Xv4-vrxv4*: A New Gene-for-Gene Interaction Identified Between *Xanthomonas campestris* pv. *vesicatoria* Race T3 and the Wild Tomato Relative *Lycopersicon pennellii

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Accepted 7 September 2000.

Strains of tomato race 3 (T3) of *Xanthomonas campestris* pv. *vesicatoria* elicit a hypersensitive response (HR) in leaves of *Lycopersicon pennellii* LA716. Genetic segregation of the resistance exhibited ratios near 3:1 in F₂ populations, which confirmed that a single dominant gene controlled the inheritance of this trait. With the aid of a collection of introgression lines, restriction fragment length polymorphism, and cleaved amplified polymorphic sequence markers, the resistance locus was located on chromosome 3 between TG599 and TG134. An avirulence gene named *avrXv4* was also isolated by mobilizing a total of 600 clones from a genomic DNA library of the T3 strain 91-118 into the *X. campestris* pv. *vesicatoria* strain ME90, virulent on *L. pennellii*. One cosmid clone, pXcvT3-60 (29-kb insert), induced HR in resistant plants. The avirulent phenotype of pXcvT3-60 was confirmed by comparing growth rates in planta and electrolyte leakages among transconjugants carrying a mutated or intact clone with the wild-type T3 strain 91-118. A 1.9-kb DNA fragment contained within a 6.8-kb active subclone was sequenced and was determined to carry an open reading frame of 1,077 bp. The predicted AvrXv4 protein exhibits high similarity to members of an emerging new family of bacterial proteins from plant and mammalian pathogens comprising AvrRxx, AvrBsT, YopJ, YopP, AvrA, and YL4O.

Additional keywords: bacterial spot, *R* genes.

Bacterial spot of tomato is caused by *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye. It occurs throughout the world wherever tomatoes are grown and environmental conditions are favorable for disease development (Stall 1995a). In Florida, bacterial spot is one of the most important diseases,

causing severe yield and quality reductions (Pohronezny and Volin 1983). Chemical control strategies are limited because of the rise of *X. campestris* pv. *vesicatoria* strains that are resistant to streptomycin and tolerant to copper compounds as well as the low efficiency of chemicals during wet weather and the endemic nature of the pathogen (Jones et al. 1995; Sahin and Miller 1996). Therefore, management of this disease is dependent upon sanitation and the use of pathogen-free seeds, seedlings, and resistant cultivars (Sahin and Miller, 1996).

X. campestris pv. *vesicatoria* strains have been divided into three groups, according to host-range specificity. The XcvT group includes strains that are pathogenic on tomato, the XcvP group includes strains that are pathogenic on pepper and the XcvTP group includes strains that are pathogenic on both plant species (Minsavage et al. 1990). Three races have been identified among XcvT and XcvTP strains on the basis of the reaction they elicit on three tomato cultivars: Hawaii 7998 (H7998), Hawaii 7981 (H7981), and Bonny Best (Jones et al. 1998; Stall 1995b). Strains that only showed hypersensitive response (HR) with H7998 were designated tomato race 1 (T1) (Wang et al. 1994). Strains that caused HR only on H7981 were designated tomato race 3 (T3). Strains unable to elicit HR on any of the three cultivars were designated tomato race 2 (T2) (Jones et al. 1995; Wang et al. 1994).

Incompatibility between the host and the pathogen is the result of the interaction between a dominant resistance gene (*R*) in the plant and a dominant avirulence gene (*avr*) in the pathogen (Flor 1971). The interaction of the genes results in the activation of several signal transduction pathways and the initiation of HR (Lamb and Dixon 1997). The neighboring cells surrounding the lesion also could synthesize antimicrobial phytoalexins, several pathogenesis-related proteins, and cell wall fortifications (Bowles 1990; Dixon 1986; Dixon and Lamb 1990).

Cloning and characterization of avirulence genes from different plant-pathogenic bacteria have yielded important information about host-pathogen interactions (Leach and White 1996). Several *avr* genes from different strains of *X. campestris* pv. *vesicatoria* have been cloned and characterized (Bonas

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Florida Agricultural Experiment Station, Journal Series R-07318.

Nucleotide and/or amino acid sequence data for *Xv4-avrXv4* can be found at the GenBank database, accession no. AF221058.

et al. 1989; Canteros et al. 1995; Minsavage et al. 1990; Ronald and Staskawicz 1988; Whalen et al. 1993). One of the objectives of this project was to determine if the resistance followed the gene-for-gene pattern. This was done by cloning and characterizing the putative avirulence gene(s) from *X. campestris* pv. *vesicatoria* race T3 that defines resistance in *Lycopersicon pennellii* LA716.

Tomato DNA markers have been used to characterize the inheritance and genomic localization of several resistance genes (Dixon et al. 1996; Jones et al. 1994; Martin et al. 1993; Moreau et al. 1998; Ori et al. 1997; Thomas et al. 1997; Yu et al. 1995). Furthermore, the existence of a saturated linkage map of tomato (Tanksley et al. 1992) and the availability of introgression lines from *L. pennellii* LA716 into *L. esculentum* (Eshed and Zamir 1994) facilitates the chromosomal localization of any given locus, especially when polymorphisms between these species can be detected at such loci. Another major objective of this project was to genetically characterize and map the resistance gene to *X. campestris* pv. *vesicatoria* race T3 derived from *L. pennellii* LA716 with DNA markers and introgression lines developed by Eshed and Zamir (1994).

RESULTS

Genetic analysis of resistance in *L. pennellii* LA716.

Screening for resistance of different tomato lines and closely related species indicated that the wild species *L. pennellii* LA716 displays an incompatible reaction with T3 strains. Infiltration of 10^8 CFU of *X. campestris* pv. *vesicatoria* T3 strains per milliliter into leaves of *L. pennellii* LA716 and *L. esculentum* H7998 indicated a differential response characterized by the development of HR in leaves of the former species 24 to 36 h after infiltration. In contrast, H7998 developed confluent necrosis 72 to 96 h after infiltration, which is characteristic of the susceptible response (data not shown). These results indicated the existence of HR-related resistance in the wild species *L. pennellii*.

A subset of 16 F₁ plants obtained from the cross between LA716 and H7998 was screened for resistance by inoculations with the *X. campestris* pv. *vesicatoria* T3 strain 91-118. By 24 to 36 h after inoculation, all the F₁ plants produced HR in the infiltrated areas. Identical results were obtained when inoculation experiments were repeated with another T3 strain, *X. campestris* pv. *vesicatoria* 97-2.

When 245 F₂ plants were screened with the same two strains for resistance, 79% (194) of them developed HR, whereas only 21% (51) remained asymptomatic 24 to 36 h after inoculation. Analysis of the segregation ratio indicated a good fit to the 3:1 Mendelian segregation ($\chi^2 = 2.287$; $P = 0.13$). The single gene associated with resistance to T3 strains of *X. campestris* pv. *vesicatoria* was designated *Xv4*.

Analysis of introgression lines indicates a chromosome-3 location.

A collection of 50 introgression lines obtained from the cross between *L. pennellii* LA716 and *L. esculentum* M80 was used to narrow down the position of the *Xv4* gene. The collection spans the entire genome of *L. pennellii* LA716 and is composed of lines carrying individual fragments of known genomic location. When the collection was screened for resistance to T3 strains, only two lines, LA3488 and LA3489,

developed the characteristic HR, which was previously observed in *L. pennellii* F₁ and F₂ populations. In order to confirm this phenotype under more natural conditions with strain *X. campestris* pv. *vesicatoria* 97-2, dip inoculations of all introgression lines were performed. As expected, LA3488 and LA3489 exhibited the lowest levels of infection, scoring 4 and 5, respectively (data not shown). Both LA3488 and LA3489 carry overlapping fragments from chromosome 3 of *L. pennellii* (Eshed and Zamir 1994).

Identification of markers linked to the resistance locus.

The restriction fragment length polymorphism (RFLP) marker locus TG134, located at one end of the chromosomal fragment carried by the introgression line LA3488, was chosen for the initial linkage analysis via RFLP. Genomic clones for four neighboring loci were converted into polymerase chain reaction (PCR)-based markers by first sequencing the termini of the clones and then designing primers suitable for PCR amplification. Of these, PCR primers for TG457 produced an *L. esculentum* amplicon of about 470 bp that behaved as a dominant locus. Primers for TG377 yielded allelic amplicons with about 1,230 and 1,030 bp. Amplicons for TG284a and TG599 were digested with *Hind*III (1,040 and 830 bp) and *Hind*III/*Eco*RI (270 and 220 bp), respectively, in order to distinguish the two alleles. Resistance and the five markers used for mapping segregated in a Mendelian fashion.

Construction of a genetic map around the resistance gene.

Linkage analysis of RFLP and cleaved amplified polymorphic sequence (CAPS) markers indicated that the new resistance gene, designated *Xv4*, is linked to all markers tested ($\log_{10}$ odds ratio 5.0, maximum distance 40.0). The data were analyzed with MapMaker 3.0 (Lander et al. 1987; Lincoln et al. 1992) and are summarized in Figure 1. Chromosome location and gene order were similar to those reported by Tanksley et al. (1992), except for markers TG457 and TG599, which switched places.

Growth in plants and electrolyte leakage.

The growth of *X. campestris* pv. *vesicatoria* strains in leaves of the resistant F₁ plants (*L. esculentum* H7998 crossed with *L. pennellii* LA716) was examined. The wild-type T3 strain 91-118 of *X. campestris* pv. *vesicatoria* increased in population size about 100-fold by 4 days after infiltration, but afterward the growth ceased to increase (Fig. 2A). In contrast, the population of the virulent *X. campestris* pv. *vesicatoria* strain ME90 increased up to 10,000-fold by 6 days after infiltration (Fig. 2A). Statistical analysis of the area under the curve indicated that strains carrying the putative *avr* gene exhibited significantly lower internal populations than strains that did not carry it.

The speed and degree of cell damage caused by *X. campestris* pv. *vesicatoria* strains in resistant F₁ plants were assessed by measuring electrolyte leakage from inoculated leaf tissue. No significant differences were observed between *X. campestris* pv. *vesicatoria* T3 and ME90 at 12 h after infiltration (Fig. 2B). However, during the following 48 h, electrolyte leakage increased significantly in tissue infiltrated with *X. campestris* pv. *vesicatoria* T3, whereas that caused by ME90 remained almost unchanged. During the final 12 h, cell damage caused by ME90 started to increase, whereas that caused by the wild-

type *X. campestris* pv. *vesicatoria* T3 ceased to increase (Fig. 2B). Statistical analysis of the area under the curve indicated that there were significant differences in the degree and speed of the damage caused by strains carrying the putative *avr* compared with those strains that did not have the gene.

Identification of the avirulence gene *avrXv4*.

In order to test whether this resistance gene was the same as the previously described T3 resistance gene *Xv3* (Scott et al. 1995) from PI 128216 and PI 126932 (*L. pimpinellifolium*)

and the *L. esculentum* cultivar H7981, test plants were inoculated with the marker-exchange mutant strain M24 (*avrXv3*⁻) of *X. campestris* pv. *vesicatoria* 91-118 (T3) and the complemented mutant M24C carrying the *avrXv3* gene (Table 1) (Astua-Monge et al. 2000). The M24 mutant is unable to cause HR on H7981 or *L. pimpinellifolium* PI 128216 and PI 126932 (Astua-Monge et al. 2000). When these strains were inoculated into parental lines, F₁ and F₂ plants, and certain resistant introgression lines, both strains exhibited the ability to elicit HR, as described before.

Cloning of the avirulence gene *avrXv4*.

A total of 600 cosmid clones from a genomic DNA library of *X. campestris* pv. *vesicatoria* tomato race 3 strain 91-118 were mobilized into the *X. campestris* pv. *vesicatoria* strain ME90 (virulent on *L. pennellii*) by conjugation, and the resulting transconjugants were inoculated into leaves of resistant tomato plants. One clone, pXcvT3-60, carried by the strain ME90, induced an HR on F₁ plants obtained from the cross between *L. esculentum* H7998 and *L. pennellii* LA716 (Table 1). The cosmid clone pXcvT3-60 contained a 29-kb fragment of *X. campestris* pv. *vesicatoria* DNA, as determined by restriction endonuclease digestion (data not shown).

When leaves of LA716 and F₁ plants were infiltrated with the *X. campestris* pv. *vesicatoria* strain ME90 carrying pXcvT3-60, the population growth exhibited a trend similar to that described before for wild-type *X. campestris* pv. *vesicatoria* T3 (Fig. 2A). Similarly, the rate of cell damage caused by the same transconjugant was identical to that caused by wild-type T3 (Fig. 2B).

Tn3-*uidA* mutagenesis.

The cosmid clone pXcvT3-60 containing the putative *avrXv4* was mutagenized by transposon insertion with Tn3-*uidA*. Three of 200 transconjugants carrying transposon derivatives pXcvT3-60::33, pXcvT3-60::49, and pXcvT3-60::133 were unable to elicit HR when inoculated into F₁ (*L. esculentum* H7998 crossed with *L. pennellii* LA716) tomato plants.

Growth curves of the *X. campestris* pv. *vesicatoria* strain ME90 carrying the mutant clone pXcvT3-60::33 indicated that insertion mutations in *avrXv4* restored the normal growth pattern of a virulent strain (Fig. 2A). Similarly, the speed at which cell damage occurred in resistant plants was drastically reduced when ME90 carrying the mutant pXcvT3-60::33 was used for inoculations (Fig. 2B).

Fig. 1. Comparative map locations of cleaved amplified polymorphic sequence (CAPS) and restriction fragment length polymorphism (RFLP) markers. Left map represents the marker order as determined by Tanksley et al. (1992). Introgression fragments from chromosome 3 of *Lycopersicon pennellii* in different lines were mapped by Eshed and Zamir (1994) and are superimposed on the map. IL3-3 = LA3488, IL3-4 = LA3489. Right map represents the marker order around the resistance gene *Xv4* as determined by MapMaker. All markers shown are CAPS, except for the RFLP probe TG134. Distance between markers and log₁₀ odds ratio score is indicated.

Table 1. Differential response of tomato genotypes to strains of *Xanthomonas campestris* pv. *vesicatoria*

Strain	<i>Lycopersicon esculentum</i> H7998 <i>Xv3</i> ⁻ , <i>Xv4</i> ^{-a}	<i>L. pennellii</i> LA716 <i>Xv3</i> ⁻ , <i>Xv4</i> ⁺	<i>L. esculentum</i> 216 <i>Xv3</i> ⁺ , <i>Xv4</i> ⁻	<i>L. esculentum</i> Fla. 7060 <i>Xv3</i> ⁻ , <i>Xv4</i> ⁻	F ₁ (<i>L. pennellii</i> LA716 × <i>L. esculentum</i> Hawaii 7998) <i>Xv3</i> ⁻ , <i>Xv4</i> ⁺
M24 (<i>avrXv3</i> ⁻ , <i>avrXv4</i> ⁺)	Sus	HR	Sus	Sus	ND ^b
M24C (<i>avrXv3</i> ⁺ , <i>avrXv4</i> ⁺)	Sus	HR	HR	Sus	ND
ME90 (<i>avrXv3</i> ⁻ , <i>avrXv4</i> ⁻)	HR	Sus	Sus	Sus	Sus ^c
91-118 (<i>avrXv3</i> ⁺ , <i>avrXv4</i> ⁺)	Sus	HR	HR	Sus	HR
Xcv-60 ^d	HR	HR	Sus	Sus	HR
Xcv-60::33 ^e	HR	Sus	Sus	Sus	Sus

^a + = presence of gene; - = absence of gene; Sus = susceptible response; HR = resistant response.

^b ND = not determined.

^c Even though ME90 has *avrXv4*, the HR response in F₁ plants to ME90 is drastically delayed, so it was considered susceptible.

^d ME90 carrying the cosmid clone pXcvT3-60 (*avrXv4*).

^e ME90 with the cosmid clone pXcvT3-60::33 carrying a transposon insertion.

Sequence analysis of *avrXv4*.

Sequence analysis of a 6.8-kb active subclone and the Tn3-*uidA* insertion mutants revealed a single open reading frame (ORF) of 1,077 bp. The sequence of this ORF and flanking regions was also confirmed by sequencing a PCR product containing the entire region. A plant-inducible promoter box (PIP box) (Fenselau and Bonas 1995) was found 89 bp upstream of the putative translational start. A putative promoter region, 108 bp upstream of the start codon and spanning 50 nucleotides, was predicted by the Web-based software Promoter Scan II (Prestridge 1995) (score = 0.87). This putative promoter region includes the PIP box described before. DNA sequence similarity searches by the Blast 2.0 algorithm (Altschul et al. 1997) did not yield any significant similarity to any known sequences.

The predicted avirulence protein is 359 amino acids in length, with an estimated molecular mass of 39.7 kDa and a pI of 9.1. The putative protein is mostly hydrophilic in nature and exhibits a high percentage of the amino acid serine (13.6% of the residues). No putative cleavage signal peptides or transmembrane domains were found. Three putative nuclear localization signals were found at residues 52 (RPRRK), 256 (RHRK), and 348 (RATKIRH). A motif search by the computer program Motif revealed the presence of at least four putative myristoylation sites at residues 9 (GLKSS), 180 (GNEIAL), 267 (GLFRNH), and 306 (GSLDTN). In addition, a region of 16 amino acids, starting at residue 341 (NISIETS-RATKIRHAL) of the AvrXv4 protein, was found to be similar to the signature pattern of the NRA motif characteristic of the bacterial extracellular solute-binding protein family 5.

A sequence homology search of the AvrXv4-predicted protein was carried out with the computer program Gapped Blast 2.0 (Altschul et al. 1997). The output of the search indicated that the AvrXv4 protein exhibited high homology to several other bacterial proteins, including AvrRxv (40% identity, 54% similarity), AvrBsT (37% identity, 50% similarity) from *X. campestris* pv. *vesicatoria* (Minsavage et al. 1990; Whalen et al. 1988), YopJ (21% identity, 39% similarity), YopP (20% identity, 40% similarity), the putative targeted effector protein (20% identity, 39% similarity) from *Yersinia* spp. (Galyov et al. 1994; Mills et al. 1997), AvrA (21% identity, 40% similarity) from *Salmonella typhimurium* (Hardt and Galan 1997), and Y4LO (38% identity, 51% similarity) from *Rhizobium* spp. (Freiberg et al. 1997). The alignment of the peptide sequence of some members of the family revealed several conserved regions scattered throughout the sequences (Fig. 3).

DISCUSSION

Segregation analysis of an F₂ population obtained from the interspecific cross between *L. esculentum* and *L. pennellii* indicated that a single dominant gene controls this novel resistance. The observed segregation ratio is a good fit to the 3:1 ratio expected for a character controlled by a single gene. The dominance of this character seems to be complete since F₁ plants showed HR responses similar in intensity and speed of development to those observed in *L. pennellii*.

Introgression lines have previously been used to locate qualitative and quantitative trait loci in interspecific crosses of *L. esculentum* and *L. pennellii* (Eshed and Zamir 1995; Eshed et al. 1996; McNally and Mutschler 1997; Moreau et al.

1998). In the present study, screening of the 50 introgression lines suggested that the gene-controlling resistance to T3 strains is located in the lower arm of chromosome 3.

Linkage analysis with RFLP probes and CAPS markers suggested that five of those markers are linked to the resistance gene in chromosome 3. Analysis of the data indicated that the *Xv4* locus is located between TG599 and TG134.

The mapping of *Xv4* was carried out with the long-term goal of cloning this gene by map-based cloning or chromosome landing (Tanksley et al. 1995). The next step would be to generate a high-density map around this gene with the as-

Fig. 2. A, Time course of growth of *Xanthomonas campestris* pv. *vesicatoria* tomato races and transconjugants in F₁ plants obtained from the cross *Lycopersicon pennellii* LA716 × *L. esculentum* H7998. (ME90) wild-type virulent strain (♦), (Xcv-60::33) ME90 carrying the mutant cosmid clone pXcv-60::33 (■), (Xcv-60) ME90 carrying the intact cosmid clone pXcv-60 (▲), and (Xcv-T3wt) wild-type strain 91-118 of *Xcv* race T3 (*). **B,** Time course of electrolyte leakage from leaves of resistant F₁ plants obtained from the cross *L. pennellii* LA716 × *L. esculentum* H7998 infiltrated with strains and transconjugants of *X. campestris* pv. *vesicatoria*. (ME-90) wild-type virulent strain (♦), (Xcv-60::33) ME90 carrying the mutant cosmid clone pXcv-60::33 (■), (Xcv-60) ME90 carrying the intact cosmid clone pXcv-60 (▲), and (Xcv-T3wt) wild-type strain 91-118 of *X. campestris* pv. *vesicatoria* race T3 (*).

sistance of a large number of markers and recombination events. Through the use of bulked segregant analysis (Michelmore et al. 1991) and amplified fragment length polymorphism (Vos et al. 1995) and/or DNA amplification fingerprints (Caetano-Anollés et al. 1991; Caetano-Anollés et al. 1996), markers closely linked to *Xv4* might be generated and used to identify individual BAC or YAC clones carrying that region of the genome.

Two lines of evidence indicate that this resistance is different from that described before in *L. esculentum* cultivar H7981 and two plant introductions of *L. pimpinellifolium*, PI 128216 and PI 126932 (Scott et al. 1995). First, the activity of *avrXv3* in *X. campestris* pv. *vesicatoria* proved to be com-

pletely independent of the ability of T3 strains to elicit HR on *L. pennellii* or its progeny carrying the newly discovered resistance gene. Second, when plants bearing *Xv3* were challenged with *X. campestris* pv. *vesicatoria* strains carrying the putative *avrXv4* gene, no HR was observed. Therefore we can conclude that this incompatible interaction involves a previously unknown resistance gene and its corresponding *avr* gene in the bacterium. We propose *Xv4* as the symbol for the resistance gene.

Mobilization of a genomic library into a virulent strain of *X. campestris* pv. *vesicatoria* was carried out with the purpose of finding a clone carrying the putative *avr* gene. One single cosmid clone was able to convert the virulent strain into a

Fig. 3. Alignment of some of the members of the AvrRxv host interaction family. The alignment was produced with CLUSTAL W (Thompson et al. 1994). Identical amino acid residues are shaded in black, similar residues are shaded in light gray, and conserved residues are shaded in dark gray.

fully avirulent one. As shown before for other *avr* genes (Minsavage et al. 1990; Whalen et al. 1993), the incorporation of heterologous *avr* genes into virulent strains of the pathogen modifies their host specificity. The resistant tomato plants used in the screening carried the resistance gene *Xv4* from *L. pennellii*, so the putative *avr* gene was designated *avrXv4*.

A series of experiments were performed in order to further substantiate that the isolated clone carried *avrXv4*. The experiments included comparing the effect of the wild-type and mutated genes on the speed and degree of damage caused to the plant with the growth rate in planta of different transconjugants. The presence of an active avirulence gene in the virulent ME90 strain and the wild-type T3 strain resulted in a lower growth rate of the bacterium in the resistant plant. This limited growth was most likely a result of the onset of HR, which has also been reported for other *X. campestris* pv. *vesicatoria* strains carrying *avr* genes such as *avrRxv* (Whalen et al. 1993). In contrast, when a mutated form of the avirulence gene was introduced into a transconjugant of the virulent strain ME90, HR was not observed and the growth rate of this transconjugant resembled that of the virulent strain.

Cook and Stall (1968) determined that electrolyte leakage is a measure of membrane disruption in a plant undergoing HR. When electrolyte leakage was measured, the speed and degree of damage caused by the transconjugants carrying the intact gene and the wild-type T3 strain were equally high because the incompatible interaction between *avrXv4* and *Xv4* rapidly led to cell death. Additionally, the onset of electrolyte leakage induced by avirulent strains correlated with the onset of the visible HR (24 to 36 h). This finding agrees with observations for other gene-for-gene interactions involving other races of *X. campestris* pv. *vesicatoria* (Minsavage et al. 1990; Whalen et al. 1993). Conversely, the speed and amount of damage caused by the virulent ME90 strain and the transconjugant carrying a mutated form of *avrXv4* gene were significantly lower, which is characteristic of compatible interactions. These results strongly indicate that *avrXv4* was the only *avr* gene restricting growth of the strain ME90 and inducing HR in resistant F₁ plants.

Sequence analysis of the putative *avr* gene indicated a single ORF, which agrees with what has been found for the majority of *avr* genes described so far (Leach and White 1996). A PIP box (TTCGC-N₁₅-TTCGC) upstream from the putative start codon of *avrXv4* was also found to resemble that described for the *hrpB* pathogenicity operon and *avrRxv* from *X. campestris* pv. *vesicatoria* (Ciesiolka et al. 1999; Fenselau and Bonas 1995). Previous studies on the role of these elements in the expression of *avrRxv*, also from *X. campestris* pv. *vesicatoria*, indicated that the PIP box was not essential for its activity (Ciesiolka et al. 1999). Mutagenesis of those regions and expression studies will be carried out in the future to determine the role of PIP boxes on *avrXv4* expression.

Sequence homology searches with the predicted protein of *avrXv4* revealed that this new *avr* gene product is part of an emerging family of bacterial proteins found in pathogens of plants and mammals. Ciesiolka et al. (1999) were, as far as we know, the first to propose the existence of this group that they refer to as the "AvrRxv-host interaction factor family." Even though sequence similarity exists among all members of this family, those originating from plant-pathogenic organisms exhibit a higher degree of similarity and share several other

features such as serine-rich regions, nuclear localization signals, and myristoylation sites. Nuclear localization signals have been found to play a role in the activity of several members of the AvrBs3 family (Bonas and Van den Ackerveken 1997; Gabriel 1997). In addition, evidence has been presented suggesting that myristoylation of the N terminus of at least three *Pseudomonas avr* gene products is required for their function as both virulence and avirulence factors (Nimchuk et al. 2000).

The sequence of the predicted AvrXv4 protein seems to have a unique motif that resembles the signature pattern of the NRA motif from the family 5 of solute-binding proteins. This family of proteins is defined on the basis of sequence similarity, the molecular size of the proteins, and their affinity for binding other peptides and nickel (Tam and Saier 1993). Overall, solute-binding proteins are part of a well-known transport system involved in chemoreception, initiation of sensory transduction pathways, and high-affinity traffic of solutes across the bacterial cytoplasmic membrane (Tam and Saier 1993).

Currently, no experimental evidence exists to support either the involvement of *avr* gene products in any of the functions associated with solute-binding proteins or the role of the above-mentioned motifs in the avirulence activity of AvrXv4. Nevertheless, future research oriented to explore the role of these putative motifs in the activity of AvrXv4 and its consequences on bacterial fitness will certainly contribute in elucidating at least part of the puzzle surrounding the function of Avr proteins in virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains used in this study are listed in Table 2. Strains of *X. campestris* pv. *vesicatoria* were grown overnight at 28°C on nutrient agar plates (Becton Dickinson, Sparks, MD, U.S.A.) or in nutrient broth for plant inoculations. *Escherichia coli* strains were grown overnight at 37°C on Luria-Bertani medium (Maniatis et al. 1982). All bacterial strains were stored at room temperature in sterile tap water or at –70°C in nutrient broth containing 30% glycerol. Plasmids were introduced into *E. coli* by transformation (Maniatis et al. 1982) and mobilized into *X. campestris* pv. *vesicatoria* strains by conjugation with pRK2073 as the helper plasmid in triparental matings (Ditta et al. 1980; Figurski and Helinski 1979). Triparental matings were made at 28°C on plates of NYG agar (Daniels et al. 1984). Antibiotics were added to the medium at the following final concentrations per milliliter: ampicillin, 100 µg; kanamycin, 25 or 50 µg; rifamycin SV, 100 µg; spectinomycin, 50 µg; and tetracycline, 12.5 µg.

Plant material, inoculum production, and plant inoculations.

F₁ and F₂ populations were derived from an interspecific cross between *L. pennellii* LA716 and *L. esculentum* accession Hawaii 7998, a susceptible tomato cultivar to race T3 of *X. campestris* pv. *vesicatoria*. Roger Chetelat, curator of the C. M. Rick Tomato Genetics Resource Center (Davis, CA, U.S.A.), provided the population of 50 introgression lines that were generated, as described by Eshed and Zamir (1994).

Xanthomonas strains used for inoculations were grown in nutrient broth for 20 h at 28°C with shaking (100 rpm) (KS10, BEA-Emprtech Corp., Hyde Park, MA, U.S.A.). Bacterial cells were pelleted by centrifugation at $1,500 \times g$ for 15 min and resuspended in sterile tap water. The concentration was adjusted to $A_{600} = 0.3$ with a spectrophotometer (Spectronic 20, Spectronic UNICAM, Rochester, NY, U.S.A.). This reading represents approximately 2 to 5×10^8 CFU/ml. Leaves were infiltrated with the bacterial suspension, as described by Hibberd et al. (1987). When whole plants were inoculated, the bacterial suspension was prepared as described above, but diluted to 10^6 CFU/ml in a sterile tap water solution containing 250 μ l of Silwet L77 (Osi Specialties Inc., Danbury, CT, U.S.A.), an organosilicon surfactant, per liter. Inoculations were carried out by dipping the foliage for 15 s in the suspension. Each treatment was replicated three times. The experiment was also carried out three times, unless otherwise indicated.

Two hundred forty-five F_2 plants, their parental lines, and 50 introgression lines were grown in the greenhouse for 4 to 5 weeks at temperatures ranging from 25 to 35°C. Infiltrated plants were moved to a growth room that was kept at a constant temperature of 22°C with a 16-h light period. Assessments for HR were carried out 24 and 36 h after inoculations. Plant leaves exhibiting HR in the infiltrated areas within this period of time were scored as resistant to bacterial spot. When dip inoculation was carried out, assessments were performed starting 4 to 5 days after inoculations. A visual scale designed for these experiments was used, with scores ranging from 0 (no symptoms) to 11 (100% of the leaf area was affected by bacterial spot). Plants with scores of 5 or below were considered resistant.

For population dynamics studies, bacterial suspensions were diluted to a concentration of 2 to 5×10^5 CFU/ml in sterile tap water. For electrolyte leakage, tomato leaves were infiltrated with bacterial suspensions of 2 to 5×10^8 CFU/ml and measurements were carried out as previously described by Hibberd et al. (1987). Unless otherwise indicated, all experiments were arranged as a completely randomized design with

three replications. All experiments were carried out twice. For electrolyte leakage and growth curve experiments, statistical analysis was conducted by analysis of variance procedure of the Statistical Analysis System (SAS Institute, Cary, NC, U.S.A.).

Molecular techniques.

Standard molecular techniques were used for the extraction of plasmid DNA, restriction endonuclease digestions of DNA, and cloning procedures (Ausubel et al. 1992; Maniatis et al. 1982). Enzymes for restriction digestion and ligation reactions were purchased from Promega (Madison, WI, U.S.A.), and the manufacturer's protocol was followed.

The genomic DNA library of *X. campestris* pv. *vesicatoria* strain 91-118 (tomato race 3) that was used for screening was constructed in the cosmid vector pLAFR3, as previously described (Minsavage et al. 1990). Individual clones were mobilized into *X. campestris* pv. *vesicatoria* strain ME90 by triparental mating (Daniels et al. 1984), and transconjugants were inoculated by leaf infiltration into F_1 (*L. esculentum* H7998 crossed with *L. pennellii* LA716) tomato plants to screen for elicitation of HR.

Subclones were constructed in plasmid vector pLAFR3 for mobilization into *X. campestris* pv. *vesicatoria* for plant testing. Insert DNA from active subclones was subsequently moved into plasmid vector pBluescriptII SK (Stratagene, La Jolla, CA, U.S.A.) for sequencing.

DNA extraction and hybridization analysis.

Plant genomic DNA was isolated from the leaf tissue of 104 F_2 plants and progenitors, as described by Prince et al. (1997). For Southern hybridization, genomic DNA was digested with *EcoRV*, according to the manufacturer's conditions (Promega). DNA fragments were resolved by agarose gel electrophoresis (Sambrook et al. 1989) and transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH, U.S.A.), as described by Southern (1975). The RFLP probes, provided by S. Tanksley of Cornell University, were labeled with digoxigenin-11 dUTP (Boehringer Mannheim, Indianapolis, IN, U.S.A.) by PCR, as described by Lanzillo (1990), and probed against the DNA

Table 2. Bacterial strains, plasmid vectors, and plasmid constructs used in this study

Designation	Relevant characteristics	Source or reference
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>		
91-118	Tomato race 3, Rif ^r , <i>avrXv3</i> , <i>avrXv4</i>	This study
97-2	Tomato race 3, <i>avrXv3</i> , <i>avrXv4</i>	This study
ME24	91-118 marker exchange, <i>avrXv3</i> mutant	This study
ME24C	91-118 marker exchange carrying pT3-5, <i>avrXv3</i>	This study
ME90	85-10 carrying Tn5, tomato race 1, pepper race 2, Rif ^r Km ^r	Bonas et al. 1991
<i>Escherichia coli</i>		
DH5 α	FrecA ϕ 80dlacZ Δ M15	Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.)
C2110	Nal ^r polA ⁻	Stachel et al. 1985
Plasmids		
pT3-5	Tc ^r pLAFR6 subclone, <i>avrXv3</i>	Astua-Monge et al. 2000
pLAFR3	Tc ^r rlx ⁺ RK2 replicon, Tc ^r	Staskawicz et al. 1987
pBluescript II KS +/-	Phagemid sequencing vector, Ap ^r	Stratagene (La Jolla, CA, U.S.A.)
pRK2073	Sp ^r Tra ⁺ , helper plasmid	Figurski and Helinski 1979
pHoKmGUS	Km ^r Ap ^r tnpA ⁻ , Tn3-uidA fusion	B. Staskawicz ^a
pSShe	Cm ^r tnpA	Stachel et al. 1985
pXcvT3-60	Tc ^r pLAFR3 cosmid clone, <i>avrXv4</i>	This study
pXcvT3-60::33, ::49, ::133	<i>avrXv4</i> ::Tn3-uidA derivatives, <i>avrXv4</i> mutant	This study

^a Department of Plant Biology, University of California, Berkeley, U.S.A.

immobilized on the Nytran membrane. Hybridization signals were developed with CSPD (disodium 3-(4-methoxyspiro)1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3, 3.1.1^{3,7}] decan-4-yl-phenyl phosphate) chemiluminescence substrate, according to the manufacturer's conditions (Boehringer Mannheim).

Construction of CAPS markers and identification of recombinants between the resistance gene and selected markers.

The termini of the RFLP probes used in this study were sequenced at the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR) (Gainesville, FL, U.S.A.). Oligonucleotides specific for the amplification of each RFLP probe were synthesized at the ICBR's DNA Synthesis Laboratory. DNA for PCR amplifications was extracted, as described before, for hybridization assays.

PCR was carried out with a DNA automated thermocycler PTC-100, equipped with a hot bonnet (M. J. Research, Watertown, MA, U.S.A.), and *Taq* DNA polymerase (Promega). Unless otherwise indicated, each 25- μ l PCR reaction contained 1 $\times$ amplification buffer (from the manufacturer), 100 μ M dNTP (each), 17.5 μ M primer (each), 1.5 mM MgCl₂, 1.25 U of *Taq* DNA polymerase, and 100 ng of template DNA. Generally, the template DNA was initially denatured at 95°C for 3 min followed by 30 PCR cycles. For most of the primer pairs, with few exceptions (Table 3), each cycle consisted of 30 s of denaturation at 95°C, 30 s of annealing at 50°C, and 1 min of extension at 72°C. For the final cycle, the extension step was extended to 5 min. Oligonucleotide sequences used in this study are shown in Table 3.

Aliquots of 20 μ l of the PCR products were mixed with 1 μ l of tracking dye and added to wells of a 1.5% agarose gel in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.2), as described by Sambrook et al. (1989). Agarose electrophoresis was performed at 5 v/cm for 1.5 h and then stained with 0.5 μ g of ethidium bromide per milliliter in water, visualized on a UV transilluminator, and photographed with Type 55 film (Polaroid Corp., Cambridge, MA, U.S.A.).

Restriction endonuclease digestion of PCR products.

Amplified DNA fragments obtained with oligonucleotides designed from probes TG284a and TG599 were digested with *Hind*III and *Hind*III-*Eco*RI, respectively, according to the manufacturer's conditions (Promega). Restriction fragments were resolved by agarose gel electrophoresis in 4% agarose gels (3% NuSieve and 1% Seakem GTG) (FMC BioProducts,

Rockland, ME, U.S.A.) in TAE buffer at 8 v/cm. The gel was stained with 0.5 μ g of ethidium bromide per milliliter in water for 30 min, destained in 1 mM MgSO₄ for 30 min, visualized by UV transillumination, and photographed as described above.

Genetic analysis.

Linkage and segregation analyses were performed with the MapMaker/EXP 3.0 software package (Lander et al. 1987; Lincoln et al. 1992). Statistical analysis was conducted with the Statistical Analysis System.

Transposon mutagenesis.

For transposon mutagenesis, the plasmid pLAFR3 carrying the target insert was transformed into *E. coli* strain DH5 α containing the transposon-carrying plasmid pHoKmGus and the plasmid pSShe carrying the transposase gene (Stachel et al. 1985). Transformants were selected on media containing chloramphenicol, tetracycline, and kanamycin. After overnight growth, pLAFR3 carrying mutated inserts was isolated and transformed into *E. coli* strain C2110. Two hundred individual transformants, selected on media containing nalidixic acid, kanamycin, and tetracycline, were mobilized into the virulent *X. campestris* pv. *vesicatoria* strain ME90 by triparental mating and screened for their inability to elicit HR in F₁ plants obtained from the cross between *L. esculentum* and *L. pennellii*.

Subcloning and DNA sequencing of *avrXv4*.

Tn3-*uidA* insertion sites were sequenced by an oligonucleotide primer complementary to the N terminus of the β -glucuronidase gene (Jefferson et al. 1987) (5'-GATTTACGG-GTTGGGGTTTCT3').

Subcloning of the 29-kb cosmid clone yielded an *Eco*RI 6.8-kb active subclone that conferred on the virulent strain ME90 an ability to elicit HR in the resistant line LA716. Restriction mapping of the 6.8-kb active subclone and the Tn3-*uidA* insertion mutants indicated the presence of a unique *Hind*III site between two of the three Tn3-*uidA* insertions (data not shown). Digestion of the *Eco*RI fragment with *Hind*III yielded two fragments of 1.2 and 5.6 kb, which were subsequently cloned into pBluescript II KS.

Sequencing the 1,971-bp region of DNA encompassing *avrXv4* was carried out with pBluescript vector, T3 and T7 primers, and Xcv60F and Xcv60R primers that were designed from the DNA sequence flanking the Tn3-*uidA* insertions. Sequencing was done at the ICBR Sequencing Facility with

Table 3. Sequence of specific oligonucleotides used for polymerase chain reaction-amplification of genomic DNA from *Lycopersicon esculentum*, *L. pennellii*, introgression lines, and F₂ population

Marker	Chromosome	Oligonucleotide sequences ^a	Modified conditions
TG50c	3	FP 5'TGGAACATGTGTCGACCTTT 3' RP 5'TATGTCCACCTCCAAAACCT 3'	2.5 mM MgCl ₂
TG377	3	FP 5'TTGGCCCTTTCTTACTCTCT 3' RP 5'CGGGTTGATTCTTAATGTACG 3'	
TG284a	3	FP 5'TGACTCCGTTGAAACAATTTA 3' RP 5'AACTGTGGGCTTGCTCTTTTG 3'	
TG457	3	FP 5'AGGCCAGGTGACTTTATTAGG 3' RP 5'TTGTGTGTGGTTTCCCT 3'	Annealing temperature 53°C
TG599	3	FP 5'TGTTGATCCTTGCTTGCTGT 3' RP 5'TTGATGTTGCAACTTCCC 3'	

^a FP = forward primer; RP = reverse primer.

the Applied Biosystems Model 373 system (Applied Biosystems, Foster City, CA, U.S.A.). The computer programs SeqAid II version 3.81, Promoter Scan II (Prestidge 1995), Motif, CLUSTAL W (Thompson et al. 1994), and Blast 2.0 (Altschul et al. 1997) were used to analyze nucleotide sequence data and predict protein products.

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