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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 because 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 ΔavrRxv 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 ΔavrRxv 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 ΔavrRxv 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 ΔavrRxv 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 *Xmn*I-*Xho*I 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 ΔavrRxv.

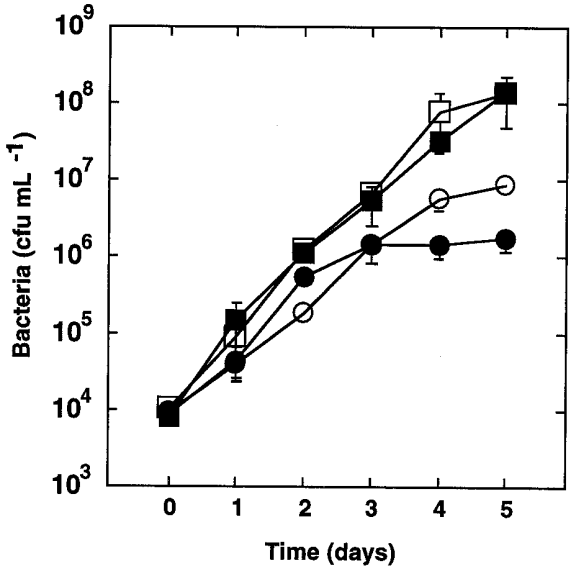

Fig. 1. Time course of growth of *Xanthomonas campestris* pv. *vesicatoria* tomato strain (XcvT) 92-14 and XcvT 92-14 ΔavrRxv in tomato lines Hawaii 7998 and Bonny Best. Leaves were infiltrated with bacterial suspensions of 10^5 CFU per ml and bacterial populations in leaves were sampled 5 days following 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 ΔavrRxv in Hawaii represented by open circles, XcvT 92-14 in Bonny Best represented by filled squares, XcvT 92-14 ΔavrRxv in Bonny Best represented by hollow squares.

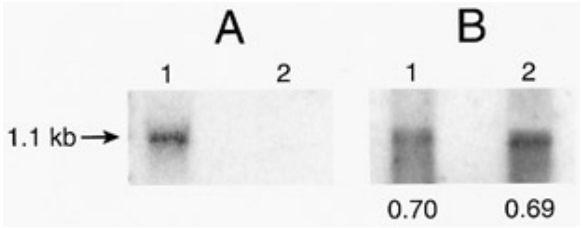

Fig. 2. Expression of *avrRxv*-transcript in *Xanthomonas campestris* pv. *vesicatoria* tomato strain (XcvT). RNA (10 µg) was fractionated by denaturing gel electrophoresis and after blotting, probed with an internal 0.9-kbp *avrRxv* fragment. Arrow represents the *avrRxv* transcript. **A,** XcvT 92-14 (lane 1) and XcvT 92-14 ΔavrRxv (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 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 Tn*5*-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-N_{15}-TTCGC (Fenselau and Bonas 1995). To learn if alterations of the sequence, rather than deletions, have

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

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

[a] β-Glucuronidase (GUS) activity after 16 h of growth in specified medium; values represent mean ± 1 standard error; sample size, 3.
[b] XcvT transconjugants carrying *uid*A 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 $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4, 0.01 mM $FeSO_4$, 10 mM fructose, 10 mM sucrose, 0.03% casamino acids, pH 6.7.

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

	Mean GUS activity ± 1 SE[a] (nM MU min^{-1} $cell^{-1}$ × 10^6)	
Transconjugant	NYG[b]	XVM2[c]
85-10 (pXV9XGUS)	30 ± 3.8	39 ± 1.5
85-10 (pL6GUS)	0.012 ± 0.009	0.010 ± 0.010
85-10 ΔhrpG (pXV9XGUS)	55 ± 8.5	67 ± 5.8
85-10 ΔhrpG (pL6GUS)	0.002 ± 0.002	0.002 ± 0.002
85-10 ΔhrpXv (pXV9XGUS)	37 ± 2.4	52 ± 1.7
85-10 ΔhrpXv (pL6GUS)	0.001 ± 0.001	0.017 ± 0.017

[a] β-Glucuronidase (GUS) activity after 16 h of growth in specified medium; values represent mean ± 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 $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4, 0.01 mM $FeSO_4$, 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

	Tomato lines		
Strain	Hawaii	Bonny Best	Mean GUS activity ± 1 SE[a] (nM MU min^{-1} $cell^{-1}$ × 10^6)
75-3	R[b]	S	5.15 ± 0.9
75-3 *hrp*A::*Tn*5 #38	0	0	9.68 ± 0.9
75-3 *hrp*B::*Tn*5 #5	0	0	9.55 ± 2.1
75-3 *hrp*C::*Tn*5 #139	0	0	6.67 ± 4.5
75-3 *hrp*D::*Tn*5 #57	0	0	9.73 ± 1.5
75-3 *hrp*E::*Tn*5 #15	0	0	13.9 ± 2.5
75-3 *hrp*F::*Tn*5 #132	0	0	3.76 ± 0.2

[a] β-Glucuronidase (GUS) activity of strains carrying pXV9XGUS after 16 h of growth on hrp-inductive XVM2 medium; values represent mean ± 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, T**GA**GC-N_{15}-TTCGC; PIP1M2, TTC**TA**-N_{15}-TTCGC; PIP2M1, TTCGC-N_{15}-TT**GA**C; PIP1&2M1, T**GA**GC-N_{15}-TT**GA**C; PIP1&2DM, **CCTCG**-N_{15}-**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 symplasmid 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

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1                                                                    50
YopP   .......... .......... .......... .......... ..........
YopJ   .......... .......... .......... .......... ..........
AvrAS  .......... .......... .......... .......... ..........
AvrRxv MCDSIRVQFR SIQKMVVKMK KFFRSLGVGG SSSSR...FQ HHIPEADSAP
AvrBsT .......... ........MK NFMRSLGFGS SRSSRSSSSN WNEQQADNDE
Y4LO   .......... .......... .......... .......... ..........

51                                                                  100
YopP   .......... .......... ...MIGPISQ INSFGGLS.E KETRSLISNE
YopJ   .......... .......... ...MIGPISQ INISGGLS.E KETSSLISNE
AvrAS  .........M IFSVQELSCG GKSMLSPTTR .NMGASLSPQ PDVSGELNTE
AvrRxv SSKASTPPAS PPPDSPPSNS AFSALPTRPR KKAEALSDAV E.SRGHLAPP
AvrBsT QTPASSPSTS PSQTS....S AFSGLPERPR KKAIALEESL N.SSNNI.PY
Y4LO   ........MQ LSRRAEIGNP SSRNLSPRIN EKVELLGQAL EHARRAGMSS

101                                                                 150
YopP   ELKNIIIQLE TDIADGSWFH KNYSRLDIEV MPALVIQANN KYPEMNLNFV
YopJ   ELKNIITQLE TDISDGSWFH KNYSRMDVEV MPALVIQANN KYPEMNLNLV
AvrAS  ALTCIVERLE SEIIDGSWIH ISYEETDLEM MPFLVAQANK KYPELNLKFV
AvrRxv SLVSYANATL DQLRRNEPIS ESLRLMDIEN LPHLVRSYDN RLNNLNLRSF
AvrBsT EMRMYAEAAL SAA..NDGSS EAITKADVEN KYYLAHAYNE RFPELHLSCH
Y4LO   SLMEYGRQVA RHLSANVQPD EKILSLDIRN LPLLAASYNR KYPDLDLRHM

151                                                                 200
YopP   TSPQDLSIEI KNVIENGVGS SRFIINMGEG G..IHFSVID YKHIN.GKTS
YopJ   TSPLDLSIEI KNVIENGVRS SRFIINMGEG G..IHFSVID YKHIN.GKTS
AvrAS  MSVHELVSSI KETRMEGVES ARFLVNMGSS G..IHISVVD FRVMD.GKTS
AvrRxv DTPGQFLHDL SRWHKTGLPL .RAVVRLDED PRRWHRVAFD VRNHESGHTT
AvrBsT DSAQSFFSEF MTSEKQA..W .RSIVRL..S PSSMHHAAID VRFKD.GKRT
Y4LO   DSPARFFDAL NDRSSDG.AW .RAVVRLADG EQ..HHVAAD VRTRAGAAPT

201                                                                 250
YopP   LILFEPVNFN SMGPAILAI. ....STKTAI ERYQLPDCHF SMVEMDIQRS
YopJ   LILFEPANFN SMGPAMLAI. ....RTKTAI ERYQLPDCHF SMVEMDIQRS
AvrAS  VILFEPAACS AFGPA.LAL. ....RTKAAL EREQLPDCYF AMVELDIQRS
AvrRxv IIALEPASAY NPDH.....M PGFVKMRENL TSQFGRKISF AVIEAEALKS
AvrBsT MLVIEPALAY GMKDGEIKVM AGYETLGKNV QNCLGENGDM AVIQLGAQKS
Y4LO   IIVMEGANFY T.......FV ASYFKLRGDS FRQLGTQAKW AFIEVGAQKS

251                                                                 300
YopP   SSECGIFSLA LAKKLYTERD SLLKIHED.. ...NIKGILS DSENPLPHN.
YopJ   SSECGIFSFA LAKKLYIERD SLLKIHED.. ...NIKGILS DGENPLPHD.
AvrAS  SSECGIFSLA LAKKLQLEFM NLVKIHED.. ...NICERLC GEEPFLPSD.
AvrRxv IGGCVIFSLD YALAAYQERS TFDQWHKDLR KKGNIKGMTP .ESQHLNELG
AvrBsT LFDCVIFSLN MALCAYQKDS VFDNLHDCLR R..NVRCFSS GERKSILHKN
Y4LO   AADCVMFGVQ FALAAYRELP TFDAWHDNLH HHGTI..... ..........

301                                                                 350
YopP   ....KLDPYL PVTFYKHTQG KKRLNEYLNT NPQGVGTVVN KK.....NET
YopJ   ....KLDPYL PVTFYKHTQG KKRLNEYLNT NPQGVGTVVN KK.....NET
AvrAS  ....KADRYL PVSFYKHTQG AQRLNEYVEA NPAAGSSIVN KK.....NET
AvrRxv VYLLKGTRLL PANFYKHAHS RRTIDELEAD QPGASGTDVR SGRAAVYKES
AvrBsT IEFIEGDKFL PPIFYKHSHS RGVVGEFISN QPEYAHKNVS TGRTNP.SED
Y4LO   .......... .......... ........AH EGDYSSDYMP RRHAGICANK

351                                  386
YopP   IFNRFDNNKS IIDGKELSVS VHKKRIAEYK TLLKV.
YopJ   IVNRFDNNKS IVDGKELSVS VHKKRIAEYK TLLKV.
AvrAS  LYERFDNNAV MLNDKKLSIS AHKKRIAEYK SLLKPZ
AvrRxv LSRRLEEFQV QR.DKTYSMS IEASRARKIR HALES.
AvrBsT LSERVENFRV RRGDLSYSMS IEASRLRKIR KTIES.
Y4LO   PFSWGE...V PPSDLLQALS LQQCN..... ......
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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
Xanthmonas campestris pv. *vesicatoria*		
75-3	Tomato race 1, Rif^r, *avrRxv*	Whalen et al. 1988
92-14	Tomato race 1, Rif^r, *avrRxv*; HR on Hawaii 7998	Whalen et al. 1993
92-14 ΔavrRxv	92-14 deletion of *avrRxv* 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 hrpFGUS	hrpF312-Tn*3*-gus fusion in 85-10; pepper race, Rif^r	Schulte and Bonas 1992
85-10 ΔhrpXv	85-10 deleted for *hrp*Xv, Rif^r	Wengelnik and Bonas 1996
85-10 ΔhrpG	85-10 deleted for *hrp*G, Rif^r	Wengelnik et al. 1996b
75-3 hrp::Tn5 #35, 5, 139, 57, 15, 132	75-3 with Tn*5* insertion in *hrp*A,B,C,D,E, and F, respectively	Bonas et al. 1991
Vectors		
pL6	pLAFR3 deleted for Plac with *trp* terminators flanking polylinker	Huynh et al. 1989
pLITMUS 38	pUC-derived backbone lacking *Xmn*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 *Bam*HI fragment containing avrRxv gene cloned from *X. campestris* pv. *vesicatoria* tomato race 1 75-3; HR on Hawaii 7998	Whalen et al. 1988
pXV9117	pXV9000 opened at *Xho*I site and deleted for 1.3 kbp in both directions; *Xho*I site reconstructed	This study
pXV9SC920	*avrRxv* deletion construct; pXV9117 with Ω inserted at *Xho*I site; WS on Hawaii 7998	This study
pXV9006, pXV9007	pL6 with 2.1-kbp *Pst*I *avrRxv* fragment in both orientations	Whalen et al. 1993 and this study
pRAJ275	1.8-kbp *uidA* gene with *Nco*I site at initiator ATG codon	Jefferson et al. 1987
pUC118RXV3	2.1-kbp *Pst*I fragment cloned into pUC118	Whalen et al. 1993
pUC118RXV3ΔN277	pUC118RXV3 deleted for *Nco*I site at bp 277	This study
pXV9XM2N	pUC118RXV3ΔN277 with *Xmn*I site at bp -601 converted to *Nco*I site	This study
pXV9XM2NGUS	pXV9XM2N with 1.5-kbp *Nco*I-*Eco*RI fragment replaced with 1.9-kbp *Nco*I-*Eco*RI *uidA* gene from pRAJ275 creating translational fusion of *avrRxv* and *uidA*	This study
pXV9XGUS	pL6 carrying 2.5-kbp *Hin*dIII-*Eco*RI fragment from pXV9XM2NGUS; translational fusion of *avrRxv* and *uidA* at bp 601	This study
pXV9LC1	pUC118RXV3ΔN277 with *Stu*I site at bp 747 converted to *Nco*I site; 100-bp *Stu*I fragment deleted	This study
pXV9LC2	pXV9LC1 with 1.3-kbp *Nco*I-*Eco*RI fragment replaced with 1.9-kbp *Nco*I-*Eco*RI *uidA* gene from pRAJ275 creating translational fusion of *avrRxv* and *uidA*	This study
pXV9LC3	pL6 carrying 2.7-kbp *Hin*dIII-*Eco*RI fragment from pXV9LC2; translational fusion of *avrRxv* and *uidA* at bp 747	This study
pL6GUS	pL6 carrying 1.9-kbp NcoI-EcoRI uidA gene from pRAJ275	D. Dahlbeck and B. Staskawicz
pMC1871	pBR322-derived backbone containing promoterless *lacZ* lacking a ribosome binding site and codons for the first 8 amino acids	Amersham Pharmacia Biotech, Piscataway, NJ
pXV9SCAT1	pXV9007 with 0.6-kbp *Hin*dIII-*Xho*I fragment replaced with 3.9-kbp *Hin*dIII-*Xho*I fragment from pMC1871S2X containing promotorless *lacZ* gene; translational fusion of *avrRxv* and *lacZ* at bp 1483	This study
pL6LACZ	pL6 carrying 3.1-kbp *Bam*HI fragment with promoterless *lacZ* 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 *avrRxv* with mutations in PIP-box sequence	This study
pXV9JM1	pUFR051 carrying *avrBsT* active 1.9-kbp subclone	This study
pBSTA	0.8-kbp *Eco*RI-*Hin*dIII *avrBsT* fragment cloned into pUC118	This study
pBSTB	0.8-kbp *Eco*RI-*Hin*dIII *avrBsT* fragment cloned into pUC119	This study
pBSTC	1.1-kbp *Eco*RI-*Kpn*I *avrBsT* fragment cloned into pUC118	This study
pBSTD	1.1-kbp *Eco*RI-*Kpn*I *avrBsT* fragment cloned into pUC119	This study
pUR288A6	1.1-kbp open reading frame (ORF) from *avrBsT* in pUR288	This study
pET3CA16	1.1-kbp ORF from *avrBsT* 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 (10^7 to 10^8 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* DH5α 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 µg/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 $NaPO_4$, pH 6.8, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and finally washed at 65°C in 0.2× SSC (1× 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 92-14ΔavrRxv. 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 pUC118RXV3ΔN277. To create a translational fusion at bp 601 in *avrRxv*, the *Xmn*I site in pUC118RXV3ΔN277 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 pUC118RXV3ΔN277 was converted into an *Nco*I site, creating pXV9LC1, followed by cloning the promotorless 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 OD_{600}, 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-cm^2 disks were macerated in 250 µl of 10 mM $MgCl_2$ 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; Barettino et al. 1994). The *Sca*I-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, AAGCTGAAAAACCCTGAACGAAG CA; PIP1MP1, TTCAGGGTTTTTCAGCTTTGAGCATTAT TGCCT; PIP1MP2, GTTCAGGGTTTTTCAGCTTTTCTAA TTATTGCCTA; PIP2LWR, GATCTAGGCAATAATGCGA AA AGCTG; PIP2MP1, CGCATTATTGCCTAGATCTTAA CGCAACGAAAC; PIPDMPLLWR, GGATCTAGGCAATA ATCGAGGAAGCTGAAAAACC; PIPDMPR UPP, CGATT ATTGCCTAGATCCCTCGGCAACGAAACCG; PIPDLL WR, TGAGCGGATAACAATTTCACACAGGAAACAGC. 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 *Nco*I and *Xmn*I and the 0.32-kbp mutagenized *Nco*I-*Xmn*I 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 *Eco*RI, *Kpn*I, and *Hin*dIII. The resulting 0.8-kbp *Eco*RI-*Hin*dIII and 1.1-kbp *Eco*RI-*Kpn*I 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 *Hin*dIII sites added to the 5′ and 3′ ends (5′ to 3′; RST17, GGGAA GCTTATGAAGAATTTTATG; RST18, CCCAAGCTTAT-GATTCAATAGTT). The resulting *Hin*dIII 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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LITERATURE CITED

Alfano, J. R., and Collmer, A. 1996. Bacterial pathogens in plants: Life up against the wall. Plant Cell 8:1683-1698.

Alfano, J. R., and Collmer, A. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: Trafficking harpins Avr proteins and death. J. Bacteriol. 179:5655-5662.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Anderson, D. M., and Schneewind, O. 1997. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. Science 278:1140-1143.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. 1995. Short Protocols in Molecular Biology. 3rd ed. John Wiley and Sons, New York.

Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S. P. 1997. Signaling in plant-microbe interactions. Science 276:726-773.

Barettino, D., Feigenbutz, M., Valcarcel, R., and Stunnenberg, H. G. 1994. Improved method for PCR-mediated site-directed mutagenesis. Nucleic Acids Res. 22:541-542.

Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V., Staskawicz, B. J., and Stall, R .E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interact. 4:81-88.

Bonas, U., and Van den Ackerveken, G. 1997. Recognition of bacterial avirulence proteins occurs inside the plant cell: A general phenomenon in resistance to bacterial diseases? Plant J. 12:1-7.

Bouzar, H., Jones, J. B., Stall, R. E., Hodge, N. C., Minsavage, G. V., Benedict, A. A., and Alvarez, A. M. 1994. Physiological, chemical, serological, and pathogenic analyses of a worldwide collection of *Xanthomonas campestris* pv. *vesicatoria* strains. Phytopathology 84: 663-671.

Dangl, J. L., Dietrich, R. A., and Richberg, M. H. 1996. Death don't have no mercy: Cell death programs in plant-microbe interactions. Plant Cell 8:1793-1807.

Daniels, M. J., Barber, C. E., Turner, D. C., Cleary, W. G., and Sawczyc, M. K. 1984. Isolation of mutants of *Xanthomonas campestris* pv. *campestris* with altered pathogenicity. J. Gen. Microbiol. 130:2447-2455.

De Feyter, R., and Gabriel, D. W. 1991. Use of the cloned DNA methylase genes to increase the frequency of transfer of foreign genes into *Xanthomonas campestris* pv. *malvacearum*. J. Bacteriol. 173:6421-6427.

Fenselau, S., and Bonas, U. 1995. Sequence and expression analysis of the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. Mol. Plant-Microbe Interact. 8:845-854.

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

Finlay, B. B., and Cossart, P. 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science 276:718-725.

Finlay, B. B., and Falkow, S. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61:136-169.

Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A., and Perret, X. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature 387:394-401.

Gabriel, D. W. 1997. Targeting of protein signals from *Xanthomonas* to the plant nucleus. Trends Plant Sci. 2:204-206.

Galan, J. E., and Bliska, J. B. 1996. Cross-talk between bacterial pathogens and their host cells. Annu. Rev. Cell Dev. Biol. 12:221-255.

Galyov, E. E., Haakansson, S., and Wolf-Watz, H. 1994. Characterization of the operon encoding the YpkA Ser/Thr protein kinase and the YopJ protein of *Yersinia pseudotuberculosis*. J. Bacteriol. 176:4543-4548.

Gopalan, S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He, S. Y., and Collmer, A. 1996. Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. Plant Cell 8: 1095-1105.

Hardt, W.-D., and Galan, J. E. 1997. A secreted *Salmonella* protein with homology to an avirulence determinant of plant pathogenic bacteria. Proc. Natl. Acad. Sci. USA 94:9887-9892.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51-59.

Horton, R. M., Cai, Z., Ho, S. N., and Pease, L. R. 1990. Gene splicing by overlap extension: Tailor-made genes using the polymerase chain reaction. BioTechniques 8:528-535.

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

Hwin, T., Ciesiolka, L., Gearlds, J., Handley, V., Bravo, M., and Whalen, M. 1996. Expression analysis of *avrRxv* in the interaction between *Xanthomonas campestris* pv. *vesicatoria* and tomato. Int. Symp. Mol. Plant-Microbe Interact., 8th.

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Knoop, V., Staskawicz, B., and Bonas, U. 1991. Expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* is not under the control of *hrp* genes and is independent of plant factors. J. Bacteriol. 173:7142-7150.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:1435-1441.

Leach, J. E., and White, F. F. 1996. Bacterial avirulence genes. Annu. Rev. Phytopathol. 34:153-179.

Loh, Y.-T., and Martin, G. B. 1995a. The *Pto* bacterial resistance gene and the *Fen* insecticide sensitivity gene encode functional protein kinases with serine-threonine specificity. Plant Physiol. 108:1735-1739.

Loh, Y.-T., and Martin, G. B. 1995b. The disease-resistance gene *Pto* and the fenthion-sensitivity gene *Fen* encode closely related functional protein kinases. Proc. Natl. Acad. Sci. USA 92:4181-4184.

McCarty, D. R., Hattori, T., Carson, C. B., Vasil, V., Lazar, M., and Vasil, I. K. 1991. The viviparous-1 developmental gene of maize encodes a novel transcription activator. Cell 66:895-905.

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

Mills, S. D., Boland, A., Sory, M.-P., van der Smissen, P., Kerbourch, C., Finlay, B. B., and Cornelis, G. R. 1997. *Yersinia enterocolitica* induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector protein. Proc. Natl. Acad. Sci. USA 94:12638-12643.

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

Monack, D. M., Mecsas, J., Ghori, N., and Falkow, S. 1997. *Yersinia* signals macrophages to undergo apoptosis and YopJ is necessary for cell death. Proc. Natl. Acad. Sci. USA 94:10385-10390.

Mudgett, M. B., and Staskawicz, B. J. 1998. Protein signalling via type III secretion pathways in phytopathogenic bacteria. Curr. Opin. Microbiol. 1:109-114.

Nakai, K., and Kanehisa, M. 1992. A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics 14:897-911.

Nakai, K., and Kanehisa, M. 1991. Expert system for predicting protein localization in gram-negative bacteria. Proteins Struct. Funct. Genet. 11:95-110.

Pallen, M. J., Dougan, G., and Frankel, G. 1997. Coiled-coil domains in proteins secreted by type III secretion systems. Mol. Microbiol. 25: 423-425.

Parker, J. E., Barber, C. E., Mi-jiao, F., and Daniels, M. J. 1993. Interaction of *Xanthomonas campestris* with *Arabidopsis thaliana:* Characterization of a gene from *X. c.* pv. *raphani* that confers avirulence to most *A. thaliana* accessions. Mol. Plant-Microbe Interact. 6:216-224.

Parker, J. E., and Coleman, M. J. 1997. Molecular intimacy between proteins specifying plant-pathogen recognition. TIBS 22:291-296.

Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E., and Wolf-Watz, H. 1996. Modulation of virulence factor expression by pathogen target cell contact. Science 273:1231-1233.

Puri, N., Jenner, C., Bennett, M., Stewart, R., Mansfield, J., Lyons, N., and Taylor, J. 1997. Expression of *avrPphB*, an avirulence gene from *Pseudomonas syringae* pv. *phaseolicola*, and the delivery of signals causing the hypersensitive reaction in bean. Mol. Plant-Microbe Interact. 10:247-256.

Rosqvist, R., Magnusson, K. E., and Wolf-Watz, H. 1994. Target cell

contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. 13:964-972.
Rüther, U., and Müller-Hill, B. 1983. Easy identification of cDNA clones. EMBO J. 2:1791-1794.
Salmeron, J. M., and Staskawicz, B. J. 1993. Molecular characterization and *hrp*-dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. tomato. Mol. Gen. Genet. 239:6-16.
Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sanger, F., Nicklen, S., and Colulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
Schesser, K., Frithz-Lindsten, E., and Wolf-Watz, H. 1996. Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membrane. J. Bacteriol. 178:7227-7233.
Schulte, R., and Bonas, U. 1992. Expression of the *Xanthomonas campestris* pv. *vesicatoria hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. J. Bacteriol. 174:815-823.
Schwartz, R. M., and Dayhoff, M. O. 1979. Pages 353-358 in: Atlas of Protein Sequence and Structure. M. O. Dayhoff, ed. National Biomedical Research Foundation, Washington, DC.
Scofield, S. R., Tobias, C. M., Rathjen, J. P., Change, J. H., Lavelle, D. T., Michelmore, R. W., and Staskawicz, B. J. 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. Science 274:2063-2065.
Sory, M. P., Boland, A., Lambermont, I., and Cornelis, G. R. 1995. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. Proc. Natl. Acad. Sci. USA 92:11998-12002.
Stall, R. E., Beaulieu, C., Egel, D., Hodge, N. C., Leite, R. P., Minsavage, G. V., Bouzar, H., Jones, J. B., Alvarez, A. M., and Benedict, A. A. 1994. Two genetically diverse groups of strains are included in *Xanthomonas campestris* pv. *vesicatoria*. Int. J. Syst. Bacteriol. 44: 47-53.
Swarup, S., Yang, Y., Kingsley, M. T., and Gabriel, D. W. 1992. An *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhosts. Mol. Plant-Microbe Interact. 5: 204-213.
Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. 1996. Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. Science 274:2060-2063.
Van den Ackerveken, G., Marois, E., and Bonas, U. 1996. Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. Cell 87:1307-1316.
Viera, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.
Wang, J. F., Stall, R. E., and Vallejos, C. E. 1994. Genetic analysis of a complex hypersensitive reaction to bacterial spot in tomato. Phytopathology 84:126-132.
Wattiau, P., Woestyn, S., and Cornelis, G. R. 1996. Customized secretion chaperones in pathogenic bacteria. Mol. Microbiol. 20:255-262.
Wengelnik, K., and Bonas, U. 1996. HrpXv, an AraC-type regulator, activates expression of five out of six loci in the *hrp* cluster of *Xanthomonas campestris* pv. *vesicatoria*. J. Bacteriol. 178:3462-3469.
Wengelnik, K., Marie, C., Russel, M., and Bonas, U. 1996a. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. *vesicatoria* essential for pathogenicity and induction of the hypersensitive reaction. J. Bacteriol. 178:1061-1069.
Wengelnik, K., Van den Ackerveken, G., and Bonas, U. 1996b. HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. Mol. Plant-Microbe Interact. 9:704-712.
Whalen, M. C., Stall, R. E., and Staskawicz, B. J. 1988. Characterization of a gene from a tomato pathogen determining hypersensitive resistance in non-host species and genetic analysis of this resistance in bean. Proc. Natl. Acad. Sci. USA 85:6743-6747.
Whalen, M. C., Wang, J. F., Carland, F. M., Heiskell, M. E., Dahlbeck, D., Minsavage, G. V., Jones, J. B., Scott, J. W., Stall, R. E., and Staskawicz, B. J. 1993. Avirulence gene *avrRxv* from *Xanthomonas campestris* pv. *vesicatoria* specifies resistance on tomato line Hawaii 7998. Mol. Plant-Microbe Interact. 6:616-627.
Worley, K. A., Wiese, B. A., and Smith, R. F. 1995. BEAUTY: An enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity search results. Genome Res. 5:173-184.
Yang, Y., and Gabriel, D. W. 1995. *Xanthomonas* avirulence/pathogenicity gene family encodes functional plant nuclear targeting signals. Mol. Plant-Microbe Interact. 8:627-631.
Yang, Y., Yuan, Q., and Gabriel, D. W. 1996. Watersoaking function(s) of XcmH1005 are redundantly encoded by members of the *Xanthomonas avr/pth* gene family. Mol. Plant-Microbe Interact. 9:105-113.
Yu, Z. H., Wang, J. F., Stall, R. E., and Vallejos, C. E. 1995. Genomic localization of tomato genes that control a hypersensitive reaction to *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye. Genetics 141:675-682.
Zhou, J., Loh, Y.-T., Bressan, R. A., and Martin, G. B. 1995. The tomato gene Ptil encodes a serine-threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. Cell 83:925-935.