JOURNAL OF BACTERIOLOGY, Mar. 2006, p. 2173–2183 Vol. 188, No. 6
0021-9193/06/$08.00+0 doi:10.1128/JB.188.6.2173–2183.2006

Regulation of Long-Chain *N*-Acyl-Homoserine Lactones in *Agrobacterium vitis*

Guixia Hao and Thomas J. Burr*

Department of Plant Pathology, NYSAES, Cornell University, Geneva, New York 14456

Received 30 August 2005/Accepted 4 January 2006

Homologs of quorum-sensing *luxR* and *luxI* regulatory genes, *avsR* and *avsI*, were identified in *Agrobacterium vitis* strain F2/5. Compared to other LuxI proteins from related species, the deduced AvsI shows the greatest identity to SinI (71%) from *Sinorhizobium meliloti* Rm1021. AvsR possesses characteristic autoinducer binding and helix-turn-helix DNA binding domains and shares a high level of identity with SinR (38%) from Rm1021. Site-directed mutagenesis of *avsR* and *avsI* was performed, and both genes are essential for hypersensitive-like response (HR) and necrosis. Two hypothetical proteins (ORF1 and ORF2) that are positioned downstream of *avsR-avsI* are also essential for the phenotypes. Profiles of *N*-acyl-homoserine lactones (AHLs) isolated from the wild type and mutants revealed that disruption of *avsI*, ORF1, or ORF2 abolished the production of long-chain AHLs. Disruption of *avsR* reduces long-chain AHLs. Expression of a cloned *avsI* gene in *A. tumefaciens* strain NT1 resulted in synthesis of long-chain AHLs. The necrosis and HR phenotypes of the *avsI* and *avsR* mutants were fully complemented with cloned *avsI*. The addition of synthetic AHLs ($C_{16:1}$ and 3-O-$C_{16:1}$) complemented grape necrosis in the *avsR*, *avsI*, ORF1, and ORF2 mutants. It was determined by reverse transcriptase PCR that the expression level of *avsI* is regulated by *avsR* but not by *aviR* or *avhR*, two other luxR homologs which were previously shown to be associated with induction of a tobacco hypersensitive response and grape necrosis. We further verified that *avsR* regulates *avsI* by measuring the expression of an *avsI*::*lacZ* fusion construct.

Quorum-sensing (QS) regulation has been identified in many gram-negative bacteria and can be associated with the expression of various genes that control diverse physiological functions, including virulence and disease, in plants and animals. Hallmarks of QS in many gram-negative bacteria are characterized by the presence of LuxR proteins that function as transcriptional regulators and LuxI proteins that function as synthases for AHL signal molecules (9). Most LuxR proteins share relatively low identity (18 to 25%) but possess five highly conserved and several other conserved amino acid residues (42). The X-ray crystal structure of the TraR protein was solved, and the conserved amino acid residues that appear to be associated with pheromone and DNA binding were identified (38, 44).

The structures of AHLs differ with regard to acyl side chain lengths, hydoxyl or oxo substitutions at the third carbon, and by the presence of double bonds. The LuxI family shares four groups of conserved sequences and several essential residues. When mutations are made in such residues, a reduction or loss of AHL synthase activity typically results (13, 28). The crystal structure of EsaI, a synthase for production of 3-O-C_6-HL in *Panteoa stewartii*, was characterized. It was confirmed as a monomeric and approximately spherical protein with a deep cleft believed to be involved in substrate binding (40). Recently, the crystal structure of LasI (synthase for 3-O-C_{12}-HL) in *Pseudomonas aeruginosa* was shown to have a six-stranded beta sheet and three alpha helices forming a V-shaped substrate-binding cleft that will accommodate longer acyl-acyl carrier protein (ACP) (10).

Agrobacterium vitis causes crown gall on grapevine, a serious disease that occurs worldwide and results in poor growth and death of vines (2). The process by which *Agrobacterium* spp. causes crown gall has been studied intensively for several years, and it involves the transfer of T-DNA carried on the Ti plasmid from the bacterium to its plant host, where it is expressed (47). In addition, *A. vitis* induces a host-specific necrosis on grape and a hypersensitive-like response (HR) on non-host plants such as tobacco (15). The HR resembles a form of programmed cell death in plants characterized by a rapid localized plant cell death that is associated with resistance against disease. Several species of gram-negative bacteria induce the HR on non-host plants. Initiation of the HR usually involves the delivery of specific effector proteins to the plant cell via a type III secretion system (1, 3) that is encoded by a set of *hrp* (HR and pathogenicity) and *hrc* (HR and conserved) genes that are also essential for induction of the HR. Such genes are often grouped in the bacterial genome on pathogenicity islands. Subsequent interaction of the effectors with plant receptors leads to disease (in a compatible host) or induction of the HR (incompatible response). Many type III secretion system effectors have been functionally identified and can act as double agents in bacterial disease and plant defense (1). Although it has been reported that *A. tumefaciens* induces responses in maize that resemble an HR (12) and that *S. meliloti* induces an oxidative burst leading to localized cell death during the early infection process of alfalfa (32), *A. vitis* is thus far the only member of the *Rhizobiaceae* that has been shown to cause a rapid collapse and cell death of infiltrated tobacco leaves (15). The *A. vitis* response resembles HRs caused by

* Corresponding author. Mailing address: Department of Plant Pathology, NYSAES, Cornell University, Geneva, NY 14456. Phone: (315) 787-2312. Fax: (315) 787-2389. E-mail: tjb1@cornell.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Relevant characteristic(s)	Reference or source
Agrobacterium vitis		
F2/5	wild type	35
M1154	F2/5 containing *aviR*::Tn*5*, Km^r	45
M1320	F2/5 containing *avhR*::Tn*5*, Km^r	14
avsR mutant	F2/5 containing *avsR*::pPVIK165, Km^r	This work
avsI mutant	F2/5 containing *avsI*::pPVIK165, Km^r	This work
ORF1 mutant	F2/5 containing ORF1::pPVIK165, Km^r	This work
ORF2 mutant	F2/5 containing ORF2::pPVIK165, Km^r	This work
Comp *avsI* mutant	*avsI* mutant containing pPZP201 carrying *avsI*, Km^r, Sp^r	This work
CompA *avsR* mutant	*avsR* mutant containing pPZP201 carrying *avsI*, Km^r, Sp^r	This work
CompB *avsR* mutant	*avsR* mutant containing pPROBE-AT carrying *avsR*, Km^r, Ap^r	This work
Agrobacterium tumefaciens		
NT1	*A. tumefaciens* C58 cured of pTiC58	39
NTL4 (pZLR4)	NT1 derivative carrying a *traG*::*lacZ* reporter fusion	5
Escherichia coli		
DH5α	See source	Invitrogen
S17-1/λpir	RK2 *tra* regulon, *pir*, host for *pir*-dependent plasmids	34
HB101	*xyl-5 mtl-1 rpsL20*λ^- *supE44 hsdS20*(r_B^- m_B^-) F^- ϕ80d*lacZ* ΔM15 *recA1 endA1 gyrA9 6 thi-1 hsdR17*	31
Plasmids		
TOPO 2.1	TA vector for PCR cloning, Ap^r	Invitrogen
pavsRI	TOPO 2.1 carrying partial *avsR* and *avsI*	This work
pVIK165	suicide vector, Km^r	19
pavsR/165	pVIK165 carrying partial *avsR*, Km^r	This work
pavsI/165	pVIK165 carrying partial *avsI*, Km^r	This work
pORF1/165	pVIK165 carrying partial ORF1, Km^r	This work
pORF2/165	pVIK165 carrying partial ORF2, Km^r	This work
pPZP201	Broad-host-range cloning vector, Sp^r	11
pPZP-avsI	pPZP201 carrying *avsI*, Sp^r	This work
pPROBE-GT	Broad host vector, Gm^r	25
pGT-aviR	pPROBE-GT carrying *aviR*, Gm^r	44
pPROBE-AT	Broad host vector, Cb^r	25
pAT-avsR	pPROBE-AT carrying *avsR*, Cb^r	This work
pKP302	Broad host vector for *lacZ* fusion, Sp^r	27
pKP-RI	pKP302 carrying *avsI* promoter region	This work

[a] Comp, complemented.

other plant-associated bacteria, in that it is dependent on bacterial cell concentration, is affected by inhibitors of plant metabolism, and results in collapse of infiltrated leaf tissue within 24 h.

We previously determined that QS regulation is involved in expression of the HR and necrosis by *A. vitis*, and two associated *luxR* homologs reside in the bacterium (14, 45). A mutation in *aviR* resulted in complete loss of necrosis and HR. The mutant also produced fewer AHLs than wild-type F2/5. A second gene, *avhR*, is also involved in HR and necrosis regulation, and its deduced protein has several substitutions in conserved amino acids of the LuxR family. In this study, we identify a third *luxR* homolog (designated *avsR*) and a *luxI* homolog, *avsI*. We show that the *avsR-avsI* pair and two downstream open reading frames (ORFs) affect production of long-chain AHLs as well as induction of the tobacco HR and grape necrosis. We also demonstrate that *avsR* regulates the expression level of *avsI*.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. Table 1 lists the strains and plasmids used in this study. *A. vitis* strain F2/5 is nontumorigenic and has been used for studying HR and necrosis in our laboratory (15). S4 is a tumorigenic strain carrying a vitopine type Ti plasmid that is comprised of three separate T-DNAs (4). F2/5 was propagated on potato dextrose agar (PDA) or in potato dextrose broth (PDB) (Difco Laboratories, Detroit, MI) at 28°C. Appropriate antibiotics were added to the media when culturing mutants. *A. tumefaciens* NT1 was grown on Luria-Bertani (LB) medium at 28°C. *Escherichia coli* was cultured on LB with appropriate antibiotics at 37°C. Assays for necrosis of grape shoot explants and for HR on tobacco leaves were done as previously described (15).

Cloning and sequencing of *avsR* and *avsI*. Based on the *A. vitis* strain S4 genome sequence (http://agro.vbi.vt.edu), *luxR-luxI* homologs were identified and subsequently amplified from F2/5 with primers avsRI-F and avsRI-R (Table 2). The fragment was ligated directly to TOPO TA PCR 2.1 vector (Invitrogen) and transformed into Top 10-competent cells (Invitrogen). The plasmids of the positive colonies were isolated and sequenced. Sequence was analyzed and compared to the corresponding S4 sequence with DNASTAR software.

Site-directed mutagenesis. To determine the effects of *avsR*, *avsI*, and downstream ORFs 1, 2, and 3 on induction of the HR, grape necrosis, and AHL production, site-directed mutants were generated. F2/5 was mated with *Escherichia coli* strain S17-1/λ*pir* carrying suicide plasmid pVIK165, into which PCR-amplified fragments of each gene of interest were cloned (19). Gene disruption occurs following a single homologous recombination event. Primer pairs into which XbaI and SacI restriction sites were engineered included avsR-F and avsR-R, avsI-F and avsI-R, ORF1-F1 and ORF1-R1, ORF2-F1 and ORF2-R1, and ORF3-F1 and ORF3-R1 (Table 2). The different internal fragments were amplified respectively from F2/5 genomic DNA. The PCR products were purified, digested, and then cloned into pVIK165. Mutants generated following

TABLE 2. Primers used in this study

Name	Sequence
Primers for sequencing	
avsRI-F	5′-CTTTCGCCGCTGCCATGACC-3′
avsRI-R	5′-AACCGGCTCGATTCCCACAT-3′
Primers for site-directed mutagenesis	
avsI-F	5′-CTCAGAGCTCTGAACAGCCGACATAAGCA-3′
avsI-R	5′-GACTCTAGAATGGCAATGTCACGAATGG-3′
avsI-int	5′-TGTCGCTACCGTCAGTTTTA-3′
avsR-F	5′-CAATGAGCTCCAACCTGTCCGGCTGTCTTTAC-3′
avsR-R	5′-AGATTCTAGAGATCACGGCGGTTTCCCAATA-3′
avsR-Int	5′-GTTTGACGATGACATTTATT-3′
ORF1-F1	5′-AACTGAGCTCGCCGAGCTTGCCCAGACACC-3′
ORF1-R1	5′-ATGCTCTAGACGGCATCGCGACCAAAATC-3′
ORF1-Int	5′-ATAAGATCGGCTCGGTTCA-3′
ORF2-F1	5′-CGCAGAGCTCCACCCTGACCGATCCGAAGAC-3′
ORF2-R1	5′-TGCATCTAGACATGCTGACCTTGCCGTTGATA-3′
ORF2-Int	5′-ATATCGACCAGACCTCCATC-3′
ORF3-F1	5′-GTAGGAGTCTGACATGGCTCCGGATTTCAC-3′
ORF3-R1	5′-ACGCTCTAGACTTCCAGCGTAGTCTTCTC-3′
Orf3-Int	5′-ATGCCTTGGCCTGTATCTCC-3′
PVIK165-P	5′-TACTCATCTTTGTTTCCTCC-3′
Primers for complementation	
avsI-F1	5′-TAGAGAATTCCAATGCCTTCAAGACGGGGAGTG-3′
avsI-R1	5′-AGATCTGCAGTCAGGCGGCGCGTGCCTGTTCCA-3′
avsR-F1	5′-GTCTTCTAGACACTTTGGGTCTTTCGTATT-3′
avsR-R1	5′-CGGTGAGCTCTTATAATGGTGCTTCC-3′
Primers for *lacZ* fusion	
avsRI-F1	5′-CACGGAGCTCTCTTATGTTAGCCAGACCTC-3′
avsRI-R1	5′-ATCTTCTAGACCTTAATTGGAAGGTGACACA-3′
Primers for RT-PCR	
aviR-F	5′-TACGGCCTAGTGCGCAGTCCCAAAC-3′
aviR-R	5′-TCAGCTGATCAGGCCGAGACGA-3′
avhR-F	5′-GATATGGCGTCCCTGCTGTCGTC-3′
avhR-R	5′-CGGGTGGCGCGGTTGAGATAATG-3′
avsR-F2	5′-CTTTCGCCGCTGCCATGACC-3′
avsR-R2	5′-CCTTTGCCACCGCTTGAGT-3′
avsI-F2	5′-AGCCGACATAAGCAGACGCAACAG-3′
avsI-R2	5′-CGAAACATCCGCTCCAAAAACAC-3′
ORF1-F2	5′-GCCGAGCTTGCCCAGACACC-3′
ORF1-R2	5′-CGGCATCGCGACCAAAATC-3′
ORF2-F2	5′-CACCCTGACCGATCCGAAGACC-3′
ORF2-R2	5′-GCCGTCAGCTTGTCAAAATCATAG-3′
852-F	5′-GCTGCCAAGCGAAGTGATCCACA-3′
852-R	5′-TCCAGCAAAAGTCGAAGCGTTACAG-3′

conjugation with F2/5 were selected on AB minimal medium (6) amended with kanamycin (50 μg/ml) and 10% mannitol. Mutations were verified by PCR using primers derived from sequences of the F2/5 chromosome that reside upstream of the insertion site (avsR-Int, avsI-Int, ORF1-Int, ORF2-Int, and ORF3-int) and from within the PVIK165 vector (PVIK-P) (Table 2). The mutants were also confirmed by the expression of *gfp* using UV microscopy. Mutants were tested for their ability to cause the HR and grape necrosis.

Detection of AHLs. AHLs were extracted from F2/5 and from derivatives having mutations in *avsR* and *avsI* as well as ORF1 and ORF2, as previously described (14). Overnight bacterial growth was harvested from four 9.0-cm-diameter petri plates of PDA or PDA amended with kanamycin and suspended in a volume of 20 ml acidified ethyl acetate-acetonitirile solution (50:50). The first extraction was done by shaking the cell suspension overnight, and the second was done by shaking for 1 h at 150 rpm. The extracts were centrifuged at 10,000 rpm for 10 min, and the supernatant was dried by rotary evaporation and finally under a stream of N_2. Extractions were made from whole cells because we previously demonstrated that F2/5 produces long-chain AHLs (45), and it has been reported that permeability of such AHLs through bacterial membranes may be limited and require active transport mechanisms (29). AHL profiles were generated using reverse-phase thin-layer chromatography overlaid with biosensor strain NTL4 (pZLR4) (5).

Complementation of mutants with AHLs. Volumes of 2 μl AHL extracted from F2/5 were added to tubes, dried in a fume hood, and then mixed with *avsI* mutant, *avsR* mutant, ORF1 mutant, or ORF2 mutant (overnight cultures) suspended to an optical density at 600 nm (OD_{600}) of 1.5 in sterile distilled water. The mixtures were incubated for 30 min to 1 h, and then the cells were infiltrated into tobacco leaves and inoculated on grape shoot explants. Controls consisted of F2/5, *avsI* mutant, *avsR* mutant, ORF1 mutant, and ORF2 mutant cultures and distilled water.

Complementation of *avsI* mutant was also attempted by adding individual synthetic AHLs to cultures. Approximately 6.5 μM of synthetic AHLs (3-O-C_8, $C_{14:1}$, $C_{16:1}$, and 3-O-$C_{16:1}$) (Sigma-Aldrich) were added to *avsI* mutant suspensions as described above. These synthetic AHLs were tested because we have determined that they are produced by F2/5 (unpublished data).

Cloning of *avsR* and *avsI* and complementation. Complementation of *avsR* mutant and *avsI* mutant was done by methods previously described in our laboratory (14). Primers, avsR-F1 and avsR-R1 as well as avsI-F1 and avsI-R1, containing EcoRI and PstI restriction sites, respectively, were designed to amplify *avsR* and *avsI* from F2/5 genomic DNA (Table 2). The genes were cloned into broad-host-range vector pPROBE-AT (25) and pPZP201 with the P*lac* promoter (11), respectively, and transformed into DH5α competent cells. Positive colonies were selected on LB plates containing appropriate antibiotics. The consensus clones (pAT-avsR and pPZP-avsI) in the recombinant plasmid were verified by restriction digestion and sequencing. During this process, two clones of *avsI* construction (complement mutant 2 [Comp.2] and Comp.3) that differed by two and three amino acid residues, respectively, from the consensus clone were also evaluated in complementation tests. Clones of pAT-avsR and pPZP-avsI were conjugated into *avsR* mutant and *avsI* mutant, respectively, by triparental mating using *E. coli* helper strain HB101 (pRK2013). Transformants were selected on AB (plus 10% mannitol) minimal medium containing carbenicillin (100 μg/ml) or spectinomycin (400 μg/ml) and kanamycin (100 μg/ml) and were verified by PCR. Resulting transconjugants were tested for their ability to cause tobacco HR and grape necrosis. Similarly, the *avsI* consensus clone was also transferred to *avsR* mutant and tested for the ability to cause HR and grape necrosis.

Plasmid pPZP-avsI was also electroporated to *A. tumefaciens* strain NT1 competent cells. Resulting transformants were verified by PCR and analyzed for AHL production as described above.

Plasmid and promoter fusion construction. To construct a *lacZ* reporter fusion, the promoter region containing an apparent *lux* box motif, positioned between the convergently oriented *avsR-avsI* pair, was amplified from F2/5 genomic DNA by primers avsRI-F1 and avsRI-R1, each having appropriate restriction sites (Table 2). The resultant 299-bp fragment was purified, digested, and cloned into pKP302 vector containing a promoterless *lacZ* with its own ribosome-binding site (27). The construct (pKP-RI) was introduced into *E. coli* DH5α and then tested for β-galactosidase activity. Plasmids pAT-avsR and pKP-RI were cotransformed into *E. coli* DH5α and assayed for β-galactosidase activity. The pPROBE-aviR consensus clone was obtained as described previously (45). Similarly, pGT-aviR was also coexpressed with the *avsI*::*lacZ* reporter and assayed for β-galactosidase activity. First, β-galactosidase activities were assessed by streaking cultures on antibiotic-amended LB plates with 5-bromo-4-chloro-3-indolyl-ā-D-galactopyranoside and with or without AHLs extracted from F2/5. The intensity of colony color was visualized after 24 to 48 h at 37°C. β-Galactosidase activity was then quantified according to the Miller method (31). The assay was repeated three times, and the average was calculated for comparison.

Sequence comparison. The sequences of the *avsR-avsI* cluster were analyzed and compared to homologous regions of *A. tumefaciens* C58 and *S. meliloti* Rm1021 genomes. DNA sequence homology searches were performed with Basic Local Alignment Search Tool algorithms provided by the National Center for Biotechnology Information. Analyses of DNA and deduced protein sequences were carried out with DNASTAR software.

RT-PCR. It was determined whether the currently identified *luxR* homologs in *A. vitis* are expressed in a hierarchical manner and whether *avsR-avsI* with ORF1 and ORF2 are expressed as an operon. Expression of *aviR*, *avhR*, *avsR*, *avsI*, ORF1, and ORF2 was measured by reverse transcriptase PCR (RT-PCR). Total RNAs were isolated from overnight PDB cultures (OD_{600} = 1.4 to 1.5, corresponding to 3×10^9 to 5×10^9 CFU/ml of F2/5, M1154, M1320, and *avsR* mutant) using the TRI-Reagent kit (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. RNA samples were treated with RNase-free DNase (Promega, Madison, WI) to prevent DNA contamination, and then PCRs were run using the treated RNA samples as templates to verify the absence of contaminating DNA. RT-PCR was conducted with a Superscript One-Step RT-PCR system plus Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Products were separated by electrophoresis on 1.0% agarose in 0.5× Tris-borate-EDTA (TBE) buffer. Primers for determining expression of *aviR* (aviR-F and aviR-R), for *avhR*

FIG. 1. Alignment of the deduced protein sequence of *avsR* with selected LuxR members. The similarities to the deduced amino acid sequence of *avsR* include AvhR (23.0%) and AviR (15.4%) from *A. vitis* (accession numbers AY519466 and AF521015), SinR (37.6%) from *S. meliloti* Rm1021 (accession number NP_385944), TraR (15.0%) from *A. tumefaciens* (accession number L08596), LuxR (19.1%) from *Vibrio fischeri* (accession number M96844), EsaR (17.5%) from *Erwinia stewartii* (accession number L32184), LasR (19.7%) from *Pseudomonas aeruginosa* (accession number D30813), PhzR (19.0%) from *Pseudomonas aeruginosa* (accession number AF190630), RhlR (21.6%) from *Pseudomonas aeruginosa* (accession number AE004768), and RhiR (18.7%) from *Rhizobium leguminosarum* (accession number M98835). *, Strictly conserved amino acid residues; Δ, substitutions at strictly conserved residues; #, substitutions at conserved residues that are involved in pheromone binding.

(avhR-F and avhR-R), for *avsR* (avsR-F2 and avsR-R2), for *avsI* ((avsI-F2 and avsI-R2), for ORF1 (ORF1-F2 and ORF1-R2), for ORF2 (ORF2-F2 and ORF2-R2), and for control 852 (852-F and 852-R) are shown in Table 2. All assays were repeated at least once.

Nucleotide sequence accession number. The sequence data of *avsR-avsI* has been submitted to GenBank under accession number DQ058009.

RESULTS

Characterization of *avsR* and *avsI*. Analysis of the *A. vitis* strain S4 genome sequence revealed the presence of a *luxR-luxI* pair on one of the chromosomal replicons (*avi1890* and *avi1889*). Corresponding genes were subsequently identified in strain F2/5 and designated *avsR* and *avsI* (for *Agrobacterium vitis* genes with high similarity to *sinR-sinI* in *S. meliloti*). *avsR* shares identity with *avi1890* of S4 sequence (96% at nucleotide [nt] and 100% at amino acid levels) and encodes a predicted 246-amino-acid protein with a molecular mass of 27.7 kDa. The putative start codon of *avsR* is ATG, and a likely ribosomal binding site, GGG, is located five nucleotides upstream from the start codon. No potential *lux* box motif is identified in the promoter region. The deduced AvsR protein possesses an autoinducer binding domain and a conserved helix-turn-helix DNA binding domain and shares greatest identity with SinR (38%) from *S. meliloti* Rm1021. It shares relatively low identity with AviR (15%) or AvhR (23%) from *A. vitis* or with other LuxR homologs compared in Fig. 1. AvsR contains only two of the five amino acid residues (Glu178 and Gly188) that were previously reported to be absolutely conserved among known functional members of the LuxR family (42). Three amino acid residues, Tyr61, Asp70, and Gly113, are replaced by Ile, Ser,

FIG. 2. Alignment of the deduced protein sequence of *avsI* with selected LuxI members. The similarities to the deduced amino acid sequence of *avsI* include SinI (70.6%) from *S. meliloti* Rm1021 (accession number NP_385945), LuxI (27.5%) from *V. fischeri* (accession number M96844), EsaI (30.8%) from *E. stewartii* (accession number L32183), LasI (19.7%) from *P. aeruginosa* (accession number AAG04821), and TraI (33.3%) from *A. tumefaciens* (accession number L22207). +, Conserved amino acid residues; α, substitutions in Comp.1; β, substitutions in Comp.2.

and Ala, respectively, as shown in Fig. 1. Two other highly conserved residues, Trp57 and Trp85, both reported to contribute to pheromone binding (44), are also replaced by Ala and Pro, respectively, in AvsR and in AvhR.

AvsI shows identity with *avi1889* of the S4 sequence (95% at nt and 99% at amino acid levels with one residue substitution, Gly135Ala). It contains 214 amino acids with a molecular mass of 23.8 kDa and possesses eight conserved residues identified in LuxI, RhlI, and EsaI homologs (13, 28) (Fig. 2). *avsI* is located 299 nt downstream of *avsR* and is convergently transcribed with *avsR*. The promoter region of *avsI* possesses a likely *lux* box motif located 74 nt upstream from the putative translational start site of *avsI* (AACTGtCGcTAcCGtCAGTT) (uppercase letters indicate perfectly inverted repeats). It has been reported that such motifs are critical for the binding of LuxR transcriptional regulators to the target gene(s) (8, 22, 43). The deduced protein, AvsI, shows a very high level of identity with SinI (71%) from Rm1021. AvsI has much lower identity with synthases TraI (33%) responsible for 3-O-C_8-HL in *A. tumefaciens* strain C58, LuxI (28%) for 3-O-C_6-HL in *Vibrio fischeri*, LasI (31%) for 3-O-C_{12}-HL in *P. aeruginosa*, and CinI (30%) for 3-OH-$C_{14:1}$-HL in *Rhizobium leguminosarum* (Fig. 2).

***avsR-avsI* controls the production of long-chain AHLs.** The mutations in *avsR* mutant and *avsI* mutant affect production of long-chain AHLs (Fig. 3A). Whereas F2/5 produces both long- and short-chain AHLs, *avsI* mutant shows no evidence for long-chain AHL production and *avsR* mutants shows reduced long-chain signal production. Thin-layer chromatography detection shows that the cloned *avsI* in *A. tumefaciens* NT1 produces the long-chain AHLs as in F2/5, whereas NT1 harboring vector alone produces no detectable signal (Fig. 3C). Taken together, it indicates that AvsI directs long-chain AHLs production, and there is an additional synthase(s) in F2/5 for short-chain AHLs. This scenario is supported by our recent finding that clones containing individual F2/5 plasmids produce short-chain, but not long-chain, AHLs (unpublished data). Therefore, the *avsR-avsI* pair resembles *sinR-sinI* in *S. meliloti*, where SinR regulates SinI leading to synthesis of multiple long-chain AHLs, including C_{12}-HL, 3-O-C_{14}-HL, 3-O-C_{16}:1-HL, $C_{16:1}$-HL, and C_{18}-HL. $C_{16:1}$-HL was shown to be the cognate AHL for SinR (24).

Genes downstream of *avsR-avsI*. ORFs residing downstream of *avsR-avsI* have homologs in C58 and Rm1021 (Fig. 4). ORF1, ORF2, and ORF3 show identity with *avi1888*, *avi1887*, and *avi1886* of the S4 genome, respectively (97% at nt and 100% at amino acid levels). ORF1 is transcribed in the same direction as *avsR-avsI* and contains 1,173 nt that encode a hypothetical protein of 390 amino acids that shares identity with AGRc3045 (35%) from C58 and SMc00167 (35%) from Rm1021. ORF2 is divergently transcribed with *avsR-avsI*, and ORF2 contains 3,426 nt encoding a deduced 1,141-amino-acid protein that has identity with AGRc3361 (42%) and SMc00525 (40%). Mutations in ORF1 and ORF2 affected long-chain

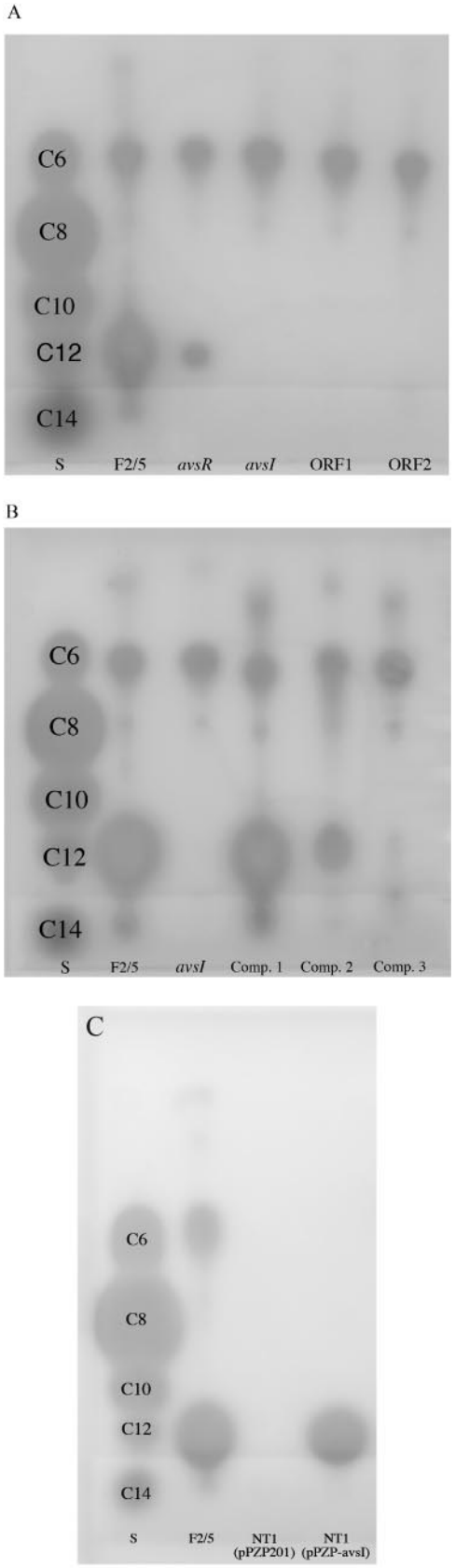


FIG. 3. Profiles of AHLs from extracts of overnight cultures of F2/5, *avsR* mutant (*avsR*), *avsI* mutant (*avsI*), ORF1 mutant, and ORF2 mutant (A) from F2/5. (B) Mutant *avsI* and complemented mutant *avsI* (marked as Comp. 1, Comp. 2, and Comp. 3, respectively) and from F2/5. (C) NT1 containing pPZP and NT1 containing pPZP-avsI. Volumes of 10 μl from each extraction were spotted and separated on C_{18} reverse-phase thin-layer chromatography plates developed with methanol-water (70:30) and were visualized following overlay with *Agrobacterium* sensor strain NTL4(pZLR4). S represents a mixture of standard unsubstituted AHLs as indicated.

AHL production similarly to the *avsI* mutant (Fig. 3A). ORF3 contains 474 nt encoding a putative 157-amino-acid bacterioferritin comigratory protein (BCP) that has identity with AGRc3362 (70%) and SMc00524 (62%). BCP is reported as a new member of the thiol-specific antioxidant protein (TSA)/alkyl hydroperoxide peroxidase C (AhpC) family, acting as a general hydroperoxide peroxidase (18). Mutation in ORF3 does not appear to affect AHL production (data not shown).

Sequence comparisons revealed that *avsI*, *avsR*, ORF1, ORF2, and ORF3 have homologs in the circular chromosome of Rm1021 and C58, except that *avsR-avsI* is not present in C58 (Fig. 4). All ORFs were first identified in the S4 genome, and subsequently their presence was confirmed in F2/5 by PCR cloning and sequencing. The relative order and orientation of the related genes are identical in F2/5, C58, and Rm1021, except for greater distances between OFR1 and ORF2 in C58 and Rm1021, as shown in Fig. 4.

Phenotypes and complementation of mutants. Mutations in *avsR*, *avsI*, ORF1, or ORF2 resulted in an HR-negative phenotype on tobacco leaves infiltrated with cells from 24-h-old cultures adjusted to an OD_{600} of about 1.5 (Fig. 5A). Grape necrosis was noticeably reduced in the *avsR* mutant but to an even greater extent by *avsI*, ORF1, and ORF2 mutants (Fig. 5B and 6A). Mutation in ORF3 did not affect HR or necrosis (data not shown). HR was complemented in *avsI* mutant by adding AHLs extracted from F2/5, whereas extracts from *avsI* mutant did not complement the mutant (data not shown); however, the complementation is not consistent, in that all of the leaf panels with the complemented *avsI* mutant did not express an HR in repeated experiments. The necrosis phenotype was consistently complemented in *avsI* mutant, *avsR* mutant, ORF1 mutant, and ORF mutants by adding AHLs extractions from F2/5 (Fig. 6A). Furthermore, HR and necrosis of *avsI* mutant and *avsR* mutant were complemented following transformation with pPZP-avsI (Fig. 5). HR and necrosis of *avsR* mutant were also complemented following transformation with pAT-avsR (data not shown). The complemented *avsI* mutant produced the same AHL profile as F2/5 (Fig. 3B, Comp. 1); complemented *avsR* mutant also exhibited a wild-type AHL profile (data not shown). During the cloning of *avsI*, one clone (Comp. 2) that differed from the consensus clone by only two amino acid substitutions (Asp108Gly and Ala166Thr) (Fig. 2) was not able to complement the HR and produced a noticeably reduced signal for the presence of long-chain AHLs (Fig. 3B). Another clone (Comp. 3) with three amino acid substitutions (Arg64Trp, Leu87Pro, and Leu158Ile) (Fig. 2) also was unable to complement the HR and produced a very faint signal for long-chain AHLs (Fig. 3B).

To identify specific AHLs that were able to complement *avsI* mutant, different synthetic AHLs were added to cul-

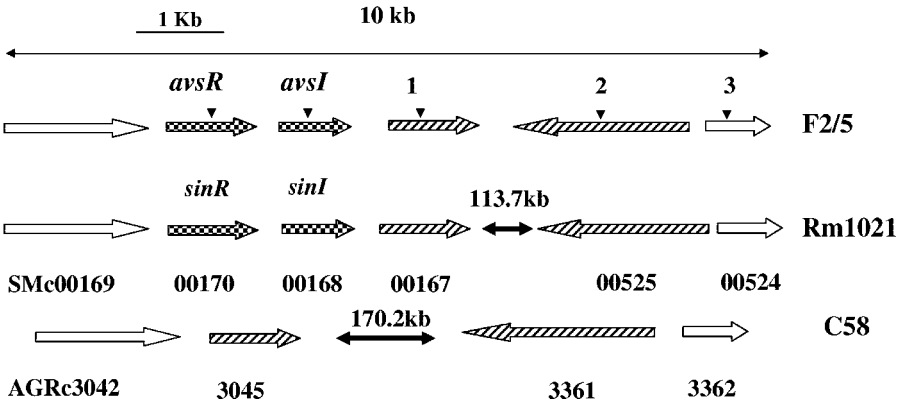
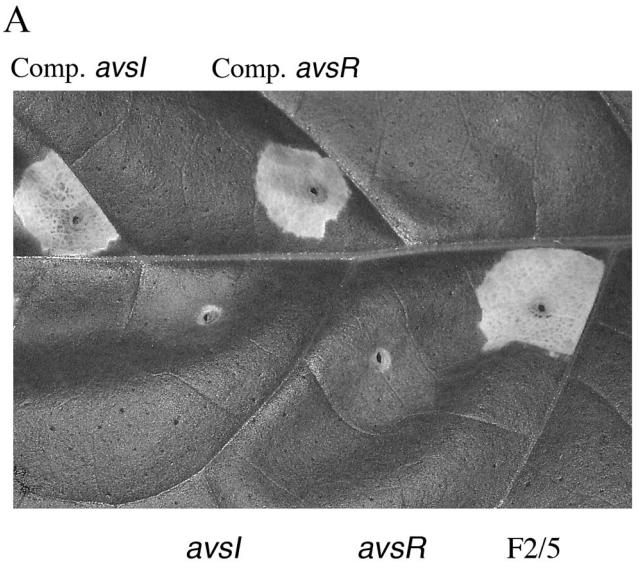


FIG. 4. Comparison of ORF organization between the *avsR-avsI* cluster and corresponding regions of genomes in *A. tumefaciens* C58 and *S. meliloti* Rm1021. The length of each arrow represents the relative ORF size and indicates the direction of transcription. Names of putative gene homologs are given under the arrows. Related gene clusters are indicated by arrow background patterns.



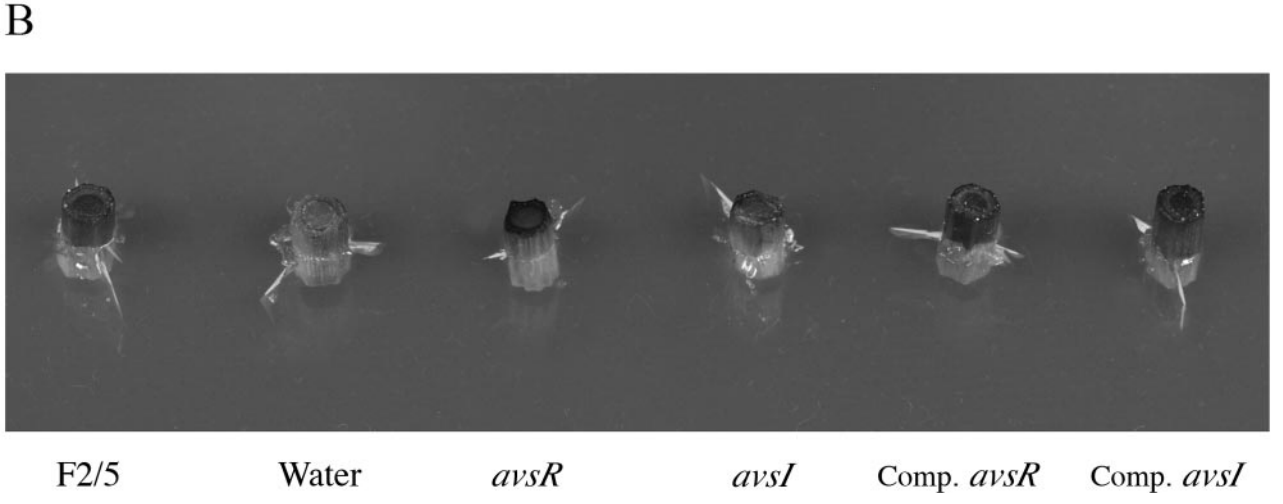


FIG. 5. Comparison of (A) tobacco HR and (B) necrosis of grape shoot explants induced by F2/5, mutants, and complemented mutants (Comp.).

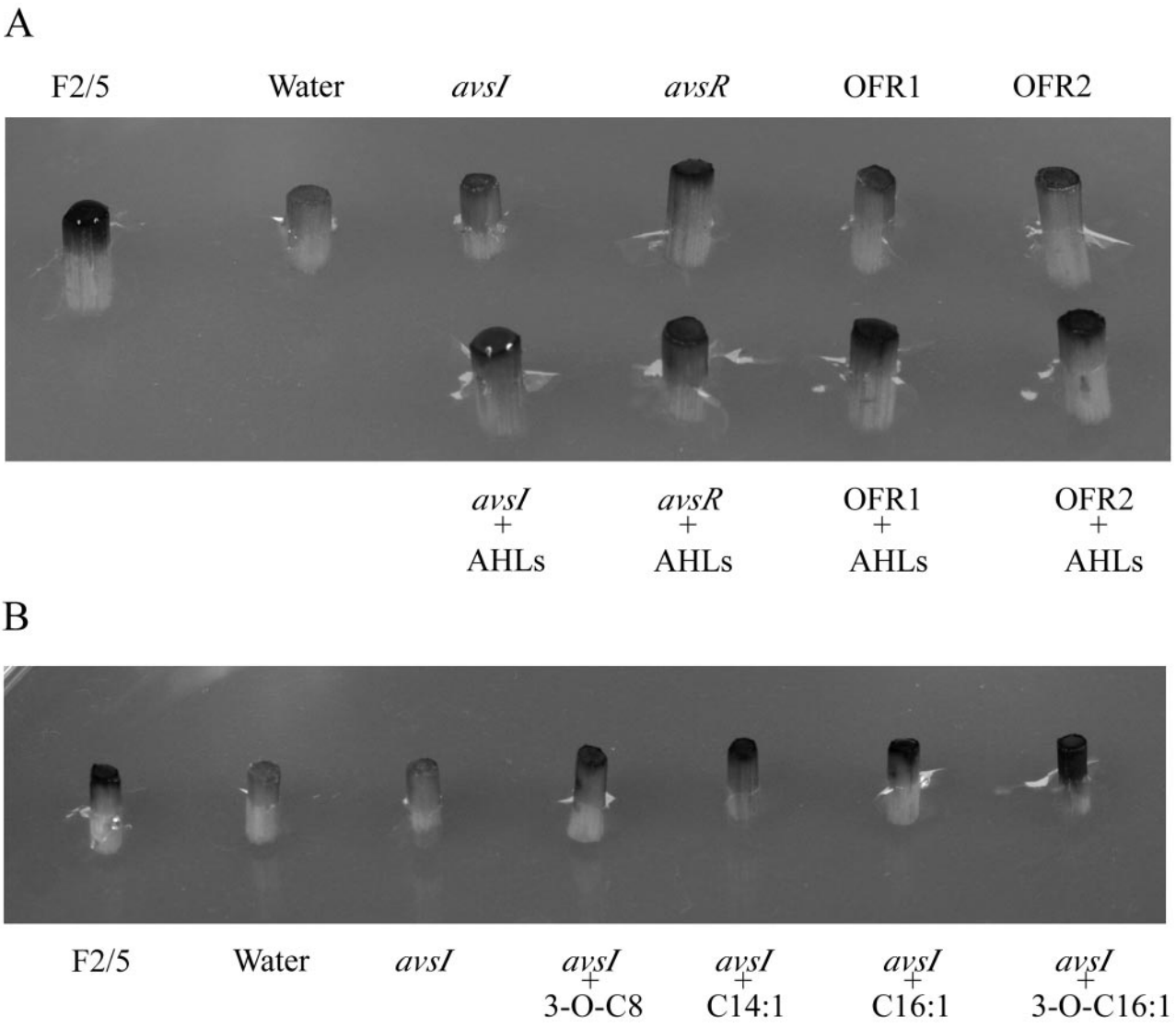


FIG. 6. (A) Necrosis of grape shoot explants induced by mutants and complemented mutants with AHLs extracted from F2/5. (B) Necrosis complementation in the *avsI* mutant with synthetic AHLs.

tures. The HR was not complemented with 3-O-C_8-HL, $C_{14:1}$-HL, $C_{16:1}$-HL, or 3-O-$C_{16:1}$-HL. However, addition of $C_{14:1}$-HL to *avsI* mutant resulted in a relatively weak necrosis phenotype, and addition of $C_{16:1}$-HL and 3-O-$C_{16:1}$-HL resulted in necrosis at wild-type levels (Fig. 6B).

***avsR* affects the expression of *avsI*.** The expressions of the putative QS-associated genes *aviR*, *avhR*, *avsR*, and *avsI* in F2/5 (HR and necrosis positive), M1154 (HR and necrosis negative), M1320 (HR negative and necrosis reduced), and *avsR* mutant (HR negative and necrosis reduced) were determined by RT-PCR (Fig. 7). *aviR* is expressed in F2/5, M1320, and the *avsR* mutant; similarly, *avhR* is expressed in F2/5, M1154, and the *avsR* mutant. The expression of *avsR* appears to be reduced in M1154 compared to F2/5 and M1320. The expression of *avsI* was shown as a faint signal in the *avsR* mutant compared to F2/5, M1154, and M1320, suggesting that the expression level of *avsI* is regulated by *avsR* but not by *aviR* or *avhR*. These results conform to the QS model that would assume a basal-level expression of the AHL synthase gene in the absence of its transcriptional regulator (24, 33). The levels of expression of ORF1 and ORF2 appear to be the same in F2/5 and the *avsR* mutant, indicating that the expressions of these putative genes are not dependent on *avsR* (data not shown).

To confirm *avsR* is involved in regulation of *avsI* expression, two plasmids (pKP-RI and pAT-avsR as well as pKP-RI and pGT-aviR) were introduced into *E. coli* DH5α which produce no detectable β-galactosidase activity. The reporter strain pKP-RI alone containing *avsI*::*lacZ* shows basal level β-galactosidase activity (Table 3). The strain harboring pKP-RI and pGT-aviR shows no difference for β-galactosidase activity, indicating *aviR* does not affect *avsI* expression, as was also shown by RT-PCR. The strain expressing both *avsR* and *avsI*::*lacZ* shows a slight increase for β-galactosidase activity. The addition an ethyl acetate extract of F2/5 results in slightly increased β-galactosidase

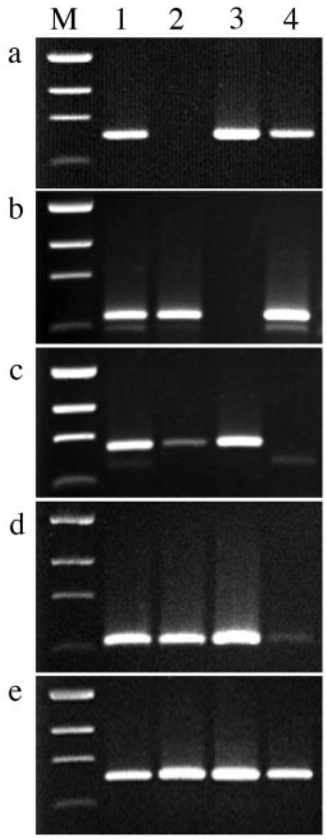


FIG. 7. Expression of (a) *aviR*, (b) *avhR*, (c) *avsR*, (d) *avsI*, and (e) a putative HR-associated gene identified in F2/5 mutant M852 (used as a positive control). Lane 1, F2/5; lane 2, M1154 (*aviR* mutant); lane 3, M1320 (*avhR* mutant); and lane 4, the *avsR* mutant as determined by RT-PCR. M, low-molecular-weight DNA ladder.

activity (Table 3). Together with RT-PCR expression, these results confirm that *avsI* is regulated by *avsR*, and it is not very clear whether AHLs are central for *avsR* function.

DISCUSSION

We have demonstrated that a complex quorum-sensing system is involved in the regulation of *A. vitis*-plant interactions that result in HR and necrosis phenotypes. Two *luxR* homologs, *aviR* and *avhR*, were previously reported, and we now report a third, *avsR*, which resides upstream from *avsI*, the first identified AHL synthase gene in *A. vitis*. The AvsR-AvsI pair shows unusually high homology to SinR-SinI in Rm1021 that regulates production of long-chain AHLs and exopolysaccharide II synthesis, i.e., mutation results in a nonmucoid colony morphology (23). AvsR-AvsI resembles SinR-SinI in the regulation of long-chain AHL production; however, a change in mucoid colony phenotypes in *avsR* and *avsI* mutants was not apparent in our culture condition. Both *avsI* mutant and *avsR* mutant can be complemented with pPZPavsI, thereby indicating that *avsR* is likely to affect HR and necrosis by regulating the expression of *avsI* and subsequent AHL production.

It is interesting that the two hypothetical proteins (ORF1 and ORF2) located directly downstream of *avsR-avsI* are also necessary for long-chain AHL production, HR, and necrosis,

TABLE 3. Effect of AvsR and AHLs on induction of *avsI*::*lacZ*

Reporter fusion	Enzyme activity	
	Without AHLs	With AHLs[a]
pKP-RI	5.61	
pKP-RI + pGT-aviR	4.41	4.34
pKP-RI + pAT-avsR	8.70	12.06

[a] Cultures were supplemented with AHLs extracted from F2/5.

and that these ORFs are not apparently regulated by *avsR*. We hypothesize that ORF1 and ORF2 encode proteins involved in the synthesis pathways of the long-chain AHLs that are essential for expression of HR and necrosis. AHLs derive their invariant homoserine lactone rings from the substrate *S*-adenosyl-L-methionine and their variable acyl chains from the acyl carrier protein (ACP) pool (28). Employing an in vitro fatty acid-3-O-AHL synthesis system, it was demonstrated that the minimum Fab-3-O-AHL biosynthetic pathway includes Fab (fatty acid biosynthesis) proteins, ACP, and the LasI synthase (17). ORF1 and ORF2 in *A. vitis* reported here may encode unique ACP or Fab proteins that interact with AvsI. Further work needs to be done to determine how ORF1 and ORF2 are involved in long-chain AHL synthesis. Thin-layer chromatography assays provided evidence that short-chain AHLs in F2/5 are not affected by the mutations in *avsR*, *avsI*, ORF1, or ORF2. Therefore, more than one AHL synthase must exist in F2/5. To support this hypothesis, we recently characterized a putative *luxI* homolog (unpublished data) residing in one of the conjugal F2/5 plasmids (36). In this case, the gene shares a high degree of similarity to *traI* of C58. It may be that as in C58, the short-chain AHLs are involved in conjugal transfer of the plasmid.

Mutational studies of AHL synthases, LuxI, RhlI, and EsaI have shown that several residues near the N terminus are essential for gene function, including Arg23, Glu42, Asp44, Asp47, Arg70, Glu101, Ser103, and Arg104 (13, 28, 40). The structural analysis of LasI revealed that Arg154 and Lys150 are associated with acyl-ACP binding and provides an explanation for the specificity between the AHL synthase and the acyl chain length (10). In our case with the deduced AvsI protein, nonfunctional clones Comp.2 and Comp.3 differed from the consensus clone in only two and three residues that have not previously been recognized as being essential for synthase activity. It will be interesting to determine more completely which residues are essential for AvsI function and whether they are also essential for SinI.

The addition of AHLs extracted from F2/5 to the *avsI* mutant culture complemented both the necrosis and the HR phenotypes. When different synthetic AHLs were added to *avsI* mutant, however, only necrosis was complemented at different efficiencies depending on the specific AHL. The strongest necrosis responses were observed following addition of $C_{16:1}$-HL and 3-O-$C_{16:1}$-HL, indicating that one or both of them may be the cognate AHL for a regulator that plays a critical role in necrosis induction. As indicated above, the complementation of HR was inconsistent and could have resulted from timing of AHL exposure to the mutant or insufficient concentration of AHL for regulation of bacterial gene expression that is re-

quired to induce the HR. In contrast, consistent complementation of HR is obtained with cloned *avsI* (pPZP-avsI). Former studies suggested that the development of HR and necrosis (disease) are similar processes, their induction requires different numbers of bacteria, and their development proceeds at different rates (1). A recent analysis of large-scale mRNA expression in *P. syringae-Arabidopsis*-compatible and -incompatible interactions documents the similarity of the responses and how they differ primarily in their timing. The report also suggests that a common signal transduction pathway underlies both responses (37). Efforts are under way in our laboratory to determine mechanisms associated with necrosis and the HR and how QS regulation is involved.

Recently, we demonstrated that the deduced AvhR protein has several substitutions in residues that were reported as conserved in known functional LuxR homologs (14). The putative AvsR protein has five amino acid substitutions located in the AHL binding domain that are predicted to be involved in pheromone binding. Phylogenetic analyses suggest that LuxR proteins comprise two families with different functions and structures (21). Family A, including TraR, requires binding of its cognate AHL, *N*-(3-oxo-octanoyl)-L-homoserine lactone, for proper protein folding and dimerization into its active form and for protection against proteolysis (30, 46). A similar scenario was also demonstrated for LuxR in *V. fischeri* and LasR in *P. aeruginosa* (20). In contrast, binding of pheromone for functionality is not necessary in family B. For example, CarR and ExpR in *Erwinia carotovora* have been shown to bind the promoter in the absence of autoinducer, and EsaR is inactivated in the presence of autoinducer (26, 41). In addition, CarR functions independently of AHL to control carbapenem antibiotic synthesis in *Serratia marcescens* (7). Therefore, an interesting question is whether the substitutions that are apparent at conserved amino acids affect binding of the cognate AHL in AvsR. The promoter region of *avsI* possesses a conspicuous *lux* box motif and was used to construct an *avsI*::*lacZ* reporter fusion. We found AvsR (and not AviR) activates *avsI*::*lacZ* expression consistently but weakly with or without adding AHLs. A similar result was observed with *sinI*; it was shown to be weakly controlled by *sinR* in microarray analysis in *S. meliloti*, although AHL production was affected in a *sinR* mutant (16). We speculate that as in *S. meliloti*, there is a complex QS network in F2/5, and *avsR-avsI* is not at the top of the QS hierarchy. AviR does not activate *avsI*::*lacZ* reporters in the presence or absence of AHLs, confirming that they do not bind the *avsI* promoter and regulate *avsI* expression. We speculate that AviR may bind on AHL for activity, because it possesses all the conserved residues of that AHL binding domain identified in TraR (44). In a previous work, we demonstrated that *aviR* regulates expression of a gene that encodes a putative dipeptidyl aminopeptidase and also has an upstream motif suggestive of a *lux* box (45). A *lacZ* fusion will also be made to the putative *lux* box promoter region of this gene to determine the cognate AHL for AviR. The identification of the cognate AHLs for other LuxR proteins in *A. vitis* that are associated with induction of HR and necrosis are under investigation. It will be important to determine whether the functionality of these regulatory proteins is dependent on AHL binding.

Gene phylogenies of LuxR and LuxI families in *Pseudomonas* species suggest a complex process of lateral transfers, ancestral duplication, and gene loss. It has been proposed that microbial species obtain *luxR-luxI* genes through horizontal gene transfer (21). We find that all of the predicted proteins flanking the *avsR-luxI* genes have corresponding homologs in Rm1021. SinR-SinI is responsible for the production of long-chain AHLs (24). AvsI not only shares unusual high homology with SinI (71%) but also affects long-chain AHL production similarly. Earlier, we reported that AviR shares high homology with ExpR of Rm1021 and AvhR shares homology with two Rm1021 *luxR* homologs (SMc00877 and SMc00878) (14, 45). This suggests that these LuxR homologs in *A. vitis* and *S. meliloti* have arisen from the same ancestral genome by duplications and followed by speciation. It is interesting that AvsR-AvsI homologs are not present in C58, though the orientation and arrangement of bordering gene homologs are conserved. It appears, therefore, that C58 either never obtained AvsR-AvsI genes from the ancestral genome or lost it during evolution. A comparison of the *avsR-avsI*, *aviR*, and *avhR* bordering genes in these three members of *Rhizobiaceae* indicates a close relationship, although there are evident rearrangements and deletions.

In *A. vitis* we have identified functional *aviR* and *avhR* that reside in the genome with no nearby *luxI* partner. Together with the presence of *avsR-avsI*, this indicates a complex quorum-sensing network that regulates interactions between the bacterium and plants. Several other bacteria also employ multiple quorum-sensing systems; some of them are known to function in a hierarchical manner to regulate diverse phenotypes (42). From RT-PCR results, we show that *aviR*, *avhR*, and *avsR* are expressed independently. We previously demonstrated that *aviR* affects the production of AHLs (44) and now have determined that it does not regulate *avsI*. Therefore, it may be that *avsI* produces the AHL(s) that serves as the cognate for *aviR*. All three of the presently known *luxR* homologs in *A. vitis* affect HR and necrosis; however, only the disruption of *aviR* completely abolishes the necrosis phenotype, and the phenotype of *avsI* mutant is highly reduced in necrosis. We predict, therefore, that function of AviR may require a specific AHL synthesized by AvsI; however, this scenario now needs to be confirmed.

A main goal of our research is to determine a basic understanding of genetic mechanisms used by *A. vitis* in plant interactions. We have begun to identify QS circuits that are involved in regulation of the interactions and will continue to focus on target genes and their products. Such results will add to our general understanding of plant-microbe interactions and may help to identify novel disease control strategies.

ACKNOWLEDGMENTS

This research was funded by NRI Competitive Grants Program/USDA award number 2002-35319-12582 and NSF Microbial Genome Sequencing Program SA6624.

We are very grateful for the invaluable S4 sequence information that was provided by members of the NSF grant team. We thank S. Winans (Cornell) for vectors pVIK165, pPZP201, and pKP302; S. Lindow (University of Californa—Berkeley) for vectors pPROBE-GT and pPROBE-AT; S. Farrand (University of Illinois) for biosensor NTL4 (pZLR4); and A. Eberhard (Cornell University) for providing AHL standards.

REFERENCES

1. **Alfano, J. R., and A. Collmer.** 2004. Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. Annu. Rev. Phytopathol. **42:**385–414.
2. **Burr, T. J., and L. Otten.** 1999. Crown gall of grape: biology and disease management. Annu. Rev. Phytopathol. **37:**58–80.
3. **Buttner, D., and U. Bonas.** 2002. Getting across-bacterial type III effector proteins on their way to the plant cell. EMBO J. **21:**5313–5322.
4. **Canaday, J., J. C. Gerad, P. Crouzet, and L. Otten.** 1992. Organization and functional analysis of three T-DNAs from the vitopine Ti plasmid pTiS4. Mol. Gen. Genet. **235:**292–303.
5. **Cha, C., Y. Gao, P. D. Shaw, and S. K. Farrand.** 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. **11:**1119–1129.
6. **Chilton, M., T. C. Currier, S. K. Farrand, A. J. Bendich, M. P. Gordon, and E. W. Nester.** 1974. *Agrobacterium tumefaciens* DNA and Ps8 bacteriophage DNA not detected in crown gall tumorigenesis. Proc. Natl. Acad. Sci. USA **71:**2672–2676.
7. **Cox, A. R., N. R. Thomson, B. Bycroft, G. S. Stewart, P. Williams, and G. P. Salmond.** 1998. A pheromone independent CarR protein controls carbapenem antibiotic synthesis in the opportunistic human pathogen *Serratia marcescens*. Microbiology **144:**201–209.
8. **Egland, K. A., and E. P. Greenberg.** 1999. Quorum sensing in Vibrio fischeri: elements of the luxI promoter. Mol. Microbiol. **31:**1197–1204.
9. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727–751.
10. **Gould, T. A., H. P. Schweizer, and M. E. Churchill.** 2004. Structure of the *Pseudomonas aeruginosa* acyl-homoserinelactone synthase LasI. Mol. Microbiol. **53:**1135–1146.
11. **Hajdukiewicz, P., Z. Svab, and P. Maliga.** 1994. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. **25:**989–994.
12. **Hansen, G.** 2000. Evidence for *Agrobacterium*-induced apoptosis in maize cells. Mol. Plant-Microbe Interact. **13:**649–657.
13. **Hanzelka, B. L., A. M. Stevens, M. R. Parsek, T. J. Crone, and E. P. Greenberg.** 1997. Mutational analysis of the *Vibrio fischeri* LuxI polypeptide: critical regions of an autoinducer synthase. J. Bacteriol. **179:**4882–4887.
14. **Hao, G., H. S. Zhang, D. S. Zheng, and T. J. Burr.** 2005. *luxR* homolog *avhR* in *Agrobacterium vitis* affects the development of a grape-specific necrosis and a tobacco hypersensitive response. J. Bacteriol. **187:**185–192.
15. **Herlache, T. C., H. S. Zhang, C. L. Reid, S. Carle, D. Zheng, P. Basaran, M. Thaker, A. T. Burr, and T. J. Bur.** 2001. Mutations that affect *Agrobacterium vitis*-induced grape necrosis also alter its ability to cause a hypersensitive response on tobacco. Phytopathology **91:**966–972.
16. **Hoang, H. H., A. Becker, and J. E. Gonzalez.** 2004. The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. J. Bacteriol. **186:**5460–5472.
17. **Hoang, T. T., S. A. Sullivan, J. K. Cusick, and H. P. Schweizer.** 2002. Beta-ketoacyl acyl carrier protein reductase (FabG) activity of the fatty acid biosynthetic pathway is a determining factor of 3-oxo-homoserine lactone acyl chain lengths. Microbiology **148:**3849–3856.
18. **Jeong, W., M. K. Cha, and I. H. Kim.** 2000. Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (BCP) as a new member of the thiol-specific antioxidant protein (TSA)/alkyl hydroperoxide peroxidase C (AhpC) family. J. Biol. Chem. **275:**2924–2930.
19. **Kalogeraki, V. S., and S. C. Winans.** 1997. Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene **188:**69–75.
20. **Kiratisin, P., K. D. Tucker, and L. Passador.** 2002. LasR, a transcriptional activator of *Pseudomonas aeruginosa* virulence genes, functions as a multimer. J. Bacteriol. **184:**4912–4919.
21. **Lerat, E., and N. A. Moran.** 2004. The evolutionary history of quorum-sensing systems in bacteria. Mol. Biol. Evol. **21:**903–913.
22. **Luo, Z. Q., and S. K. Farrand.** 1999. Signal-dependent DNA binding and functional domains of the quorum-sensing activator TraR as identified by repressor activity. Proc. Natl. Acad. Sci. USA **96:**9009–9014.
23. **Marketon, M. M., S. A. Glenn, A. Eberhard, and J. E. Gonzalez.** 2003. Quorum sensing controls exopolysaccharide production in *Sinorhizobium meliloti*. J. Bacteriol. **185:**325–331.
24. **Marketon, M. M., M. R. Gronquist, A. Eberhard, and J. E. Gonzalez.** 2002. Characterization of the *Sinorhizobium meliloti sinR/sinI* locus and the production of novel *N*-acyl homoserine lactones. J. Bacteriol. **184:**5686–5695.
25. **Miller, W. G., J. H. Leveau, and S. E. Lindow.** 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol. Plant-Microbe Interact. **13:**1243–1250.
26. **Minogue, T. D., M. Wehland von Trebra, F. Bernhard, and S. Beck von Bodman.** 2002. The autoregulatory role of EsaR, a quorum-sensing regulator in *Pantoea stewartii ssp. stewartii*: evidence for a repressor function. Mol. Microbiol. **44:**1625–1635.
27. **Pappas, K. M., and S. C. Winans.** 2003. A LuxR-type regulator from Agrobacterium tumefaciens elevates Ti plasmid copy number by activating transcription of plasmid replication genes. Mol. Microbiol. **48:**1059–1073.
28. **Parsek, M. R., A. L. Schaefer, and E. P. Greenberg.** 1997. Analysis of random and site-directed mutations in rhII, a *Pseudomonas aeruginosa* gene encoding an acylhomoserine lactone synthase. Mol. Microbiol. **26:**301–310.
29. **Pearson, J. P., C. Van Delden, and B. H. Iglewski.** 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J. Bacteriol. **181:**1203–1210.
30. **Qin, Y., Z. Q. Lou, A. J. Smyth, P. Gao, S. Beck von Bodman, and S. K. Farrand.** 2000. Quorum-sensing signal binding results in dimerization of TraR turnover rates in whole cells. EMBO J. **19:**5212–5221.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Santos, R., D. Herouart, S. Sigaud, D. Touati, and A. Puppo.** 2001. Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. Mol. Plant-Microbe Interact. **14:**86–89.
33. **Seed, P. C., L. Passador, and B. H. Iglewski.** 1995. Activation of the *Pseudomonas aeruginosa lasI* gene by LasR and the *Pseudomonas* autoinducer PAI: an autoinduction regulatory hierarchy. J. Bacteriol. **177:**654–659.
34. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad-host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. BioTechnology **1:**784–791.
35. **Staphorst, J. L., F. G. H. van Zyl, B. W. Strijdom, and Z. E. Groenewold.** 1985. Agrocin-producing pathogenic and nonpathogenic biotype-3 strains of *Agrobacterium tumefaciens* active against biotype-3 pathogens. Curr. Microbiol. **12:**45–52.
36. **Szegedi, E., S. Sule, and T. J. Burr.** 1999. *Agrobacterium vitis* F2/5 contains tartrate and octopine utilization plasmids which do not encode functions for tumour inhibition on grapevine. J. Phytopathol. **147:**665–669.
37. **Tao, Y., Z. Xie, W. Chen, J. Glazebrook, H. Chang, B. Han, T. Zhu, G. Zou, and F. Katagiri.** 2003. Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. Plant Cell **15:**317–330.
38. **Vannini, A., C. Volpari, C. Gargioli, E. Muragli, R. Cortese, R. De Francesco, P. Neddermann, and S. D. Marco.** 2002. The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. EMBO J. **21:**4393–4401.
39. **Waston, B., T. C. Currier, M. P. Gordon, M.-D. Chilton, and E. W. Nester.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **123:**255–264.
40. **Watson, T. W., T. D. Minogue, D. L. Val, S. Beck von Bodman, and M. E. A. Churchill.** 2002. Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol. Cell **9:**685–694.
41. **Welch, M., D. E. Todd, N. A. Whitehead, S. J. McGowan, B. W. Bycroft, and G. P. Salmond.** 2000. N-acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in Erwinia. EMBO J. **19:**631–641.
42. **Whitehead, N. A., A. M. L. Barnard, H. Slater, N. J. L. Simpson, and G. P. Salmond.** 2001. Quorum-sensing in gram-negative bacteria. FEMS Microbiol. Rev. **25:**365–404.
43. **Whiteley, M., and E. P. Greenberg.** 2001. Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. J. Bacteriol. **183:**5529–5534.
44. **Zhang, R., T. Pappas, J. L. Brace, P. C. Miller, T. Qulmassov, J. M. Molyneaux, J. C. Anderson, J. K. Bashkin, S. C. Winans, and A. Joachimiak.** 2002. Structure of bacterial quorum-sensing transcription factor complexed with pheromone and DNA. Nature **417:**971–974.
45. **Zheng, D., H. S. Zhang, S. Carle, G. X. Hao, M. R. Holden, and T. J. Burr.** 2003. A luxR homolog, aviR, in *Agrobacterium vitis* is associated with induction of necrosis on grape and a hypersensitive response on tobacco. Mol. Plant-Microbe Interact. **16:**650–658.
46. **Zhu, J., and S. C. Winans.** 2000. The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance and dimerization. Proc. Natl. Acad. Sci. USA **98:**1507–1512.
47. **Zhu, J., P. M. Oger, B. Schrammeijer, P. J. J. Hooykaas, S. K. Farrand, and S. C. Winans.** 2000. The bases of crown gall tumorigenesis. J. Bacteriol. **182:**3885–3895.