An Sfp-Type PPTase and Associated Polyketide and Nonribosomal Peptide Synthases in *Agrobacterium vitis* Are Essential for Induction of Tobacco Hypersensitive Response and Grape Necrosis

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An Sfp-type phosphopantetheinyl transferase (PPTase) encoding gene *F-avi5813* in *Agrobacterium vitis* F2/5 was found to be required for the induction of a tobacco hypersensitive response (HR) and grape necrosis. Sfp-type PPTases are post-translation modification enzymes that activate acyl-carrier protein (ACP) domains in polyketide synthases (PKS) and peptidyl-carrier protein (PCP) domains of nonribosomal peptide synthases (NRPS). Mutagenesis of PKS and NRPS genes in *A. vitis* led to the identification of a PKS gene (*F-avi4330*) and NRPS gene (*F-avi3342*) that are both required for HR and necrosis. The gene immediately downstream of *F-avi4330* (*F-avi4329*) encoding a predicted aminotransferase was also found to be required for HR and necrosis. Regulation of *F-avi4330* and *F-avi3342* by quorum-sensing genes *avhR*, *aviR*, and *avsR* and by a *lysR*-type regulator, *lhnR*, was investigated. It was determined that *F-avi4330* expression is positively regulated by *avhR*, *aviR*, and *lhnR* and negatively regulated by *avsR*. *F-avi3342* was found to be positively regulated by *avhR*, *aviR*, and *avsR* and negatively regulated by *lhnR*. Our results suggest that a putative hybrid peptide-polyketide metabolite synthesized by *F-avi4330* and *F-avi3342* is associated with induction of tobacco HR and grape necrosis. This is the first report that demonstrates that NRPS and PKS play essential roles in conferring the unique ability of *A. vitis* to elicit a non-host-specific HR and host-specific necrosis.

Agrobacterium vitis is a host-specific plant pathogen that causes crown gall of grape affecting vine growth and production worldwide (Burr and Otten 1999). *A. vitis* possesses unique features compared with other *Agrobacterium* spp.; both tumorigenic and nontumorigenic strains cause a host- and tissue-specific necrosis on grape (Burr et al. 1987) and a hypersensitive response (HR) when infiltrated into leaf panels of nonhost plants such as tobacco (Herlache et al. 2001). Grape necrosis is most evident on certain tissues such as young grape

roots. Grape necrosis may play roles in survival of the bacterium in soil and in invasion into grapevines (Burr et al. 1987). HR has been shown to represent a defense response of plants against pathogen attack. Several HR elicitor proteins produced by plant-pathogenic bacteria have been identified and are generally delivered to plants via the type III secretion system (T3SS) (Büttner and He 2009). No *hrp* gene clusters or a T3SS have been found in the genome of *A. vitis* strain S4. Therefore, it appears that the HR and necrosis caused by *A. vitis* are T3SS independent.

We previously reported several genes in *A. vitis* strain F2/5 involved in HR and necrosis, including quorum-sensing-associated *luxR* homologs (*aviR*, *avhR*, and *avsR*), one *luxI* homolog (autoinducer synthase), *avsI* (Hao and Burr 2006; Hao et al. 2005; Zheng et al. 2003), and an *lysR*-type transcriptional regulator gene, *lhnR*, and an upstream operon, *lhnABC* (Zheng et al. 2012). In addition, a type I polyketide synthase (PKS) gene cluster was found to be associated with but not absolutely required for HR and necrosis (Carle 2008). To further elucidate the role of PKS and related genes in HR and necrosis, we initially investigated genes encoding phosphopantetheinyl transferases (PPTases). PPTases belong to a superfamily of post-translational modification enzymes that have been divided into two types based on gene structure and substrate specificity (Lambalot et al. 1996). The first are AcpS-type enzymes, consisting of 120 to 140 amino acids that are involved in fatty acid biosynthesis; and the second are Sfp-type enzymes, 220 to 240 amino acids in length, which participate in biosynthesis of secondary metabolites by activating the peptidyl-carrier protein (PCP) domain of the nonribosomal peptide synthetase (NRPS) and acyl carrier protein (ACP) domain of PKS (Lambalot et al. 1996; Walsh et al. 1997). In preliminary experiments, disruption of a gene encoding a putative Sfp-type PPTase resulted in HR- and necrosis-negative phenotypes, suggesting the presence of a PKS or NRPS required for these phenotypes. PKS and NRPS share architectural and organizational similarities and catalyze the biosynthesis of polyketides or nonribosomal peptides and peptide-polyketide hybrid molecules in bacteria and fungi. Polyketides and nonribosomal peptides are two large families of secondary metabolites with structural complexities that have diverse biological activities and pharmacological properties (Cane and Walsh 1999; Gokhale et al. 2007; Hertweck 2009; Schwarzer et al. 2003). Among the known polyketides and nonribosomal peptides, a number of them produced by phytopathogenic bacteria have phytotoxin, antibiotic activities, or both. For example, coronatine from *Pseudomonas*

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syringe, a non-host-specific phytotoxin, is an important virulence factor (Bender et al. 1999) consisting of the polyketide coronafacic acid and the cyclized amino acid coronamic acid, which are synthesized by PKS and NRPS individually and coupled into a hybrid polyketide-amino acid metabolite by a discrete ligase (Du et al. 2001). Another example, albicidin produced by the sugarcane leaf scald pathogen *Xanthomonas albilineans*, is a phytotoxin that blocks DNA replication in chloroplasts (Birch and Patil 1987) and shows antibiotic activity to a range of gram-positive and gram-negative bacteria (Birch and Patil 1985). Albicidin is synthesized by PKS-NRPS hybrid enzymes and a number of modifying enzymes (Huang et al. 2001; Royer et al. 2004). There are no reports of polyketide, nonribosomal peptide, or polyketide-peptide hybrid molecules or derivatives being involved in plant HR induction. From mining the genome sequence of *A. vitis* strain S4, we identified three PKS and two NRPS genes in chromosomes that were also found present in strain F2/5 by polymerase chain reaction (PCR) analyses.

In this article, we report the identification, organization, and regulation of genes encoding PPTase, PKS, and NRPS in *A. vitis* genome and elucidate their role in HR and necrosis induction.

RESULTS

An Sfp type PPTase in *A. vitis* is associated with induction of HR and grape necrosis.

Two PPTase gene homologs were identified in the *A. vitis* S4 genome. One (*avi9644*) resides on plasmid P130 and another (*avi5813*) on chromosome II. A PCR analysis indicated that only *avi5813* is present in strain F2/5 and was designated as *F-avi5813*; no sequence corresponding to *avi9644* was identified in F2/5. The organization of *F-avi5813* in F2/5 relative to surrounding genes is shown in Figure 1A, and is the same as

in S4. Its deduced sequence includes 244 amino acid residues having 97.5% sequence identity to *avi5813*. *F-avi5813* also shares identity with the PPTase gene (ZP_09284730) from *P. psychrotolerans* L19 (35.7%) and with EGH96826 from *P. syringae* pv. *lachrymans* M302278 (33.2%). Sequence identity between *F-avi5813* and PPTase (Atu_4416) in *A. tumefaciens* C58 is 30.8% and the identities between *F-avi5813* and those from other sequenced Rhizobiaceae are lower. Based on the gene size and sequence similarity, *F-avi5813* belongs to the Sfp-type PPTase group. Mutant of *F-avi5813* was generated by insertion of vector pVIK165 sequence through a single homologous recombination region comprising amino acid residues 24 to 162. Disruption of *F-avi5813* did not affect F2/5 growth in media; however, it resulted in the loss of capacity to cause tobacco HR and grape necrosis (Fig. 1B). Complementation of the *F-avi5813* mutant restored the HR and necrosis phenotypes (Fig. 1B). From these results, we predicted that *F-avi5813* is involved in biosynthesis of polyketides and nonribosomal peptides that are required for grape necrosis and tobacco HR.

Identity of PKS and NRPS genes and their effects on HR and necrosis.

Two NRPS and three PKS gene homologs were identified on chromosome I and chromosome II of strain S4. Based on PCR analyses, all five genes were also present in F2/5. Two of the PKS genes, *avhE* and *avhF*, (corresponding to *avi5624* and *avi5625*, residing within a gene cluster on chromosome II of S4) have been characterized previously by our lab (Carle et al. 2008) and found to be associated with but not absolutely required for HR and necrosis. Therefore, two NRPS genes (*F-avi5730* and *F-avi3342*) and one PKS gene (*F-avi4330*) in F2/5 were mutagenized by gene disruption. The *F-avi5730* (residing on chromosome II) mutant did not affect tobacco HR and grape necrosis

Fig. 1. A, Organization of *F-avi5813* phosphopantetheinyl transferase (PPTase) and flanking genes and **B**, tobacco hypersensitive response (HR) and necrosis of grape shoot explants caused by F2/5, mutant $\Delta F-avi5813$, and complemented mutant $\Delta F-avi5813^+$.

whereas *F-avi3342* and *F-avi4330*, both residing on chromosome I, were required for expression these phenotypes.

F-avi3342 comprises 4,005 bp and encodes a polypeptide with 1,334 amino acid residues. The deduced sequence contains condensation (C), adenylation (A), PCP, and thio-esterase domains in a single module of NRPS (Fig. 2A). It shares 99% identity with *avi3342* from S4 and is similar (61% identity) to the NRPS gene (Arad_8972) from *A. radiobacter* K84, and 46% with YP_693504 from *Alcanivorax borkumensis*. The A domain of an NRPS is responsible for recognition of a specific substrate (amino acid or hydroxyl acid) and activates it as its aminoacyl adenylate. The specificity-conferring amino acid residues (signature) of the A domain in NRPS have been identified based on the analysis of crystal structure of gramicidin S synthetase-1 (GrsA) (Conti et al. 1997). General rules for deducing substrate specificity of the A domain in an NRPS have been developed by comparing the signature with GrsA (Stachelhaus et al. 1999). The signature of *F-avi3342* has been identified and the A domain is predicted to be glycine activat-

ing according to signature comparison with other glycine-activating NRPS (Fig. 2B). *F-avi3342* was mutagenized by interruption of the sequence encoding the 134th to 334th amino acid residues. The mutant does not induce tobacco HR and grape necrosis (Fig. 2C). Therefore, *F-avi3342* encodes a putative NRPS that is required for tobacco HR and grape necrosis.

The organization of *F-avi4330* and flanking genes is shown in Figure 3A. *F-avi4330*, which shares 98% identity with *avi4330* from S4, is a predicted PKS gene that consists of 7,572 bp and encodes a polypeptide of 2,523 amino acid residues. It contains keto-synthase, acyltransferase (AT), dehydratase (DH), enoylreductase, ketoreductase, and ACP domains. The two immediate downstream genes, *F-avi4329* and *F-avi4328*, that are co-transcribed as a operon according to reverse-transcription PCR analysis (results not shown), encode a putative aminotransferase protein and a short-chain dehydrogenase protein, respectively according to genome annotation. *F-avi4330*, *F-avi4329*, and *F-avi4328* are similar in sequence and structure to *rkpA*, *rkpG*, and *rkpH*, respectively, in the *rkp-1*

Fig. 2. **A**, Organization of *F-avi3342* and flanking genes; **B**, comparison of A-domain signature and prediction of amino acid activated by *F-avi3342*; and **C**, tobacco hypersensitive response (HR) and necrosis of grape shoot explants caused by F2/5, mutant $\Delta F-avi3342$, and complemented mutant $\Delta F-avi3342^+$.

region of *Sinorhizobium fredii* HH103 and *S. meliloti* 1021 (Fig. 3A). The similarities of deduced sequences between *F-avi4330*, *F-avi4329*, and *F-avi4328* and *rkpA*, *rkpG*, and *rkpH* are 58, 63, and 49%, respectively. To generate the *F-avi4330* mutant, vector pVIK165 was inserted at the coding region of the 69th to 311th amino acid residues, and the flanking genes *F-avi4331*, *F-avi4329*, and *F-avi4328* were also individually mutated and tested for ability to cause tobacco HR and grape necrosis. The *F-avi4331* and *F-avi4328* mutants did not affect either phenotype whereas the *F-avi4330* and *F-avi4329* mutants were HR and necrosis minus (Fig. 3B). The *F-avi4329* mutant was complemented (Fig. 3C). These results indicate

that *F-avi4330*, a PKS gene, and its immediate downstream *F-avi4329*, an aminotransferase-encoding gene, are required for induction of tobacco HR and grape necrosis.

Regulation of *F-avi4330* and *F-avi3342* gene expression.

We previously reported that *luxR* homologs *aviR*, *avhR*, and *avsR* and an *lysR*-type transcriptional regulator gene, *lhmR*, in F2/5 are involved in regulation of HR and necrosis (Hao and Burr 2006; Hao et al. 2005; Zheng et al. 2003, 2012). To determine whether these genes were involved in regulation of *F-avi4330* and *F-avi3342*, the promoter regions of *F-avi4330* and *F-avi3342* were amplified and cloned into the promoter-

Fig. 3. A, Organization of *F-avi4330* locus in F2/5 compared with *rkp-1* loci in *Sinorhizobium fredii* HH103/S. *meliloti* 1021 (S.f./S.m.). B, Tobacco hypersensitive response (HR) and necrosis of grape shoot explants caused by F2/5 and mutants $\Delta F-avi4331$, $\Delta F-avi4330$, $\Delta F-avi4329$, and $\Delta F-avi4328$. C, Tobacco HR and necrosis of grape shoot explants caused by F2/5, mutant $\Delta F-avi4329$, and complemented mutant $\Delta F-avi4329^+$.

probe vector, pPROBE-AT (pAT) (Miller et al. 2000) to create *F-avi3342* promoter-green fluorescent protein (*gfp*) fusion (pAT-P3342-*gfp*) and *F-avi4330* promoter-*gfp* fusion (pAT-P4330-*gfp*) constructs (Fig. 4). The constructs were individually transformed into F2/5 and into the *aviR*, *avhR*, *avsR*, and *lhnR* mutants. Fluorescence levels for the *avhR* mutant carrying pAT-P3342-*gfp* or pAT-P4330-*gfp* were approximately 25 and 9% less, respectively, than those expressed in F2/5. Levels in the *aviR* mutant were approximately 1,120% (11.2-fold) and 530% (5.3-fold) less than those in F2/5 (Fig. 4). Interestingly, the fluorescence level in the *lhnR* mutant carrying pAT-P3342-*gfp* was 160% (1.6-fold) greater than that in F2/5, in contrast to the *avsR* mutant, where fluorescence was only 15% that of F2/5 (Fig. 4). Conversely, fluorescence in the *lhnR* mutant harboring pAT-P4330-*gfp* was 38% of that in F2/5 whereas, in the *avsR* mutant, the value was 150% (1.5-fold) greater than F2/5 (Fig. 4). The differences of *gfp* expression between mutants and F2/5 reflect that *avhR* and *aviR* upregulate *F-avi3342* and *F-avi4330*, with the regulation activity of *aviR* being much stronger than that of *avhR*. It also indicates that *lhnR* downregulates expression of *F-avi3342* and upregulates *F-avi4330* whereas *avsR* upregulates *F-avi3342* and downregulates *F-avi4330*.

Sequence analyses of *F-avi3342* and *F-avi4330* promoter regions identified putative recognition binding sites (RBS) characteristic of genes regulated by *lysR*-type transcriptional regulators (LTTR). The RBS in the promoter region of *F-*

avi3342 has a partial dyad symmetry, ATTGT-N₁₁-ACAAT, containing a T-N₁₁-A motif. It is located at position -180 from the start codon ATG, and a repeat of the upstream arm of the dyad symmetry and two such repeats occur downstream of the RBS (Fig. 5A). The putative RBS in the promoter region of *F-avi4330* has a partial dyad symmetry, TCTGA-N7-TCAGA, containing a T-N₁₁-A motif present at -32 from the start codon ATG, and a repeat of the upstream arm of the dyad symmetry is found upstream of the RBS (Fig. 5B).

To further evaluate regulation of *F-avi3342* and *F-avi4330* by *lhnR*, the promoter-*gfp* fusion constructs, pAT-P3342-*gfp* and pAT-P4330-*gfp*, were individually introduced into *Escherichia coli* with or without *plhnR* and, subsequently, *gfp* expression was measured. The results were consistent with those obtained in F2/5 and F2/5 mutants (Fig. 5C), confirming that *lhnR* downregulates expression of *F-avi3342* and upregulates *F-avi4330*.

Effects of killed *Agrobacterium vitis* cell suspensions and culture supernatants on tobacco HR and grape necrosis.

When F2/5 cell suspensions were incubated at 65°C for 15 min, incubated with kanamycin (50 µg/ml) or carbenicillin (100 µg/ml) at 28°C for 1 h, or centrifuged to remove the cells, the treated cells and culture supernatants were not able to cause HR and necrosis (Table 1). Although cell-free F2/5 supernatant did not cause tobacco HR, when F2/5 cells were suspended in

Fig. 4. Descriptions of constructs of *F-avi3342* and *F-avi4330* promoter-green fluorescent protein (*gfp*) fusion. Fluorescence measurements of *Agrobacterium vitis* F2/5, *avhR* mutant (M1320), *aviR* mutant (M1154), *lhnR* mutant (M901), and *avsR* mutant (Δ *avsR*) carrying pAT-P3342-*gfp* or pAT-P4330-*gfp*, respectively.

F2/5 cell-free supernatant, fewer cells were required to cause HR than when suspended in water (Table 2). In contrast, cell-free supernatants of *F-avi3342*, *F-avi4330*, *F-avi4329*, and *F-avi5813* did not affect the numbers of F2/5 cells required to cause HR, and F2/5 culture supernatant was not able to complement the mutants for tobacco HR (Table 2). These results suggest that a secreted compound in supernatant is associated with the ability of F2/5 to cause the HR.

DISCUSSION

We previously reported three quorum-sensing-associated, *luxR* homologs and an LTTR member, *lhnR*, and its immediate upstream operon that were involved in regulation of grape

necrosis and tobacco HR (Hao and Burr 2006; Hao et al. 2005; Zheng et al. 2003, 2012). In this study, we identified *F-avi5813*, an Sfp-type PPTase-encoding gene in F2/5 that is required for induction of tobacco HR and grape necrosis. Sfp-type PPTases are known to function in secondary metabolism through modification of NRPS and PKS (Lambalot et al. 1996; Walsh et al. 1997). Therefore, screening and characterization of NRPS and PKS genes present in chromosomes of *A. vitis* were performed and have led to identification of an NRPS (*F-avi3342*) and a PKS (*F-4330*) that are essential for tobacco HR and grape necrosis. Disruption of either *F-avi3342* or *F-avi4330* resulted in HR- and necrosis-negative phenotypes. This suggests that both *F-avi3342* and *F-avi4330* act on a pathway for biosynthesis of a compound responsible for HR and necrosis. Based on the

A

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tacggggttattgcaagtcggacactttacaaacgtgccgtgtgctgttttcattgtctcacggtt
tcaggccttggtgctgctgatttgtatatagtttgtgaattttatagcaaaatctgtgttctttta

      T      -11N-      A      -180
ttcgtgattgtatcgttttgcgacaatttgggttttattgctttgtatttattctaatttgtgttg
agcagttgaattcatgacatgctgatgtgctttattttgagtggcagttagtattttgcgtttcc
tagcatcgtaatcgtttgattgtgcttttggattgttttctggttttggccgatatttcag
tgagggtttttcaATG → F-avi3342
```

B

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gattagatttgcagcgcagaccgggggtttgcgctgtcacgaatcgtatcgtcctgttgggaa
tgtttgttcagggtgggtgttgataccgacattgcaatttggcgggttcctttcaaagggtctaaa
accgccatccgttttgtcaagaaacgacgaaatcgtccatgaggacaaaggcacgaaccgtatc
ggataacctgctcattttaagaggaaattcgaaacgggcatgttgggttttcgctctcatatccg
gcaggatagaagtgggtttgcggggcgggtttctctctggtgccacgtctcatcagggttaaaaa
atcgttctacgccataagtttccggcggttttctttcatgaggcgaggcctgcctatgtgaat
cctgattgacgccctatgtcgcttctctaagtgaaggacaattatacttgaggaca

      T      -11N-      A      -32
tctgaaaggctatcagacctcgctaaactgttgagatccgtacgaatagATG → F-avi4330
```

C

Fig. 5. DNA sequences of **A**, *F-avi3342* and **B**, *F-avi4330* promoter regions. The putative recognition binding site is boxed and T-N₁₁-A motif is shown above the box. Two arms of a dyad symmetry element and repeats of the upstream arm of the dyad are indicated with horizontal arrows. **C**, Fluorescence measurement of *Escherichia coli* carrying *F-avi3342* or *F-avi4330* promoter-*gfp* fusion construct with or without pPZP-*lhnR* (*plhnR*).

may be similar to cyclosporin. We predict that the polyketide intermediate is first synthesized from a starter unit by catalysis of the *F-avi4330*-encoded PKS and then converted into an amino acid by the *F-avi4329*-encoded aminotransferase (Fig. 6). The specific amino acid is subsequently incorporated into the product by the *F-avi3342*-encoded NRPS. The ACP in PKS (*F-avi4330*) and PCP in NRPS (*F-avi3342*) are activated by PPTase *F-avi5813* (Fig. 6). Verification of the hybrid polyketide-peptide compound synthesized by *F-avi3342* and *F-avi4330* remains to be determined by biochemical analysis, and whether there are genes encoding tailoring enzymes involv-

ing this compound biosynthesis also remain to be determined. A homolog of *F-avi3342* occurs in *A. radiobacter* K84 and the locus of *F-avi4330* exists within the *rkp-1* region of *Sinorhizobium* spp., which is required for production of capsular polysaccharide in *S. fredii* HH103 and *S. meliloti* 1021 (Hidalgo et al. 2010; Müller et al. 2009, Parada et al. 2006). However, homologs of *F-avi3342* and *F-avi4330* together in the same genome have not been reported for other Rhizobiaceae spp. Production of such a hybrid PK-NRP molecule may explain why *A. vitis* has the unique ability among Rhizobiaceae spp. investigated thus far to cause HR and grape necrosis.

Table 3. Bacterial strains and plasmids used in this study

Bacterial strains	Relevant characteristics ^a	Reference
<i>Agrobacterium vitis</i>		
F2/5	Wild type	Staphorst et al. 1985
M1154	F2/5 <i>aviR</i> ::Tn5, Km ^r	Zheng et al. 2003
M1320	F2/5 <i>avhR</i> ::Tn5, Km ^r	Hao et al. 2005
M901	F2/5 <i>lhnR</i> ::Tn5, Km ^r	Harlache et al. 2001
<i>ΔavsR</i>	F2/5 <i>avsR</i> ::pPVIK165, Km ^r	Hao and Burr 2006
<i>ΔF-avi5813</i>	<i>F-avi5813</i> ::pPVIK165, Km ^r	This work
<i>ΔF-avi5813</i> ⁺	<i>ΔF-avi5813</i> carrying pPZP-5813, Km ^r , Sp ^r	This work
<i>ΔF-avi3342</i>	<i>F-avi3342</i> ::pPVIK165, Km ^r	This work
<i>ΔF-avi4330</i>	<i>F-avi4330</i> ::pPVIK165, Km ^r	This work
<i>ΔF-avi4329</i>	<i>F-avi4329</i> ::pPVIK165, Km ^r	This work
<i>ΔF-avi4329</i> ⁺	<i>ΔF-avi4329</i> carrying pWSK-4329, Km ^r , Sp ^r	This work
<i>ΔF-avi5730</i>	<i>F-avi5730</i> ::pPVIK165, Km ^r	This work
<i>Escherichia coli</i>		
DH5α	See source	Invitrogen
JM109	See source	Promega Corp.
S17-1/λpir	RK2 <i>tra</i> regulon, <i>pir</i> , host for <i>pir</i> -dependent plasmids	Simon et al. 1983
Plasmids		
pPROBE-AT	Broad host range vector, Cb ^r	Miller et al. 2000
pPZP201	Broad-host range cloning vector, Sp ^r	Hajdukiewicz et al. 1994
<i>p/lhnR</i>	pPZP201 carrying <i>lhnR</i> , Sp ^r	Zheng et al. 2012
pVIK165	Suicide-vector, Km ^r	Kalogeraki and Winans 1997
pWSK29	Low-copy-number vector, Cb ^r	Wang and Kushner 1991
pPZP-5813	pPZP201 carrying <i>avi5813</i> , Sp ^r	This work
pWSK-4329	pWSK-29 carrying <i>F-avi4329</i> , Cb ^r	This work
pAT-P3342	pPROBE-AT carrying <i>F-avi3342</i> promoter, Cb ^r	This work
pAT-P4330	pPROBE-AT carrying <i>F-avi4330</i> promoter, Cb ^r	This work

^a Km^r, Sp^r, and Cb^r indicate kanamycin, spectinomycin, and carbenicillin resistance, respectively.

Table 4. Primers used in this study

Gene	Primer	Primer sequence ^a
For mutagenesis		
<i>F-avi3342</i>	F-avi3342-F:	5'-ATGAGCTCTCTGGTATCATTGGGCACATC-3'
	F-avi3342-R:	5'-AGTCTAGAGAATGTCCTCATAGCGATAGC-3'
<i>F-avi4328</i>	F-avi4328-F:	5'-ACGAGCTCCTCGTTGTGCGTGTGAGAC-3'
	F-avi4328-R:	5'-AGTCTAGATGACATTGATGTGCGAGTTGCTG-3'
<i>F-avi4329</i>	F-avi4329-F:	5'-ACGAGCTCAGCTCGTCGACGAGCAGCTG-3'
	F-avi4329-R:	5'-AGTCTAGATGAACCTCGTCATGGATCACCAG-3'
<i>F-avi4330</i>	F-avi4330-F:	5'-ATGAGCTCTGTGATGGATGGAATCTATCAG-3'
	F-avi4330-R:	5'-AGTCTAGATCATAGACGGCATGCAGCAGAAC-3'
<i>F-avi4331</i>	F-avi4331-F:	5'-ACGAGCTCGTCTGATGATCCTCGTTCTG-3'
	F-avi4331-R:	5'-AGTCTAGAACTGTCTTTCTGTCTCGAC-3'
<i>F-avi5730</i>	F-avi5730-F:	5'-ATGAGCTCTTGTCTGATGCAGCACATTG-3'
	F-avi5730-R:	5'-AGTCTAGATCGATCATTGCATCACTATGG-3'
<i>F-avi5813</i>	F-avi5813-F:	5'-AAGAATTCCTTGATCTCGCATCGCGCTG-3'
	F-avi5813-R:	5'-AATCTAGATGCGCTGTCTATGACCTGTTCTG-3'
Vector	pVIK165-R:	5'-TACTCATCTTTGTTTCTCC-3'
For complementation		
<i>F-avi4329</i>	F-avi4329-FF:	5'-AGAAGCTTTGGTTCTTGCTATCCGTTACAC-3'
	F-avi4329-RR:	5'-ACGAGCTCAGTTCAGGTGCTCAAGGACGAG-3'
<i>F-avi5813</i>	F-avi5813-FF:	5'-ACAAGCTTGGTTCTTGCTATCCGTTACAC-3'
	F-avi5813-RR:	5'-AAGAATTCGCTAAATTATAACGCAGCCTCATC-3'
For promoter- <i>gfp</i> fusion		
<i>F-avi3342</i>	P3342-F:	5'-ACGAGCTCACGGGTTATTGCAAGTCGGTC-3'
<i>F-avi4330</i>	P4330-F:	5'-AGAAGCTTGATTAGATTTGCAAGCGCAGAC-3'
	P4330-R:	5'-ATGAGCTCGATCTCAACAGTTTAGCGAG-3'

^a Restriction sites are underlined.

Promoter-*gfp* fusions were used to determine that expression of *F-avi3342* and *F-avi4330* are regulated by *avhR*, *aviR*, *avsR*, and *lhnR* in different ways (Fig. 4). Both *avhR* and *aviR* upregulate expression of *F-avi3342* and *F-avi4330* whereas *lhnR* downregulates *F-avi3342* and upregulates *F-4330*. In contrast to *lhnR*, *avsR* upregulates *F-avi3342* but downregulates *F-4330* (Fig. 4). These results indicate that the expression levels of *F-avi3342* and *F-avi4330* affect tobacco HR and grape necrosis and transcriptional regulators *avhR*, *aviR*, *avsR*, and *lhnR* play essential roles in regulation of *F-avi3342* or *F-avi4330*. The results also suggest that expression levels of *F-avi3342* and *F-avi4330* may be correlated with the production of the putative hybrid polyketide-peptide metabolite and subsequent induction of HR and necrosis. In other bacteria, it has also been shown that LuxR and LysR-type transcriptional factors regulate polyketide or nonribosomal peptide biosynthesis. For example, production of enacyloxins, the antimicrobial compounds synthesized by a hybrid modular PKS gene cluster in cryptic *Burkholderia ambifaria*, are positively regulated by two LuxR-type regulators (Mahenthiralingam et al. 2011). In addition, SylA, a member of the LuxR family, positively regulates biosynthesis of syringolin A, a phytotoxin synthesized by a mixed nonribosomal peptide/polyketide synthetase in *Pseudomonas syringae* pv. *syringae* (Ramel et al. 2012); and the *pltR*, a putative LysR-type regulator, positively regulates biosynthesis of pyoluteorin, an antifungal polyketide, in *Pseudomonas* sp. M18 (Yan et al. 2007). An RBS of LTTR exists in the promoter region of *F-avi3342* at –180 bp from the start codon and another RBS in the promoter region of *F-avi4330* at –32 bp from its start codon (Fig. 5A and B). Co-transformation of promoter-*gfp* fusion constructs and *lhnR* into *E. coli* confirmed that *lhnR* suppresses *F-avi3342* and enhances *F-avi4330* expression (Fig. 5C). These results suggest that *lhnR* directly targets promoters of *F-avi3342* and *F-avi4330* by binding to their RBS and that regulation by *LhnR* may depend on the location of an RBS in the promoter of the target gene. No motifs indicative of lux boxes were found in the *F-avi3342* and *F-avi4330* promoters; therefore, how *avhR*, *aviR*, and *avsR* proteins interact with *F-avi3342* and *F-avi4330* remains to be determined.

We now predict that a putative polyketide-peptide hybrid compound is produced by *A. vitis* and appears to be acting as a phytotoxin associated with grape (host) necrosis and induction of tobacco (nonhost) HR. Living cells of *A. vitis* are essential for induction of tobacco HR and grape necrosis whereas cells killed using various methods and culture supernatants were unable to do so (Table 1). Promoter-*gfp* fusion analyses and reverse-transcription PCR (results not shown) indicated that the related biosynthetic genes were transcribed under in vitro conditions even though we obtained no direct evidence that a specific elicitor molecule was secreted in culture media. This scenario is similar to colibactin, a genotoxin produced by certain strains of *E. coli*. Colibactin is a polyketide-peptide hybrid compound that causes double-strand DNA breaks (Nougayrède et al. 2006). The genes responsible for colibactin biosynthesis were discovered in the *pks* island and were transcribed under in vitro conditions. The cytopathic activity of colibactin is contact dependent and the killed bacteria, bacterial culture supernatants, and bacterial lysates were not cytopathic (Nougayrède et al. 2006). In our experiments, it was found that bacterial cultural supernatant enhances biological activity of F2/5 (i.e., fewer cells are required to induce HR when suspended in the supernatant) (Table 2). This may indicate that a level of the putative hybrid polyketide-peptide compound is produced in vitro but is too low to induce the plant response. It may also suggest that the production of the specific elicitor molecule is enhanced by stimuli of plant signal molecules when *A. vitis* is in contact with plant cells.

It has been reported that production of some phytotoxins requires plant signal molecules. For example, synthesis of the phytotoxin syringomycin by *P. syringae* pv. *syringae* required plant signal molecules arbutin and D-fructose for activation of biosynthetic gene *syrB* (Mo et al. 1995; Qingley and Gross 1994; Wang et al. 2006). Further research is needed to determine whether plant signals are involved in production of the putative hybrid polyketide-peptide by *A. vitis*.

MATERIALS AND METHODS

Bacterial stains, media, and plasmids.

Bacterial strains used in this study are listed in Table 3. *A. vitis* strain F2/5 is nontumorigenic and was used previously in our laboratory to study HR and grape necrosis (Herlache et al. 2001). F2/5 was propagated on potato dextrose agar (PDA) or in potato dextrose broth (Difco Laboratories, Detroit) at 28°C. Appropriate antibiotics were added to the media for culturing mutants. *E. coli* were cultured on Luria-Bertani (LB) medium with appropriate antibiotics at 37°C.

Sequence analyses.

Genes targeted for mutation and DNA fragments amplified from F2/5 were cloned into vector pGEM-T and were sequenced at The Cornell University Life Sciences Core Laboratories Center. Genes in F2/5 that correspond to those in S4 were designated as F followed by the gene ID number of S4. DNA sequence homologies were evaluated with the Basic Local Alignment Search Tool algorithm provided by the National Center for Biotechnology Information. Analyses of DNA and deduced protein sequences were carried out with DNASTAR software.

Mutagenesis by targeted gene disruption.

Specific mutants were generated through disruption with suicide vector pVIK165 (Kalogeraki and Winans 1997). Internal fragments of target genes were amplified from F2/5 genomic DNA via PCR employing primers listed in Table 4. Restriction sites were introduced into forward and reverse primers. PCR products were purified, digested, and then ligated into suicide vector pVIK165 (Kalogeraki and Winans 1997) at corresponding restriction sites. The constructs were transformed into *E. coli* strain S17-1/ λ pir and selected on LB agar plus kanamycin (50 μ g/ml). Constructs were transferred to F2/5 via conjugal mating, and targeted gene disruptions by insertion of vector pVIK165 sequence occurred following a single homologous recombination. The resulting mutants contained two truncated copies of the target gene separated by the inserted plasmid. Mutants were selected on AB minimal medium (Chilton et al. 1974) amended with kanamycin (50 μ g/ml) and 10% mannitol. Mutations were verified by PCR using primers derived from sequences of the F2/5 and from the pVIK165 vector (Table 4). Mutants were tested for their ability to cause the tobacco HR and grape necrosis.

Complementation of mutants.

Complementation of the $\Delta F-avi5813$ and $\Delta F-avi4329$ mutants were performed using methods previously described (Hao et al. 2005). Full-length targeted sequences including their promoter regions were amplified from F2/5 genomic DNA; the PCR product of *F-avi5813* was cloned into a broad-host-range vector, pPZP201 (Hajdukiewicz et al. 1994), and PCR product of *F-avi4329* was cloned into a low-copy-number vector, pWSK29 (Wang and Kushner 1991). The plasmid constructs were transformed into DH5 α -competent cells. Positive colonies were selected on LB agar containing spectinomycin (100 μ g/ml). The recombinant plasmids

that were designated as pPZP-5813 and pWSK-4329 were verified by restriction analyses and sequencing. The pPZP-5813 and pWSK-4329 were conjugated into ΔF -*avi5813* and ΔF -*avi4329*, respectively, by conjugation. Transconjugants were selected on AB (plus 10% mannitol) minimal medium containing spectinomycin (400 µg/ml) and kanamycin (100 µg/ml) and verified by PCR. Expression of the recombinant *F-avi5813* and *F-avi4329* in the complementation constructs are driven by their native promoters. Complemented ΔF -*avi5813* and ΔF -*avi4329* were tested for their ability to cause tobacco HR and grape necrosis.

Construction and assay of *F-avi3342* and *F-avi4330* promoter-*gfp* fusions.

As indicated previously, three quorum-sensing regulatory genes and an *lysR*-type regulatory gene were shown to be associated with HR and necrosis. To determine whether expression of *F-avi3342* and *F-avi4330* was regulated by these genes, promoter-*gfp* fusions were initially constructed. The promoter regions of *F-avi3342* (337 bp) and *F-avi4430* (479 bp) were amplified from F2/5 genomic DNA and cloned into the promoter probe vector, pPROBE-AT (Miller et al. 2000), to create *F-avi3342* promoter-*gfp* fusion (pAT-P3342-*gfp*) and *F-avi4330* promoter-*gfp* fusion (pAT-P4330-*gfp*). Both of the resulting constructs are transcriptional fusions. The constructs were introduced into F2/5 and mutants M1320 (Tn5 mutant of *avhR*), M1154 (Tn5 mutant of *aviR*), M901 (Tn5 mutant of *lhnR*), and Δ *avsR*. The promoter-*gfp* fusion constructs were also introduced into *E. coli* strain JM109 with or without co-transformation with *plhnR* (Table 3) to further elucidate regulation of *F-avi3342* and *F-avi4330* by *lhnR*. *A. vitis* strains were grown on PDA amended with kanamycin (50 µg/ml) and carbenicillin (100 µg/ml) at 28°C for 3 days and *E. coli* on LB agar with kanamycin (50 µg/ml) and carbenicillin (100 µg/ml) at 37°C overnight. Subsequently, bacterial cells containing promoter-*gfp* fusion constructs were suspended in water and cell concentrations were adjusted to an optical density at 600 nm (OD₆₀₀) of 0.5 (approximately 5 × 10⁸ CFU/ml). Then, 200 µl of the cell suspensions were transferred to black 96-well Cliniplates (Labsystems, Franklin, MA, U.S.A) and fluorescence assays were performed using a Multi-mode Microplate reader Synergy 2 (Biotek Instruments Inc., Winooski, VT, U.S.A.). Fluorescence was measured with an FITC filter set (excitation of 485 ± 20 and emission of 508 ± 20) with a sensitivity of 55 on a scale of 100. Fluorescence data were analyzed using the Gene5 Data Analysis Software (v1.06; Biotek Instruments Inc.). Fluorescence readings of F2/5 or JM109 with empty vector pPROBE-AT were set as background. The *avsR* mutant was generated employing suicide vector pVIK165, which also contains a *gfp*. Therefore, to determine *gfp* expression driven by the *F-avi3342* or *F-avi4430* promoter in Δ *avsR*, fluorescence of Δ *avsR* not carrying the promoter-*gfp* fusion constructs was measured as background. Readings were subtracted from background and at least three independent experiments were done with 12 replicates each that were averaged.

Tobacco HR and grape necrosis assay.

HR was determined by infiltration of tobacco leaves with *A. vitis* cell suspensions, as previously described (Herlache et al. 2001). Necrosis assays were done on young grape shoot explants (Herlache et al. 2001). Shoots were cut into 0.5-cm sections and supported on end in water agar (2%) with basal end up. Cell suspensions (5 µl) of F2/5 and F2/5 mutants were placed on the basal end of each explant and necrosis was observed 5 to 7 days after inoculation.

Effects of killed *A. vitis* cell suspensions and culture supernatants on tobacco HR and grape necrosis.

F2/5 was grown on PDA plates at 28°C for 2 days and then cells were suspended in water by vortexing. Cell densities were adjusted at OD₆₀₀ to 2.0 and suspensions were either i) heated at 65°C for 15 min, ii) treated with kanamycin (50 µg/ml) or carbenicillin (100 µg/ml) at 28°C for 1 h with shaking (200 rpm), or iii) centrifuged, and the supernatants were filtered through a 0.25-µm filter. The treated suspensions were compared with an untreated suspension of F2/5 for ability to cause HR and necrosis.

In addition, the effects of mutant and F2/5 cell-free supernatants on the concentrations of F2/5 cells required to cause HR was determined. F2/5 cells grown on PDA plates were suspended in water or in the cell-free supernatants and adjusted to different ODs. The supernatant was prepared as mentioned above. The suspensions were infiltrated into tobacco leaves to determine ability to cause HR.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Virginia Tech and Virginia Bioinformatics Institute's *A. vitis* genome: agro.vbi.vt.edu/public