A *luxR* Homolog, *aviR*, in *Agrobacterium vitis* Is Associated with Induction of Necrosis on Grape and a Hypersensitive Response on Tobacco

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Submitted 28 June 2002. Accepted 17 March 2003.

A Tn5 mutant of *Agrobacterium vitis* F2/5 (M1154) differs from the wild-type strain in that it has lost its abilities to cause necrosis on grape and a hypersensitive-like response (HR) on tobacco. The Tn5 insertion occurred in an open reading frame (ORF) *aviR* that is homologous to genes encoding the LuxR family of transcriptional regulators, thereby suggesting that the HR and necrosis are regulated by a quorum-sensing system. Fewer *N*-acyl-homoserine lactone autoinducers were detected in extracts from M1154 compared with extracts from F2/5 and from *aviR*-complemented M1154. The complemented mutant regained full ability to cause grape necrosis and HR. Eighteen ORFs located on a 36.6-kb insert in cosmid clone CPB221, which includes *aviR*, were sequenced and aligned with homologous genes from *A. tumefaciens* C58 and *Sinorhizobium meliloti* Rm1021. The order of several clustered genes is conserved among the bacteria; however, rearrangements are also apparent. Reverse transcriptase-polymerase chain reaction analysis indicated that ORF2 and ORF14 may be regulated by an *aviR*-encoded transcriptional regulator. Single site-directed mutations in each of the ORFs, however, had no effect on expression of HR or necrosis as compared with the wild-type parent.

Additional keywords: crown gall

Agrobacterium vitis, the primary cause of crown gall disease on trunks and canes of grapes, also causes a localized necrosis on grape roots (Burr and Otten 1999). Necrosis is a host-specific response that is induced by tumorigenic and nontumorigenic strains of the bacterium. *A. vitis* also causes a hypersensitive-like response (HR) on tobacco leaves that are infiltrated with cell concentrations of $\geq 5.0 \times 10^8$ CFU per ml (Herlache et al. 2001). The HR is similar to that induced by several other gram-negative bacterial plant pathogens in that tissue collapse occurs within 14 to 18 h after infiltration and the reaction is inhibited by inhibitors of eukaryotic metabolism (Herlache et al. 2001). Tn5 mutants generated from nontumorigenic *A. vitis* F2/5 with altered necrosis properties have generally also lost their ability to induce the HR, suggesting that the underlying mechanisms of both phenotypes may be related. We previously reported that one F2/5 mutant, M6, which is necrosis-altered and HR-minus, carries the Tn5 insertion in a gene predicted to be involved in fatty acid biosynthesis (Herlache et al. 2001).

Of the Tn5 mutants generated thus far, only one, M1154, is completely negative for necrosis and HR.

In this paper, we report that the Tn5 insertion in M1154 disrupted a putative gene, *aviR*, that is homologous to genes encoding members of the LuxR family of transcriptional regulators. LuxR together with cognate LuxI proteins (synthases for production of *N*-acyl-homoserine lactone (HSL) autoinducer molecules) are hallmarks of quorum-sensing regulatory systems that have been identified in several gram-negative bacteria (Fuqua et al. 1996; Melissa and Bassler 2001; Whitehead et al. 2001). We also demonstrate that F2/5 produces several distinguishable HSLs and that the *aviR* mutation affects the number HSLs that are detectable using a bio-sensor assay. In addition, a 36.6-kb region that includes *aviR* was sequenced and was compared with homologous regions in *A. tumefaciens* C58 and *Sinorhizobium meliloti* Rm1021. Evidence is presented that *aviR* is associated with regulation of two ORFs located in this region.

RESULTS

Identity of the Tn5 mutation in M1154.

An *EcoRI* fragment containing the Tn5 insert in M1154 was cloned and sequenced, revealing the presence of a putative gene, *aviR*, that encodes a predicted 246-amino acid protein with a calculated molecular mass of 28.9 kDa. The likely start codon of *aviR* is GTG and a putative ribosomal binding site (GGA) is located eight nucleotides upstream from the start codon. BLAST analysis indicated the similarity between the deduced protein sequences of *aviR* and several members of LuxR-type transcription regulators (Fig. 1). The *aviR* amino acid sequence possesses all of the highly conserved residues of the LuxR family (Whitehead et al. 2001). It has highest identity (56.5%) to ExpR, a LuxR homolog in *S. meliloti* that is associated with regulation of genes involved in the production of symbiotically-active forms of EPS II (Pellcock et al. 2002). It also shares relatively high identity (40.1%) to AGR-c-4942, a putative HSL receptor from *A. tumefaciens* C58 (Wood et al. 2001). The Tn5 insertion in M1154 is located in the 3' region of *aviR*, four amino acid residues upstream of the putative helix-turn-helix motif (Fig. 1).

To confirm that the Tn5 insertion in *aviR* is responsible for the loss of necrosis and HR phenotypes, M1154 was complemented with *aviR* that was cloned into vector pPROBE-GT and transformed into M1154. Complemented M1154 was fully restored in its ability to cause HR on tobacco and necrosis on grape (Fig. 2).

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Fig. 1. Alignment of deduced *aviR* amino acid sequence with diverse members of the LuxR family. The different LuxR homologs and their percent similarity to the deduced amino acid sequence of *aviR* include: AGR-c-4942 from *A. tumefaciens* C58 (36.7 %, accession number NC003062), Expr-SM from *S. meliloti* Rm1021 (56.5%, accession number NC003047), CepR from *Burkholderia cepacia* (22.6%, accession number AJ422183), EsaR from *Erwinia stewartii* (17.7%, accession number L32184), ExprR from *Pectobacterium carotovorum* (17.3%, accession number X 80475), LasR from *Pseudomonas aeruginosa* (19.2%, accession number D30813), LuxR from *Vibrio fischeri* (19.4%, accession number M96844), PhzR from *Pseudomonas aeruginosa* (21.5%, accession number AF190630), RhIR from *Pseudomonas aeruginosa* (23.9%, accession number AE 004768), RhIR from *Rhizobium leguminosarum* (23.9%, accession number M98835), SdiA from *Salmonella typhimurium* (22.9%, accession number U86651), VanR from *Vibrio anguillarum* (19.6%, accession number U69677), and YenR from *Yersinia enterocolitica* (18.0%, accession number X76082).

HSL production by F2/5 and M1154.

At least five species of HSLs from F2/5 were visualized on thin-layer chromatography (TLC) plates that were overlaid with biosensor strain NTL4(pZLR4) (Fig. 3). The *aviR* mutation affected the detection of several species, i.e., profiles of F2/5 and complemented M1154 were similar, whereas that of M1154 had fewer and weaker signals. One group of HSLs detected from extracts of F2/5 are strongly nonpolar, remaining close to the origin and corresponding to HSL standards having acyl side chains of 14 to 16 carbons. Two other predominant species chromatographed similarly to the standards, 3-hydroxydecanoyl-HSL and 3-hydroxyoctanoyl-HSL, and one migrated further than 3-hydroxyhexanoyl-HSL, suggesting the presence of a shorter acyl side chain.

Analysis of DNA sequences flanking *aviR*.

The 36.6-kb insert that contains *aviR* in cosmid clone CPB221 was sequenced. The insert contains 18 putative ORFs and one insertion element (IS869) that was reported to reside on Ti plasmids and on the chromosome of other strains of *A. vitis* (Paulus et al. 1991). ORF18 is incomplete. The overall GC content of the insert is 57.6%, which is similar to that of the *A. tumefaciens* C58 genome (58%) (Wood et al. 2001). A GC content of 49.7% exists within a 2-kb segment between ORF1 and ORF2 that includes IS869. Sequence comparisons reveal that the deduced proteins from most of the ORFs in the insert are homologs of genes in *A. tumefaciens* C58 and *S. meliloti* Rm1021, except for that encoded by ORF1, which is similar to genes that encode L-ascorbate oxidase in *Ralstonia solanacearum* and *Xylella fastidiosa*. Gene homologs and their putative functions are summarized in Table 1.

The arrangement of ORFs in the CPB221 insert was compared with corresponding regions of the C58 and Rm1021 genomes (Capela et al. 2001; Wood et al. 2001) (Fig. 4). The order is conserved for several clusters of ORFs; however, rearrangements through translocation, recombination, and deletion are apparent. All of the homologous genes in C58 exist on its circular chromosome, except for that corresponding to ORF7 (AGR-L-1188), which is present in its linear chromosome.

Gene regulation by *aviR*.

Expression of mRNAs that correspond to seven ORFs on the CPB221 insert were determined by Reverse transcriptase-polymerase chain reaction (RT-PCR). Expression was first determined for ORFs 8 to 12. As predicted, ORF11 (*aviR*) is not expressed in M1154 but produces a strong signal in complemented M1154 (Fig. 5A). No differences in expression were observed for ORF8, 9, 10, or 12, suggesting that *aviR* is not involved in their regulation. Subsequently, expression of ORF2 and ORF14 were determined. No RT-PCR product for ORF2 was detected in M1154, and there was only a weak signal for ORF14 (Fig. 5B). In complemented M1154, both ORF2 and ORF14 generated band intensities that were as strong or stronger than those observed in F2/5. The experiments were repeated with identical results. All of the ORFs gave strong signals in PCR, suggesting that the variable band intensities observed in RT-PCR are related to the level of transcription (Fig. 5C).

Site-directed mutants of ORF2 and ORF14.

Two different site-specific mutants of ORF 2 and a mutant of ORF14 were generated as described below. When evaluated for their abilities to cause grape necrosis and HR on tobacco, they induced responses that did not differ from those caused by F2/5 (data not shown). Therefore, mutations in these genes alone do not produce obvious effects on the ability of F2/5 to cause necrosis in grape and the HR on tobacco.

DISCUSSION

The discovery that M1154 carries a Tn5 insertion in *aviR*, a homolog of genes belonging to the LuxR family of transcriptional regulators, strongly suggests that mechanisms associated with the HR and grape necrosis in *A. vitis* are regulated by quorum-sensing. Quorum-sensing represents a means by which bacteria sense their population densities through the production and detection of bacterial pheromones and subsequently regulate the expression of specific genes in a population density-dependent manner. Quorum-sensing systems in several bacterial species have been identified and shown to be involved in

Fig. 2. Phenotypic comparisons of **A**, hypersensitive-like response and **B**, grape necrosis induced by F2/5, M1154, and complemented M1154.

Fig. 3. Profiles of *N*-acyl-homoserine lactones (HSLs) extracted from F2/5, M1154, and complemented M1154. HSLs were separated on C18 reverse-phase thin layer chromatography plates developed with methanol/water (70:30) and were visualized following overlay with *Agrobacterium* reporter strain NTL4(pZLR4). S1 consists of a mixture of standard unsubstituted HSLs that have various acyl side chain lengths as designated. S2 consists of a mixture of 3-hydroxy HSLs with various acyl side chain lengths.

regulation of diverse physiological activities, including symbiosis, virulence, competence, conjugation, mobility, antibiotic production, secondary metabolite production, and biofilm formation (Whitehead et al. 2001). The vast majority of quorum-sensing systems known thus far in gram-negative bacteria employ homologs of LuxI and LuxR proteins, which have been studied extensively in the marine bacterium *Vibrio fischeri* (Visick and McFall-Ngai 2000). LuxI is a synthase that functions in production of pheromones that are typically derivatives of HSLs, whereas LuxR is a modular protein (Slock et al.

1990) that functions as a transcriptional regulator. At high cell densities, bacteria produce threshold levels of HSLs that diffuse or may be transported via efflux pumps (Pearson et al. 1999) across bacterial membranes, where they bind cognate LuxR receptors that subsequently regulate expression of target genes.

The LuxR homolog TraR from *A. tumefaciens* C58 has been the focus of extensive research. Similar to other LuxR homologs, an amino domain of TraR is associated with HSL binding, and a carboxyl terminus helix-turn-helix domain inter-

Table 1. Comparison of genes in the cosmid clone CPB221 insert from *Agrobacterium vitis* F2/5 with *A. tumefaciens* C58,^a *Sinorhizobium meliloti* Rm1021,^b and other bacteria

F2/5 ORF	Deduced size (aa)	Homolog			
		Gene	Putative products	Size (aa)	Identity (%)
ORF1	717	Rsp1530 ^c	L-ascorbate oxidase	717	40.4
		XF2677 ^d	L-ascorbate oxidase	721	34.7
ORF2	709	AGR-c-1636	Dipeptidyl aminopeptidase	699	80.3
		SMc00114	Peptidyl aminopeptidase B	712	77.6
ORF3	126	AGR-c-1639	Hypothetical protein	133	50.0
		Smc02317	Hypothetical signal peptide	129	46.8
ORF4	260	AGR-c-1646	Membrane protein	391	51.2
		SMc00793	Permease protein	376	50.4
ORF5	440	AGR-c-5015	NAD-glutamate dehydrogenase	1,585	46.3
		SMc04085	Hypothetical protein	1,595	44.2
ORF6	419	AGR-c-5017	Integral membrane protein	458	61.7
		SMc04087	Membrane protein	463	62.6
ORF7	208	AGR-L-1188	Hypothetical protein	225	40.9
		SMc03887	Amino acid efflux protein	215	38.5
ORF8	2,832	AGR-c-4949	B-1,2-glucan synthetase	2832	66.6
		SMc04382	B-1,2-glucan synthetase	2832	63.8
ORF9	428	AGR-c-4953	Hippurate hydrolase	393	63.1
		SMc02256	Hippurate hydrolase 2	389	56.8
ORF10	585	AGR-c-4944	β -1,2-glucan exporter	598	75.4
		SMc03900	β -1,2-glucan exporter	616	75.7
ORF11	248	AGR-c-4942	HSL receptor	267	40.1
		SMc03896	Transcriptional regulator	184	56.5
ORF12	1,155	AGR-c-4940	Pyruvate carboxylase	1,174	87.5
		SMc03895	Pyruvate carboxylase	1,152	84.5
ORF13	147	Atu2786 ^c	Hypothetical protein	146	82.9
		SMc03791	Hypothetical protein	144	77.8
ORF14	661	AGR-c-4980	Acetyl-CoA synthetase	657	83.9
		SMc04093	Acetyl-CoA synthetase	648	82.3
ORF15	232	AGR-c-4981	Hypothetical protein	266	74.6
		SMc04094	Hypothetical protein	232	74.8
ORF16	220	AGR-c-4983	Hypothetical protein	219	75.5
		SMc02812	Hypothetical protein	220	77.2
ORF17	878	AGR-c-4985	Leucyl-tRNA synthetase	876	77.2
		SMc02804	Leucyl-tRNA synthetase	876	75.1
ORF18	79 ^f	AGR-c-4986	Hypothetical protein	182	29.1
		SMc02803	Hypothetical protein	168	41.2

^a According to sequence of accession number NC 003062, except where noted.

^b According to sequence of accession number NC 003047.

^c From *Ralstonia solanacearum*, accession number CAD18681.

^d From *Xylella fastidiosa* 9a5c, accession number AAF85474.

^e According to sequence of accession number NC 003304.

^f Incomplete open reading frame.

acts with gene promoter regions (Fuqua et al. 1996). It has been established that binding of TraR with its cognate HSL, *N*-3-oxooctanoyl-L-homoserine lactone (AAI), facilitates a conformational change resulting in the formation of dimers, i.e., the active form of the regulator (Qin et al. 2000; Zhu and Winans 2001). Gene regulation by TraR dimers follows binding of the regulator to an 18-bp inverted repeat promoter region (*tra* box) located upstream of the gene coding regions. Position and integrity of the *tra* box were shown to be essential to TraR interaction and for subsequent gene expression (Lou and Farrand 1999). The fate of TraR that is not bound to AAI (monomeric form) in bacterial cells has also been addressed. TraR monomers are highly sensitive to proteolysis (Zhu and Winans 2001), leading to rapid turnover in the absence of AAI (Zhu and Winans 1999). It was also demonstrated that monomers can associate with the inner face of the bacterial membrane and that binding of AAI facilitates their release and dimerization (Qin et al. 2000). In addition, the structure of functional TraR was recently revealed (Zhang et al. 2002) and has allowed the visualization of the TraR domains that are associated with AAI and DNA binding.

LuxR homologs typically share only about 18 to 25% amino acid sequence identity, and only five residues are conserved among all of them (including *aviR*) (Whitehead et al. 2001). Comparison of gene arrangements in Figure 4 shows that *luxR* homologs exist in *A. tumefaciens* and *S. meliloti* at the same relative position at which *aviR* is found in *A. vitis*, i.e., between genes *chvA* (*ndvA*) and *pyc*. Although the function of the C58 gene is unknown, functional ExpR in *S. meliloti* is involved in regulation of *exp* genes that encode production of symbiotically active EPS II in a density-dependent manner (Pellock et al. 2002). It was determined that *expR* in strain Rm1021 is

inactivated by an insertion sequence and that a spontaneous mutation resulting in its removal and subsequent *expR* reading frame restoration rendered the gene functional (Pellock et al. 2002). It is most interesting that *aviR* and *expR* share an unusually high degree of similarity (56.5%). Whether the genes are cross functional in these different bacterial species remains to be determined.

We demonstrated that F2/5 may produce five or more HSLs and that the *aviR* mutation greatly alters the HSL profile. Thus, similar to the *Rhizobium leguminosarum* *cinRI* (Lithgow et al. 2000) and the *S. meliloti* *sinRI* quorum-sensing systems (Marketon et al. 2002), the putative transcriptional regulator encoded by *aviR* may regulate transcription of more than one HSL synthase gene or a single gene that directs the synthesis of multiple HSLs. A dominant feature of the F2/5 profile is the presence of one or more signals that reside near the origin of the TLC plates and presumably have long acyl side chains. Such HSLs, with side chains ranging from 12 to 18 carbons, were recently identified in *S. meliloti*, and their production was shown to be associated with the *sinI* gene (Marketon et al. 2002). At least one of these HSLs now appears to be involved in regulation of symbiotically active EPS II production (Marketon et al. 2003). The identity of *luxI* gene homologs in *A. vitis*, the number and chemical nature of the HSLs that are produced, and their involvement in regulation of the HR and necrosis remain to be determined.

Gene *aviR* is flanked by several ORFs that may have biological functions in *A. vitis*-plant interactions. For example, upstream of *avR1* are located ORF8 and ORF10, which are homologs of *A. tumefaciens* genes *chvA* and *chvB* and *S. meliloti* genes *ndvA* and *ndvB*. These genes encode proteins involved with export and synthesis of β -1,2-glucan, respectively, which are known to func-

Fig. 4. Comparison of open reading frame (ORF) organization within the 36.6-kb insert in CPB221 to corresponding regions in *A. tumefaciens* C58 and *S. meliloti* Rm1021. The length of each arrow represents the relative ORF size and points in the direction of transcription. Names of putative gene homologs are given above the arrows. The location of the Tn5 insertion in ORF11 (*aviR*) is indicated by a dark arrow. Similar background patterns indicate related gene clusters in the bacteria.

tion in the attachment of the bacteria to plant cells (Cangelosi et al. 1989; Ielipi et al. 1990; Stanfield et al. 1988). ORF12 is a homolog of *pyc* (encoding pyruvate carboxylase), which has been identified in several members of the family *Rhizobiaceae*, including *S. meliloti* (Dunn et al. 2001) and *A. tumefaciens*. The *pyc* homologs in *A. vitis*, *A. tumefaciens*, and *S. meliloti* are all located immediately downstream of the *luxR*-like genes. This enzyme is associated with growth of the bacteria in different nutrient environments and *pyc* mutants are not able to grow with glucose or pyruvate as sole carbon sources (Dunn et al. 2001). It is also interesting that homologs of ORF9 (putative hippuricase genes, *hip*) occur in *A. tumefaciens* and *S. meliloti*, although at different relative positions. Hippuricase cleaves N-benzoylglycine into glycine and benzoic acid and is a key gene used in the identification of the human enteritis pathogen *Campylobacter jejuni* (Hani and Chan 1995). Hippuricase is also related to plant enzymes that regulate auxin signaling by cleaving amino acids from inactive, conjugated indole-3-acetic acid (Bartel and Fink 1995). Although our results thus far indicate that ORF8, 9, 10, and 12 are not regulated by *aviR*, these ORFs may be associated with *A. vitis*-plant interactions.

Our results suggest that ORF2 and ORF 14 are regulated by *aviR*. ORF2 is homologous to *dap* genes that encode dipeptidyl aminopeptidases, which remove dipeptides from the amino termini of peptides and proteins (Roao et al. 1998). Such enzymes may play important roles in intracellular protein cleavage, cell growth, and enzyme processing (Ogasawara et al. 1998). Further evidence to support that *aviR* may regulate ORF2 is that a 32-bp palindrome, TCGcTGGcaCCGGCCA TGGCCGGgACCAcCGA, resides 101 bp upstream from the putative ORF2 start codon and, therefore, may function as a *lux* box promoter element (Egland and Greenberg 1999). Further analysis is necessary to determine the transcriptional start site of *dap* and whether this palindrome is, in fact, a functional *lux* box.

The expression of ORF14 is reduced in M1154, indicating that it may also be regulated by *aviR*. ORF14 is homologous to genes that encode acetyl-CoA synthases, enzymes which are associated with production of acetyl-CoA from acetate (Kumari et al. 2000). In addition to their role in acetate metabolism, acetate pathways are known to be involved in regulation of signal transduction by two-component regulatory systems (McCleary et al. 1993), in flagellar gene expression (Pruss and Wolfe 1994), and in nutrient starvation (Nystrom 1994). Although *aviR* appears to be associated with regulation of *dap* and *acs*, site directed mutants of these genes did not have obvious effects on the ability of F2/5 to cause HR or grape necrosis. Further research will be necessary to conclusively determine whether these genes are regulated by *aviR* and their functions in *A. vitis*.

The ability of *A. vitis* to cause a host-specific necrosis and hypersensitive response is unusual among members of the family *Rhizobiaceae*. We provide the first evidence that these responses in *A. vitis* are regulated by quorum-sensing. Identification of additional genes that are regulated by *aviR* will facilitate the discovery of mechanisms that are involved in these bacterial-plant interactions.

MATERIALS AND METHODS

Bacterial strains and phenotype assays.

A. vitis strain F2/5 was propagated on potato dextrose agar (PDA) or potato dextrose broth (PDB) (Difco Laboratories, Detroit) at 28°C. M1154 was cultured on PDA amended with kanamycin (50 µg/ml) at 28°C. *A. tumefaciens* NTL4(pZLR4) (Shaw et al. 1997) was grown on Luria-Bertani agar (LBA) amended with carbenicillin (100 µg/ml) and gentamycin (30 µg/ml). *E. coli* strains were grown at 37°C on LBA or Luria-Bertani broth amended with appropriate antibiotics. Assays for

necrosis on grape shoot explants and for the HR on tobacco leaves were done as previously described (Herlache et al. 2001). All sequencing was done with an ABI automated DNA sequencer with fluorescent-dye-labeled dideoxy terminator at Cornell University DNA Services (Ithaca, NY, U.S.A.).

Cloning and sequencing of the Tn5-disrupted gene.

Genomic DNA of M1154 was digested with *Eco*RI, ligated into plasmid vector pBluescript SK⁻, and then transformed into *E. coli* JM109 cells, as described previously (Herlache et al. 2001). Transformants containing Tn5 insertions were selected

Fig. 5. A, and B, Comparison of expression of mRNAs from F2/5, M1154, and complemented M1154 that correspond to seven open reading frames (ORFs) that reside on the CPB221 insert as determined by Reverse transcriptase-polymerase chain reaction (RT-PCR) and C, PCR products from the ORFs. Lanes in A and B with the overloading of I correspond to F2/5, II to M1154, and III to complemented M1154. The band in A that corresponds to 1 kb is identified. In B and C, M= 1-kb DNA ladder. Lanes 1 = ORF12, lanes 2 = ORF11 (*aviR*), lanes 3 = ORF10, lanes 4 = ORF9, lanes 5 = ORF8, lanes 6 = ORF2, and lanes 7 = ORF14.

on LBA plates containing X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 50 μ g/ml), IPTG (isopropyl- β -thiogalactopyranoside, 100 μ g/ml), kanamycin (50 μ g/ml), and ampicillin (100 μ g/ml). Plasmids harboring an *Eco*RI fragment containing the Tn5 insertion were characterized by restriction analysis and sequencing.

Detection of HSLs.

A previous report concerning production of HSLs from *S. meliloti* suggested that extraction from whole cells may increase the recovery of those with relatively long carbon chains (Marketon et al. 2002). Therefore, we used a modification of that protocol. F2/5, M1154, and complemented M1154 were grown for 4 days on PDA or PDA amended with appropriate antibiotics. Approximately 3 cc of bacterial growth were harvested from agar plates and were extracted twice with 10 ml of an ethyl-acetate/acetonitrile mixture (50:50). The first extraction was done for a minimum of 6 h and the second for 1 h on a rotary shaker at 120 rpm. The extract was centrifuged at 10,000 rpm for 10 min, and then, the supernatant was dried by rotary evaporation (Rotavapor, Büchi Analytical, New Castle, DE, U.S.A.) and finally under a stream of N_2 . Dried extracts were suspended twice in ethyl-acetate (99.8% high-performance liquid chromatography grade), dried again, and then suspended in a final volume of 40 μ l of ethyl acetate.

A volume of 1 to 4 μ l of ethyl-acetate extract was spotted on C18 reverse phase TLC plates (Whatman, Clifton, NJ, U.S.A.) and was developed with methanol/water (70:30). Plates were air-dried and then overlaid with strain NTL4(pZLR4) suspended in AB minimal medium (Chilton et al. 1974) containing 0.9% agar and 0.07% X-Gal (vol/vol), as previously described (Cha et al. 1998). HSL standards (provided by A. Eberhard, Ithaca College, Ithaca, NY, U.S.A.) that were used as comparisons on TLC plates included the unsubstituted and 3-hydroxy derivatives of hexanoyl-HSL, octanoyl-HSL, and decanoyl-HSL. Standards also included tetradecanoyl-HSL, hexadecanoyl-HSL, octadecanoyl-HSL, and 3-hydroxydodecanoyl-HSL.

Analysis of *aviR*-containing clone CPB221.

Based on sequence analysis of the region flanking the Tn5 insertion in the *Eco*RI fragment described above, primers M1154-F and M1154-R (Table 2) were designed. A 1.3-kb DNA fragment was then amplified by PCR from F2/5 genomic DNA and was used to probe an F2/5 cosmid library (Herlache et al. 2001). A clone, CPB221, carrying a 36.6-kb insert including *aviR*, was completely sequenced using the following strategy. DNA was digested with *Eco*RI and was subcloned into plasmid vector pUC18. Subclones were selected based on size differences and were sequenced from both ends. Those with large inserts were digested with restriction enzymes *Hind*III or *Bam*HI and were cloned into vector pUC18. If there was no suitable restriction site for further subcloning, a primer walking strategy was used, employing primers designed from ends of sequenced fragments. The order of each fragment within the cosmid clone was determined by identifying overlapping sequences, by comparison of PCR products, and with the assistance of DNA analysis software (DNASTAR, Madison, WI, U.S.A.). DNA sequence homology searches were performed with Basic Local Alignment Search Tool (BLAST) algorithm provided by the National Center for Biotechnology Information. Analysis of DNA and deduced protein sequences were carried out with DNASTAR software. The sequence of the insert in CPB221 has been deposited in GenBank under accession number AF51015.

Complementation of M1154.

A *Pst*I restriction site was incorporated into primer AVR-F, and a *Bam*HI site was incorporated into primer AVR-R (Table 2). A 1,105-bp fragment was then amplified from F2/5 genomic DNA template that contained the entire coding sequence of *aviR* (including putative start and stop codons) and a promoter region 333 bp upstream from the putative start codon. The PCR product was digested with *Pst*I and *Bam*HI and was ligated to a broad host range promoter probe vector pPROBE-GT (Miller et al. 2000). It was then transformed into *E. coli* DH5 α competent cells, and transformants were selected

Table 2. Primers used in polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR

Primer	Sequence	Gene target	Fragment size
PYC-F PYC-R	5'-TGCCGTGCATCTGTTCGAAC-3' 5'-TCATAGAAACGGGTGATGTACGC-3'	ORF12	514 bp
M1154-F M1154-R	5'-TGCGTGTGAGTGTCAATCATCTC-3' 5'-TGCCTTAAGCATCAGCGGATAGC-3'	ORF12/11	1,353 bp
AVR-F ^a AVR-R ^b	5'-ACCTGCAGCGGCAAGATATTGC-3' 5'-ATGGATCCAGGCAAGTCAGCAGAACG-3'	ORF11	1,105 bp
M1154-F AVR-R1	5'-TGCGTGTGAGTGTCAATCATCTC-3' 5'-GAAATTATCAGCTGATCAGGC-3'	ORF11	752 bp
ChvA-F ChvA-R	5'-TCATCGACGCAATTTCCAGTG-3' 5'-CGACGATGGCAATCGTCTGTC-3'	ORF10	966 bp
HIP-F HIP-R	5'-CTGCCAATCACTGAAGAGAC-3' 5'-AAGCTGCGGATGGTCAGCAC-3'	ORF9	561 bp
ChvB-F ChvB-R	5'-ACTCCAGATCGCTACCATC-3' 5'-CTGTGAGCGACGACAACGAC-3'	ORF8	1,080 bp
ACS-F ACS-R	5'-GTCGCGTCGATGACGTGCTG-3' 5'-TCAGATCGTCGACAACGCCAG-3'	ORF14	334 bp
DAP-F DAP-R	5'-TCGACCACAGCAATGATCATG-3' 5'-TCATAATCATAGAGCTGGCTG-3'	ORF2	783 bp

^a The underlined CTGAAG was added to create a *Pst*I restriction site.

^b The underlined GGATCC was added to create a *Bam*HI restriction site.

on LBA containing gentamicin (15 µg/ml). The plasmid construct was verified by restriction digestion and examination for fluorescence under UV light. The construct was then conjugated into M1154 by triparental mating using *E. coli* helper strain HB101(pRK2013). Transformants were selected on AB minimal medium containing kanamycin (50 µg/ml) and gentamicin and were verified by PCR and fluorescence under UV light. Complemented M1154 clones were screened for their ability to cause the HR and grape necrosis.

PCR and RT-PCR.

PCR was carried out using genomic or plasmid DNA as templates. Total volumes of reaction mixtures were 50 µl that consisted of 1 µl of DNA, 20 pmol of each primer, 1 µl of dNTP (10 mM each), 3.0 µl MgCl₂ (2.5 mM), 5 µl of 10× buffer, and 2 units of Taq DNA polymerase (Promega, Madison, WI, U.S.A.). A standard program was used, in most cases, that included denaturing at 94°C for 3 min, 30 cycles of denaturing at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, followed by extension at 72°C for 5 min and holding at 4°C. Depending on the melting temperature of the primer and expected size of PCR products, annealing temperature and extension times were adjusted.

The effects of the *aviR* mutation on the expression of seven ORFs on CPB211 were determined. ORFs were selected based on their relative similarity to genes of known functions and on their potential for being involved in plant-microbe interactions. Total RNAs used as templates for RT-PCR analyses were isolated from liquid cultures of F2/5, M1154, and complemented M1154 using the TRI-Reagent Kit (Molecular Research Center, Cincinnati, OH, U.S.A.), following the manufacturer's protocol. RNA samples were treated with RNase-free DNase (Promega) to prevent DNA contamination, and PCR reactions were run using the treated RNA samples as templates to verify the absence of contaminating DNA. RT-PCR was conducted with Superscript One-Step RT-PCR system plus Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, U.S.A.), following the manufacturer's protocol. Products were separated by electrophoresis on 0.7% agarose gel in 0.5× Tris-Borate EDTA buffer. Primers for ORFs that were investigated are shown in Table 2. All assays were repeated at least once.

Site-directed mutagenesis.

Site-directed mutants of ORF2 and 14 were generated by mating F2/5 with *E. coli* S17-1/λpir carrying suicide plasmid pVIK165 into which a PCR-amplified fragment of the ORF to be disrupted was cloned (Kalogeraki and Winans 1997). Gene disruption occurs following a single homologous recombination event, whereby the entire plasmid inserts into a portion of the target gene. For ORF2 (2,130 bp), one mutant was generated by amplifying a 654-bp product that represents nucleotides 94 through 748 with primers F5'-CACCGAGCTCGGCA-TAACCCGCATTGACGAC-3' containing a *SacI* restriction site and R5'-ACCCTCTAGAAGCCGGGGTCTTTTCCTC-ATAG-3' containing a *XbaI* restriction site. For ORF14 (2,118 bp), a 866-bp product representing nucleotides 623 through 1,489 was amplified with primers F5'GTGCGAGCTCTTCG-GTGGTGTGTTGGC-3' containing a *SacI* restriction site and R5'GAACTCTAGAGTCGCGCGGAACCGGGCTTGAG-3' containing a *XbaI* restriction site. Insertions of vector were verified by selection for kanamycin resistance, the positive expression of *gfp* with UV microscopy, and by PCR.

A second ORF2 mutant was made by amplifying a 783-bp fragment from F2/5 with primers DAP-F and DAP-R (Table 2), which corresponds to nucleotides 477 through 1,257 of the ORF (2,130 bp). The PCR product was ligated into vector pGEMT-Easy (Promega) and then transformed into *E. coli*

JM109. The ORF2 fragment was cut from the vector using *PstI* and *SpeI* and was ligated into the *PstI* and *XbaI* sites of pVIK165. Matings and selection were done as described above.

ACKNOWLEDGMENTS

We thank S. Lindow, University of California-Berkeley, for providing vector pPROBE-GT; S. Winans, Cornell, for vector pVIK165; S. Farrand, University of Illinois, for strain NTL4(pZLR4); and A. Eberhard, Ithaca College, for HSL standards.

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