

# Resistance of Tomato and Pepper to T3 Strains of *Xanthomonas campestris* pv. *vesicatoria* Is Specified by a Plant-Inducible Avirulence Gene

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**Tomato race 3 (T3) of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) elicits a hypersensitive response (HR) in leaves of *Lycopersicon esculentum* near-isogenic line (NIL) 216 and pepper genotypes. One cosmid clone (35 kb) selected from a genomic library of a T3 strain induced an HR in all resistant plants. A 1.5-kb active subclone containing the putative avirulence (*avr*) gene, designated *avrXv3*, was sequenced. The *avrXv3* gene encodes a 654-bp open reading frame (ORF) with no homology to any known gene. Expression studies with a fusion of this gene and *uidA* indicated that *avrXv3* is plant inducible and controlled by the hypersensitivity and pathogenicity (*hrp*) regulatory system. Mutational analysis and transcription activation assays revealed that *AvrXv3* has transcription activation activity in yeast, and that the putative domain responsible for that activity is located at the C terminus of the *AvrXv3* protein. *Agrobacterium tumefaciens*-mediated transient expression confirmed the direct role of *AvrXv3* in eliciting the HR in tomato NIL 216 and supported the hypothesis that *Avr* proteins must be present inside the plant host cell to trigger the HR.**

The gene-for-gene model, proposed by Flor in the 1940s, suggested the existence of an incompatibility system between plants and pathogens controlled by single dominant genes (Flor 1971). In this model, the interaction between a pathogen expressing an avirulence (*avr*) gene product and a host expressing the appropriate resistance (*R*) gene product results in cell death and limited spread of the pathogen (Bent 1996).

Minsavage et al. (1990) demonstrated the gene-for-gene relationship for *Xanthomonas campestris* pv. *vesicatoria* (Xcv) in pepper and tomato, and identified three different groups among Xcv strains: 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). On tomato, three races, designated T1, T2, and T3, have been identified based on their reaction on three tomato cultivars: Hawaii 7998 (H7998), Hawaii 7981 (H7981), and Bonny Best (Jones et al. 1998, 1995; Stall 1995). T1 strains carrying the avirulence gene *avrRxv* only induce hypersensitive reactions (HRs) on the genotype H7998 (Whalen et al. 1993) and T2 strains do not induce an HR on any of the indicator cultivars, while T3 strains carry the gene *avrXv3* and induce an HR on the tomato genotype H7981 and all pepper cultivars tested (Minsavage et al. 1996). In addition, the ability of T1 and T2 strains to cause HR on pepper (nonhost resistance) is controlled by a different *avr* gene designated *avrBsT* (Minsavage et al. 1990).

Three tomato genotypes with high levels of resistance to race T3 of the bacterial spot pathogen were found by Scott et al. (1995). However, the resistance found in *Lycopersicon pimpinellifolium* PI 128216 and PI 126932 and *L. esculentum* cv. H7981 appears to be controlled by the interaction of the same two genes, *avrXv3* and *Xv3* (Minsavage et al. 1996; Scott et al. 1995).

The role of *Avr* proteins in plant-pathogen interactions has been one of the most interesting aspects. Currently, only a few of these gene products have been associated with a biochemical function such as enzymatic activity, increased fitness, induction of host-specific symptoms, or transcription activation activity (Duan et al. 1999; Kearney and Staskawicz 1990; Swords et al. 1996; Zhu et al. 1998). Based on recent evidence, *Avr* proteins are suggested to be delivered by a bacterial delivery system capable of introducing *Avr* proteins into plant cells (Hyun-Han et al. 1998; Rossier et al. 1999; Van den Ackerveken et al. 1996).

Studies on virulence mechanisms of gram-negative, mammalian pathogens revealed the existence of a novel secretion system now known as the type III protein secretion pathway (Hueck 1998). In plant-pathogenic bacteria, a cluster of genes known as hypersensitivity and pathogenicity (*hrp*) genes have been found to be involved in regulating both compatible and incompatible interactions (Bonas 1994). Sequence similarity of at least nine *hrp* genes with components of the type III secretion pathway described in mammalian pathogens first indicated the

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possibility that *hrp* genes could be involved in the secretion of a broad range of virulence factors, elicitors, and perhaps even Avr proteins. Evidence for *hrp*-dependent secretion of Avr proteins has recently been presented for AvrB, AvrPto, AvrBs3, and AvrRxv (Hyun-Han et al. 1998; Rossier et al. 1999).

The objective of this work was to characterize the new avirulence gene *avrXv3* from Xcv race T3 involved in specifying resistance in *L. pimpinellifolium* PI 128216 and PI 126932 and *L. esculentum* cv. H7981. An attempt was made to study the functional domains of this *avr* gene product and their involvement in eliciting the HR in tomato.

## RESULTS

### Cloning of the avirulence gene *avrXv3*.

To clone the gene responsible for avirulence activity, a total of 500 clones from a genomic DNA library of Xcv T3 strain 91-118 were mobilized into Xcv strain 87-13 by conjugation. In inoculation tests, one transconjugant, carrying the cosmid clone designated pT3-176, caused an HR in pepper and tomato. Cosmid pT3-176 contained a 35-kb insert of Xcv DNA. Subcloning of the 35-kb insert into pUFR051 yielded a 1.5-kb active subclone, designated pT3-5, capable of eliciting an HR when mobilized into Xcv and inoculated into resistant host plants. Insert DNA from the active subclone was subsequently moved into plasmid vector pLAFR6 for transposon mutagenesis and pBluescript KS for sequencing.

### DNA sequence analysis.

Sequence analysis of the subclone pT3-5 containing the putative *avrXv3* revealed a single open reading frame (ORF) of 654 bp encoding a 218 amino acid (aa) protein of 24.5 kDa (GenBank accession number AF190120). An imperfect plant-inducible promoter (PIP-box) sequence motif (Fenselau and Bonas 1995) was found 66 bp upstream of the putative translational start site. When genomic DNA from several strains of Xcv was hybridized with an internal fragment of *avrXv3*, only T3 strains exhibited a positive hybridization signal (Fig. 1).

A sequence homology search of *avrXv3* and its predicted protein did not yield any significant homology to any known



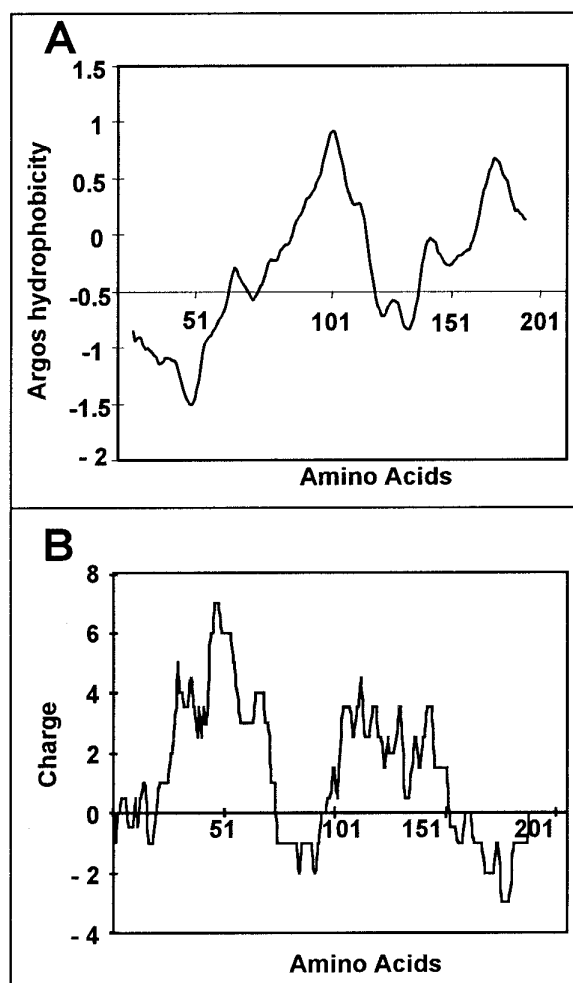
**Fig. 1.** Southern hybridization analysis of different strains of *Xanthomonas campestris* pv. *vesicatoria*. Genomic DNA was digested with *EcoRI*, immobilized on a membrane, and hybridized to an *avrXv3*-specific probe. ( $\lambda$ ) Lambda DNA digested with *HindIII*: Lane 1, XV153, T1 strain, phenotypic group A; Lane 2, XV155, T1 strain, phenotypic group A; Lane 3, XV157, T1 strain, phenotypic group A; Lane 4, XV330, T1 strain, phenotypic group A1; Lane 5, XV338, T1 strain, phenotypic group A1; Lane 6, XV1085, T1 strain, phenotypic group A1; Lane 7, XV56, T2 strain; Lane 8, XV141, T2 strain; Lane 9, XV144, T2 strain; Lane 10, XV1111, T2 strain; Lane 11, BV5-4a, T2 strain; Lane 12, MME, T2 strain; Lane 13, XV938, T3 strain; Lane 14, XV1220, T3 strain; Lane 15, XV1484, T3 strain; Lane 16, 91-118, T3 strain; Lane 17. 97-2, T3 strain.

gene or protein. Analysis of the predicted protein sequence indicated clustering of amino acids with similar hydrophobic properties and charge (Fig. 2).

### Mutagenesis of *avrXv3*.

The regions spanning the three most prominent clusters of hydrophobic and charged residues in AvrXv3 were chosen as targets for deletion (Fig. 2). The predicted AvrXv3 protein is mostly hydrophilic and positively charged; however, the C terminus is negatively charged.

Polymerase chain reaction (PCR)-based deletion mutagenesis yielded three different mutant proteins lacking 47 aa (MutA), 49 aa (MutB), and 52 aa (MutC) at the N terminus, middle portion, and C terminus of the predicted AvrXv3 protein, respectively. Addition of histidine (His) tags at either the C or N terminus of the AvrXv3 protein yielded two larger proteins of about 27 kDa (data not shown). The sequence of all mutants was confirmed by sequence analysis.



**Fig. 2.** **A**, Distribution of hydrophobic residues in predicted protein of *avrXv3*. Argos hydrophobicity values were calculated as described by Shaw (1995). Solid line: average of hydrophobicity values for 20 amino acid residues. **B**, Distribution of charged residues in predicted protein of *avrXv3*. Charge values were calculated as described by Shaw (1995). Solid line: average of charge values for 20 amino acid residues.

To test the avirulence activity of the *avrXv3* alleles, the three deletion mutants, the wild-type gene, and the His-tagged constructs were cloned into pLAFR6, and introduced into the virulent strain of Xcv ME-90 (Bonas et al. 1991). Even though all modified genes were under control of their natural promoter, only the wild-type construct of *avrXv3* conferred on this strain the ability to elicit the HR in the resistant tomato NIL 216 (Table 1).

Clone pT3-5 encompassing *avrXv3* was also mutagenized with Tn3-*uidA*. Sixteen of 300 transconjugants carrying transposon derivatives were unable to elicit the HR when inoculated into tomato NIL 216 and pepper cv. Early Calwonder. The relative position and orientation of six Tn3-*uidA* insertions were determined by restriction endonuclease digestion analysis (data not shown). A preliminary screening for the presence of  $\beta$ -glucuronidase (GUS) activity in the transconjugants grown in NYG or XVM2 broth revealed activity only in mutant derivatives designated 144, 245, and 249 (data not shown). Tn3-*uidA* derivative pT3-5::249 was sequenced. The *uidA* gene was fused in frame 277 nucleotides downstream of the putative translational start of the *avrXv3* protein. Xcv transconjugants carrying pT3-5::249 were used for in planta and media protein expression studies.

Growth experiments and electrolyte leakage (Fig. 3A and B) in plants showed that the virulent Xcv strain 93-1 became avirulent (HR) when the clone pT3-5 was introduced by conjugation. Marker exchange mutagenesis of Xcv T3 strain 91-118 with pT3-5::249 resulted in the generation of the mutant ME24, fully virulent on the tomato NIL 216. Growth of ME24 on pepper cv. Early Calwonder resembled more that of the avirulent T3 strain than that of the virulent strain (Fig. 3A). However, electrolyte leakage data indicated that the marker exchange mutant had lost its ability to elicit the typical HR on pepper cv. Early Calwonder (Fig. 3B).

#### Expression of the *avrXv3* gene.

The ORF of *avrXv3* was cloned into the expression vector pET-15b. IPTG induction of transformed cells of *Escherichia*

**Table 1.** Comparison of activity of wild-type *avrXv3* and its mutant alleles in avirulence activity assays, *Agrobacterium tumefaciens*-mediated transient expression, and transcription activation activity in yeast

Constructs	HR activity in tomato <sup>v</sup>		HR by transient expression in tomato <sup>x</sup>		Transcription activation activity in yeast <sup>y</sup>
	NIL 216	Fla. 7060	NIL 216	Fla. 7060	
AvrXv3	+	-	+	-	21.29 ± 0.08 b
MutA	-	-	-	-	21.32 ± 0.05 b
MutB	-	-	-	-	22.59 ± 0.08 b
MutC	-	-	-	-	5.41 ± 0.01 d
HisT3	-	-	+	-	28.55 ± 0.02 a
T3His	-	-	+	-	31.41 ± 0.06 a
RF	NA <sup>z</sup>	NA	NA	NA	10.30 ± 0.1 c
P	NA	NA	NA	NA	10.14 ± 0.04 c

<sup>v</sup>HR = hypersensitive response. NIL = near-isogenic line. Constructs were introduced into the virulent *Xanthomonas campestris* pv. *vesicatoria* (Xcv) strain ME-90 under control of their original promoter.

<sup>x</sup> Constructs were introduced into *Agrobacterium tumefaciens* C58C1 under control of the 35S promoter.

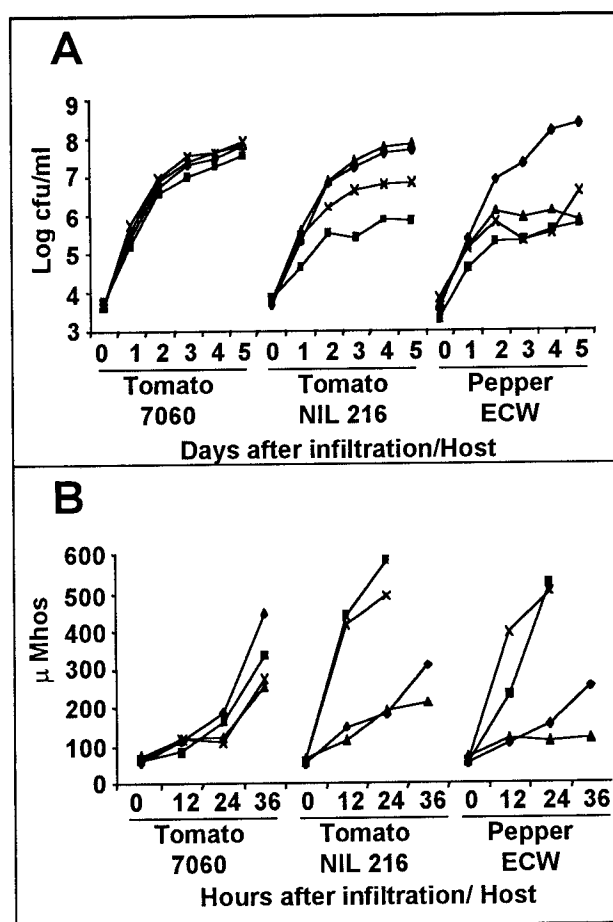
<sup>y</sup> Constructs were introduced into yeast strain EGY48 under control of a yeast constitutive promoter.  $\beta$ -Galactosidase activity  $\pm$  1 standard error is expressed in Miller units. Different letters next to each number indicate statistically significant differences by Duncan test ( $\alpha = 0.05$ ).

<sup>z</sup> Not applicable.

*coli* strain BL21 (DE3) pLysS carrying the fusion clone resulted in production of a fusion protein of expected size (data not shown).

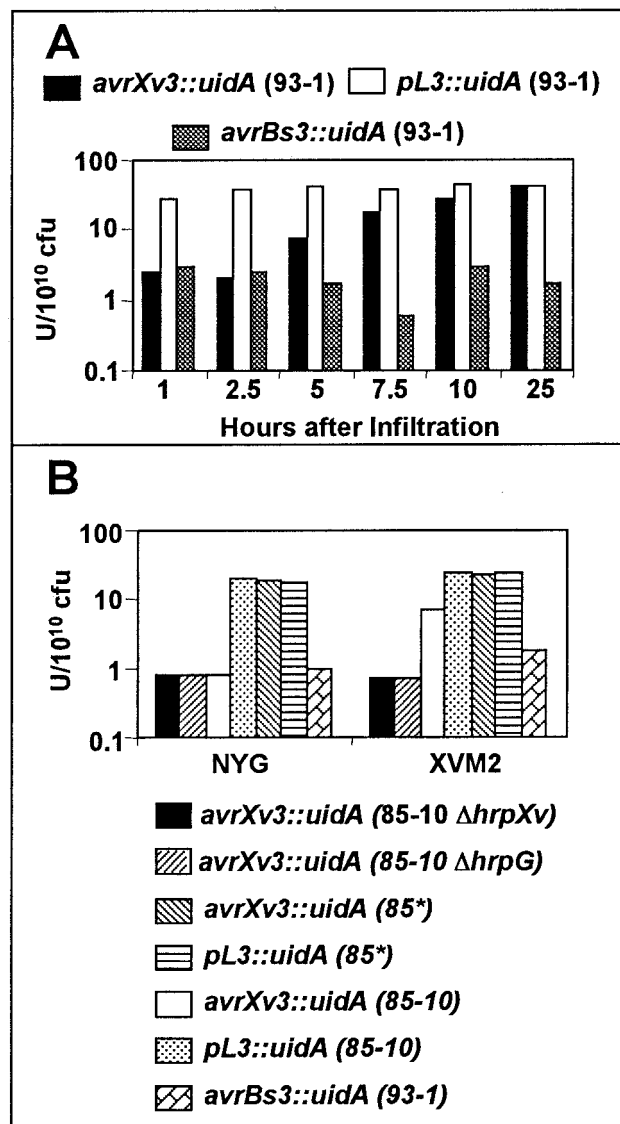
A chicken polyclonal antibody was raised against the putative AvrXv3 protein. Initial efforts to detect production of the *avrXv3* protein in Xcv strains were complicated by the presence of many nonspecific bands on Western blots (immunoblots) when purified total IgY extracts were used as primary antiserum (data not shown). This antiserum was affinity-purified to avoid the problem of nonspecific bands. Affinity-purified antibody detected AvrXv3 and AvrXv3-His fusion proteins of expected sizes expressed in Xcv strain 93-1 transconjugants and *E. coli*, respectively (data not shown).

GUS activity of Xcv transconjugants carrying various *uidA* fusion constructs was determined in media and in planta induction experiments. Results indicated that *avrXv3* is strongly induced in the pepper cultivars Early Calwonder (ECW) and ECW30R, and the tomato cultivars Fla. 7060 and NIL 216 (Fig. 4A), as well as in XVM2 broth (Fig. 4B). The level of



**Fig. 3.** A, Growth of *Xanthomonas campestris* pv. *vesicatoria* wild-type and mutant strain transconjugants infiltrated at  $10^5$  CFU per ml into leaves of tomato (genotypes Fla. 7060 and NIL 216) and pepper cv. Early Calwonder, and incubated at 28°C. B, Electrolyte leakage from tomato (genotypes Fla. 7060 and NIL 216) and pepper cv. Early Calwonder after infiltration of leaves with *X. campestris* pv. *vesicatoria* wild-type and mutant strain transconjugants at  $10^8$  CFU per ml and incubation at 28°C. Transconjugants used were pUFR051::93-1 virulent T2 (◆), pT3-5::93-1 avirulent T2 carrying *avrXv3* (■), pUFR051::ME24 virulent T3 (▲), and pUFR051::91-118 avirulent T3 (×).

GUS activity was comparable to the level exhibited by transconjugants expressing *uidA* constitutively behind the *lacZ* promoter. In planta, induction of the *avrXv3* gene occurred within 5 h post inoculation and GUS activity was similar to or higher than the level detected in transconjugants carrying pUXV1006::61 (*avrBs3-uidA* fusion) 25 h post inoculation.



**Fig. 4. A,** Activity of  $\beta$ -glucuronidase (GUS) expressed in *Xanthomonas campestris* pv. *vesicatoria* (Xcv) transconjugants grown in the tomato near-isogenic line (NIL) 216. Transconjugants of the virulent Xcv strain 93-1 carrying translational fusions pT3-5::249 (*avrXv3::uidA*), pUXV1006::61 (*avrBs3::uidA*), and plasmid-borne GUS gene under control of *lacZ* promoter (*pL3::uidA*) were infiltrated into tomato NIL 216. GUS activity is reported as U per 10<sup>10</sup> CFU (nanomoles methylumbelliferone [MU] produced per min per 10<sup>10</sup> CFU). The same trend was observed with pepper cvs. Early Calwonder (ECW) and ECW30R, and tomato genotype Fla. 7060. **B,** GUS activity expressed in Xcv transconjugants grown in different media. Bacteria were grown at 28°C with shaking for 18 h in NYG (Daniels et al., 1984) or XVM2 (Wengelnik et al. 1996a) broth. pT3-5::249 (*avrXv3::uidA*) in Xcv 85-10  $\Delta$ *hrpXv*, pT3-5::249 (*avrXv3::uidA*) in Xcv 85-10  $\Delta$ *hrpG*, pT3-5::249 (*avrXv3::uidA*) in Xcv 85\*, GUS gene under control of *lacZ* promoter (*pL3::uidA*) in Xcv 85\*, pT3-5::249 (*avrXv3::uidA*) in 85-10, *pL3::uidA* in 85-10, and pUXV1006::61 (*avrBs3::uidA*) in Xcv 93-1.

When the same GUS fusion constructs were introduced into Xcv strain 85\* expressing a constitutively active *hrpG* gene (Wengelnik et al. 1999), a high level of GUS expression was observed for the *avrXv3* construct (Fig. 4B). In contrast, when GUS fusion constructs were introduced into the mutant Xcv strains 85-10  $\Delta$ *hrpXv* and 85-10  $\Delta$ *hrpG*, which are unable to express *hrpX* and *hrpG* (Wengelnik and Bonas 1996; Wengelnik et al. 1996b), respectively, only a low level of GUS activity was detected (Fig. 4B).

#### *avrXv3* induces the HR when expressed inside the plant cell.

*Agrobacterium tumefaciens*-mediated expression of *avrXv3* inside plant cells led to HR in the resistant tomato NIL 216, but not in the susceptible Fla. 7060, 48 h after inoculation (Table 1). The confluent necrosis observed in the resistant genotype as a result of transient expression resembles the HR induced by race T3 of Xcv expressing *avrXv3*. Furthermore, HR in the resistant tomato NIL 216 only occurred when the 35S promoter controlled the expression of *avrXv3*. *A. tumefaciens* carrying the empty binary vector pMD-1 did not induce the development of any reaction on either NIL 216 or Fla. 7060. When mutated constructs of *avrXv3* were introduced into the plant cells by *A. tumefaciens*-mediated transient expression, only the HisT3 and T3His mutants were able to elicit the HR (Table 1).

#### *AvrXv3* has putative transcription activation activity.

Wild type and the mutated forms of *avrXv3* fused to the LexA DNA binding domain were expressed in the yeast strain EGY48. The transcription activation activity of each construct was determined by measurement of  $\beta$ -galactosidase activity. *AvrXv3* exhibited significant transcription activation activity, compared with the negative controls, the homeodomain of the *Drosophila* protein Bicoid (RF), and the plasmid pEG202 (P) without the insert (Table 1).

Deletion of acidic regions located at the N terminus and middle portion of the *AvrXv3* protein did not alter its transcription activation activity. However, the deletion of 52 aa at the C terminus of MutC made this mutant unable to induce transcription of the  $\beta$ -galactosidase reporter gene. The addition of His tags to either end of the *AvrXv3* protein did not alter its transcription activation activity (Table 1).

#### DISCUSSION

A gene-for-gene system has been identified involving T3 strains of Xcv, three different cultivars of tomato, and all cultivars of pepper tested (Minsavage et al. 1996). We cloned and characterized the *avr* gene involved in the interaction and designated it *avrXv3*. Southern hybridization analysis indicates that this *avr* gene is limited to T3 strains.

Bacterial growth and electrolyte leakage experiments in planta indicated that mobilization of the active *avr* gene into a previously virulent strain resulted in a lower growth rate of the bacterium in the resistant tomato and pepper genotypes. This limited growth was most likely due to the onset of the HR, as has also been reported for other Xcv strains carrying *avr* genes, such as *avrRxv* (Whalen et al. 1993). Interestingly, when *avrXv3* was mutated by marker exchange in the original strain 91-118, bacterial growth in pepper was not restored to the fully compatible level as in tomato NIL 216. This may in-

dicating the presence of at least one additional pepper-specific *avr* gene or contribution of *avrXv3* to virulence of strain 91-118 in pepper.

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 due to rapid cell death caused by the incompatible interaction between *avrXv3* and *Xv3*. Furthermore, the onset of electrolyte leakage induced by avirulent strains correlated with the onset of the visible HR (24 h). This finding agrees with observations made of other gene-for-gene interactions involving other races of Xcv (Minsavage et al. 1990; Whalen et al. 1993). Conversely, the speed and amount of damage caused by the virulent strains were characteristically reduced in the compatible interactions. These results indicate that *avrXv3* alone was able to restrict growth of Xcv strain 93-1 and induce the HR in resistant plants.

The monocistronic nature observed in the *avrXv3* gene agrees with what has been found for the majority of *avr* genes described so far (Leach and White 1996). A PIP-box upstream of the start codon of *avrXv3* resembles that described for the *hrpB* and other pathogenicity operons of Xcv (Fenselau and Bonas 1995). However, previous studies on the role of this element in the expression of the *avrRxv* gene from Xcv indicated that the PIP-box was not essential for its activity (Ciesiolka et al. 1999). In *avrXv3*, the PIP-box may be involved in the regulation of gene expression once the bacterium is inside the plant. Analysis of *avrXv3-uidA* fusions indicated that the expression of *avrXv3* was strongly induced in tomato, pepper, and the medium XVM2, which is known to mimic the intercellular space of plant cells (Wengelnik et al. 1996a). In addition, constitutive expression of GUS in transconjugants of the Xcv strain 85\* (Wengelnik et al. 1999) carrying the *avrXv3-uidA* fusion and a mutated form of *hrpG* suggested that expression of *avrXv3* is controlled by the *hrp* gene regulatory system. Furthermore, the lack of induction of GUS expression observed in the mutant Xcv strains 85-10  $\Delta$ *hrpG* and 85-10  $\Delta$ *hrpXv* carrying the *avrXv3-uidA* fusion indicated that *hrpXv* is necessary for the activation of transcription of *avrXv3*. These results are the first report of a plant-inducible *avr* gene found in a plant-pathogenic bacterium. The direct role of the PIP-box in the expression of *avrXv3* still remains to be confirmed.

In this study, *A. tumefaciens*-mediated transient expression of *avrXv3* indicated that the gene product must be present inside the host cell to trigger the HR. These results agree with what has been shown for several other Avr proteins (Bonas and Van den Ackerveken 1997; de Feyter et al. 1998; Duan et al. 1999; Gopalan et al. 1996; Leister et al. 1996; Parker and Coleman 1997; Scofield et al. 1996; Tang et al. 1996; Van den Ackerveken et al. 1996).

The clustering of negative residues in a particular region of a protein along with interspersed hydrophobic amino acids could be associated with domains involved in activation of transcription (Goodrich et al. 1996; Ptashne and Gann 1997). Experiments designed to compare the ability of different deletion mutants to activate transcription in yeast indicated that the C terminus of AvrXv3 encoded an active transcription activation domain. The presence of a transcription activation domain at the C terminus of the AvrXv3 protein draws a parallel with previous reports on similar findings in the AvrXa10

protein (Zhu et al. 1998). However, in contrast to AvrXa10, nuclear localization signals (NLSs) are absent in the AvrXv3 protein, and a lack of correlation between transcription activation and avirulence was observed with mutant alleles of AvrXv3. The inability of MutA and MutB to cause HR in tomato may be explained by the disruption of potential DNA-binding or protein-binding domains required for avirulence. On the other hand, the small size of the AvrXv3 protein and its potential ability to diffuse into the nucleus may explain the lack of an NLS.

Tagging of bacterial *avr* genes has been commonly used to monitor protein expression and secretion. The short, synthetic epitope tag known as FLAG has been successfully used to construct C-terminal fusions with several Avr proteins such as AvrB, AvrPto, AvrRxv, AvrXa10, and AvrBs3 without causing any apparent alterations in their secretion or avirulence activity (Gopalan et al. 1996; Rossier et al. 1999; Van den Ackerveken et al. 1996; Young et al. 1994). Addition of the His tags at either terminus of the AvrXv3 protein did not alter its ability to elicit the HR in tomato, assessed by transient expression, or to activate transcription in yeast. However, when either construct was introduced into a virulent strain of Xcv, the resulting transconjugants were unable to elicit the HR in the resistant host. These results may indicate that modifications of the termini could be interfering with the secretion of AvrXv3 either by modifying an unknown signal for secretion recognized by the Hrp system or by altering the three-dimensional structure of the protein needed for secretion. It is also important to mention that the His tags used in this work contained six histidine residues flanked by stretches of 4 aa and 33 aa (pET-15b), or six histidine residues preceded by 44 aa (pET-22b). Therefore, the higher complexity and length of the His tag, compared with the FLAG, could explain why secretion was inhibited when the His tag was used.

## MATERIALS AND METHODS

### Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 2. Strains of Xcv were grown at 28°C on plates of nutrient agar (BBL; Becton Dickinson and Company, Cockeysville, MD) or in nutrient broth for plant inoculations. Strains of *E. coli* were routinely cultivated at 37°C on Luria-Bertani (LB) medium (Maniatis et al. 1982). Plasmids were introduced into *E. coli* by transformation (Maniatis et al. 1982) and mobilized into *Xanthomonas* 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). *Xanthomonas* spp. transconjugants were grown at 28°C in nutrient, NYG, or XVM2 broth (Wengelnik et al. 1996a) for gene induction and protein expression experiments. Antibiotics were added to the media at the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 25 or 50 µg/ml; rifamycin SV, 100 µg/ml; spectinomycin, 50 µg/ml; and tetracycline, 12.5 µg/ml.

### Plant material and plant inoculations.

Seeds of pepper (*Capsicum annuum*) cv. Early Calwonder and NIL ECW30R, tomato cvs. Fla. 7060, NIL 216, and H7998 were planted in Plugmix (W. R. Grace & Co., Cam-

bridge, MA). After 2 weeks, the emerged seedlings were transferred to Metromix 300 (W. R. Grace & Co.) in 10-cm plastic pots. Seedlings were grown in the greenhouse at temperatures ranging from 25 to 35°C (night/day). Tomato plants were grown for 4 to 5 weeks and then the terminal bud was removed above the fully expanded sixth true leaves. Plants were inoculated approximately 7 days after topping and transferred to a growth room kept at a constant temperature of either 25 or 28°C with a daily 16-h light period.

Bacterial cultures for plant inoculations were grown in nutrient broth for 18 h at 28°C with shaking (100 rpm; KS10; BEA-Enprotech, Hyde Park, MA). Cells were pelleted by centrifugation (4,000 × *g*, 15 min) and resuspended in sterile tap water. For electrolyte leakage in pepper and tomato, the concentration of cells was adjusted to an OD<sub>600</sub> = 0.3 (2–5 ×

10<sup>8</sup> CFU per ml) with a spectrophotometer (Spectronic 20; Spectronic-UNICAM, Rochester, NY). Bacterial suspensions were diluted to a concentration of 2–5 × 10<sup>5</sup> CFU per ml in sterile tap water for population dynamics studies. Leaves of pepper and tomato were infiltrated with a hypodermic syringe and needle as previously described (Hibberd et al. 1987).

#### Molecular genetic techniques.

Standard molecular techniques were used for the extraction of genomic and plasmid DNA, restriction endonuclease digestions of DNA, cloning procedures, and Southern blot hybridization analyses (Ausubel et al. 1992; Maniatis et al. 1982). Enzymes for restriction digestion and ligation reactions were purchased from Promega (Madison, WI) and used following the manufacturer's protocol. For Southern hybridiza-

**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 <sup>r</sup> , <i>avrXv3</i> <sup>+</sup>	This study
97-2	Tomato race 3, <i>avrXv3</i> <sup>+</sup>	This study
87-13, 93-1	Tomato race 1, Rif <sup>r</sup>	This study
ME24	91-118 marker exchange, <i>avrXv3</i> mutant	This study
85-10	Pepper race 2, tomato race 1, Rif <sup>r</sup>	Minsavage et al. 1990
85*	85-10, E44K exchange mutant, <i>hrpG</i> <sup>*</sup> , Rif <sup>r</sup>	Wengelnik et al. 1999
85-10 $\Delta$ <i>hrpG</i>	85-10 deleted for <i>hrpG</i> , Rif <sup>r</sup>	Wengelnik et al. 1996b
85-10 $\Delta$ <i>hrpXv</i>	85-10 deleted for <i>hrpXv</i> , Rif <sup>r</sup>	Wengelnik and Bonas 1996
ME-90	85-10 carrying Tn5, Rif <sup>r</sup> Km <sup>r</sup>	Bonas et al. 1991
XV153, XV155, XV157	Tomato race 1, phenotypic group A, <i>avrXv3</i> absent	
XV330, XV338, XV1085	Tomato race 1, phenotypic group A1, <i>avrXv3</i> absent	
56, 141, 144, 1111, BV5-4a, MME	Tomato race 2, <i>avrXv3</i> absent	
XV938, XV1220, XV1484	Tomato race 3, <i>avrXv3</i> <sup>+</sup>	
<i>X. gardneri</i>		
XG444, XG451, XCGA2	<i>avrXv3</i> mutant	
<i>Saccharomyces cerevisiae</i>		
EGY48	MAT $\alpha$ , <i>trp1</i> , <i>his3</i> , <i>ura3</i> , <i>6ops-LEU2</i>	R. Brent <sup>y</sup>
<i>Agrobacterium tumefaciens</i>		
C58C1	Carrying pGV2260, Rif <sup>r</sup>	B. Staskawicz <sup>z</sup>
<i>Escherichia coli</i>		
DH5 $\alpha$	F <sup>-</sup> <i>recA</i> $\Phi$ 80 <i>dlacZ</i> $\Delta$ M15	Bethesda Research Laboratories, Gaithersburg, MD
C2110	Nal <sup>r</sup> <i>polA</i> mutant	Stachel et al. 1985
HB101	F <sup>-</sup> <i>recA</i>	Boyer and Roulland-Dussoix 1969
BL21(DE3)pLysS	Host strain for pET-15b	Novagen, Madison, WI
Plasmids		
pLAFR3	Tc <sup>r</sup> <i>rlx</i> <sup>+</sup> RK2 replicon, Tc <sup>r</sup>	Staskawicz et al. 1987
pL3 <i>uidA</i>	Constitutive <i>lacZ-uidA</i> fusion	B. Staskawicz
pLAFR6	pLAFR1 with <i>trp</i> terminators	B. Staskawicz
pUFR051	pLAFR3 with pUC19 polylinker	de Feyter and Gabriel 1991
pBluescript II KS +/-	Phagemid sequencing vector, Ap <sup>r</sup>	Stratagene, La Jolla, CA
pET15-b	His tag expression vector, Ap <sup>r</sup>	Novagen, Madison, WI
pET15-b24	<i>avrXv3</i> expression clone	This study
pRK2073	Sp <sup>r</sup> Tra <sup>+</sup> , helper plasmid	Figurski and Helinski 1979
pHoKmGUS	Km <sup>r</sup> Ap <sup>r</sup> <i>mpA</i> <sup>-</sup> , Tn3- <i>uidA</i> fusion	B. Staskawicz
pSShe	Cm <sup>r</sup> <i>mpA</i>	Stachel et al. 1985
pUXV1006::61, ::74	<i>avrBs3</i> Tn3- <i>uidA</i> derivatives	Bonas et al. 1989
pT3-176	Tc <sup>r</sup> cosmid clone, <i>avrXv3</i> <sup>+</sup>	This study
pT3-5	Tc <sup>r</sup> pLAFR6 subclone, <i>avrXv3</i> <sup>+</sup>	This study
pT3-5::24, ::47, ::144, ::221, ::245, ::249	<i>avrXv3</i> Tn3- <i>uidA</i> derivatives	This study
pMD-1	Binary vector, 35S, Km <sup>r</sup>	B. Staskawicz
pEG202	LexA-fusion, <i>HIS3</i> , Ap <sup>r</sup>	R. Brent
pMW106	<i>URA3</i> , 2 $\mu$ , 8ops- <i>lacZ</i> , Km <sup>r</sup>	R. Brent
pRFHM1	<i>HIS3</i> , homeodomain of Bicoid, Ap <sup>r</sup>	R. Brent
pSH17-4	<i>HIS3</i> , LexA-Gal4p, Ap <sup>r</sup>	R. Brent

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tions, DNA probes were labeled with  $\alpha^{32}\text{P}$  with the Random Primed DNA Labeling kit (Boehringer Mannheim, Indianapolis, IN) and probed against the DNA immobilized on a Biotyne nylon membrane (Life Technologies, Grand Island, NY).

A total genomic DNA library of Xcv strain 91-118 (tomato race 3) was constructed in the cosmid vector pLAFR3 as previously described (Minsavage et al. 1990). Individual clones were mobilized into Xcv strain 87-13 or 93-1 by triparental mating (Ditta et al. 1980; Figurski and Helinski 1979) and transconjugants were inoculated into pepper and tomato to screen for elicitation of an HR.

The cosmid pT3-176 containing a 35-kb insert of Xcv DNA was selected for further manipulation. A 5.5-kb *Bam*HI-*Eco*RI DNA fragment from pT3-176 was subcloned into pBluescript KS +/- (Stratagene, La Jolla, CA) for further analysis. Deletion subclones were generated by restriction digestion of the pBluescript clone with *Xho*I, *Sal*I, or *Cla*I (one flanking site for each within the polylinker), removal of excised fragments, and self-ligation (recircularization) of the remaining vector-insert DNA. Various fragments were subcloned into pUFR051 to assess the HR-inducing activity in Xcv. The smallest active subclone generated by *Cla*I deletion (2.2-kb insert) was further subcloned by digestion with *Pst*I and subsequent recircularization of the plasmid. The insert DNA was cloned into pBluescript for sequencing, and also into pLAFR6 for Tn3-*uidA* insertional mutagenesis tests. The pLAFR6 clone, designated pT3-5, elicited an HR when mobilized into Xcv and inoculated into resistant host plants.

#### DNA sequence analysis.

Sequencing of both strands of a 1,524-bp (pT3-5) DNA fragment encompassing *avrXv3* was initiated with pBluescript vector T3 and T7 primers. Sequencing was done at the ICBR sequencing facility (University of Florida, Gainesville, FL) with the Applied Biosystems (Foster City, CA) model 373 system. To complete sequencing, custom primers were synthesized at the ICBR facility with an Applied Biosystems model 394 DNA synthesizer. The computer program SeqAid II version 3.81 was used to analyze nucleotide sequence data and predicted protein products.

The analysis of distribution of hydrophobic and charged residues throughout the predicted AvrXv3 protein was carried out as described by Shaw (1995). A search for homology of the nucleotide sequence of *avrXv3* and the amino acid sequence of the predicted AvrXv3 was carried out on the World Wide Web with the BLAST 2.0 algorithm (Altschul et al. 1997).

Tn3-*uidA* insertion sites were sequenced with an oligonucleotide primer complementary to the N terminus of the GUS gene (Jefferson et al. 1987, 5'GATTTCACGGGTTGGGGT TCT3').

#### Protein expression in *E. coli*.

Custom oligonucleotide primers were synthesized, RST88 (5'CCGC TCGAGATGACAAGTAGTATCAATC3') and RST89 (5'CCGCTCGAGCTACTTAACGAGATTTGTTA C3'), which allowed amplification of a DNA fragment containing the 654-bp ORF of *avrXv3* from Xcv target DNA by the PCR. PCR amplification of DNA was done in a DNA thermocycler (M. J. Research, Watertown, MA) with *Taq*

DNA polymerase (Promega, Madison, WI). Each 50- $\mu$ l PCR contained 1 $\times$  amplification buffer (from the manufacturer), 100  $\mu$ M concentrations of each dNTP, 17.5  $\mu$ M concentrations each primer, 1.5 mM MgCl<sub>2</sub>, 1.25 U of *Taq* DNA polymerase, and 100 ng of template DNA. For this particular primer pair, each cycle consisted of 30 s of denaturation at 95°C, 30 s of annealing at 56°C, and 1 min of extension at 72°C. An initial cycle with an annealing temperature of 48°C was also added. For the final cycle, the extension step was prolonged to 5 min. Restriction endonuclease *Xho*I sites were incorporated into the primer sequences to facilitate cloning of the gene in-frame into the expression region of plasmid vector pET-15b. PCR products were treated with proteinase K and cleaned as previously described (Crowe et al. 1991) prior to digestion with *Xho*I for cloning into the fusion vector.

The fusion plasmid pET15-b24 was transformed into *E. coli* host BL21 (DE3) pLysS and sequenced to confirm that the correct fusion had been generated. Fusion protein was expressed in *E. coli* at 37°C for 3 to 5 h after induction of early log phase cultures by the addition of 0.1 mM IPTG (isopropyl  $\beta$ -thiogalactopyranoside). Fusion protein was affinity purified from French-pressed cell suspensions (30 ml of 4-h induced culture) in the presence of urea (6 M) and phenylmethylsulfonyl fluoride (0.5 mM) with the His-Bind Resin and Buffer Kit (Novagen, Madison, WI) following the manufacturer's protocol. Protein extracts were desalted with Microcon 100 microconcentrator spin columns (Amicon, Beverly, MA) and stored at -70°C in 10 mM phosphate-buffered saline (PBS, pH 7.2).

Protein concentrations were measured with BioRad protein assay dye reagent (BioRad Laboratories, Hercules, CA). Recovery of fusion protein was verified by solubilization in Laemmli buffer (Maniatis et al. 1982), boiling for 5 min. and running on 12% discontinuous sodium dodecyl sulfate-polyacrylamide ready-gels (BioRad Laboratories). For analytical protein analyses, *E. coli* or *Xanthomonas* cells were harvested by centrifugation, washed once in sterile, distilled water, and solubilized in Laemmli buffer. Gels were stained in Coomassie blue R-250 or transferred to polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories) for Western blot analysis with a BioRad Mini Trans-blot chamber according to the manufacturer's protocol.

#### Preparation of antisera and immunoblotting.

The AvrXv3 fusion protein purified from *E. coli* was used for immunization of chickens for polyclonal antibody (IgY) production. Two 6- to 8-month-old laying Leghorn hens were injected subcutaneously along the wing vein and in one footpad with 100  $\mu$ l of purified protein in PBS at a concentration of 100  $\mu$ g/ml. Additional injections were made after 2 and 12 weeks to boost specific antibody titer. Eggs were collected daily, marked for identification, and stored at 4°C until a period of 2 weeks after the final immunization. Peak titers of 1/1,000 were detected in eggs of the immunized hens approximately 9 days post-immunization as determined in enzyme-linked immunosorbent assays (ELISAs; data not shown).

Initially, crude IgY extracts were prepared from every third egg by dispersing the yolks in 4 volumes of 0.1 M phosphate buffer at pH 7.6 (Polson et al. 1985). Sample dilutions of 1/100 and 1/1,000 were used as the primary antisera in indi-

rect ELISAs to identify peak antibody titers. Briefly, wells of MicroFluor B microtiter plates were coated with 100  $\mu$ l of purified fusion protein (1  $\mu$ l/ml in coating buffer) by incubation at 4°C overnight. The wells were washed 4  $\times$  125  $\mu$ l with PBST (PBS buffer with 0.05% Tween 20). Dilutions of the crude yolk extracts (100  $\mu$ l) were added to each well and the plates were incubated for 2 h at room temperature. Wells were washed 4  $\times$  125  $\mu$ l with PBST and then 100  $\mu$ l of 1/1,000 diluted anti-chicken IgG-alkaline phosphatase conjugate (Sigma Chemical, St. Louis, MO) was added. Plates were incubated at room temperature for 2 h and the wells were then washed 4  $\times$  125  $\mu$ l with PBST before 100  $\mu$ l of substrate solution (4-methylumbelliferyl phosphate 0.33 mg/ml, MUP, Sigma Chemical Company) was added. Fluorescence in the wells was measured after incubation of the plates for 30 min at room temperature.

IgY was purified from individual eggs selected from high titer (peak) sample times by polyethylene glycol precipitation as previously described (Polson et al. 1985) or by the commercially available EGGstract IgY Purification System (Promega, Madison, WI). Titer of individual eggs was determined by ELISA and one preparation was selected for use in immunoblot assays. The anti-fusion antiserum was affinity purified to the fusion protein immobilized on nitrocellulose (Smith and Fisher 1984) and antibodies recovered in the pH 2.3 eluate were used for immunoblot tests at a dilution of 1/100 relative to the original purified extract.

Western blot analysis was carried out as described by Maniatis et al. (1982). Blots were reacted for 20 h at 4°C with primary IgY antiserum and bound antibody was detected with anti-chicken IgG-alkaline phosphatase conjugate and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate tablets (Boehringer Mannheim, Indiana, IN).

### Mutagenesis of *avrXv3*.

Insertion mutagenesis was performed with Tn3-*uidA* as previously described (Bonas et al. 1989). Individual insertion derivatives were analyzed by restriction enzyme digestion profiles and mobilized into Xcv strain 93-1 for HR tests in plants. Selected insertion derivatives were used to generate marker exchange gene replacement mutants in the wild-type Xcv strain 91-118 as described by Bonas et al. (1991).

The positions of insertions in derivatives selected for protein expression experiments were confirmed by sequencing. GUS (*UidA*) activity was determined in Xcv transconjugants after growth in NYG or XVM2 broth and in leaves of pepper and tomato by measurement of fluorescence with 4-methylumbelliferyl  $\beta$ -D-glucuronide as substrate (MUG; Sigma Chemical Company) as previously described (Schulte and Bonas 1992; Wengelnik and Bonas 1996).

Samples of Xcv grown in plant tissue for various lengths of time were obtained by macerating an area of 1 cm<sup>2</sup> of inoculated leaf tissue in 1 ml of sterile tap water. An aliquot of 500  $\mu$ l of the macerated tissue suspension was centrifuged for 2 min at 12,000  $\times$  g in a microcentrifuge tube to pellet bacterial cells. The pellets were resuspended in 50  $\mu$ l of sterile, distilled water and frozen at -70°C until all samples from all time points were accumulated. The samples were then thawed quickly and 450  $\mu$ l of MUG extraction buffer (Jefferson 1987; Jefferson et al. 1987) was added to each tube. Reactions were

incubated at 37°C and terminated at regular time intervals with 0.2 M Na<sub>2</sub>CO<sub>3</sub>. Fluorescence was measured with a Cytofluor II fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham, MA) calibrated against known standard concentrations of methyl-umbelliferone (MU; Sigma Chemical Company). MicroFluor B flat bottom microtiter plates (Dynatech Laboratories, Chantilly, VA) were used.

Appropriate dilutions of plant extracts and broth cultures were plated onto media to determine the CFU of bacteria to calculate *UidA* activity as units per CFU value. This value was calculated based on the number of nanomoles of MU produced per minute per CFU of bacteria.

PCR-based deletion mutagenesis was performed on the clone pT3-5 to create in-frame deletions of approximately 50 aa in three different sites along the sequence of this gene. This clone carries the entire ORF of *avrXv3* and its original promoter region. First, pT3-5 was digested with *Hind*III and *Eco*RI and the fragment containing the promoter region and the ORF of *avrXv3* was transferred to pBluescript KS, resulting in pBS:T3 $\Delta$ Pst. The targets for deletion were chosen by identifying putative domains defined by the distribution of hydrophobic and charged residues in the predicted protein (Fig. 2). A set of eight oligonucleotide primers was synthesized at the ICBR DNA Synthesis Laboratory (University of Florida, Gainesville) to make the in-frame deletions of the three putative domains (Table 3). Separate PCRs were carried out with pBS:T3 $\Delta$ Pst as template and the following primer combinations: P1/P2, P3/P8, P1/P4, P5/P8, P1/P6, and P7/P8. To ease the process of screening for the right construct, unique restriction sites for the endonucleases *Xho*I, *Ava*I, and *Kpn*I were engineered as silent mutations in primers P3, P5, and P7, respectively (Table 3). PCR was carried out as described above. The annealing temperature used for all primers was 60°C.

Subsequently, PCR products were diluted 100-fold and an aliquot of 2  $\mu$ l of each product was combined as follows: P1/P2 + P3/P8, P1/P4 + P5/P8, and P1/P6 + P7/P8. The mixtures were used as templates for a second PCR with the primers P1/P8 and the same conditions described above. The resulting modified constructs were designated *mutA*, *mutB*, and *mutC*. These mutants were digested with *Hind*III and *Eco*RI and cloned into pLAFR6.

To determine the effect of possible changes in the three-dimensional structure of the *AvrXv3* on its activity, the termini of the predicted protein were also modified by the addition of six His residues. First, the ORF of *avrXv3* was cloned into pET15b as described before. The resulting construct was designated pET15b:HisT3. Secondly, primers P1 and RST88b (5'CCGCTCGAGCTTCTTAACGAGATTTGTTAC3'), which eliminate the stop codon of the gene, were used to isolate the ORF of *avrXv3* and its original promoter from pBS:T3 $\Delta$ Pst. The resulting fragment was digested with *Hind*III and *Xho*I and cloned into pET22b, in frame with an His tag at the 3' end of the ORF. This construct was designated pET22b:T3His. PCR was carried out as described before. All mutants were sequenced at the ICBR sequencing facility.

### Avirulence activity in Xcv background.

The constructs pLAFR6:*mutA*, pLAFR6:*mutB*, and pLAFR6:*mutC* were introduced into the virulent Xcv strain ME-90 by triparental mating and inoculated into the tomato



**Table 3.** Sequence of specific oligonucleotides used for polymerase chain reaction-based deletion mutagenesis of *avrXv3*<sup>z</sup>

ID	Oligonucleotide sequence	Restriction enzyme site added
P1	5'GCGCGCAATTAACCCTCACTAAAG3'	–
P2	5'GTAACGATTGATACTACTTGTTCATGG3'	–
P3	5'GACAAGTAGTATCAATCGTTCGCTCGAGTGGAGCAGGTCG3'	<i>XhoI</i>
P4	5'AACGCCCTTGATCGGCTTATTTCG3'	–
P5	5'ATAAGCCGATCAAGGGCGTTGTTATGCCCGAGAATCGC3'	<i>AvaI</i>
P6	5'TTTAGCGGCATACCCCTGCGAACG3'	–
P7	5'CGCAGGGGTATGCCGCTAAAGAAAAGGGTACCGTAAGG3'	<i>KpnI</i>
P8	5'CG CGCGTAATACGACTCACTATAG3'	–

<sup>z</sup> Restriction sites added by each oligonucleotide are also indicated.

NILs 216 and Fla. 7060 as described above. To generate pLAFR6:T3His, pET22b:T3His was digested with *HindIII* and *PstI* and the insert was cloned into pLAFR3. The construct HisT3 was cloned into a previously constructed plasmid, pLAFR3:Pt3, under control of the original promoter region of *avrXv3*. The resulting constructs, pLAFR3:T3His and pLAFR3Pt3:HisT3, were introduced into Xcv strain ME-90 by triparental mating and inoculated into the tomato NILs 216 and Fla. 7060 as described above.

#### A. *tumefaciens*-mediated transient expression.

The binary vector pMD-1, kindly provided by B. Staskawicz, was used for all *A. tumefaciens*-mediated transient expression assays. The ORFs of *avrXv3*, *mutA*, *mutB*, and *mutC* were isolated by PCR with the primers RST89b (5'CCGTCTAGAAATGACAAGTAGTATCAATC3') and RST88c (5'CCGGGATCCCTTCTTAACGAGATTGTTAC3'), which add the restriction sites *XbaI* upstream of the start codon and *BamHI* downstream of the stop codon, respectively. Following digestion with the appropriate enzyme combination, constructs were cloned into pMD-1. The ORF of *avrXv3* was also cloned into the binary vector pO4541, which lacks the 35S promoter. HisT3 was isolated from pET15b:HisT3 by PCR with the primers HIST3-F (5'CCGGAATTCATGGGCAGCA GCCATCAT3') and RST88c, which add *EcoRI* and *BamHI* sites, respectively. T3His was isolated from pET22b:T3His by PCR with the primers RST89c (5'CCGGAATTCATGACAA GTAGTATCAATC3') and T3HIS-R (5'GCTGGATCCAG TTATTGCTCAGCGG3'), which add *EcoRI* and *BamHI* sites, respectively. Following digestion with the appropriate enzyme combination, constructs were cloned into pMD-1. All constructs were maintained in *E. coli* and transferred to *A. tumefaciens* by triparental mating as described before.

The *A. tumefaciens* strain C58C1 containing the Ti-plasmid pGV2260 (Deblaere et al. 1985) and individual transconjugants were grown overnight at 28°C in YEB medium (Kapila et al. 1997) amended with 10 mM *N*-morpholino-ethanesulfonic acid (MES) pH 5.6 (Sigma Chemical Company), 20 µM acetosyringone (Sigma Chemical Company), and the appropriate antibiotics. After overnight growth, the concentration was adjusted to an  $A_{600} = 0.5$ – $0.6$ , bacteria were pelleted, washed with MMA medium pH 5.6 (Murashige and Skoog's medium, Gibco BRL, amended with 10 mM MES, sucrose 20 g/liter, and 200 µM acetosyringone) and resuspended in the same medium to a final concentration of  $A_{600} = 0.05$ . Bacterial suspensions were kept at 25°C for 1 h and then used for infiltration. Fully expanded leaves of the tomato cultivar Fla. 7060 and NIL 216 were infiltrated with the bacterial suspension as described before. After infiltration, plants were incubated at

22°C under continuous light for 48 h or until symptoms developed. Unless otherwise indicated, inoculations were done three times.

#### Transcription activation activity.

The yeast strain EGY48 (*Saccharomyces cerevisiae*) carrying the plasmid pMW106, which contains the *lacZ* gene under control of LexA-regulated promoters, was used for testing transcription activation activity. The vector pEG202, containing the LexA DNA binding domain sequence under control of the constitutive yeast ADH1 promoter and a polylinker region at the C terminus, was used for generating fusions with the wild type and modified AvrXv3 proteins. The ORF of all mutant genes was isolated by PCR with the following primer combinations: *mutB* and *mutC* with RST89c/RST88, *mutA* with RST89c/RST88c, *HisT3* with HIST3-F/RST88c, and *T3His* with RST89c/T3HIS-R. The plasmids pMW106 and pEG202 were maintained in yeast by selection for uracil and His auxotrophy, respectively, and in *E. coli* by selection for resistance to ampicillin and kanamycin, respectively. The plasmid pSH17-4 carrying the LexA fused to Gal4p activation domain was used as a positive control for transcription activation, and pRFHM1 carrying the non-activating fusion between LexA and the *Drosophila* protein Bicoid was used as negative control for transcription activation.

Expression of the expected fusion proteins in yeast was determined by standard Western blot analysis (Ausubel et al. 1992) with a polyclonal antibody raised against the LexA DNA-binding domain (kindly provided by E. Golemis). The membranes were reacted for 1 h at 25°C with primary Anti-LexA polyclonal antiserum followed by 1 h of incubation with the anti-rabbit IgG-alkaline phosphatase conjugate (Sigma Chemical Company). Bound antibody was detected with NBT/BCIP substrate tablets (Boehringer Mannheim, Indianapolis, IN). Methods for yeast manipulations were as described by Golemis and Brent (1997).  $\beta$ -Galactosidase activity assays were performed as described by Clontech Laboratories (Palo Alto, CA). Roger Brent (Department of Molecular Biology, Massachusetts General Hospital, Boston), kindly provided all plasmids and yeast strains used in this study. Unless otherwise indicated, all experiments were arranged in a completely randomized design with three replications. All experiments were done twice. Statistical analysis was conducted with the Statistical Analysis System (SAS Institute, Cary, NC).

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