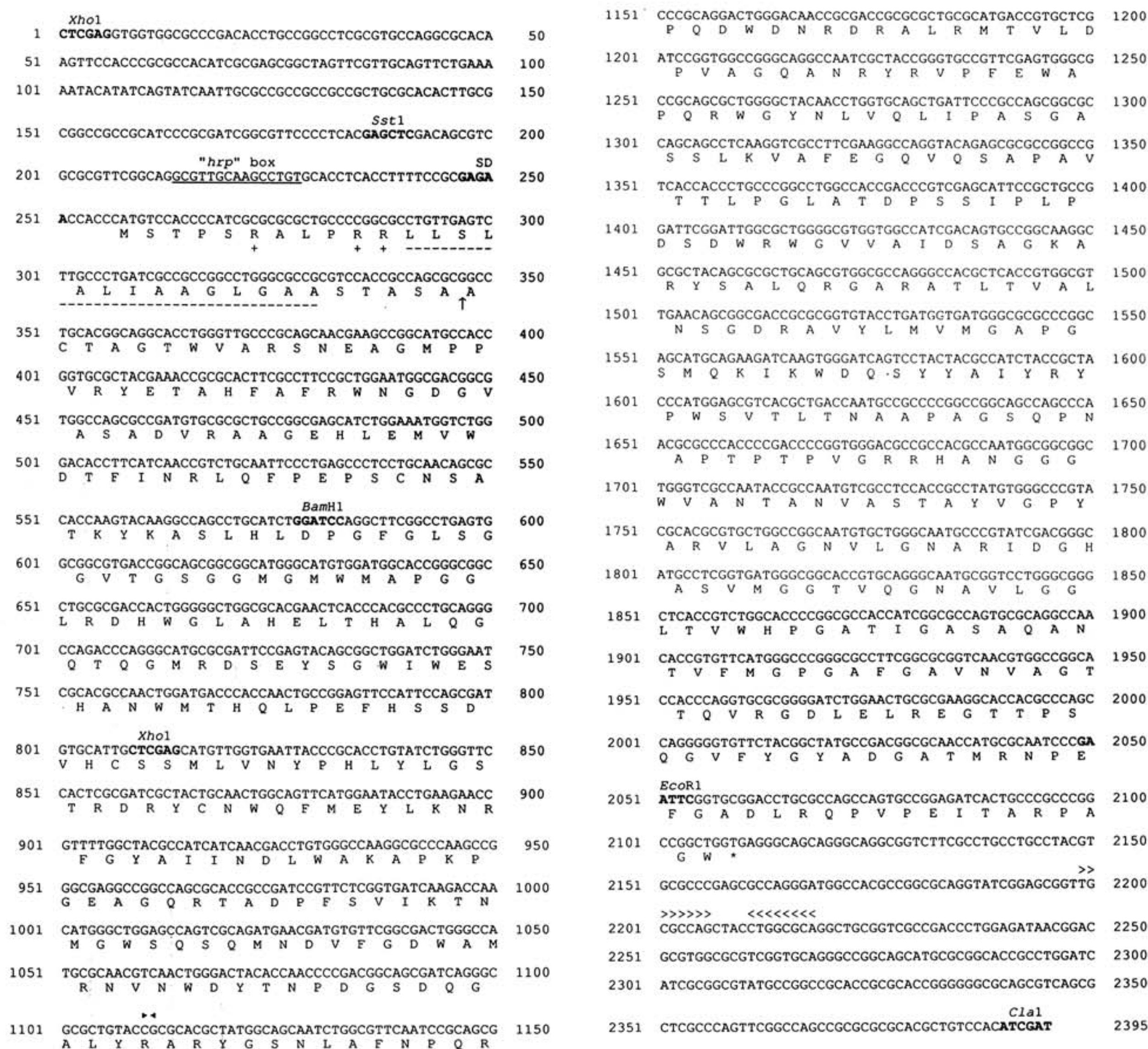

Fig. 4. Nucleotide sequence of 2.4-kb *Xho*I/*Cla*I fragment of pIJ3130 (compare Fig. 3C) and derived amino acid sequence of avirulence gene *avrXca*. The "*hrp* box" consensus motif and possible Shine-Dalgarno (SD) sequence are shown. Also, three basic arginine residues (+) preceding 14 hydrophobic amino acids (−) and a consensus bacterial peptidase recognition site (↑) are indicated. The position of Tn*5lac*-B20 insertion R2 (compare Fig. 3B) is also shown (▶◀). The arrowheads indicate a possible transcription termination site.

growth in Col-0 plants. The mutant plasmid Z3 gave reduced growth similar to wild-type pIJ3130, whereas bacteria carrying the mutant plasmid V2 grew to levels similar to the control *X. c.* pv. *campestris* 8004(pIJ3200) (data not shown).

The insertions R8, R2, V2, and T2 were oriented such that transcription from right to left (as drawn in Fig. 3B) would give β-galactosidase activity. In three independent experiments, β-galactosidase levels of 350–500 units (Miller 1972) were obtained. Such levels indicate active transcription in *X. c.* pv. *campestris* (Tang *et al.* 1991). β-Galactosidase levels were unaffected by growth of bacteria in complete or minimal media (Arlat *et al.* 1991) and were similar when the plasmids were introduced into *X. c.* pv. *campestris* 8420, which carries a 20-kb deletion in the *hrp* gene cluster (Liddle 1992). The mutant plasmids were used for localized mutagenesis of the genome of *X. c.* pv. *raphani* 1067, giving mutants defective in the indigenous gene by marker exchange (Turner *et al.* 1985). The behavior of mutants was indistinguishable from that of wild type *X. c.* pv. *raphani* 1067 in both turnip and Col-0.

Sequencing.

The nucleotide sequence was determined of the 2.4-kb *Cla*I-*Xho*I fragment (Fig. 3C), which was thought from the mutagenesis data to contain the putative avirulence gene. Analysis of the sequence with the FRAME program (Bibb *et al.* 1984) showed the presence of an open reading frame (ORF) of 1,851 bp, which would encode a protein of 617 amino acid residues and a mass of 66,566 Da (Fig. 4). This ORF has the same orientation as the avirulence gene deduced above. Since no other potential ORFs were detected in the region, we think that it represents the avirulence gene, which has been designated *avrXca*. Partial sequencing of DNA from pIJ3130::Tn*5lac*-R2 verified that the mutation lies within the coding region (Fig. 4). A "*hrp* box" consensus sequence (Fellay *et al.* 1991) was found approximately 40 bp upstream of the presumed start codon (Fig. 4). Also, the N-terminal region of the protein contains a sequence of 14 hydrophobic amino acid residues preceded by three basic residues and followed by a consensus bacterial peptidase recognition site (Sjöström *et al.* 1987). The sequence of *avrXca* has been deposited in the EMBL database with the accession number M99059.

Presence of *avrXca*-related sequences in *Xanthomonas*.

A 1.2-kb *Eco*RI-*Xho*I DNA fragment from pIJ3130 that lies wholly within the presumed coding region of *avrXca* (Figs. 3B and 4) was used as a hybridization probe against Southern blots of *Bam*HI- and *Eco*RI-digested DNA of a range of *Xanthomonas* strains and other phytopathogenic bacteria. Most of the *Xanthomonas* strains with the exception of *X. campestris* pathovars *graminis* and *holcicola* showed hybridizing bands, although some polymorphism was apparent (Fig. 5). Members of other genera showed no hybridization.

Subcloning of the avirulence gene.

To confirm that the sequenced region of pIJ3130 contained the avirulence gene, a 3.6-kb *Cla*I fragment (Fig. 3D) was subcloned into pIJ3200 to give pIJ3132. When pIJ3132 was introduced into *X. c.* pv. *campestris* 8004, the bacterium became avirulent to Col-0, with concomitant reduction in growth *in planta* (Fig. 6A). Virulence to turnip was unaffected (results not shown).

Interaction of *avrXca* with other *A. thaliana* accessions.

X. c. pv. *campestris* 8004 containing either pIJ3132 or the vector pIJ3200 was inoculated at 10^6 cfu/ml into leaves of 32 *A. thaliana* accessions and some *Brassica* lines. In most, *X. c.* pv. *campestris* 8004(pIJ3200) was virulent, whereas 8004(pIJ3132) was avirulent to *A. thaliana*. However, accession Kas-1 was susceptible to 8004(pIJ3132), and there was little difference in the growth of the two bacterial strains in this accession (Fig. 6B). The two bacterial strains were equally virulent on *B. campestris* 'Golden Ball,' 'Green Top Stone,' and 'Snowball'; *B. napus* 'Westar,' 'Maris Haplona,' and 'Cobra'; and *Raphanus sativus* 'Scarlet Globe,' 'Sparkler,' and 'Champion' (data not shown).

DISCUSSION

Results presented in this paper and by others (Simpson and Johnson 1990; Tsuji *et al.* 1991) show that the interaction of *A. thaliana* and *X. campestris* can serve as a useful model system for the analysis of recognition and signalling components both in the pathogen and the plant.

In this study, clear differences among *A. thaliana* accession lines in their response to independent wild isolates of *X. c.* pv. *campestris* were not observed. In contrast, Tsuji *et al.* (1991) identified an *X. c.* pv. *campestris* strain that incited chlorosis in *A. thaliana* Pr-0 plants but was symptomless in Col-0 plants, even though it grew to the same extent in both lines. The resistance or "tolerance" reaction of Col-0 was attributed to the presence of a single dominant locus. The overall low frequency of variation in the *A. thaliana* reaction phenotype to *X. c.* pv. *campestris* may reflect the natural mode of *X. c.* pv. *campestris* pathogenesis. The bacterium normally invades the xylem

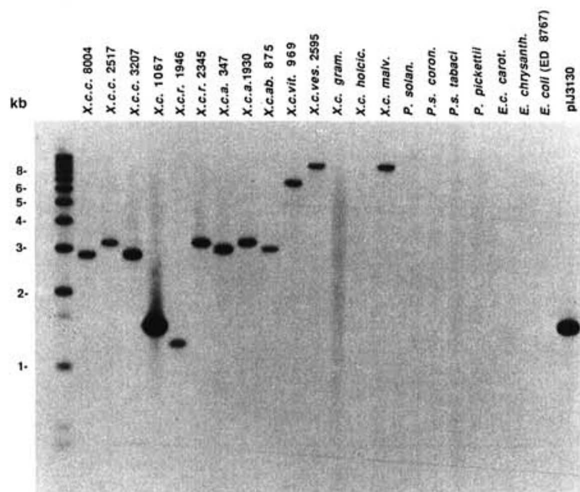

Fig. 5. Southern blot analysis of *Eco*RI/*Bam*HI-digested genomic DNA isolated from different phytopathogenic bacteria probed with a 1.2-kb *Eco*RI/*Xho*I internal fragment (see Fig. 3B) of *avrXca*.

vessels (Shaw and Kado 1988; Bretschneider *et al.* 1989), whereas plant defense mechanisms have been studied most extensively in metabolically active nonvascular tissues. However, a "vascular hypersensitive response" to incompatible *X. campestris* strains has been described (Kamoun *et al.* 1992), although the phenomenon has not been studied in detail. In this respect, it is perhaps significant that resistance in *A. thaliana* was found only with an *X. c.* pv. *raphani* strain (described in this study) and an *X. c.* pv. *armoraciae* strain (Parker *et al.*, in press). These pathovars cause leaf spot diseases and, although closely related to *X. c.* pv. *campestris*, readily invade the leaf

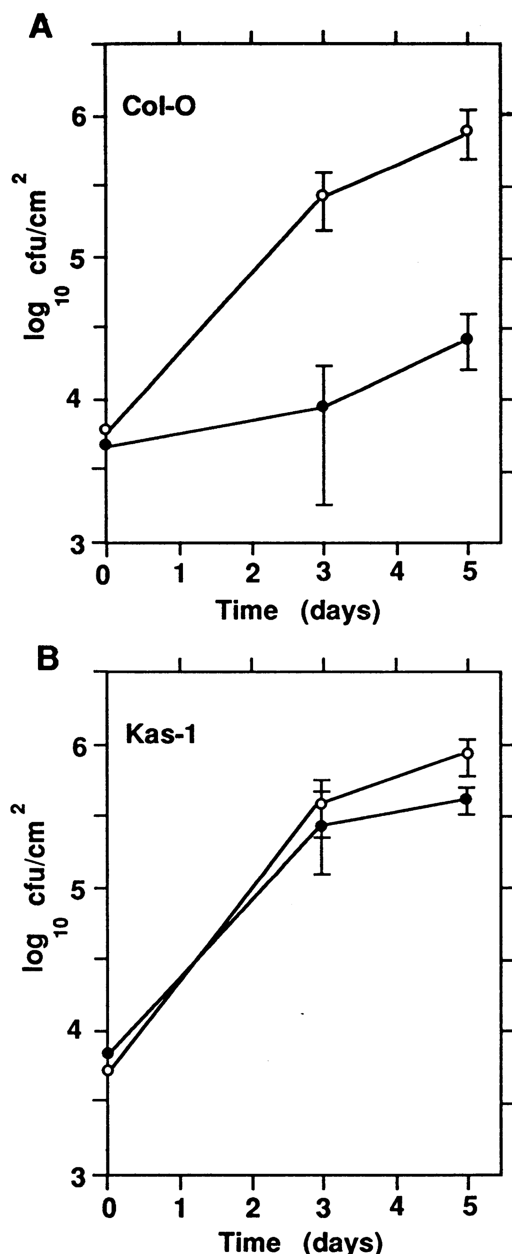

Fig. 6. Growth of *Xanthomonas campestris* pv. *campestris* 8004/pIJ3200 (○) and *X. c.* pv. *campestris* 8004/pIJ3132 (●) (8004 containing pIJ3200 with 3.6-kb *Cla*I fragment derived from pIJ3130; compare Fig. 3B) in *Arabidopsis thaliana* Col-0 **A,** and Kas-1 **B,** plants.

mesophyll tissue through the stomata (Hunter *et al.* 1987; Kamoun *et al.* 1992).

A genomic DNA clone isolated from *X. c.* pv. *raphani* 1067 rendered *X. c.* pv. *campestris* 8004 avirulent to *A. thaliana* Col-0 and most other *A. thaliana* accessions but did not affect virulence of *X. c.* pv. *campestris* 8004 to turnip and other *Brassica* plants tested. The genotype specificity of this interaction leads us to conclude that we have identified an avirulence gene, which has been designated *avrXca*.

Interestingly, inoculation of Col-0 plants with high concentrations of *X. c.* pv. *campestris* 8004 containing *avrXca* did not incite rapid plant cell necrosis characteristic of a hypersensitive response, which is commonly observed in race- or cultivar-specific and nonhost incompatible reactions (Keen 1990). Hypersensitive plant cell death has also been shown in *A. thaliana* plants in response to two different avirulence genes isolated from *P. s.* pv. *tomato* (Dong *et al.* 1991; Whalen *et al.* 1991) and from *P. s.* pv. *maculicola* (Debener *et al.* 1991).

Conjugation of *avrXca* into a virulent *X. c.* pv. *armoraciae* strain produced the same phenotype as *X. c.* pv. *campestris* 8004/*avrXca* on Col-0 and turnip plants (C. E. Barber, J. E. Parker, and M. J. Daniels, unpublished). This suggests that the attenuation of disease symptoms and bacterial growth without concomitant visible plant cell necrosis is intrinsic to *avrXca* function and not an effect of the recipient *X. campestris* strain in which it is expressed. Thus, it appears that the reaction of *A. thaliana* observed with *avrXca* is a novel resistance response quite distinct from hypersensitivity and also different from the tolerance phenomenon described previously (Tsuji *et al.* 1991). Sequence analysis of *avrXca* did not reveal homology with known sequences in the data bases at the nucleotide or amino acid level. A perfect *hrp* box consensus motif (Fellay *et al.* 1991) was found approximately 40 bp upstream of the putative start codon. The *hrp* box, defined only by sequence, has been found upstream of *P. syringae* pv. *phaseolicola hrp* operons, which require *hrpR* and *S* for expression (Fellay *et al.* 1991), and certain *P. syringae* avirulence genes (Tamaki *et al.* 1988; Kobayashi *et al.* 1990; J. M. Salmeron and B. J. Staskawicz, personal communication), but its function is unknown.

Expression of *avrXca* determined with β-galactosidase fusions was apparently not dependent on functional *hrp* genes, or on the nutritional status of the bacteria, in contrast to *avrB* of *P. s.* pv. *glycinea* (Huynh *et al.* 1989) and *avrPto* from *P. s.* pv. *tomato* (J. M. Salmeron and B. J. Staskawicz, personal communication). However, a more detailed analysis of its mode of expression at the RNA and protein level will need to be performed to clarify this. The N-terminal portion of the *avrXca* product has the features of a prokaryotic signal peptide (Sjöström *et al.* 1987). This suggests translocation of the protein across the bacterial inner membrane into the periplasmic space or possible secretion to the external medium. This would facilitate direct interaction of the *avrXca* product with plant cells. Experiments are in progress to determine whether the product is processed and exported. There was no evidence that products of other characterized avirulence genes were secreted from bacteria. Sequences hybridizing

to *avrXca* were identified in other strains of *X. campestris*, including *X. c.* pv. *campestris* 8004, but not in *Pseudomonas* or *Erwinia* species. The presence of hybridizing DNA in *X. c.* pv. *campestris* 8004 suggests that it contains an allele of *avrXca* that is inactive by the criteria of inhibition of symptom development and bacterial growth in Col-0 plants. Isolation and characterization of the gene from *X. c.* pv. *campestris* 8004 would help elucidate its function. Marker exchange of a mutant *avrXca*::Tn*5* allele into the wild-type strain *X. c.* pv. *raphani* 1067 did not increase its virulence towards Col-0 or turnip plants. This may be due to the presence of additional avirulence genes in *X. c.* pv. *raphani* 1067 that are recognized by these plants. Alternatively, it is possible that mutation of *avrXca*, as well as destroying the avirulence function, reduces pathogenic fitness. This has been observed with *avrBs2*, an avirulence gene isolated from *X. c.* pv. *vesicatoria* (Kearney and Staskawicz 1990). The low overall virulence of *X. c.* pv. *raphani* 1067 on all *A. thaliana* and *Brassica* plants tested so far suggests it may be deficient in some other virulence function that would make it difficult to test these possibilities in *X. c.* pv. *raphani* 1067.

The near-isogenic nature of *X. c.* pv. *campestris* 8004 and the transconjugant containing only *avrXca* allowed us to search for sensitive *A. thaliana* accessions that specifically do not recognize this gene. A search among 32 accessions for disease symptom expression in response to *X. c.* pv. *campestris* 8004/*avrXca* revealed only one, Kas-1, which consistently showed symptoms and supported growth to levels similar to *X. c.* pv. *campestris* 8004 containing only the cosmid vector. Analysis of segregating populations derived from crosses between Col-0 and Kas-1 plants will be performed to determine the inheritance of resistance as a first step towards understanding the genetic basis of resistance in *A. thaliana* to *X. campestris*.

MATERIALS AND METHODS

Bacterial cultures.

X. c. pv. *campestris* strain 8004 has been described previously (Daniels *et al.* 1984). *X. c.* pv. *campestris* strains 240, 404, 528, 529, 1025, 1129, 1143, 1146, 1147, 1685, 1711, 2031, 2517, 3207, 3290; *X. c.* pv. *aberrans* strains 875 and 2986; and *X. c.* pv. *raphani* strains 1067, 1946, 2345, and 2586 were obtained from The National Collection of Plant Pathogenic Bacteria (Harpenden, U.K.). *X. c.* pv. *campestris* strains 2669 and A were a gift from R. Stall (University of Florida, Gainesville). The pathovar status of strain 1067 was confirmed as *raphani* by fatty acid profiling (performed by R. Stall). A spontaneous rifampin-resistant mutant of *X. c.* pv. *raphani* strain 1067 was obtained for laboratory experiments. Bacteria were cultured at 30° C on nutrient (NYG) agar (Turner *et al.* 1984) containing rifampin (50 μg/ml) and tetracycline (5 μg/ml) as appropriate.

Plant material and cultivation.

A. thaliana accessions Aa-0, Al-0, Be-0, Bla-10, Bla-12, Bur-0, Ge-1, Hs-0, Kas-1, Kil-0, Li-6, Ll-0, Ms-0, Oy-0, No-0, Po-1, Se-0, Sy-0, Tu-1, and Zü-1 were obtained from the Arabidopsis Information Service (Kranz and Kirchheim 1987). Accessions Columbia (Col-0), Landsberg-*erecta* (La-*er*), and Ws-0 were given by C. Dean (Cambridge Laboratory, Norwich, U.K.); Fe-1, Hi-0, Per-C, and Tsu-0 by E. Holub (Horticultural Research International, East Malling, U.K.); Bch-1, Di-0, and Pr-0 by S. Somerville (Michigan State University, East Lansing); and RLD by A. Slusarenko (University of Zürich, Switzerland). JI-1 is a local isolate. Seeds were sown in a 3:1:1 mixture of John Innes no.1 compost, vermiculite, and chick grit and allowed to germinate under an 8-hr light period in a growth chamber at 23–24° C and 75% relative humidity. Plants were illuminated at 150–200 $\mu E \cdot s^{-1} \cdot m^{-2}$ for 8 hr each day. Under these short-day conditions, leaf development was promoted and flowering delayed. This was necessary to provide sufficient leaf material to inoculate and because early results showed that the response of plants to bacterial infection was not consistent once flowering had been initiated. Individual seedlings were transferred to 4 × 4 cm pots after 3 wk and used for inoculation after a further 3–4 wk. *B. campestris, B. napus*, and *R. sativus* plants were grown in the glasshouse at 20–25° C under a 16-hr light period as described previously (Conrads-Strauch *et al.* 1990). Plants 4- to 5-wk old were taken for bacterial inoculations.

Inoculation of plants and bacterial growth curves.

Fresh overnight cultures of bacteria (grown with tetracycline for strains containing pIJ3200 derivatives) were harvested by centrifugation, and the bacteria were resuspended in 10 mM $MgCl_2$ at 10^8 cfu/ml. Dilutions of the suspensions were made to give 10^7 and 10^6 cfu/ml. The bacterial suspensions were infiltrated into one half of fully expanded *A. thaliana* leaves using a 1-ml plastic syringe pressed to the leaf underside, and the inoculated leaves were marked with nontoxic ink. Symptoms were scored daily.

The concentration of viable bacteria in inoculated leaves was determined by punching 0.2-cm diameter disks from the infected area of four or five individual leaves from each plant. The combined disks were homogenized in distilled water, and 10-fold dilutions were plated on NYG-rifampin agar. Uninoculated leaves were also tested for the systemic spread of bacteria from the inoculation site.

Recombinant DNA techniques.

Standard methods were used for DNA subcloning, restriction mapping, gel electrophoresis, and Southern blotting (Maniatis *et al.* 1982). A cosmid library was prepared with DNA from *X. c.* pv. *raphani* 1067 partially digested with *Sau*3A, enriched for 20- to 30-kb fragments, and cloned into the *Bam*HI site of pIJ3200 (Liu *et al.* 1990), essentially as described by Daniels *et al.* (1984). The library was maintained and transferred by conjugation as described by Daniels *et al.* (1984).

Mutagenesis with Tn*5lac* and assay of β-galactosidase were performed as described by Arlat *et al.* (1991). The chain termination method was used for DNA sequencing (Sanger *et al.* 1977) with nested deletion templates (Henikoff 1984).

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LITERATURE CITED

Arlat, M., Gough, C. L., Barber, C. E., Boucher, C., and Daniels, M. J. 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 4:593-601.

Bibb, M. J., Findlay, P. R., and Johnson, M. W. 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein coding sequences. Gene 30:157-166.

Bonas, U., Stall, R. E., and Staskawicz, B. 1988. Genetic and structural characterization of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*. Mol. Gen. Genet. 218:127-136.

Bretschneider, K. E., Gonella, M. P., and Robeson, D. J. 1989. A comparative light and electron microscopical study of compatible and incompatible interactions between *Xanthomonas campestris* pv. *campestris* and cabbage (*Brassica oleracea*). Physiol. Mol. Plant Pathol. 34:285-297.

Conrads-Strauch, J., Dow, J. M., Milligan, D. E., Parra, R., and Daniels, M. J. 1990. Induction of hydrolytic enzymes in *Brassica campestris* in response to pathovars of *Xanthomonas campestris*. Plant Physiol. 93:238-243.

Daniels, M. J., Barber, C. E., Turner, P. C., Sawczyc, M. K., Byrde, R. J. W., and Fielding, A. H. 1984. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. EMBO J. 3:3323-3328.

Daniels, M. J., Fan, M. J., Barber, C. E., Clarke, B. R., and Parker, J. E. 1991. Interaction between *Arabidopsis thaliana* and *Xanthomonas campestris*. Pages 84-89 in: Advances in Molecular Genetics of Plant-Microbe Interactions, vol 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Davis, K. R., Schott, E., and Ausubel, F. M. 1991. Virulence of selected phytopathogenic pseudomonads in *Arabidopsis thaliana*. Mol. Plant-Microbe Interact. 4:477-488.

Debener, T., Lehnackers, H., Arnold, M., and Dangl, J. L. 1991. Identification and molecular mapping of a single *Arabidopsis thaliana* locus determining resistance to a phytopathogenic *Pseudomonas syringae* isolate. Plant J. 1:289-302.

De Feyter, R., and Gabriel, D. W. 1991. At least six avirulence genes are clustered on a 90-kilobase plasmid in *Xanthomonas campestris* pv. *malvacearum*. Mol. Plant-Microbe Interact. 4:423-432.

Dong, X., Mindrinos, M., Davis, K. R., and Ausubel, F. M. 1991. Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell 3:61-72.

Ellingboe, A. H. 1981. Changing concepts in host-pathogen genetics. Ann. Rev. Phytopathol. 19:125-143.

Fellay, R., Rahme, L. G., Mindrinos, M. N., Frederick, R. D., Pisi, A., and Panopoulos, N. J. 1991. Genes and signals controlling the *Pseudomonas syringae* pv. *phaseolicola*-plant interaction. Pages 45-52 in: Advances in Molecular Genetics of Plant-Microbe Interactions, vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

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 specificty towards both host and non-host species. Physiol. Mol. Plant Pathol. 40:1-15.

Flor, H. H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9:275-296.

Gabriel, D. W., Burges, A., and Lazo, G. R. 1986. Gene-for-gene recognition of five cloned avirulence genes from *Xanthomonas campestris* pv. *malvacearum* by specific resistance genes on cotton. Proc. Natl. Acad. Sci. USA 83:6415-6419.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359.

Herbers, K., Conrads-Strauch, J., and Bonas, U. 1992. Race-specificity of plant resistance to bacterial spot disease determined by repetitive motifs in a bacterial avirulence protein. Nature 356:172-174.

Hitchin, F. E., Jenner, C. E., Harper, S., Mansfield, J. W., Barber, C. E., and Daniels, M. J. 1989. Determinant of cultivar specific avirulence cloned from *Pseudomonas syringae* pv. *phaseolicola* race 3. Physiol. Mol. Plant Pathol. 34:309-322.

Hunter, J. E., Dickson, M. H., and Ludwig, J. W. 1987. Source of resistance to black rot of cabbage expressed in seedlings and adult plants. Plant Dis. 71:263-266.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.

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.

Kamoun, S., Kamdar, H. V., Tola, E., and Kado, C. I. 1992. Incompatible interactions between crucifers and *Xanthomonas campestris* involve a vascular hypersensitive response: Role of the *hrpX* locus. Mol. Plant-Microbe Interact. 5:22-33.

Kearney, B., and Staskawicz, B. J. 1990. Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene *avrBs2*. Nature 346:385-386.

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

Keen, N. T., and Buzzel, R. I. 1991. New disease resistance genes in soybean against *Pseudomonas syringae* pv. *glycinea*: Evidence that one of them interacts with a bacterial elicitor. Theor. Appl. Genet. 81:133-138.

Keen, N. T., Tamaki, S., Kobayashi, D., Gerhold, D., Stayton, M., Shen, N., 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.

Kelemu, S., and Leach, J. E. 1990. Cloning and characterization of an avirulance gene from *Xanthomonas campestris* pv. *oryzae*. Mol. Plant-Microbe Interact. 3:59-65.

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.

Kobayashi, D. Y., Tamaki, S. J., and Keen, N. T. 1990. Molecular characterization of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. Mol. Plant-Microbe Interact. 3:94-102.

Konz, C., Chua, N. H., and Schell, J., eds. 1992. Methods in *Arabidopsis* Research. World Scientific Publishing, Singapore.

Kranz, A. R., and Kirchheim, B. 1987. Genetic resources in *Arabidopsis*. Arabidopsis Inf. Serv. 24:1-386.

Liddle, S. A. 1992. Strategies for studying pathogenicity genes of *Xanthomonas campestris* pv. *campestris*. Ph.D. thesis. University of East Anglia, Norwich, U.K.

Liu, Y.-N., Tang, J.-L., Clarke, B., Dow, J. M., and Daniels, M. J. 1990. A multipurpose broad host range cloning vector and its use to characterize an extracellular protease gene of *Xanthomonas campestris* pathovar *campestris*. Mol. Gen. Genet. 220:433-440.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Laboratory, Cold Spring Harbor, NY.

Meyerowitz, E. M. 1989. *Arabidopsis*, a useful weed. Cell 56:263-269.

Miller, J. 1972. Experiments in Molecular Genetics. Cold Spring Laboratory, Cold Spring Harbor, NY.

Minsavage, G. V., Dahlbeck, D., Whalen, M. C., Kearney, B., Bonas, U., Staskawicz, B. J., and Stall, R. E. 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria*-pepper interactions. Mol. Plant-Microbe Interact. 3:41-47.

Osbourn, A. E., Clarke, B. R., and Daniels, M. J. 1990. Identification and DNA sequence of a pathogenicity gene of *Xanthomonas campestris* pv. *campestris*. Mol. Plant-Microbe Interact. 3:280-285.

Parker, J. E., Barber, C. E., Fan, M.-J., and Daniels, M. J. Interaction of *Arabidopsis thaliana* with *Xanthomonas campestris*. In: *Arabidopsis thaliana* as a Model for Plant-Pathogen Interactions. K. R. Davis and R. Hammerschmidt, eds. American Phytopathological Society, St. Paul, MN. (In press)

Ronald, P. C., and Staskawicz, B. J. 1988. The avirulence gene *avrBs1* from *Xanthomonas campestris* pv. *vesicatoria* encodes a 50-kD protein. Mol. Plant-Microbe Interact. 1:191-198.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Shaw, J. J., and Kado, C. I. 1988. Whole plant wound inoculation for consistent reproduction of black rot of crucifers. Phytopathology 78:981-986.

Simpson, R. B., and Johnson, L. J. 1990. *Arabidopsis thaliana* as a host for *Xanthomonas campestris* pv. *campestris*. Mol. Plant-Microbe Interact. 3:233-237.

Sjöström, M., Wold, S., Wieslander, A., and Rilfors, L. 1987. Signal peptide amino acid sequences in *Escherichia coli* contain information related to final protein localization. A multivariate data analysis. EMBO J. 6:823-831.

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.

Swanson, J., Kearney, B., Dahlbeck, D., and Staskawicz, B. 1988. Cloned avirulence gene of *Xanthomonas campestris* pv. *vesicatoria* complements spontaneous race change mutants. Mol. Plant-Microbe Interact. 1:5-9.

Tamaki, S., Dahlbeck, D., Staskawicz, B., and Keen, N. T. 1988. Characterization and expression of two avirulence genes cloned from *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 170:4846-4854.

Tang, J.-L., Gough, C. L., and Daniels, M. J. 1990. Cloning of genes involved in negative regulation of production of extracellular enzymes and polysaccharide of *Xanthomonas campestris* pathovar *campestris*. Mol. Gen. Genet. 222:157-160.

Tang, J.-L., Liu, Y.-N., Barber, C. E., Dow, J. M., Wootton, J. C., and Daniels, M. J. 1991. Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*. Mol. Gen. Genet. 226:409-417.

Tsuji, J., Sommerville, S. C., and Hammerschmidt, R. 1991. Identification of a gene in *Arabidopsis thaliana* that controls resistance to *Xanthomonas campestris* pv. *campestris*. Physiol. Mol. Plant Pathol. 38:57-65.

Turner, P., Barber, C., and Daniels, M. 1984. Behaviour of the transposons Tn*5* and Tn*7* in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 195:101-107.

Turner, P., Barber, C., and Daniels, M. 1985. Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. Mol. Gen. Genet. 199:338-343.

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

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

Williams, P. H. 1980. Black rot: A continuing threat to world crucifers. Plant Dis. 64:736-742.