Relationship Between Avirulence Gene (*avrA*) Diversity in *Ralstonia solanacearum* and Bacterial Wilt Incidence

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Bacterial wilt, caused by *Ralstonia solanacearum*, is a serious disease of tobacco in North and South Carolina. In contrast, the disease rarely occurs on tobacco in Georgia and Florida, although bacterial wilt is a common problem on tomato. We investigated whether this difference in disease incidence could be explained by qualitative characteristics of avirulence gene *avrA* in the *R. solanacearum* population in the southeastern United States. Sequence analysis established that wild-type *avrA* has a 792-bp open reading frame. Polymerase chain reaction (PCR) amplification of *avrA* from 139 *R. solanacearum* strains generated either 792-bp or ≈960-bp DNA fragments. Strains that elicited a hypersensitive reaction (HR) on tobacco contained the 792-bp allele, and were pathogenic on tomato and avirulent on tobacco. All HR-negative strains generated a ≈960-bp DNA fragment, and wilted both tomato and tobacco. The DNA sequence of *avrA* in six HR-negative strains revealed the presence of one of two putative miniature inverted-repeat transposable elements (MITEs): a 152-bp MITE between nucleotides 542 and 543, or a 170-bp MITE between nucleotides 461 and 462 or 574 and 575. Southern analysis suggested that the 170-bp MITE is unique to strains from the southeastern United States and the Caribbean. Mutated *avrA* alleles were present in strains from 96 and 75% of North and South Carolina sites, respectively, and only in 13 and 0% of the sites in Georgia and Florida, respectively. Introduction of the wild-type allele on a plasmid into four HR-negative strains reduced their virulence on both tobacco and tomato. Inactivation of *avrA* in an HR-positive, avirulent strain, resulted in a mutant that was weakly virulent on tobacco. Thus, the incidence of bacterial wilt of tobacco in the southeastern United States is partially explained by which *avrA* allele dominates the local *R. solanacearum* population.

Additional keywords: insertion sequence.

Bacterial wilt is a major disease of tobacco in North and South Carolina (Lucas 1975). In contrast, the disease rarely

occurs on tobacco in Georgia and Florida, although it is common on tomato in tobacco-growing counties (Fortnum and Martin 1998; Kelman and Person 1961). Kelman and Person (1961) found considerable variation in wilt disease severity caused by *Ralstonia solanacearum* strains from these four states on different hosts. Their investigation revealed that Georgia strains of *R. solanacearum* have low virulence on tobacco, and that bacterial wilt on tomato does not necessarily indicate a hazard for tobacco or peanut production in Georgia. When a wilt-susceptible cultivar of tobacco was planted into a tomato-transplant field in Georgia with a history of 90% bacterial wilt incidence, not a single plant exhibited wilt symptoms (Kelman and Person 1961). In contrast, *R. solanacearum* strains from tobacco in North Carolina, South Carolina, Georgia, Florida, and Virginia were highly virulent on both tobacco and tomato in greenhouse tests.

Numerous studies have assessed the genetic diversity of *R. solanacearum* strains collected from diverse geographic areas and host species (Cook et al. 1989). There also have been studies on the phenotypic and genotypic variation of *R. solanacearum* populations within localized geographic regions (Dookun et al. 2001; Gillings and Fahy 1993; Horita and Tsuchiya 2001; Poussier et al. 1999; Smith et al. 1995). Previously we reported that repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR) could be used to differentiate *R. solanacearum* populations isolated from different geographical areas of the southeastern United States (Robertson et al. 2001). However, it was not possible to associate a specific genetic marker with either aggressiveness or geographic location. Subsequent to these observations, we hypothesized that, given its potential in mediating plant–pathogen interactions, the avirulence gene *avrA* may be useful in discriminating *R. solanacearum* populations and explain differences observed in bacterial wilt incidence in the Carolinas and Georgia or Florida.

In *R. solanacearum* AW1, *avrA* is responsible for eliciting a hypersensitive response (HR) on tobacco (Carney and Denny 1990). *AvrA* was cloned, and its ability to shift strain K60 from being pathogenic on tobacco to eliciting an HR was demonstrated. The transconjugants also were nonpathogenic on tobacco. Inactivating *avrA* in AW1 prevented this strain from eliciting an HR on tobacco, but it did not become pathogenic on tobacco. Therefore, *avrA* is one factor that restricts the host range of *R. solanacearum* (Carney and Denny 1990). Avirulence genes in a pathogen determine race specificity by limiting the range of host cultivars and, occasionally, host species and genera that the pathogenic strain may attack (Gabriel 1999). Therefore, loss or inactivation of an avirulence gene

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often extends the host range of a pathogen to include plants previously found to be resistant (Kearney and Staskawicz 1990a). However, numerous reports have shown that inactivation of *avr* genes eliminates the HR but does not enable the strains to induce disease symptoms on the same host (Leach and White 1996). The objective of this study was to investigate the sequence diversity of *avrA* in *R. solanacearum* strains from the southeastern United States in relation to geographic distribution and the incidence of wilt disease on tomato and tobacco.

In addition to regulating resistance (*R*)-gene-specific interactions, *avr* genes have been reported to contribute to pathogen virulence on susceptible hosts. The first such evidence was provided by Kearney and Staskawicz (1990b), who reported that *Xanthomonas campestris* pv. *vesicatoria* containing an inactivated *avrBs2* gene was less virulent on a susceptible pepper cultivar compared with the wild-type strain. In addition, a marker exchange *avrBs2* mutant of *X. campestris* pv. *vesicatoria* exhibited a 100-fold reduction in its ability to colonize susceptible plant tissue. Similarly, the *avrE* gene from *Pseudomonas syringae* pv. *tomato* restored pathogenicity to the "disease-specific" locus (*dsp*) mutant strain of *Erwinia amylovora* that was avirulent on pear fruit (Bogdanove et al. 1998). Although the mechanisms describing these observations are unknown, *avr* gene products mediate the suppression of inducible host defenses (Abramovitch et al. 2003; Vivian and Gibbon 1997; Yang et al. 2000). When expressed ectopically in plants lacking the corresponding *R* gene, *avr* genes can induce such phenotypes as chlorosis, necrosis, canker formation, cell division, and enlargement and cell death (Kjemtrup et al. 2000). Interestingly, other type III effectors from animal-bacterial pathogens exhibit similar cytotoxic effects on the host cells (van't Slot and Knogge 2002).

Plant resistance to bacterial wilt is often geographically specific (Jack and Robertson 1997; Michel et al. 1996; Prior et al. 1994). It has been hypothesized that environmental conditions and genetic diversity of the pathogen over a wide geographic area may account for these observations. Here we have shown that diversity of the avirulence gene, *avrA*, impacts the incidence of bacterial wilt as a function of geography.

RESULTS

AvrA is a novel protein with characteristics of effectors secreted by Hrp type III systems.

Previous research indicated that, in pBC73, which contains 4.5 kb of AW1 genomic DNA, the 5' end of *avrA* is within the terminal 200 bp that separate the end of the fragment from a unique internal *EcoRI* site and that the gene must be less than 2 kb long (Carney and Denny 1990). A 3.1-kb *PstI* fragment from pBC73, which contains *avrA*, was subcloned into pRK404 to produce pBC738 (Fig. 1A), and a 2.3-kb region was sequenced on both strands (GenBank accession number AY517472). DNA sequence analysis indicated that it has one open reading frame (ORF) with 263 codons and, 214 nucleotides downstream, a second ORF with 305 codons (Fig. 1A). We concluded that the smaller ORF corresponds to *avrA*, because its 5' end is appropriately situated, the ORF spans the unique *EcoRI* site, and its codon usage is similar to all ORFs in the genome of *R. solanacearum* strain GMI1000. The G + C content of *avrA* is 60.6%, which is only moderately lower than the ≈67% typical of this species. Seven base pairs upstream of *avrA* is a potential ribosome binding site, and 69 bp upstream is a near consensus plant-inducible promoter (PIP-box) sequence of TTCGT-N₁₅-TTCGT (consensus nucleotides are underlined) (Cunnac et al. 2004; Fenselau and Bonas 1995). A PIP-box is present upstream of three transcriptional units in

the *hrp* gene cluster and six additional avirulence gene homologues in GMI1000 (Cunnac et al. 2004; Fenselau and Bonas 1995; Salanoubat et al. 2002). Five bases downstream of the *avrA* stop codon is a potential stem-loop sequence that could be a transcriptional terminator. This sequence was used to design PCR primers to amplify a 793-bp fragment that extends from 4 bases upstream of the *avrA* start codon through the last amino acid codon.

Analysis of the predicted AvrA protein showed it to be very hydrophilic, lacking both a signal peptide and transmembrane domains. No large α -helices or β -sheet structures were predicted, and no motifs were found at an E value < 0.01 using reverse position-specific BLAST (Altschul et al., 1997). Like

Fig. 1. DNA sequence analysis of *Ralstonia solanacearum* *avrA* in strain AW1. **A**, The 3.1-kb *PstI* fragment of pBC73 that was subcloned into pBlueScript (hatched bands) for use as a sequencing template. The two open arrows represent the complete open reading frames (ORFs) present in the 2.3-kb sequenced region (heavy line). Restriction enzymes abbreviations: E, *EcoRI*; P, *PstI*; S, *Sall*; Sa, *Sau3A* (only relevant sites are shown). Restriction sites labeled above the figure are in the vector, whereas those below the figure are in the cloned *R. solanacearum* DNA. **B**, Alignment of AvrA from AW1 with its homolog in strain GMI1000 (ORF number RS04833). Symbols below the alignment indicate identical amino acids (*), conserved substitutions (: and .), and gaps (-). The two proteins were aligned using ClustalW with default settings.

many type III secreted effectors in *P. syringae*, the N-terminal third of AvrA is amphipathic, rich in serine residues, and low in acidic residues (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). Also typical of many effectors, the putative AvrA protein has no homolog in GenBank, except for ORF RS04833 in *R. solanacearum* GMI1000 (Salanoubat et al. 2002). On the GMI1000 Web site, RS04833 is annotated as being AvrA because we provided French scientists the DNA sequence of AW1 *avrA* prior to this report. Surprisingly, alignment of the amino acid sequences for AvrA from AW1 and GMI1000, revealed that they are only 60% identical, mostly because the latter protein has 33 fewer residues (Fig. 1B). In comparison, the putative proteins encoded by ORFs downstream of *avrA* in both strains are 93% identical (and exactly the same size); ORF04832 in GMI1000 is annotated as a putative lipoprotein gene.

The size of *avrA* correlates with HR-inducing activity.

A collection of *R. solanacearum* strains isolated from soil and from wilted tomato or tobacco plants in the southeastern United States was screened for the presence of *avrA* and the ability to induce the HR in tobacco (Table 1). PCR amplification of *avrA* from each of the 139 strains generated either an ≈800-bp DNA fragment, corresponding to the wild-type allele in AW1, or an ≈960-bp fragment. The ≈960-bp allele was present in strains from 96% of the North Carolina sites and 75% of the South Carolina sites, but in only 13% of the sites in Georgia and none of the sites in Florida. All of the tomato strains, except for K60 from North Carolina, contained the wild-type allele. All of the strains from tobacco in Georgia

also contained the wild-type allele, but almost all of the strains from tobacco in North and South Carolina contained the ≈960-bp allele. Of the 100 strains of *R. solanacearum* isolated from soil, only 7 contained the wild-type allele.

When infiltrated into tobacco leaves, all the strains in the collection that elicited an HR within 16 to 24 h after inoculation contained the wild-type allele. In contrast, all of the HR-negative strains (necrosis in 48 to 96 h) contained the ≈960-bp allele of *avrA*. Pathogenicity assays on tobacco and tomato were performed using a selection of strains from tomato, tobacco, and soil to determine whether there was a correlation between HR and pathogenicity. In all cases, the HR-positive strains were pathogenic on tomato but avirulent on tobacco, whereas all HR-negative strains wilted both tomato and tobacco.

AvrA alleles from HR-negative strains contain one of two insertion sequences.

The ≈960-bp fragments with the *avrA* alleles from six HR-negative strains (SC06, SC111, NC116, GA102, GA122, and GA142) (Table 2) were cloned, sequenced, and compared with the wild-type, HR-inducing *avrA* allele from AW1. Nucleotide homology ranged from 95.3 to 99.3% (and amino acid homology from 84.4 to 97.9%) except in regions containing one of two insertions (Figs. 2 and 3). A 155-bp DNA insertion was found in strain GA102 between what would be nucleotides 542 and 543 in wild-type *avrA* (counting from the ATG start codon). This insertion has a low G + C content of 54.5%, a 16-bp imperfect terminal inverted repeat, and a 3-bp direct repeat of the insertion site in *avrA* (Figs. 2B and 3A). The 152-bp

Table 1. Summary of *Ralstonia solanacearum* strains used in this study

State	No. of strains from				No. of strains containing <i>avrA</i>		No. of sites	No. sites containing 972-bp <i>avrA</i>
	Tobacco	Tomato	Soil	Total	792-bp	972-bp		
NC	2	1	37	40	1	39	24	23
SC	11	4	19	34	10	24	20	15
GA	3	10	42	55	16	39 ^z	25	3
FL	0	12	0	12	12	0	9	0

^z Of these strains, 34 were from soil from the same field with a history of bacterial wilt of tobacco.

Table 2. Partial list of *Ralstonia solanacearum* strains used in the study with their hypersensitivity reaction on tobacco and DNA fragment size of the polymerase chain reaction amplified *avrA* gene¹

Isolate	State	Host	Size of <i>avrA</i> gene (bp)	Nucleotide homology (%) ^u	Amino acid identity (%) ^v	HR on tobacco	Source ^w
SC06	SC	Tobacco	972	98.9	97.7 ^x	–	Robertson et al. 2001
SC14	SC	Tomato	792	ND	ND	+	This study
SC17-1 ^y	SC	Tobacco	792	97.9	94.9	+	This study
SC17-1M ^z	...	...	≈2,800	...	...	...	This study
SC108	SC	Soil	972	ND	ND	–	This study
SC111	SC	Soil	972	99.1	97.9	–	This study
342	NC	Tobacco	972	ND	ND	–	T. Denny, UGA
K60	NC	Tomato	972	ND	ND	–	Robertson et al. 2001
NC116	NC	Soil	972	97.8	95.3	–	This study
NC136	NC	Soil	972	ND	ND	–	This study
108-3	GA	Tobacco	792	ND	ND	+	T. Denny, UGA
AW1	GA	Tomato	792	100	100	+	Carney and Denny 1990
GA102	GA	Soil	947	99.3	84.4	–	This study
GA122	GA	Soil	972	98.5	95.1	–	This study
GA142	GA	Soil	972	98.6	95.1	–	This study
RS14	FL	Tomato	792	ND	ND	+	T. Momol, UFL
RS19	FL	Tomato	792	ND	ND	+	T. Momol, UFL

¹ Representative strains were chosen for each state and each *avrA* allele. States: NC = North Carolina, SC = South Carolina, and GA = Georgia; HR = hypersensitive response; ND = not done.

^u Nucleotide homology to *avrA* in AW1 (%) excluding inserted DNA.

^v Amino acid identity with *avrA* in AW1 (%) excluding inserted DNA.

^w Name of donor, affiliation (UFL = University of Florida; UGA = University of Georgia).

^x Homology of the first 43 amino acids. A single base insertion between nucleotides 132/133 results in a reading frame shift.

^y Nalidixic acid resistant mutant of SC17.

^z *avrA* knockout strain.

sequence resembles a miniature inverted-repeat transposable element (MITE) (Brugger et al. 2002), and has two divergent ORFs that are 144 and 141-bp long; however, neither has any known coding potential. This putative MITE is not homologous to any sequence in the GenBank database. We searched for known insertion sequence (IS) elements that are related to the 152-bp MITE at the IS Finder Web site and found that the left and right ends (Fig. 3) are 93.8 and 100%, respectively, identical to the 18-bp imperfect terminal inverted repeat in ISRs01 (GenBank AF239240) (Poussier et al. 2003). Little sequence similarity was detected between the central regions of the 152-bp MITE and ISRs01. The 3-bp direct repeat, TTA, was identical to the direct repeat reported for one mobile copy of ISRs01 in strain GMI1000 (Poussier et al. 2003). Insertion of the 152-bp MITE creates a stop codon at the insertion point (Fig. 3A), which results in a severely truncated AvrA protein.

In the remaining five strains, a 180-bp insertion was found between nucleotides 574 and 575 in SC111, and between nucleotides 461 and 462 in SC06, NC116, GA122, and GA142. The insertion is in the inverse orientation in SC06 compared with the other four strains. This larger insertion has an even lower G + C content of 52.2%, a putative 170-bp MITE with a 13-bp inverted terminal repeat, a 10-bp direct repeat of the insertion site in *avrA* (Figs. 2C and 3B), and two ORFs, 138 and 133 bp long, with no coding capacity, in the same orientation but different reading frames. No IS elements related to the 170-bp MITE were found using the IS Finder Web site. A BLASTn search of the GenBank database using the putative 170-bp MITE as the query revealed that the only significant homology (>99%) is to a 170-bp sequence within the 722-bp intergenic region upstream of *phcA* in *R. solanacearum* AW1

(Brumbley and Denny 1993). Each insertion of the 170-bp MITE would result in altered or truncated *avrA* proteins (Fig. 3B). The *avrA* allele in strain SC06 also contained a single base insertion between nucleotides 132 and 133 which resulted in a reading frame shift. Therefore, the presence of the

Fig. 3. Miniature inverted-repeat transposable element (MITE) DNA **A**, 152-bp and **B**, 170-bp sequences showing the terminal inverted repeats and direct repeats. The nucleotide sequences of the insertion elements are shown in upper case, the inverted repeats are underlined, and the direct repeats are italicized. The primer sites for IS-1 and IS-2 are outlined and their 5' to 3' orientation is shown. The stop codon created by insertion of each MITE is in bold. Only one insertion site of the 170-bp MITE is shown.

Fig. 2. Diagram showing **A**, wild-type *avrA* allele, **B**, 155-bp insertion in *avrA* in GA102 that results in the 947-bp allele, and **C**, 180-bp insertion in strains such as SC06, SC111, NC116, GA122, and GA142 that results in the 972-bp allele.

170-bp MITE in this allele may be meaningless, because the wild-type protein already is destroyed (Brumbley and Denny 1993). Multiplex PCR with primers Avr-F, Avr-R, and IS-2 was used to screen all HR-negative strains for the presence of the 170-bp MITE. Because IS-2 hybridizes to the junction between the MITE and the duplicated *avrA* sequence (Fig. 2C), PCR amplification generates 972- and a 518-bp products corresponding to *avrA* and the MITE plus the 3' end of *avrA*, respectively (Fig. 4), only when the MITE is present between nucleotides 461 and 462. The 170-bp MITE was present in *avrA* in all of the North Carolina strains, all Georgia strains except GA102, and in all but five of the South Carolina strains (Fig. 4). Sequence analysis of *avrA* in one of these five strains, SC111, showed the 170-bp MITE to be present in the gene, but at a different insertion site; that is, between nucleotides 574 and 575.

R. solanacearum strains from the southeastern United States carry multiple copies of the 179-bp MITE.

A PCR fragment containing the 170-bp MITE was used to probe *EcoRI*-digested genomic DNA from 14 *R. solanacearum* strains from the Carolinas, Georgia, and Florida. All the strains were found to contain a copy of this MITE on at least 10 restriction fragments (Fig. 5). The variation in signal intensity could be due to different numbers of the MITE on the different fragments or due to differences in homology. The results also showed that strains K60 and 342 have very similar restriction fragment length polymorphism fingerprints, but that the other 12 strains are unique (Fig. 5).

Fig. 4. Polymerase chain reaction (PCR) amplification, using the primers Avr-F, Avr-R, and IS-2 of the *avrA* gene in selected *Ralstonia Solanacearum* strains from North Carolina, South Carolina, Georgia, and Florida. Lane 1 = 100-bp marker, lane 2 = SC06, lane 3 = SC14, lane 4 = SC108, lane 5 = SC111, lane 6 = 342, lane 7 = K60, lane 8 = NC116, lane 9 = NC136, lane 10 = 108-3, lane 11 = AW1, lane 12 = GA102, lane 13 = GA122, lane 14 = RS14, lane 15 = RS19, and lane 16 = PCR negative control.

Table 3. Hypersensitivity reaction on tobacco and the area under disease progress curve (AUDPC) on tobacco and tomato of the transconjugants and their respective parents

Strain ^y	<i>avrA</i> size (bp)	HR ^z	AUDPC ^x	
			Tobacco	Tomato
AW1	792	+	0.00 d	63.13 a
SC06-1	972	–	52.63 ab	52.25 ab
SC06-1(pRK404)	972	–	32.63 c	59.25 a
SC06-1(pBC738)	972/792	+	3.13 d	0.38 d
K60-1	972	–	32.63 c	48.75 abc
K60-1(pRK404)	972	–	35.5 bc	27.5 bcd
K60-1(pBC738)	972/792	+	0.00 d	23.75 cd
NC116-1	972	–	32.63 c	55.25 a
NC116-1(pRK404)	972	–	51.25 abc	36.63 abc
NC116-1(pBC738)	972/792	+	1.75 d	4.38 d
GA102-1	947	–	57.38 a	62.88 a
GA102-1(pRK404)	947	–	32.63 bc	62.25 a
GA102-1(pBC738)	947/792	+	0.00 d	7.38 d

^x AUDPC was based on the measurement of the wilt index over time. Means within columns followed by different letters are significantly different by Fisher's protected least significant difference ($P < 0.05$).

^y pRK404 = empty vector; pBC738 = pRK404::*avrA*.

^z Hypersensitive response (HR) elicited (+) or not elicited (–) within 16 h.

The wild-type *avrA* allele reduces virulence on both tobacco and tomato.

The HR and pathogenicity on tobacco and tomato of four HR-negative *R. solanacearum* strains before and after the introduction of wild-type *avrA* on a plasmid was assessed. As expected based on the results of Carney and Denny (1990), introduction of pBC738 endowed the transconjugants with the ability to elicit an HR when infiltrated into tobacco leaves and virtually eliminated pathogenicity when inoculated on tobacco roots (Table 3). Thus, the wild-type *avrA* allele is dominant to the mutated alleles. Unexpectedly, the presence of multiple copies of wild-type *avrA* in *trans* (5 to 10 copies/cell) resulted in a significant reduction of pathogenicity on tomato (Table 3). This suggests that overproduction of AvrA interferes with normal pathogenic processes or triggers a resistance response in normally susceptible tomato.

avrA does not contribute to pathogenicity on tomato.

The HR and pathogenicity on tobacco and tomato of the *avrA* mutant, SC17-1M was compared with its parent, SC17-1, to determine the role of its effector protein on these traits. Unlike its parent strain, SC17-1M was HR negative and weakly pathogenic on tobacco. Thus, as was observed earlier for the *avrA* mutant strain AW1-31 (Carney and Denny 1990), inactivation of *avrA* in SC17-1 did not make it as pathogenic as a natural tobacco-pathogenic strain (e.g., SC06). When tested on tomato, there was no statistical difference in the pathogenicity of the four strains ($P = 0.2230$), even though SC17-1M was somewhat more aggressive than its wild-type parent (Table 4). Therefore, *avrA* did not contribute to the ability of SC17-1 to cause disease on tomato.

Fig. 5. Southern blot analysis of *EcoRI*-digested genomic DNA from 14 *Ralstonia solanacearum* strains probed with the 170-bp miniature inverted-repeat transposable element. Lane 1 = Lambda DNA/*HindIII* marker, lane 2 = RS19, lane 3 = RS14, lane 4 = GA122, lane 5 = GA102, lane 6 = AW1, lane 7 = 108-3, lane 8 = NC136, lane 9 = NC116, lane 10 = K60, lane 11 = 342, lane 12 = SC111, lane 13 = SC108, lane 14 = SC14, and lane 15 = SC06.

Table 4. Hypersensitivity reaction on tobacco and the area under disease progress curve (AUDPC) on tobacco and tomato of *Ralstonia solanacearum* SC17-1 and the *avrA* knockout mutant, SC17-1M

Strain	<i>avrA</i> size (bp)	HR ^y	AUDPC ^x	
			Tobacco	Tomato ^z
AW1 (control)	792	+	0.0 c	57.38
SC06 (control)	972	–	52.65 a	52.25
SC17-1	792	+	0.0 c	31.25
SC17-1M	2,800	–	16.0 b	59.13

^x AUDPC was based on the measurement of disease severity over time. Means within columns followed by different letters are significantly different by Fisher's protected least significant difference ($P < 0.05$).

^y Hypersensitive response (HR) elicited (+) or not elicited (–) within 16 h.

^z Analysis of variance of the AUDPC data was not significant ($P = 0.2230$).

DISCUSSION

Millions of dollars are lost each year due to bacterial wilt in North and South Carolina. In Georgia and Florida, bacterial wilt rarely is reported on tobacco although it is commonly found on tomato. This difference has been investigated since the early 1950s (Kelman 1953; Kelman and Person 1961). We investigated how diversity of the *avrA* gene in *R. solanacearum* populations relates to the observed incidence of bacterial wilt in the southeastern United States. PCR amplification of *avrA* from 139 strains of *R. solanacearum* from this area generated either 792-bp (wild-type) or $\approx$ 960 bp DNA fragments. The modified avirulence gene was present in strains from the majority of the sites sampled in the Carolinas; however, it rarely was found in the sites sampled in Georgia and was not found in Florida. We interpret these data to indicate that the incidence of bacterial wilt of tobacco in this region is explained partially by which *avrA* allele occurs more frequently in the local *R. solanacearum* population.

R. solanacearum strains that contained the wild-type *avrA* allele (792 bp) elicited an HR (HR-positive) on tobacco that was light brown and necrotic and appeared 16 to 24 h after inoculation. All HR-negative strains contained the modified allele ($\approx$ 960-bp) and caused chlorosis of the infiltrated tissue 36 to 72 h after inoculation, followed by necrosis 48 to 96 h post inoculation. We found all of the HR-positive strains to be pathogenic on tomato and avirulent on tobacco. In contrast, Prior and Steva (1990) reported that *R. solanacearum* strains from the French West Indies were both pathogenic and induced HR on tobacco. We believe this observation resulted from the method of inoculation used. The phenotype we report (i.e., HR positive and pathogenic negative) was obtained using the root-drenching method of inoculation in the pathogenicity bioassay. However, if we used stem inoculation, as reported by Prior and Steva (1990), all of our HR-positive strains also wilted tobacco (wilt indices ranged from 2/5 to 5/5).

Cloning and sequencing of the *avrA* gene from six of the HR-negative strains revealed the presence of one of two DNA insertion sequences, 170 or 152 bp in length. Both of these DNA fragments possess features similar to MITEs (Brugger et al. 2002). They are small (<500 bp), bound by terminal inverted repeats (TIRs), 13 and 16 bp, respectively, and are flanked by a target site duplication (TSD), 10 and 3 bp, respectively, of the *avrA* gene. They also do not code for any functional protein. MITE-like elements have been reported in a few other prokaryotes, namely, RUP (repeat unit of pneumococcus) elements in *Streptococcus* spp. (Oggioni and Claverys 1999), Neisseria miniature insertion sequences (*nemis*) in *Neisseria* spp. (Mazzone et al 2001), and an unnamed element in *Photobacterium luminescens* (Mahillon et al. 1999).

Sequence similarities exist between some MITEs and class II elements. This implies that a MITE could be mobilized by transposase activity encoded by the analogous class II element (Buisine and Tang 2002; Feschott and Mouches 2000; Oggioni and Claverys 1999; Redder et al. 2001). Using the "bottom-up approach" (Feschotte et al. 2002), we identified the likely partner transposase, ISRs01, of the 152-bp MITE. ISRs01 belongs to the IS5 family and also duplicates the trinucleotide TTA upon insertion into *phcA* in *R. solanacearum* strain GMI1000 (Poussier et al 2003). This TSD indicates that the 152-bp MITE is a *Tourist*-like MITE and that ISRs01 belongs to the superfamily of transposase *Tc1/Mariner* (Feschotte and Mouches 2000; Jiang et al. 2003). It is possible that the 152-bp MITE was derived from ISRs01. The *Emigrant* family of MITEs, in *Arabidopsis*, arose from *Lem1*, which encodes a putative transposase (Feschotte and Mouches 2000).

Although we found no IS related to the 170-bp MITE, sequence analysis revealed that AW1 has this MITE upstream of *phcA*, and Southern analysis (Fig. 5) showed that many additional copies of this MITE are present in this and other strains from the southern United States. Brumbley and Denny (1993) similarly noted the presence of a repeated sequence in some *R. solanacearum* strains when they probed a Southern blot of genomic DNAs using a labeled fragment containing *phcA* and its upstream region (i.e., including the 170-bp MITE). It is significant that, in the same experiment, they did not detect a repeated sequence in strains from around the globe classified as race 1/biovars 3 or 4 (GMI1000, UW130, and UW143), race 2/biovar 1 (UW9, UW20, and UW127), and race 3/biovar2 (UW19, UW23, and UW150). Furthermore, the 170-bp MITE is not present in the GMI1000 genomic sequence. Thus, it is possible that the 170-bp MITE is unique to *R. solanacearum* strains from the southeastern United States and Caribbean basin. Likewise, it is possible that an IS encoding the transposase for mobilizing the 170-bp MITE might be unique to this population. In *P. luminescens*, a MITE-like element was found to have homology to a new IS found as an intact copy in the *P. luminescens* genome (Mahillon et al. 1999).

Mobile genetic elements play an important role in generating genetic diversity by facilitating gene loss or acquisition and genomic rearrangement via homologous recombination (Mahillon and Chandler 1998). Strains that contain insertion sequences in identical positions are likely to be closely related (Mahillon and Chandler 1998). Multiplex PCR of *avrA* in all the southeastern *R. solanacearum* strains containing the 972-bp allele revealed that the 170-bp DNA insertion was inserted between nucleotides 461 and 462 in the *avrA* gene in 89 of the 94 strains tested. In five of the South Carolina strains, the 518-bp DNA fragment was not amplified, implying that either this element was not present in these strains, or it was located at a different site in the gene. These five strains were collected from two fields in Williamsburg County. Cloning and sequencing *avrA* from one of the five strains that did not amplify the 518-bp DNA fragment, SC111, found the 170-bp MITE to be present and located between nucleotides 574 and 575. Therefore, the strains examined probably are closely related, but they are not strictly clonal because the MITEs are present on different-sized restriction fragments.

This is not the first report of a DNA insertion in an *avr* gene resulting in virulence on a resistant host. An insertion of 104 bp in the *avrPphE* allele in *Pseudomonas syringae* pv. *phaseolicola* race 8 was found to be one of the two routes to virulence of this pathogen (Stevens et al. 1998). Similarly, a 1.2-kb insertion sequence, IS476, in the *avrBs1* locus in spontaneous mutants of *X. campestris* pv. *vesicatoria* Race 2 (Kearney et al. 1988) was shown to result in a race shift and, consequently, pathogenicity on resistant cultivars of field-grown pepper (Kousik and Ritchie 1996). However, this is, to our knowledge, the first report showing a relationship between geographic origin of isolation and the presence of IS elements at a specific genetic locus.

It has been demonstrated that *avr* genes may be involved in phenotypes other than mediating gene-for-gene interactions with plant disease resistance genes. For example, Jackson and associates (1999) showed *virPphA* in *P. syringae* pv. *phaseolicola* was a virulence determinant on bean but acted as an *avr* gene in soybean. The genetic insertions in the *avrA* locus allow *R. solanacearum* to evade the recognition and defense response of tobacco; therefore, it is possible that *avrA* acts in a similar fashion; that is, as a virulence determinant on susceptible hosts and an avirulence determinant on tobacco. Quantitative losses of virulence have been observed for insertion mutants of several *P. syringae* *avr* genes, leading to the suggestion that

most pathogens carry an array of factors that contribute to virulence (Lorang et al. 1994) in addition to triggering plant disease resistance genes (Dangl and Holub 1997). For example, the avirulence gene *avrBs2*, which is highly conserved among strains of *X. campestris*, is needed for full virulence of the pathogen on susceptible hosts (Kearney and Staskawicz 1990b). Nevertheless, our results suggest that *avrA* is not a virulence determinant on tomato because strains containing the 972-bp allele were not significantly less virulent on tomato than strains containing the wild-type allele when tested under controlled environmental conditions.

Some bacterial *avr* genes increase virulence only when present in the appropriate species, strain, or pathovar or a pathovar on a particular host species or variety (White et al. 2000). For example, the virulence effects of *avrA* and *avrE* have been observed only in *P. syringae* pv. *tomato* strain PT23 (Lorang et al. 1994; White et al. 2000). *AvrRpt2* of *P. syringae* pv. *tomato* DC3000 has a virulence effect on No-0 land race of *Arabidopsis* but not on land race Col-0 (White et al. 2000). *R. solanacearum* has a wide host range; therefore, it is possible that *avrA* may be a virulence factor on another host plant species. A number of the strains used in this study were isolated from soil from a Georgia field planted to green pepper with a history of bacterial wilt on tomato. All of the strains that contained the modified *avrA* allele were very aggressive on tobacco under controlled environment conditions; however, no wilt symptoms were observed on pepper in the field. It remains to be determined whether either a second avirulence gene is involved in the pepper-*R. solanacearum* interaction or whether the insertion in *avrA* results in a loss of virulence on pepper.

Investigations into the avirulence loci of *R. solanacearum* may aid in our understanding of both the host range and survival exhibited by this diverse pathogen. This will have significant implications for the development of novel control strategies for this aggressive pathogen with a worldwide distribution.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions.

Five plasmids were used: pGEM-T Easy (Promega Corp., Madison, WI, U.S.A.), pBC738 (*avrA*, Tc^r) (Carney and Denny 1990), pRK2013 (Ditta et al. 1980), pRK404 (Ditta et al. 1985), and pBluescript II (Stratagene, La Jolla, CA, U.S.A.). *R. solanacearum* strains were grown routinely on either CPG or TTC media (Denny and Hayward 2001) or in 1/10 BG broth at 28°C (Carney and Denny 1990). Spontaneous nalidixic acid-resistant *R. solanacearum* strains SC06-1, SC17-1, NC116-1, GA102-1, and NC25-1 were selected by plating >10⁹ cells on CPG plates containing the antibiotic at 100 µg ml⁻¹. Media used for the selection of transconjugants were supplemented with the following antibiotics: nalidixic acid at 100 µg ml⁻¹ and tetracycline at 15 µg ml⁻¹. *Escherichia coli* was cultured at 37°C in Luria-Bertani (LB) broth (Sambrook et al. 1989). *E. coli* transformants were plated on LB agar plates containing ampicillin at 50 µg ml⁻¹, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) at 40 µg ml⁻¹, and 40 µM isopropyl-beta-D-thiogalactopyranoside (IPTG).

Plant assays.

Inoculum from each of the *R. solanacearum* strains was prepared as previously described from a stock culture that had been maintained in sterile distilled water at room temperature (≈25°C) (Robertson et al., 2001). Typical fluidal colonies of 48-h cultures grown on TTC medium were used to inoculate 50 ml of 1/10 BG broth. The cultures were grown overnight at 28°C with constant shaking (200 rpm). The broth cultures

were pelleted at 10,000 × *g* for 10 min and the pellet suspended in sterile distilled water. Bacterial cell density was determined spectrophotometrically and adjusted to an optical density at 600 nm (OD₆₀₀) = 0.2 (≈10⁸ CFU ml⁻¹).

For HR assays, *R. solanacearum* inoculum was infiltrated into the intercellular spaces of fully expanded tobacco leaves (*Nicotiana tabacum*, 'K326') (Carney and Denny 1990). Inoculated plants were placed in a controlled environment chamber at 30°C with a 12-h photoperiod. Four inoculations on two plants were performed for each isolate. Plant reactions were scored daily for up to 4 days after inoculation.

Pathogenicity of *R. solanacearum* strains on the tomato (*Lycopersicon esculentum* 'Marion') and the tobacco K326 was assessed as described previously (Robertson et al. 2001). Briefly, 6-week-old tobacco seedlings, with four to six leaves, were transplanted into individual 3-in. pots and inoculated 24 h later by drenching the soil with 20 ml of inoculum (OD₆₀₀ = 0.2). Each pot was placed on a petri dish to avoid cross contamination. Five replications in a complete randomized block design were used and the experiment was repeated three times. Wilting was assessed every 3 days from 7 days after inoculation for a total of 21 days using the following scale (Robertson et al. 2001): 0 = healthy, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = whole plant wilted, and 5 = plant dead. Disease progress curves were constructed from the wilt index data, and the area under the disease progress curve (AUDPC) was calculated for each strain using the method of Shaner and Finney (1977). The AUDPC was subjected to analysis of variance (ANOVA; SAS Institute, Cary, NC, U.S.A.), and Fisher's protected least significant difference (LSD) was calculated for all *F* values that indicated significance (*P* < 0.05).

General DNA procedures.

Genomic DNA was prepared using the proteinase K-sarcosyl lysis miniprep of Boucher and associates (1987) and was quantified using a Biophotometer (Brinkman Instruments, Inc., Westbury, NY, U.S.A.). Plasmid DNA isolations were performed using Qiagen kits (Qiagen Inc., Chatsworth, CA, U.S.A.). Southern blotting and hybridization were done using standard techniques (Sambrook et al. 1989). For each isolate, 5 µg of genomic DNA was restricted with *EcoRI*, resolved on a 1% agarose gel, and transferred to a positively charged nylon membrane. The 170-bp MITE was amplified and the PCR product gel purified using the GeneClean spin kit (Bio101, Vista, CA, U.S.A.) and then labeled using [α-³²P]-dCTP and the Multiprime labeling system (Promega Corp.). The probe was hybridized overnight at 65°C, washed at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate, and exposed to autoradiographic X-ray film.

PCR amplification, cloning, and DNA sequencing.

Primers, synthesized by Life Technologies (Rockville, MD, U.S.A.), were designed to the 5' and 3' end of the *avrA* gene (Avr-F: 5'-CAAGATGAGAAGAATTGGAAAC and Avr-R: 5'-CTCGCTGTCGCTATCGTTGCC; the AvrA start codon is underlined), and to both ends of the 170-bp MITE (IS-1: 5'-CGTCTTATACACATCTC and IS-2: 5'-GGGTCAGAGGCATCGTC). Each 25-µl PCR reaction consisted of: 1.5 mM MgCl₂, 400 µM dNTPs, primers at 50 and 100 pmol for *avrA* and MITE primer sets, respectively, 1.0 unit of *Taq* DNA polymerase (Promega Corp.) and 25 ng of genomic DNA. The thermocycler conditions using the *avrA* primer set were 3 min of denaturation at 94°C; followed by 30 cycles of 94°C for 1 min, 60°C for 1.5 min, and 72°C for 1 min; with a final extension of 10 min at 72°C. Cycling conditions for the MITE primer set were 3 min at 96°C; followed by 40 cycles of 94°C for 30

s, 60°C for 30 s, and 72°C for 30 s; with a final extension for 10 min at 72°C. Multiplex PCR reactions contained 50 pmol each of the primers *avrA*-F, *avrA*-R, and IS-2, and the other components as above. The cycling conditions were 3 min at 94°C; followed by 22 cycles of 94°C for 1 min, 58°C for 1.5 min, and 72°C for 1 min; and finally 10 min at 72°C. Amplified products were resolved in a 1% (wt/vol) agarose gel stained with ethidium bromide and visualized under ultraviolet light.

The original DNA sequence of the 2.3-kb region of the AW1 genome containing *avrA* in pBC738 was determined as described previously (Carney and Denny 1990). To sequence *avrA* alleles from other strains, the desired fragments were PCR amplified from genomic DNAs, gel purified using the GeneClean spin kit, ligated into pGEM-T Easy using the manufacturer's protocol (Promega Corp.), and transformed by heat shock into *E. coli* DH10B. Transconjugants with recombinant plasmids were selected by plating on LB agar containing ampicillin, IPTG, and X-Gal as described above. The DNA sequence of the inserts was determined using a BigDye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI 373A automated DNA sequencer. Analysis of DNA sequence data was performed using Gene-Jockey (version 1.2; Biosoft, Cambridge, U.K.). Sequence homology searches were performed using BLASTn and BLASTx algorithms through the NCBI (Altschul et al. 1990).

Bacterial conjugations.

Triparental matings to introduce pBC738 into spontaneous nalidixic-acid resistant strains of *R. solanacearum* were performed as described previously (Carney and Denny 1990) using *E. coli* containing pRK2013 as the helper strain. Transconjugants were selected on BG plates containing nalidixic acid and tetracycline. The presence of the wild-type *avrA* gene in each transconjugant was confirmed by PCR amplification as described above.

Site-directed mutagenesis of *avrA*.

The *Pst*I fragment containing *avrA* from pBC738 was ligated into a derivative of pBluescript SK lacking an *Eco*RI site to create pAR12 (Amp^r, *avrA*). Insertion of the streptomycin-spectinomycin (Sp/Sm^r) resistance cassette from a Tn5-derived minitransposon (de Lorenzo et al. 1990) into the *Eco*RI site within *avrA* created pAR28 (Amp^r, *avrA*:Sp/Sm^r). The *Pst*I fragment from pAR28 was subcloned into pRK404 (Tet^r) to create pAR31 (Tet^r, *avrA*:Sp/Sm^r). Site-directed gene replacement of *avrA* was accomplished by transferring pAR31 from *E. coli* DH10B into *R. solanacearum* SC17-1. The resultant transconjugants were selected BG plates containing nalidixic acid, tetracycline, and streptomycin. *R. solanacearum* SC17-1M, which had undergone homologous recombination between the plasmid-borne mutant *avrA* gene and the wild-type *avrA* gene, was chosen by screening for Nal^r Sp/Sm^r Tet^r colonies after passage in broth culture without antibiotics.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- EMBnet ClustalW website: www.ch.embnet.org/software/ClustalW.html
- INRA (Institute for Agronomy Research) *Ralstonia solanacearum* GMI1000 website: sequence.toulouse.inra.fr/R.solanacearum.html
- IS Finder website: www-is.biotoul.fr/
- Kazusa DNA Research Institute Codon Usage database: www.kazusa.or.jp/codon/
- National Center for Biotechnology Conserved Domain database search: www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi