Regulation of Expression of Avirulence Gene *avrRxv* and Identification of a Family of Host Interaction Factors by Sequence Analysis of *avrBsT*

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Resistance in tomato line Hawaii 7998 as well as in several nonhost plants to *Xanthomonas campestris* pv. *vesicatoria* tomato strain (XcvT) is mediated in part by the avirulence gene *avrRxv*. Analysis of growth of wild-type and *avrRxv* deletion strains indicates that *avrRxv* plays a crucial role in the ability of XcvT 92-14 to induce resistance on Hawaii 7998. We used *avrRxv* reporter gene fusions and Northern (RNA) blot analysis to test several growth environments for inductive potential. We found that *avrRxv* is constitutively expressed at high levels and that growth in planta, in tobacco conditioned medium, and in *hrp*-inductive medium XVM2 did not affect the high levels of expression. In addition, *hrp* structural and regulatory mutant backgrounds had no effect. We mutated the bipartite plant inducible promoter (PIP)-box sequence and found that *avrRxv* activity appears to be independent of an intact PIP-box element. We present the sequence of the *avrRxv* homologue called *avrBsT* and align the six *AvrRxv* host interaction factor family members including mammalian pathogen virulence factors YopJ and YopP from *Yersinia* spp. and *AvrA* from *Salmonella typhimurium*, and open reading frame Y4LO with unknown function from the symbiont *Rhizobium* sp.

Additional keywords: sequence overlap extension, site-directed mutagenesis.

Many recent studies of pathogens that associate with vastly diverse hosts illustrate the conservation of molecular determinants of pathogenicity. Specialized secretion systems and virulence factors targeted to host cells appear to play a role in pathogenic attack of hosts whether the host is a mammal or plant (reviewed in Leach and White 1996; Galan and Bliska 1996; Alfano and Collmer 1996; Baker et al. 1997; Finlay and Falkow 1997; Mudgett and Staskawicz 1998). In plant bacterial-pathogen interactions, the molecules for which there is evidence of host cell targeting were originally identified be-

cause they induce resistance in the host (Yang and Gabriel 1995; Gopalan et al. 1996; Scofield et al. 1996; Tang et al. 1996; Van Den Ackerveken et al. 1996). A dual role in virulence was uncovered for a subset of these so-called avirulence genes (Swarup et al. 1992; Yang et al. 1996; Van Den Ackerveken et al. 1996). It has been known for many years that the resistance-inducing activity of bacterial pathogens of plants is controlled in a simple genetic fashion by avirulence genes in the pathogen and corresponding resistance genes in the host (Leach and White 1996). Now that several resistance genes have been cloned and found to be active in such signal transduction functions as ligand binding (Scofield et al. 1996; Tang et al. 1996) and phosphorylation (Loh and Martin 1995a, 1995b; Zhou et al. 1995) a likely model is that avirulence gene products serve directly or indirectly to produce the ligand with which the resistance gene product interacts (Baker et al. 1997). It is predicted that secretion of avirulence gene products will resemble that of mammalian virulence factors (Fenselau and Bonas 1995; Alfano and Collmer 1997; Parker and Coleman 1997; Mudgett and Staskawicz 1998).

Studies of regulation of expression of avirulence genes in *Pseudomonas* spp. have illustrated functional interaction between genes for pathogenicity and genes for avirulence. In *Xanthomonas* spp., it appears that there may be more than one regulatory pathway for pathogenicity and avirulence genes. One pathway involves the putative promoter motif called the PIP-box (for plant inducible promoter; Fenselau and Bonas 1995). The regulatory proteins HrpXv (AraC homologue) and HrpG (response regulator protein for two component sensor systems) regulate expression of *hrp* genes, and in some *hrp* loci this regulation is thought to be mediated by PIP-box sequences (Wengelnik and Bonas 1996; Wengelnik et al. 1996b). Genes without PIP-boxes such as *avrBs3* (Knoop et al. 1991) and *hrpA* (Wengelnik and Bonas 1996) must use a second pathway.

Xanthomonas campestris pv. *vesicatoria* tomato strain (XcvT) is the causal agent of bacterial spot disease, a significant problem for tomato growers. Recent molecular phylogenetic studies have placed members of the traditional XcvT group into two species: *X. axonopodis* pv. *vesicatoria* (XcvT race 1 75-3 and 92-14) and *X. vesicatoria* (XcvT race 2 89-1)

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(Stall et al. 1994; Bouzar et al. 1994). For simplicity in this report, we will use the traditional nomenclature and the abbreviation XcvT for both groups. Several avirulence genes have been cloned from XcvT race 1 75-3, including *avrRxv* (Whalen et al. 1988), *avrBsT*, and *avrBs2* (Minsavage et al. 1990), which induce resistance on tomato and pepper, respectively. This present work focuses on *avrRxv* and *avrBsT*. Resistance in tomato to XcvT race 1 is mediated in part by the avirulence gene *avrRxv*, and has been shown to be genetically complex (Whalen et al. 1988, 1993; Wang et al. 1994; Yu et al. 1995). Additionally, sequence comparisons of AvrRxv have revealed similarity with three mammalian pathogen virulence factors (Hwin et al. 1996; Leach and White 1996). Avirulence gene *avrBsT* induces a characteristic hypersensitive response (HR) in all pepper lines tested to date (Minsavage et al. 1990). Spontaneous loss of the plasmid-borne *avrBsT* allows XcvT race 1 75-3 to then cause disease on a subset of normally resistant pepper lines, suggesting simple genetic control of nonhost, *avrBsT*-specific resistance (Minsavage et al. 1990).

For a full mechanistic understanding of the function of *avrRxv*, knowledge of regulation of expression and identification of homologues is crucial. In the studies described herein, we address *avrRxv* function first by analyzing control of expression and second by sequence analysis of *avrBsT*. We tested the hypothesis that the regulation of expression of *avrRxv* reflects its role in a host interaction pathway. To discover conditions under which *avrRxv* is maximally expressed, we used *avrRxv* reporter gene fusions and Northern (RNA) blot analysis and tested several growth environments for inductive potential. We found that *avrRxv* is constitutively expressed at high levels and that growth in planta, in tobacco conditioned medium, and in hrp-inductive medium did not affect the high levels of expression. In addition, the regulation of *avrRxv* expression was not altered in *hrp* mutant backgrounds and appears to be independent of the PIP-box motif. We present the sequence of the AvrRxv homologue AvrBsT and the alignment of the six-member AvrRxv family, including mammalian pathogen virulence factors.

RESULTS

Inoculation phenotypes and analysis of growth in planta of *avrRxv* deletion mutant.

To test whether the avirulence gene *avrRxv* was solely responsible for induction of resistance on Hawaii 7998, we created a deletion mutant of *avrRxv*. When the mutant strain XcvT 92-14 Δ avrRxv was inoculated into leaves of Hawaii 7998, a susceptible response (watersoaking) resulted. The subclone of *avrRxv*, pXV9007, complemented the mutation to yield a resistance response (HR). In leaves of Hawaii 7998, the final population size of XcvT 92-14 Δ avrRxv was about 10-fold more than that of wild-type XcvT 92-14 (Fig. 1). In contrast, in leaves of the susceptible line Bonny Best, the final population size of XcvT 92-14 Δ avrRxv was indistinguishable from that of XcvT 92-14 (Fig. 1), indicating the mutation had no effect on growth of XcvT 92-14. Comparison of growth of the XcvT 92-14 deletion mutant in Hawaii 7998 with growth in the susceptible line Bonny Best demonstrated that the final population size was about 10-fold less in Hawaii 7998 than in Bonny Best (Fig. 1).

Northern blot analysis of *avrRxv* expression.

The sequence of *avrRxv* predicts an open reading frame (ORF) of 1,121 bp (Whalen et al. 1993). To assess whether a transcript is produced that is consistent with that prediction, RNA from XcvT 92-14 and XcvT 92-14 Δ avrRxv grown to mid-log phase in the rich medium NYG was analyzed by RNA gel blotting. A transcript that hybridized to the 0.9-kbp *XmnI-XhoI* internal fragment probe from *avrRxv* was observed with a size of about 1.1 kb in XcvT 92-14 (Fig. 2A), consistent with the predicted *avrRxv* ORF. No hybridization was apparent with RNA from the deletion mutant XcvT 92-14 Δ avrRxv.

Fig. 1. Time course of growth of *Xanthomonas campestris* pv. *vesicatoria* tomato strain (XcvT) 92-14 and XcvT 92-14 Δ avrRxv in tomato lines Hawaii 7998 and Bonny Best. Leaves were infiltrated with bacterial suspensions of 10^5 CFU per ml and bacterial populations in leaves were sampled 5 days following infiltration. Values are means from three repetitions and vertical bars represent ± 1 SE. XcvT 92-14 in Hawaii 7998 represented by filled circles, XcvT 92-14 Δ avrRxv in Hawaii represented by open circles, XcvT 92-14 in Bonny Best represented by filled squares, XcvT 92-14 Δ avrRxv in Bonny Best represented by hollow squares.

Fig. 2. Expression of *avrRxv*-transcript in *Xanthomonas campestris* pv. *vesicatoria* tomato strain (XcvT). RNA (10 μ g) was fractionated by denaturing gel electrophoresis and after blotting, probed with an internal 0.9-kbp *avrRxv* fragment. Arrow represents the *avrRxv* transcript. **A**, XcvT 92-14 (lane 1) and XcvT 92-14 Δ avrRxv (lane 2) grown in NYG medium to mid-log phase. **B**, XcvT 92-14 grown in NYG (lane 1) and XVM2 (lane 2) media. Numbers below the lanes represent ratio of intensity of signal on autoradiograph of Northern (RNA) blot to intensity of ethidium-stained RNA in gel.

Reporter gene analysis of regulation of expression of *avrRxv*.

Different environmental conditions were tested for maximal induction of expression of *avrRxv* with β -glucuronidase (GUS) and β -galactosidase reporter gene constructs *uidA* and *lacZ*, respectively. To discover conditions under which *avrRxv* is optimally expressed, leaves of Hawaii 7998 were infiltrated with XcvT 92-14 carrying one of two reporter gene constructs, pXV9XGUS (*avrRxv* promoter) or pL6GUS (promoterless control). The activity of the *avrRxv* promoter remained relatively constant during the course of the experiment, suggesting that *avrRxv* is not induced in the resistant plant within 24 h (data not shown). To further test if molecules of plant origin influence expression of *avrRxv*, conditioned medium from tobacco suspension cells (TobCM) was used as a growth medium for XcvT 92-14 carrying pXV9XGUS or pL6GUS. After 4 h of growth there was no significant difference in *avrRxv* expression in either medium alone (MXTD), TobCM, or rich medium NYG (data not shown). Molecules of plant origin appear to have no effect on expression of *avrRxv*, which appears to be expressed equally well in complex and synthetic media.

A synthetic medium, XVM2, was recently described that induced expression of *X. campestris* pv. *vesicatoria* pepper strain (Xcv) pathogenicity loci (Wengelnik et al. 1996b). When XcvT 92-14 carrying one of the two *avrRxv* reporter gene constructs, pXV9XGUS or pXV9LC3, was grown in NYG and XVM2 for 16 h, *avrRxv* expression was slightly greater in XVM2 (Table 1). In contrast, when Xcv 85-10 carrying an *hrpF-uidA* fusion was grown in NYG and XVM2, *hrpF* promoter activity was about 1,000 times greater in XVM2 than in NYG (Table 1). That we obtained the expected level of XVM2 induction from the *hrpF-uidA* fusion strain (Wengelnik and Bonas 1996) indicates that our assay was both accurate and sufficiently sensitive to monitor induction. We tested different genetic backgrounds for the ability of XVM2 to induce *avrRxv* expression and found that GUS activity levels were not significantly different in XcvT 89-1 or XcvT 92-14. A third *avrRxv* reporter gene fusion with *lacZ* called pXV9SCAT1 was tested for induction in XVM2. Similar to previous results with *avrRxv-uidA* fusions, pXV9SCAT1 was not induced in XVM2 (data not shown). To verify the lack of

Table 1. Expression of *avrRxv* in *Xanthomonas campestris* pv. *vesicatoria* 92-14 transconjugants and 85-10 mutant strain grown in rich medium and in hrp-inductive synthetic medium

Strain ^b	Mean GUS activity $\pm$ 1 SE ^a (nM MU min ⁻¹ cell ⁻¹ $\times$ 10 ⁶)	
	NYG ^c	XVM2 ^d
92-14 (pXV9XGUS)	76 $\pm$ 4.9	124 $\pm$ 2.6
92-14 (pXV9LC3)	123 $\pm$ 4.1	166 $\pm$ 2.9
92-14 (pL6GUS)	0.001 $\pm$ 0.001	0.005 $\pm$ 0.003
85-10 hrpF::GUS	0.011 $\pm$ 0.001	12 $\pm$ 0.0

^a β -Glucuronidase (GUS) activity after 16 h of growth in specified medium; values represent mean $\pm$ 1 standard error; sample size, 3.

^b XcvT transconjugants carrying *uidA* fusion plasmids; pXV9XGUS and pXV9LC3, *avrRxv-uidA* reporter gene fusions; pL6GUS, promoterless control; or Xcv 85-10 hrpF::GUS mutant strain.

^c NYG, rich medium; nutrient yeast glycerol medium.

^d XVM2, hrp-inductive medium; 20 mM NaCl, 10 mM (NH₄)₂SO₄, 5 mM MgSO₄, 1 mM CaCl₂, 0.16 mM KH₂PO₄, 0.32 mM K₂HPO₄, 0.01 mM FeSO₄, 10 mM fructose, 10 mM sucrose, 0.03% casamino acids, pH 6.7.

induction demonstrated by reporter gene analyses, we analyzed *avrRxv* transcript levels in XcvT 92-14 grown in NYG and XVM2. No difference in transcript levels was apparent (Fig. 2B). All of our results indicate that regulation of expression of *avrRxv* differs from that of *hrp* genes.

We tested the effect of mutations in *hrp* regulatory loci *hrpG* and *hrpXv* on expression of *avrRxv* and induction in XVM2. Expression of all strains was about the same in XVM2 and NYG, demonstrating that mutations in *hrpG* and *hrpXv* have no effect on *avrRxv* expression (Table 2). Functional *hrp* structural loci are necessary for induction of HR in Hawaii 7998 by XcvT 75-3 (Table 3). To study the interaction between *hrp* gene products and *avrRxv* expression, levels of GUS activity of Tn5-insertion *hrpA,B,C,D,E*, and *F* mutant strains carrying pXV9XGUS were compared. Our experiments suggest that GUS activity of pXV9XGUS was not significantly different in *hrp* mutant strains and wild type (Table 3).

Role of the PIP-box in expression of *avrRxv*.

avrRxv has a perfect PIP-box starting at bp 465 with the sequence TTCGC-N₁₅-TTCGC (Fenselau and Bonas 1995). To learn if alterations of the sequence, rather than deletions, have

Table 2. Expression of *avrRxv* in *Xanthomonas campestris* pv. *vesicatoria* 85-10 wild-type and *hrp* mutant strains, 85-10 Δ hrpG and 85-10 Δ hrpXv grown in rich medium and in hrp-inductive synthetic medium

Transconjugant	Mean GUS activity $\pm$ 1 SE ^a (nM MU min ⁻¹ cell ⁻¹ $\times$ 10 ⁶)	
	NYG ^b	XVM2 ^c
85-10 (pXV9XGUS)	30 $\pm$ 3.8	39 $\pm$ 1.5
85-10 (pL6GUS)	0.012 $\pm$ 0.009	0.010 $\pm$ 0.010
85-10 Δ hrpG (pXV9XGUS)	55 $\pm$ 8.5	67 $\pm$ 5.8
85-10 Δ hrpG (pL6GUS)	0.002 $\pm$ 0.002	0.002 $\pm$ 0.002
85-10 Δ hrpXv (pXV9XGUS)	37 $\pm$ 2.4	52 $\pm$ 1.7
85-10 Δ hrpXv (pL6GUS)	0.001 $\pm$ 0.001	0.017 $\pm$ 0.017

^a β -Glucuronidase (GUS) activity after 16 h of growth in specified medium; values represent mean $\pm$ 1 standard error; pXV9XGUS sample size, 3; pL6GUS sample size, 2.

^b NYG, rich medium; nutrient yeast glycerol medium.

^c XVM2, hrp-inductive medium; 20 mM NaCl, 10 mM (NH₄)₂SO₄, 5 mM MgSO₄, 1 mM CaCl₂, 0.16 mM KH₂PO₄, 0.32 mM K₂HPO₄, 0.01 mM FeSO₄, 10 mM fructose, 10 mM sucrose, 0.03% casamino acids, pH 6.7.

Table 3. Response of tomato lines to inoculation with *Xanthomonas campestris* pv. *vesicatoria* 75-3 wild-type and *hrp* mutants and expression of *avrRxv* in transconjugants grown in hrp-inductive synthetic medium

Strain	Tomato lines		Mean GUS activity $\pm$ 1 SE ^a (nM MU min ⁻¹ cell ⁻¹ $\times$ 10 ⁶)
	Hawaii	Bonny Best	
75-3	R ^b	S	5.15 $\pm$ 0.9
75-3 <i>hrpA::Tn5</i> #38	0	0	9.68 $\pm$ 0.9
75-3 <i>hrpB::Tn5</i> #5	0	0	9.55 $\pm$ 2.1
75-3 <i>hrpC::Tn5</i> #139	0	0	6.67 $\pm$ 4.5
75-3 <i>hrpD::Tn5</i> #57	0	0	9.73 $\pm$ 1.5
75-3 <i>hrpE::Tn5</i> #15	0	0	13.9 $\pm$ 2.5
75-3 <i>hrpF::Tn5</i> #132	0	0	3.76 $\pm$ 0.2

^a β -Glucuronidase (GUS) activity of strains carrying pXV9XGUS after 16 h of growth on hrp-inductive XVM2 medium; values represent mean $\pm$ 1 standard error; sample size, 3.

^b Plant response to infection; R, resistant response; S, susceptible response; 0, no response.

an effect on *avrRxv* phenotype, site-directed mutagenesis was performed, creating the following mutations: PIP1M1, TGAGC-N₁₅-TTCGC; PIP1M2, TTCTA-N₁₅-TTCGC; PIP2M1, TTCGC-N₁₅-TTGAC; PIP1&2M1, TGAGC-N₁₅-TTGAC; PIP1&2DM, CCTCG-N₁₅-CCTCG. None of these mutations had any effect on *avrRxv* activity on Hawaii 7998, confirming that an intact PIP-box sequence is not essential for activity.

Sequence analysis of *avrRxv* homologue *avrBsT*.

Analysis of avirulence genes in the tomato pathogen strain XcvT race 1 75-3 uncovered the avirulence gene *avrBsT* in addition to *avrRxv*. Similar to *avrRxv*'s ability to induce resistance on several plant species that are not normal hosts for XcvT, *avrBsT* induces resistance in pepper (Minsavage et al. 1990). To learn about the molecular basis of the avirulence activity encoded in *avrBsT*, a 1.9-kbp fragment with full activity carried by pXV9JM1 was subcloned and sequenced. Sequence analysis revealed one major ORF that extends from nucleotide 241 to 1290 encoding a putative protein with a predicted molecular mass of 39.3 kDa (Fig. 3). To verify the absence of stop codons, we constructed two expression plasmids that have the full-length ORF from pXV9JM1, pUR288A6 and pET3CA16 (Table 4). Induction with isopropylthiogalactoside (IPTG) of *Escherichia coli* cultures resulted in overexpression of the expected size proteins from pUR288A6 and pET3CA16, verifying the absence of stop codons in the *AvrBsT* sequence from bp 241 to 1290.

We analyzed the nucleotide sequence to locate possible promoters. Within the sequenced region 258-bp upstream of the first start codon there is no full bipartite PIP-box sequence although there is half of a PIP-box sequence at bp 43. A possible -35 promoter sequence identical to that in *hrpA1* starts at bp 112 (TTCATC) and a possible -10 sequence resembling that in *hrpA1* starts at bp 148 (Wengelnik et al. 1996a); however, the order and spacing make them unlikely candidates for promoter elements. No typical purine-rich region is present just upstream of the first start codon at bp 241. We examined the possibility of secondary structure (stems and loops) in the mRNA surrounding the translation start codon AUG in both *avrBsT* and *avrRxv* nucleotide sequences and found that the region surrounding the AUG in both genes was not completely embedded in a stem. The putative amino acid sequence of *AvrBsT* is primarily hydrophilic, with a pI of 8.1, and a high percentage of the polar amino acid serine (14% of residues).

To find regions of high similarity, *AvrBsT* was compared with the nonredundant protein data bases. Interestingly, *AvrRxv* has the highest level of sequence similarity to *AvrBsT*. The amino acid sequences are 41% identical and 61% similar when conservative amino acid substitutions are taken in account. Three stretches within the alignment have levels of identical amino acids greater than 70%: the extreme N-terminal 17 residues, residue 48 to 64, and the extreme C-terminal 20 residues. There are potential nuclear localization signal (NLS) sequences, RPRKK and RXRKIR[KH], within two of these domains.

Four other gene products from bacterial species are significantly similar to *AvrBsT* and *AvrRxv* (Fig. 3). An ORF of unknown function called Y4LO from the large symbiont of the nitrogen-fixing symbiont *Rhizobium* sp. strain NGR234 (Freiburg et al. 1997) is 34% identical and 56% similar to *AvrRxv*. YopJ and YopP, secreted proteins from species of the mammalian pathogen genus *Yersinia* (Galyov et al. 1994;

Mills et al. 1997) are about 23% identical and 48% similar to *AvrRxv*. A YopJ homologue with unknown function called *AvrA* from *Salmonella typhimurium* (Hardt and Galan 1997) is 25% identical and 46% similar to *AvrRxv*. To distinguish the *S. typhimurium* *avrA* gene product from that of *Pseudomonas syringae* *avrA*, we will designate the former as *AvrAS* herein. Like *AvrRxv* and *AvrBsT*, YopJ subfamily members all have a putative NLS sequence (HRRK). None of the family members were unambiguously similar to protein motifs in the program Identify.

DISCUSSION

The results of the present study expand our knowledge of the function of the avirulence gene *avrRxv* from the bacterial pathogen of tomato XcvT. In planta bacterial growth analysis

1	50
YopP	
YopJ	
AvrAS	
AvrRxv	MCDSIRVQFR SIQKVVVKMK KFFRSGLGVGG SSSSR...FQ HHIPEDASAP
AvrBsT	MK NFMRSLGFSGS SRSSRSSSSN WNEQQADNDE
Y4LO	
51	100
YopP	 MIGPISQ INSPGGLS.E KETRSLISNE
YopJ	 MIGPISQ INISGGLS.E KETSSLSISNE
AvrAS	M IFSVQELSCG GKSMLSPTTR .NMGASLSPQ PDVSGELNTE
AvrRxv	SSKASTPPAS PPDSPSPNS AFSALPTRPR KKAALSDAV E.SRGLHAPP
AvrBsT	QTPASSPSTS PSQTS...S AFSGLPERPR KKAALDESL N.SSNNI.PY
Y4LO	MQ LSRAEIGNP SSRNLSPRIN EKVELLQAL EHARRAGMSS
101	150
YopP	ELKNIIITQLE TDIADGSWFH KNYSRDLIEV MPALVIQANN KYPEMNLMFV
YopJ	ELKNIIITQLE TDISDGSWFH KNYSRMDIEV MPALVIQANN KYPEMNLMFV
AvrAS	ALTCIVERLE SEIIDGSWFH ISYEETDLEM MPFLVAQANK KYPEMNLMFV
AvrRxv	SLVSYANATL DQLRNEPIS ESLRLMDIEN LPHLVSVDN RLNNLNLRSF
AvrBsT	EMRYAAEAL SAA..NDGSS EATTKADVEN KYLLAHAYNE RFELHLSCM
Y4LO	SLMEYGRQVA RHLSANVQPD EKILSLDIEN LPILAASYNR RYPDLDLRHM
151	200
YopP	TSPQDLSTIEI KNVIENGVS SRFIINMGEG G..IHFSVID YKHIN.GKTS
YopJ	TSPLDLSTIEI KNVIENGVS SRFIINMGEG G..IHFSVID YKHIN.GKTS
AvrAS	MSVHELVSII KETRMGVES ARFLVNMGGG G..IHISVVD FRVMD.GKTS
AvrRxv	DTPGQFLHDL SRWHKGLPL .RAVVRLDED PRWRHVAFD VRNHSQHT
AvrBsT	DSAQCFSEF MTSEKQA..W RSIVRL..S PSSMHAAD VRFKD.GKRT
Y4LO	DSPARFFDAL NDRSSDG.AW .RAVVRLADG EQ..HVAAD VRTRAGAAPT
201	250
YopP	LILFEPVNFN SMGPAILAI.STKTAI ERYQLPDCHE SMVEMDIQRS
YopJ	LILFEPANFN SMGPAILAI.RTKTAI ERYQLPDCHE SMVEMDIQRS
AvrAS	LILFEPACNS AFGPA.LAL.RTKTAI ERYQLPDCYF AMVELDIQRS
AvrRxv	VIALFEPASAY NPDH....M PGFVKMRENL TSQFGKRSIF AVIEAALKS
AvrBsT	MLVIEPALAY GMKDGKIKVM AGYETLQKIV QNCLGENDGM AVIQLGAQKS
Y4LO	IIVMEGANFY T.....FV ASYFKLRGDS FRQLGTQAKW AFIEVGAQKS
251	300
YopP	SSECGIFSLA LAKKLYTERD SLKKIHED...NIRGILS DSENPLPHN.
YopJ	SSECGIFSLA LAKKLYTERD SLKKIHED...NIRGILS DSENPLPHN.
AvrAS	SSECGIFSLA LAKKLQLEFM NLVKIHED...NICERLC GEEFPLPSD.
AvrRxv	IGGCVFISLD YALAAQERS TFDQWHLDR KKNIKGMTF .ESQHLNELG
AvrBsT	LPDCVFISLN MALCAYQKDS VFDNLHDCILR R..NVRCFSS GERKSILEKN
Y4LO	AADCVMFVGQ FALAAYREL TFDADHNLH HHGTI.....
301	350
YopP	...KLDPYL PVTFYKHTQG KKRLNEYLMT NPQGVGTVMN KK.....NET
YopJ	...KLDPYL PVTFYKHTQG KKRLNEYLMT NPQGVGTVMN KK.....NET
AvrAS	...KADRYL PVSFYKHTQG AQRLNEYVEA NPAAGSIVM KK.....NET
AvrRxv	VLLLGTRLL PANFYKHAHS RRTIDLEAD QFGASGTDVR SGRAVYKES
AvrBsT	IEFIEGDKFL PPIFYKSHS RGVVGEFISN QPEYAHKNVS TGRITNP.SED
Y4LO	AH EGDYSSDYMP RRHAGICANK
351	386
YopP	IFNRFDNKNS IIDGKELSVS VEKKRIAEYK TLLKV.
YopJ	IVNRFDNKNS IVDGKELSVS VEKKRIAEYK TLLKV.
AvrAS	LYERFDNNAV MLNDKLSIS AEKKRIAEYK SLKPZ
AvrRxv	LSERLEEFQV QR.DKTYSMS IEASRAKRIT HALE.
AvrBsT	LSERWENFRV RRGDLVSMS IEASRLAKRIT KTIES.
Y4LO	PFSWGE...V PPSDLLQALS LQQCN.....

Fig. 3. Alignment of the *AvrRxv*-host interaction factor family. Alignment of YopJ, YopP, *AvrAS*, *AvrBsT*, *AvrRxv*, and Y4LO produced with Pileup (GCG, Madison, WI). Identical amino acid residues in at least two sequences out of the six are indicated in boldface.

suggests that *avrRxv* plays a crucial role in the ability of XcvT 92-14 to induce resistance on Hawaii 7998, but that XcvT 92-14 may carry additional factors that contribute to the full level of resistance (Fig. 1). Accordingly, resistance in tomato to XcvT race 1 and to XcvT race 2 carrying *avrRxv* is genetically complex (Whalen et al. 1993; Wang et al. 1994; Yu et al. 1995). It is possible that a full resistance response in Hawaii

7998 requires recognition of *avrRxv* as well as other factors in XcvT.

To begin to understand the molecular basis of the function of *avrRxv*, we attempted to correlate differential expression levels (and hence gene regulation) with different growth conditions. Under all conditions tested, *avrRxv* appears to be constitutively expressed. Molecules of plant origin appear to have

Table 4. Bacterial strains, vectors and plasmid constructions

Strain, vector or plasmid	Relevant characteristics ^a	Source or reference
<i>Xanthmonas campestris</i> pv. <i>vesicatoria</i>		
75-3	Tomato race 1, Rif ^r , <i>avrRxv</i>	Whalen et al. 1988
92-14	Tomato race 1, Rif ^r , <i>avrRxv</i> ; HR on Hawaii 7998	Whalen et al. 1993
92-14 Δ <i>avrRxv</i>	92-14 deletion of <i>avrRxv</i> and replacement with Ω fragment with pXV9SC920; WS on Hawaii 7998	This study
89-1	Tomato race 2, Rif ^r ; WS on Hawaii 7998	Whalen et al. 1993
85-10	Pepper race, Rif ^r	Minsavage et al. 1990
85-10 <i>hrpFGUS</i>	<i>hrpF312-Tn3-gus</i> fusion in 85-10; pepper race, Rif ^r	Schulte and Bonas 1992
85-10 Δ <i>hrpXv</i>	85-10 deleted for <i>hrpXv</i> , Rif ^r	Wengelnik and Bonas 1996
85-10 Δ <i>hrpG</i>	85-10 deleted for <i>hrpG</i> , Rif ^r	Wengelnik et al. 1996b
75-3 <i>hrp::Tn5</i> #35, 5, 139, 57, 15, 132	75-3 with <i>Tn5</i> insertion in <i>hrpA</i> , B, C, D, E, and F, respectively	Bonas et al. 1991
Vectors		
pL6	pLAFR3 deleted for <i>Plac</i> with <i>trp</i> terminators flanking polylinker	Huynh et al. 1989
pLITMUS 38	pUC-derived backbone lacking <i>XmnI</i> site in Ap ^r gene	New England Biolabs, Beverly, MA
pUFR051	pLAFR3 with pUC19 polylinker	De Feyter and Gabriel 1991
pUR288	LacZ fusion vector	Rüther and Müller-Hill 1983
pETh-3c	T7 RNA Pol-based expression vector	McCarty et al. 1991
Plasmids		
pRK2013	Km ^r , Tra ⁺ Mob ⁺ , ColE1 replicon	Figurski and Helinski 1979
pXV9000	pLAFR3 with 8.0-kbp <i>Bam</i> HI fragment containing <i>avrRxv</i> gene cloned from <i>X. campestris</i> pv. <i>vesicatoria</i> tomato race 1 75-3; HR on Hawaii 7998	Whalen et al. 1988
pXV9117	pXV9000 opened at <i>Xho</i> I site and deleted for 1.3 kbp in both directions; <i>Xho</i> I site reconstructed	This study
pXV9SC920	<i>avrRxv</i> deletion construct; pXV9117 with Ω inserted at <i>Xho</i> I site; WS on Hawaii 7998	This study
pXV9006, pXV9007	pL6 with 2.1-kbp <i>Pst</i> I <i>avrRxv</i> fragment in both orientations	Whalen et al. 1993 and this study
pRAJ275	1.8-kbp <i>uidA</i> gene with <i>Nco</i> I site at initiator ATG codon	Jefferson et al. 1987
pUC118RXV3	2.1-kbp <i>Pst</i> I fragment cloned into pUC118	Whalen et al. 1993
pUC118RXV3ΔN277	pUC118RXV3 deleted for <i>Nco</i> I site at bp 277	This study
pXV9XM2N	pUC118RXV3ΔN277 with <i>Xmn</i> I site at bp -601 converted to <i>Nco</i> I site	This study
pXV9XM2NGUS	pXV9XM2N with 1.5-kbp <i>Nco</i> I- <i>Eco</i> RI fragment replaced with 1.9-kbp <i>Nco</i> I- <i>Eco</i> RI <i>uidA</i> gene from pRAJ275 creating translational fusion of <i>avrRxv</i> and <i>uidA</i>	This study
pXV9XGUS	pL6 carrying 2.5-kbp <i>Hind</i> III- <i>Eco</i> RI fragment from pXV9XM2NGUS; translational fusion of <i>avrRxv</i> and <i>uidA</i> at bp 601	This study
pXV9LC1	pUC118RXV3ΔN277 with <i>Stu</i> I site at bp 747 converted to <i>Nco</i> I site; 100-bp <i>Stu</i> I fragment deleted	This study
pXV9LC2	pXV9LC1 with 1.3-kbp <i>Nco</i> I- <i>Eco</i> RI fragment replaced with 1.9-kbp <i>Nco</i> I- <i>Eco</i> RI <i>uidA</i> gene from pRAJ275 creating translational fusion of <i>avrRxv</i> and <i>uidA</i>	This study
pXV9LC3	pL6 carrying 2.7-kbp <i>Hind</i> III- <i>Eco</i> RI fragment from pXV9LC2; translational fusion of <i>avrRxv</i> and <i>uidA</i> at bp 747	This study
pL6GUS	pL6 carrying 1.9-kbp <i>Nco</i> I- <i>Eco</i> RI <i>uidA</i> gene from pRAJ275	D. Dahlbeck and B. Staskawicz
pMC1871	pBR322-derived backbone containing promoterless <i>lacZ</i> lacking a ribosome binding site and codons for the first 8 amino acids	Amersham Pharmacia Biotech, Piscataway, NJ
pXV9SCAT1	pXV9007 with 0.6-kbp <i>Hind</i> III- <i>Xho</i> I fragment replaced with 3.9-kbp <i>Hind</i> III- <i>Xho</i> I fragment from pMC1871S2X containing promoterless <i>lacZ</i> gene; translational fusion of <i>avrRxv</i> and <i>lacZ</i> at bp 1483	This study
pL6LACZ	pL6 carrying 3.1-kbp <i>Bam</i> HI fragment with promoterless <i>lacZ</i> from pMC1871	This study
pRXV36A	Insert from pUC118RXV3 deleted to bp 532 cloned into pL6	Whalen et al. 1993
pXV9TH1, pXV9TH2, pXV9TH3, pXV9TH4, pXV9TH5	pL6 carrying <i>avrRxv</i> with mutations in PIP-box sequence	This study
pXV9JM1	pUFR051 carrying <i>avrBsT</i> active 1.9-kbp subclone	This study
pBSTA	0.8-kbp <i>Eco</i> RI- <i>Hind</i> III <i>avrBsT</i> fragment cloned into pUC118	This study
pBSTB	0.8-kbp <i>Eco</i> RI- <i>Hind</i> III <i>avrBsT</i> fragment cloned into pUC119	This study
pBSTC	1.1-kbp <i>Eco</i> RI- <i>Kpn</i> I <i>avrBsT</i> fragment cloned into pUC118	This study
pBSTD	1.1-kbp <i>Eco</i> RI- <i>Kpn</i> I <i>avrBsT</i> fragment cloned into pUC119	This study
pUR288A6	1.1-kbp open reading frame (ORF) from <i>avrBsT</i> in pUR288	This study
pET3CA16	1.1-kbp ORF from <i>avrBsT</i> in pETh-3c	This study

^a HR, hypersensitive resistant response; WS, watersoaking susceptible response.

no effect on expression of *avrRxv*. In addition, *avrRxv* is expressed equally well in complex or synthetic media and is not suppressed in rich medium (Fig. 2, Table 1). We conclude, therefore, that it is not involved in responses to nutritional status (e.g., carbon source response) of the pathogen.

Among the synthetic media we tested was XVM2, which induces expression of Xcv pathogenicity loci; XVM2-induction potential reportedly ranges from approximately 25-fold for *hrpE* to approximately 6,000-fold for *hrpF* (Wengelnik and Bonas 1996). XVM2 has little to no effect on *avrRxv* expression, analyzed with reporter genes fused at three different locations in *avrRxv* and Northern blot analysis (Table 1; Fig. 2). Two recently discovered *hrp* regulatory genes, *hrpG* and *hrpXv*, have been shown to control expression of all *hrp* loci (Wengelnik et al. 1996b; Wengelnik and Bonas 1996). Mutations in *hrpG* and *hrpXv* had no effect on basal levels of expression of *avrRxv* and induction in XVM2 (Table 2). In *hrpB*, *hrpC*, *hrpD*, and *hrpF*, the PIP-box is said to confer control by XVM2 and the HrpG-HrpXv regulatory cascade (Fenselau and Bonas 1995; Wengelnik et al. 1996b). Previous deletion analysis had suggested that the *avrRxv* PIP-box was not essential for *avrRxv* activity (Whalen et al. 1993). Interestingly, none of the site-directed mutations in the PIP-box had any effect on *avrRxv* activity in leaves of Hawaii 7998, indicating that an intact PIP-box sequence is not essential for activity. All of our results indicate that the regulation of expression of *avrRxv* differs from that of *hrp* loci with a PIP-box motif in their promoters and from *hrp* loci in general. Since none of the *hrp* regulatory or structural loci has an effect on expression of *avrRxv* (Tables 2 and 3), the prevention of resistance induction by *avrRxv* in *hrp* mutant backgrounds is not at the transcriptional level but may be due to blockage of secretion of an *avrRxv*-associated elicitor. No functional analog of an anti-sigma factor repressor appears to negatively regulate expression of *avrRxv*. Perhaps physical assembly of the *hrp* secretory apparatus or presence of what Pallen et al. (1997) termed a molecular cork (Finlay and Cossart 1997) controls secretion of avirulence gene products, thereby controlling their ability to function.

Only two previous studies have addressed the regulation of expression of *X. campestris* avirulence genes. *avrBs3* does not contain a PIP-box motif in its promoter and was found to be constitutively expressed (Knoop et al. 1991). The avirulence gene *avrXca* from *X. campestris* pv. *raphani*, instead of a PIP-box, has a perfect *hrp*-box consensus motif (Parker et al. 1993). However, *avrXca* was not induced in minimal medium or in planta in the manner found in *hrp*-box regulated loci or in Xcv *hrp* genes. Therefore, *avrRxv* is the third *Xanthomonas* spp. avirulence gene that appears to be constitutively expressed.

Sequence analysis of the avirulence gene *avrBsT* indicates that it encodes a putative hydrophilic protein with a molecular mass of 39.3 kDa (Fig. 3). AvrBsT has the highest level of sequence similarity to AvrRxv and there are three domains that are highly similar. The location of the N-terminal domain may indicate some common post-translational function or processing such as protein-protein interaction, secretion, or cleavage. In mammalian pathogens, the secretion signal does not have a peptide consensus sequence or secondary structure (Rosqvist et al. 1994; Sory et al. 1995; Pettersson et al. 1996; Schesser et al. 1996). Recently, it was shown for two translocated proteins from *Yersinia* that the secretion signal resides in

the mRNA (Anderson and Schneewind 1997). Analysis of the nucleotide sequence around the putative translational start codons of *avrRxv* and *avrBsT* reveals high level of identity in the two genes, but in neither *avrRxv* nor *avrBsT* is the entire AUG imbedded in a stem structure. Two other possible roles for the conserved domains include a protease recognition domain such as found in AvrPphB (Puri et al. 1997) or chaperone interaction domain as described for Yop proteins (Wattiau et al. 1996). AvrRxv- and AvrBsT-specific antisera will be used in the future to assess subcellular localization and molecular weight variances that suggest processing and protein interactions.

Interestingly, there are two potential basic NLS sequences present in AvrRxv and AvrBsT. If these NLS sequences truly function to allow transport into the host nucleus, as NLS sequences have been demonstrated to do in AvrBs3 family members (reviewed in Gabriel 1997; Bonas and Van den Ackerveken 1997), then AvrRxv and AvrBsT may also function within the host cell nucleus. These NLS are embedded in two basic domains and some of the amino acids may play a dual role in DNA binding as well as nuclear localization. YopJ subfamily members also contain an NLS sequence in their C-terminal region. Localization studies are critical to understanding the function of these host interaction factors.

Four other gene products from bacterial species that interact with host organisms are significantly similar to AvrBsT and AvrRxv and form a larger and less conserved AvrRxv family. This family contains an ORF of unknown function called Y4LO from *Rhizobium* sp., and three secreted proteins from mammalian pathogen *Yersinia* spp. and *S. typhimurium* (Fig. 3). Four out of six members of the AvrRxv family play known important roles in controlling the outcome of host interactions (Monack et al. 1997; Minsavage et al. 1990; Whalen et al. 1988, 1993; Mills et al. 1997) and five out of six contain NLS sequences, perhaps suggesting a similar mechanism of host interaction. It may be fruitful to speculate whether there is a relationship between the induction of an HR in plant tissues and induction of programmed cell death in macrophages (Dangl et al. 1996; Monack et al. 1997; Mills et al. 1997). The killing of macrophages early in the infection process is advantageous to establishment of infection by *Yersinia*. However, the HR and associated cell death are strongly correlated with prevention of pathogen establishment in plant tissues. It is likely that recognition of avirulence gene products by hosts and correlated triggering of resistance responses must be consequences of the evolution of improved fitness in an undiscovered aspect of pathogenicity. The possible convergence of function in factors from plant pathogens, plant symbionts, and mammalian pathogens significantly broadens the impact of studies on the roles of these similar gene products in host interactions.

MATERIALS AND METHODS

Growth of plants, plant inoculations, and in planta bacterial growth curves.

Plants were grown from seed in greenhouses in plastic pots with standard potting soil. Growth chambers were set with 16-h photoperiods at 24°C. Reactions of plants to infection with XcvT strains were determined by infiltrating approximately 10 µl of a bacterial suspension (10⁷ to 10⁸ CFU per ml) into leaf-

lets and scored as described (Whalen et al. 1993). To determine levels of bacterial growth in planta, leaves of plants were vacuum infiltrated with bacterial suspensions of 10^5 CFU per ml and sampled as described (Whalen et al. 1988).

Bacterial strains, media, and plasmids.

Bacterial strains, vectors, and plasmid constructions are described in Table 4 and below. *E. coli* DH5 α was routinely used. Strains of XcvT were subcultured at 30°C on nutrient yeast glycerol (NYG) medium (Daniels et al. 1984), nutrient broth, or XVM2 medium (Wengelnik et al. 1996a), and *E. coli* strains at 37°C on Luria medium (Miller 1972). Bacto agar at 1.5% (wt/vol) was added to media for plate cultures. Antibiotics (Sigma, St. Louis, MO) were used for selection at the following concentrations, in μ g/ml: tetracycline (Tc), 10; rifampicin (Rif), 100; spectinomycin (Sp), 100; ampicillin (Ap), 50; kanamycin (Km), 25. Cosmid pL6 (Huynh et al. 1989) was used as a vector to assess avirulence activity and for reporter gene constructs. Triparental matings with the helper plasmid pRK2013 (Table 4) were used to mobilize clones of DNA from *E. coli* into XcvT.

Nucleic acid techniques.

Standard techniques for DNA subcloning, plasmid preparations, genomic DNA isolation, 32 P-labeling of probes, DNA gel blot hybridizations, and agarose gel electrophoresis were used (Ausubel et al. 1995; Sambrook et al. 1989). To verify junctions of all reporter gene fusion constructs (Table 4) and products of site-directed mutagenesis procedures, double-stranded plasmid templates were sequenced by the dideoxy chain-termination method (Sanger et al. 1977). Both strands of *avrBsT* was sequenced from single-stranded templates as described (Viera and Messing 1987). Sequence information was obtained with M13 forward and M13 reverse primers in either manual (Sequenase DNA Sequencing Kit; USB, Cleveland, OH) or automatic (ABI Prism Dye Primer Cycle Sequencing Kit and ABI Prism Model 377 Sequencer; Perkin Elmer, Norwalk, CT) sequencing reactions and fractionation.

To isolate RNA from XcvT cells, strains were grown overnight in 5 ml of medium to cell densities of 3 to 9×10^8 CFU per ml and the hot phenol RNA isolation procedure outlined in Salmeron and Staskawicz (1993) was followed. RNA size standards were 0.24 to 9.4 kb ladder (Gibco BRL, Gaithersburg, MD). RNA was blotted to Hybond N⁺ membranes (Amersham, Arlington Heights, IL), hybridized in 0.5 M NaPO₄, pH 6.8, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and finally washed at 65°C in 0.2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS. Hybridizing bands on Northern blots were visualized either by autoradiography or with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Intensities of hybridizing bands on phosphorimages and intensities of ethidium bromide stained total RNA loaded on gels that produced the blots were quantified with ImageQuant software (Molecular Dynamics).

Deletion strain construction.

To assess avirulence activity of XcvT 92-14 without *avrRxv*, a deletion strain was constructed from the active subclone pXV9000. Cesium-purified pXV9000 DNA was digested with *Xho*I, treated with exonuclease BAL31, blunt-ended, and religated with *Xho*I linkers. Clone pXV9117 had 1.3 kbp of

DNA, both 5' and 3' of the original *Xho*I site, deleted and had lost avirulence activity (Table 4). To clone in a selectable marker, pXV9117 was digested with *Xho*I, blunt-ended, and ligated with the Ω fragment, producing pXV9SC920. The deletion clone pXV9SC920 was introduced into XcvT 92-14 and site-directed gene replacement was accomplished as described (Whalen et al. 1988), producing XcvT 92-14 Δ avrRxv. Southern blot analysis verified gene replacement.

Construction of reporter gene fusions.

GUS and β -galactosidase reporter gene constructs were made at three different sites in *avrRxv* downstream of the third initiator codon (Table 4). The negative control pL6GUS was constructed with an intact *uidA* translation start codon and oriented in pL6 so that there was no external promoter activity, based on the sensitive ice reporter gene assay (D. Dahlbeck and B. Staskawicz, *personal communication*). Reporter gene fusions were cloned into pL6 and conjugated into XcvT or Xcv for expression analyses.

In preparation for use of the *Nco*I site at the ATG initiator codon of *uidA* (Jefferson et al. 1987), the *Nco*I site in pUC118RXV3 at bp 277 was removed, creating pUC118RXV3 Δ N277. To create a translational fusion at bp 601 in *avrRxv*, the *Xmn*I site in pUC118RXV3 Δ N277 was replaced with *Nco*I, creating pXV9XM2N. The promoterless *uidA* from pRAJ275 was cloned into that site, resulting in pXV9XM2NGUS, and then the *avrRxv*:GUS fusion was cloned into pL6, making the promoter probe plasmid pXV9XGUS. To create a second *uidA* translational fusion, the *Stu*I site at bp 747 in pUC118RXV3 Δ N277 was converted into an *Nco*I site, creating pXV9LC1, followed by cloning the promoterless *uidA* into that site, creating pXV9LC2. The *avrRxv*:GUS fusion from pXV9LC2 was cloned into pL6, making a second promoter probe plasmid called pXV9LC3. A third *avrRxv* reporter gene fusion, called pXV9SCAT1, was constructed at bp 1483 with a promoterless *lacZ* gene from pMC1871 (Table 4). To create a promoterless *lacZ* fusion control plasmid, the 3.1-kbp partial *lacZ* gene was isolated from pMC1871 and cloned into pL6, creating pL6LACZ.

Reporter gene activity assays.

To assay GUS reporter gene activity in vitro, freshly plated XcvT transconjugants were harvested from selection plates and resuspended in water. Cells were washed and, based on OD₆₀₀, were resuspended at a density of 10^8 CFU per ml in 3 to 4 ml of specified medium and grown at 30°C with vigorous agitation. *hrp* mutant strain transconjugants were harvested after overnight growth on specified solid medium and resuspended in water. Cells in 0.5-ml samples in triplicate were pelleted and resuspended in 0.5-ml GUS lysis buffer. GUS activity assays used the fluorescent substrate methylumbelliferyl glucuronate (MUG, Sigma) according to standard protocols (Jefferson et al. 1987). Fluorescence was monitored on a fluorimeter (DynaQuant 200; Hoefer, San Francisco, CA). For GUS assays on XcvT cells grown in plant leaves, four 0.5-cm² disks were macerated in 250 μ l of 10 mM MgCl₂ and, before resuspension in lysis buffer, pelleted as described earlier. For analysis of XcvT cell number in leaf disks, dilution plating of macerate was performed. To test GUS activity of cells grown in conditioned medium, suspension cultures of tobacco cv. Xanthi grown in Murashige and Skoog (MS)-

based plant growth medium (MTXD: 1× MS salts, 3% sucrose, 1× B5 vitamins, 0.02% myo-inositol, 0.013% asparagine, 4 mg of PCPA (p-chlorophenoxyacetic acid) per liter, 500 µg of kinetin per liter, pH 5.8) were used with cells removed by filtration with sterilized Whatman #1 filter paper. β-Galactosidase assays were performed as described in Miller (1972) on cells prepared essentially as described for GUS assays. All reporter gene activities were statistically analyzed by analysis of variance (ANOVA; JMP Statistical Package, SPSS Inc., Chicago).

PCR-mediated site-directed mutagenesis.

To test the effect of specific mutations in the regulatory sequences in *avrRxv* controlling transcription, PCR-mediated site-directed mutagenesis was performed with sequence overlap extension (SOE; Ho et al. 1989; Horton et al. 1990; Baretino et al. 1994). The *ScaI*-linearized plasmid pUC118RXV3 was used as template (Table 4). Amplifications were performed with an 8:1 unit mixture of *Taq* DNA polymerase (Perkin Elmer) and *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Primers (Operon Technologies, Alameda, CA) were designed with Oligo 5.0 (National Biosciences, Plymouth, MN). To amplify the two reactant fragments for SOE, two sets of primers were used, consisting of an outside M13 primer that annealed to vector sequences and an inside primer. Inside primers were designed to overlap and to carry mismatches (underlined) that incorporated the desired mutation: 5' to 3'; PIP1LWR, AAGCTGAAAAACCCTGAACGAAGCA; PIP1MP1, TTCAGGGTTTTTCAGCTTTGAGCATTATGTCCT; PIP1MP2, GTTCAGGGTTTTTCAGCTTTTCTAA TTATTGCCTA; PIP2LWR, GATCTAGGCAATAATGCGA AA AGCTG; PIP2MP1, CGCATTATTGCCTAGATCTTAA CGCAACGAAAC; PIPDMPLWR, GGATCTAGGCAATA ATCGAGGAAGCTGAAAAACC; PIPDMPR UPP, CGATT ATTGCCTAGATCCCTCGGCAACGAAACCG; PIPDLL WR, TGAGCGGATAACAATTTACACAGGAAACAGC. Amplification products were gel purified and 30 to 50 ng of both left and right fragment combined for SOE reactions. SOE reactions were carried out with the following cycling parameters: 1 min at 94°C; 8 cycles of 1 min at 94°C, 1 min at 35 to 45°C, 5 min at 72°C; 1 cycle of 10 min at 72°C. After SOE, outside primers were added and 20 to 25 cycles were performed of 1 min at 94°C, 1 min at 55 to 59°C, 5 min at 72°C. To facilitate analysis of site-directed mutagenesis products, *avrRxv* was cloned into pLITMUS38 (New England Biolabs, Beverly, MA), creating pXV9JGRT1. SOE amplification products were digested with *NcoI* and *XmnI* and the 0.32-kbp mutagenized *NcoI-XmnI* fragments were cloned into pXV9JGRT1. Plasmids from two single-colony purified isolates of each construct were sequenced in triplicate to verify the presence of the mutation and the absence of other sequence changes. Sequenced mutant inserts were cloned into pL6, creating pXV9TH1, pXV9TH2, pXV9TH3, and pXV9TH4 (Table 4) and the mutant series was tested for avirulence activity.

avrBsT sequence and ORF verification.

The smallest active subclone of *avrBsT* in pUFR051, called pXV9JM1, contained a 1.9-kbp insert (Table 4). To create subclones for sequencing, pXV9JM1 was digested with *EcoRI*, *KpnI*, and *HindIII*. The resulting 0.8-kbp *EcoRI*-

HindIII and 1.1-kbp *EcoRI-KpnI* fragments were each cloned into pUC118 and pUC119, creating pBSTA, pBSTB, pBSTC, and pBSTD, respectively (Table 4). For sequencing the entire 1.9-kbp active fragment, internal primers were used in addition to M13 primers.

To determine if the largest predicted translation product of *avrBsT* was produced in *E. coli*, the largest ORF bp 241 to 1290 was amplified from pXV9JM1 by PCR with *HindIII* sites added to the 5' and 3' ends (5' to 3'; RST17, GGGAA GCTTATGAAGAATTTTATG; RST18, CCCAAGCTTATGATTCAATAGTT). The resulting *HindIII* fragment was cloned into pUR288, creating pUR288A6, and into pETh-3c, creating pET3CA16 (Table 4), and transformed into *E. coli* JM109 and *E. coli* BL21(DE3) pLysS, respectively. To analyze production of fusion proteins, cells were grown in Luria broth (LB) with ampicillin or ampicillin and chloramphenicol, respectively. Four-milliliter cultures induced with 1 mM IPTG were grown 2 to 5 h following induction; bacteria were harvested, resuspended in Laemmli buffer (Laemmli 1970), and boiled. Samples were run on a 10% polyacrylamide stacking gel and stained with Coomassie blue R-250.

Sequence analysis and alignment.

The predicted translation products of *avrBsT* and *avrRxv* were compared with the nonredundant data bases available at NCBI with the BLASTP algorithm with low sequence complexity filtering and BLOSUM62 similarity matrix (Altschul et al. 1990) with BEAUTY post-processing (Worley et al. 1995). Presence of known protein motifs was assessed with Identify (available on-line from Stanford University) and presence of protein sorting motifs with PSORT (available on-line from Osaka University; Nakai and Kanehisa 1991, 1992). The alignment was produced with Pileup (Genetics Cooperative Group [GCG], Madison, WI). Conservative substitutions are based on the scaled PAM250 matrix used as default (Schwartz and Dayhoff 1979). The predicted translation product of *avrBsT* was analyzed with MacVector (Eastman Kodak Company, Rochester, NY) and the program Stemloop (GCG) was used to assess RNA secondary structure.

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