Inhibition of Grape Crown Gall by *Agrobacterium vitis* F2/5 Requires Two Nonribosomal Peptide Synthetases and One Polyketide Synthase

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Agrobacterium vitis nontumorigenic strain F2/5 is able to inhibit crown gall disease on grapevines. The mechanism of grape tumor inhibition (GTI) by F2/5 has not been fully determined. In this study, we demonstrate that two nonribosomal peptide synthetase (NRPS) genes (*F-avi3342* and *F-avi5730*) and one polyketide synthase gene (*F-avi4330*) are required for GTI. Knockout of any one of them resulted in F2/5 losing GTI capacity. We previously reported that *F-avi3342* and *F-avi4330* but not *F-avi5730* are required for induction of grape tissue necrosis and tobacco hypersensitive response. *F-avi5730* is predicted to encode a single modular NRPS. It is located in a cluster that is homologous to the siderophore vicibactin biosynthesis locus in *Rhizobium* species. Individual disruption of *F-avi5730* and two immediate downstream genes, *F-avi5731* and *F-avi5732*, all resulted in reduced siderophore production; however, only *F-avi5730* was found to be required for GTI. Complemented *F-avi5730* mutant ($\Delta F-avi5730^+$) restored a wild-type level of GTI activity. It was determined that, over time, populations of $\Delta F-avi4330$, $\Delta F-avi3342$, and $\Delta F-avi5730$ at inoculated wound sites on grapevine did not differ from those of $\Delta F-avi5730^+$ indicating that loss of GTI was not due to reduced colonization of wound sites by mutants.

Crown gall caused by *Agrobacterium vitis* seriously affects grape production in several regions of the world (Burr and Otten 1999). Although the disease can be partially managed through varietal selection and cultural practices, there are no effective chemical or biological controls. On other crops, such as stone fruits, crown gall caused by *A. tumefaciens* has been successfully controlled by application of *A. radiobacter* K84 and its genetic derivative K1026 (Farrand 1990). However, K84 and K1026 are not effective for control of grape crown gall caused by *A. vitis*. Consequently, research has led to evaluation of other candidate *Agrobacterium* biological control strains, including F2/5 (originally isolated in South Africa by Staphorst et al. [1985]), E26 (Liang et al. 1990), VAR03-1 (Kawaguchi et al. 2008), and ARK1 (Kawaguchi 2013; Kawaguchi and Inoue 2012). In general, the strains provide varying levels of crown gall suppression in greenhouse experiments, they have not been studied extensively and their mechanisms of tumor inhibition are unknown. Bacterial strains of other species, including *Rahnella*

aquaticus HX2 (Chen et al. 2007), *Serratia plymuthica* IC1270, *Pseudomonas fluorescens* Q8r1-96, *P. fluorescens* B-4117 (Dandurishvili et al. 2011), and *Bacillus subtilis* 14B (Hammami et al. 2009) have also been shown to provide various levels of tumor inhibition (Eastwell et al. 2006).

Our laboratory has focused research on the mechanism associated with grape tumor inhibition (GTI) conferred by nontumorigenic *A. vitis* F2/5. We determined that F2/5 is able to inhibit tumors caused by strains of *A. vitis* on grape but not on other plant species, such as tobacco, and that GTI activity is dependent on the timing of application of F2/5 to grape wounds in relation to tumorigenic strain inoculation; optimum tumor inhibition was achieved by applying F2/5 at same time or prior to tumorigenic strains (Burr and Reid 1994; Kaewnum et al. 2013). In these same studies, it was shown that optimum GTI requires concentrations of F2/5 at wound sites to be equal to or greater than populations of tumorigenic strains. We reported that GTI required living cells and was not associated with antibiosis; heat-killed or sonication-lysed cell preparations lost GTI function, whereas antibiosis-minus mutants of F2/5 retained GTI as well as wild type (Burr et al. 1997). In addition, it was demonstrated that GTI by F2/5 does not appear to result from inhibition of attachment of tumorigenic *A. vitis* strains at grape wounds (Burr et al. 1997). Most recently, we found that F2/5 does not inhibit growth of the pathogen in wounded grape tissue but inhibits transformation by tumorigenic *A. vitis* strains on grape (Kaewnum et al. 2013).

We have identified specific regulatory genes in F2/5 that are involved in expression of GTI, including caseinolytic protease (*clp*) genes *clpA* (*F-avi2537*) and *clpP1* (*F-avi1696*) and the quorum-sensing transcription factor gene *aviR* (*F-avi4374*). Mutations of *clpA* and *clpP* resulted in complete loss of GTI, whereas the *aviR* mutant expressed reduction of GTI activity (Kaewnum et al. 2013). Several other quorum-sensing related genes including *avsR*, *avsI*, and *avhR* are associated with tobacco hypersensitive response (HR) and grape necrosis phenotypes (Hao and Burr 2006; Zheng and Burr 2013) but not with GTI (Kaewnum et al. 2013). The one or more genes specifically regulated by ClpAP1 have not been identified; however, two genes, *F-avi3342* and *F-avi4330*, were found to be positively regulated by *aviR* (Zheng and Burr 2013). *F-avi3342* encodes a putative nonribosomal peptide synthetase (NRPS), whereas *F-avi4330* encodes a putative polyketide synthase (PKS). These results indicated that NRPS and PKS may be associated with GTI. To investigate this hypothesis, several NRPS and PKS genes in F2/5 were tested for their association with GTI. PKSs and NRPSs in bacteria and fungi catalyze the biosynthesis of polyketides and nonribosomal peptides, two large families of

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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). Some compounds synthesized by PKS or NRPS (or both) were reported to be involved with biological control activity in a number of bacteria. For example, the polyketide diffidin produced by *Bacillus amyloliquefaciens* (Chen et al. 2009) and an antimicrobial compound synthesized by catalysis of NRPS in *Pseudomonas fluorescens* (Kremmydas et al. 2013) were shown to be associated with suppression of pathogens.

The results of our research, together with the properties of bacterial NRPS and PKS, lead us to focus on characterization of these two types of genes in this study. Here, we present evidence that NRPS and PKS in F2/5 are involved in GTI.

RESULTS

PKS and NRPS genes are required for GTI.

The association of PKS and NRPS genes in F2/5 on GTI was determined by identifying them within the genome, making individual mutants and testing them for GTI (Table 1). Two plasmid genes, *F-avi7306* (NRPS) and *F-avi7309* (PKS), are located in a gene cluster homologous to the ATS siderophore

biosynthesis locus in *A. tumefaciens* C58 (Rondon et al. 2004). Two other plasmid PKS genes, P-2476 and P-2477, were not detected in strain S4 or within several strains of *A. vitis*, as determined by polymerase chain reaction (PCR) (not shown). It was discovered that three chromosomal genes including one PKS gene (*F-avi4330*) and two NRPS genes (*F-avi3342* and *F-avi5730*) are required for GTI (Table 1). It was previously determined that *F-avi4330* and *F-avi3342* were required for induction of the grape necrosis and tobacco HR, whereas *F-avi5730* is not (Zheng and Burr 2013).

Inoculation of $\Delta F-avi4330$ in combination with *A. vitis* tumorigenic strain CG49 resulted in grape tumor formation similar to inoculation with CG49 alone, indicating *F-avi4330* is required for GTI (Fig. 1; Table 2). The *F-avi4330* downstream gene *F-avi4329*, encoding an amino acid transferase, is required for the tobacco HR and grape necrosis phenotypes but not for GTI (Fig. 1). This suggests that *F-avi4329* is not a component of the pathway leading to production of GTI activity.

F-avi3342 is an NRPS gene that was previously characterized (Zheng and Burr 2013) and is shown in this paper to be required for GTI (Fig. 2; Table 1). Complementation of the mutant with full-length *F-avi3342* restored the tobacco HR and grape necrosis phenotypes and partially restored GTI (Fig. 2; Table 1). Following inoculation of grape with a mixture of the

Table 1. Association of polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) genes present in F2/5 with grape crown gall tumor inhibition (GTI)

Gene	Annotation	Size (aa)	Location	Required for GTI	Reference
<i>F-avi4330</i>	PKS	2,523	Chromosome 1	+	This study
<i>F-avi5624</i>	PKS	2,448	Chromosome 2	–	Carle et al. 2008
<i>F-avi5625</i>	PKS	2,368	Chromosome 2	–	Carle et al. 2008
<i>F-avi7309</i>	PKS	1,536	Plasmid	–	This study
<i>P2476</i>	PKS	6,952	Plasmid	–	This study
<i>P2477</i>	PKS	3,019	Plasmid	–	This study
<i>F-avi3342</i>	NRPS	1,334	Chromosome 1	+	This study
<i>F-avi5730</i>	NRPS	1,327	Chromosome 2	+	This study
<i>F-avi7306</i>	NRPS	1,506	Plasmid	–	This study

Fig. 1. Organization of *F-avi4330* and flanking genes and effects of F2/5, $\Delta F-avi4330$, and $\Delta F-avi4329$ on grape tumor inhibition. Vertical arrows indicate approximate locations in genes in which the suicide vector pVIK165 is integrated.

complemented mutant ($\Delta F\text{-}avi3342^+$) and tumorigenic strain CG49, the average size of tumors that formed was significantly smaller than that from the treatment of $\Delta F\text{-}avi3342$ and CG49 mixture (Fig. 2).

F-avi5730 is an NRPS gene comprised of 3,981 bp encoding a putative polypeptide with 1,327 amino-acid residues. The deduced sequence contains adenylation (A), peptide carrier protein, and thioesterase (TE) domains in a single NRPS module (Fig. 3A). The N-terminal domain shows significant sequence similarity to a condensation (C) domain and contains all seven signature sequences (C1 to C7). The reported catalytic reaction center of C domains has the consensus sequence HHXXXD, in which the second histidine and the terminal aspartic acid residues are critical for catalytic activity (Keating et al. 2000; Marahiel et al. 1997). Within *F-avi5730*, however, the core sequence is HPLIGD, in which the second histidine is replaced with a proline, suggesting that the C domain is not active and is designated as C*. The deduced amino acid sequence shares 93.8% identity with *avi5730* from S4 and is similar (44.5% identity) with an NRPS gene, *vbsS*, in *Rhizobium etli* CFN42. The deduced sequences of *avi5730* and *vbsS* contain the same core sequence HPLIGD in the C domain. The signature sequence of *F-avi5730* was identified and the specificity of amino acid that is activated by its A domain was predicted by using software NRPSpredictor2 (Röttig et al. 2011). The signature sequence of *F-avi5730*, DGESSGGMTG, is identical to that of *VbsS*, and the predicted activating amino acid of *F-avi5730* A domain is glutamic acid or glutamine, whereas that of *VbsS* was determined to be N⁵-((R)-3-hydroxybutyryl)-N⁵-hydroxy-D-ornithine (Fig. 3B) (Heemstra et al. 2009). No other NRPSs with highly homologous signature sequence to *VbsS* have been reported.

F-avi5730 and flanking genes are homologous to the siderophore vibriobactin synthesis (*vbs*) locus carried in *Rhizobium* species (Fig. 3A and C). The biosynthetic pathway for vibriobactin has been reported (Heemstra et al. 2009). The *vbs* locus homologous region in F2/5, designated *F-vbs*, contains all the genes required for vibriobactin biosynthesis, except for the gene encoding phosphopantetheyltransferase, which resides outside of this region (Fig. 3A and C). To determine whether *F-vbs* is involved in siderophore synthesis and is required for GTI, mutants of *F-avi5730* ($\Delta F\text{-}avi5730$) and two downstream genes, *F-avi5731* and *F-avi5732* ($\Delta F\text{-}avi5731$ and $\Delta F\text{-}avi5732$), homologs of *vbsO* and *rhoI* that are essential for vibriobactin biosynthesis (Carter et al. 2002; Heemstra et al. 2009), were evaluated for siderophore production and GTI. The three mutants produced significantly less siderophore than F2/5 (Fig. 4B), but only $\Delta F\text{-}avi5730$ was required for GTI. Introduction of a plasmid carrying the full length of *F-avi5730* into $\Delta F\text{-}avi5730$ restored GTI (Fig. 4C; Table 2).

Survival of PKS and NRPS mutants in grape wounds.

To determine if the mutations in *F-avi4330*, *F-avi3342*, and *F-avi5730* affected their ability to survive in grape wounds and, thus, impact GTI, the mutants were inoculated on wounds and populations determined over time. Populations of the mutants were compared with complemented $\Delta F\text{-}avi5730$ ($\Delta F\text{-}avi5730^+$) having restored GTI, as it also provided a second antibiotic marker (spectinomycin) to help facilitate isolation on culture medium. The results showed that the populations of the GTI-negative mutants were not significantly different from GTI-positive $\Delta F\text{-}avi5730^+$ (Fig. 5). Therefore, loss of GTI in the mutants did not result from reduced ability to survive in wound sites.

As indicated, *clpA* and *clpP1* mutants of F2/5 are GTI-negative. To determine whether transcription of *F-avi4330*, *F-avi3342*, and *F-avi5730* is regulated by the *A. vitis* ClpAP regulatory system,

promoter-*gfp* fusion constructs of these three genes were introduced into the *clpA* ($\Delta clpA$) and *clpP1* ($\Delta clpP1$) mutants and into F2/5, respectively, to compare *gfp* expression by measuring fluorescence. Significant differences between *gfp* expressions in $\Delta clpA$, $\Delta clpP1$, or F2/5 for any one of the promoter-*gfp* fusion constructs were not observed (data not shown). Therefore, transcription of these three genes does not appear to be regulated by the ClpAP system.

DISCUSSION

A main goal of this research is to improve our understanding of the mechanism by which strain F2/5 inhibits crown gall disease on grape to help facilitate its use as an effective disease control. We previously reported that NRPS gene *F-avi3342* and PKS gene *F-avi4330* are required for *A. vitis* F2/5 to cause grape necrosis and tobacco HR and, in this paper, show that both genes are also required for GTI. In addition, NRPS gene *F-avi5730* that is not associated with necrosis or HR was also determined to be required for GTI.

Previously, we hypothesize that *F-avi4330*-encoded PKS and *F-avi3342*-encoded NRPS are required for biosynthesis of a peptide-polyketide hybrid that is required for necrosis and tobacco HR (Zheng and Burr 2013). Based on information reported in this paper, we modified the previous model for HR and necrosis (Zheng and Burr 2013) to include GTI, as shown in Figure 6. The determination that *F-avi4330* and *F-avi3342* are both required for HR, necrosis, and GTI strongly suggests there is a common precursor (metabolite immediate) that is required for all three phenotypes. We further determined that *F-avi4329*, the downstream gene of *F-avi4330*, is required for necrosis and HR but not for GTI. Analysis of the *F-avi4329* sequence reveals that it encodes a putative aminotransferase that is a 7-keto-8-aminopelargonic acid (KAPA) synthase, homologous to BioF in the biotin biosynthetic pathway of *E. coli* (Cronan 2010). The substrate of KAPA synthase is pimeloyl-CoA or pimeloyl-ACP. We therefore predict that the *F-avi4330* encoding PKS is able to catalyze the production of pimeloyl-ACP by using malonyl-CoA as a start unit and having two rounds of extension, condensation, reduction, and dehydration. It is possible that pimeloyl-ACP is the common precursor for HR, necrosis, and GTI (Fig. 6). In addition to forming KAPA, pimeloyl-ACP may also have different fates, such as being released from ACP catalyzed by TE. According to this model, it is possible that two nonproteinogenic amino acids are synthesized and further form into two polyketide-peptide hybrids (A and B), both under catalysis of the *F-avi3342*-encoded NRPS (Fig. 6). Our current model predicts that polyketide-peptide hybrid A is required for grape necrosis and tobacco HR, while polyketide-peptide hybrid B is further processed under catalysis of the *F-avi5730*-encoded NRPS to form an intermediate

Table 2. Effects of F2/5 and the mutants on grape crown gall formation^a

Treatment	Grape tumor formation
CG49	+
CG49 + F2/5	–
CG49 + <i>F-avi3342</i>	+
CG49 + <i>F-avi3342</i> ⁺	reduced
CG49 + <i>F-4330</i>	+
CG49 + <i>F-4329</i>	–
CG49 + <i>F-avi5730</i>	+
CG49 + <i>F-avi5730</i> ⁺	–
CG49 + <i>F-avi5731</i>	–
CG49 + <i>F-avi5732</i>	–

^a Eight plants were inoculated for each treatment. Gall formation was rated 6 to 8 weeks after inoculation. Experiments were repeated at least three times.

that is required for GTI (Fig. 6). When attempting to complement $\Delta F\text{-}avi3342$ by introducing a plasmid carrying the full-length *F-avi3342*, tobacco HR, grape necrosis, and GTI were partially restored (Fig. 2). This suggests that the enzyme activity of NRPS encoded by plasmid-born *F-avi3342*

does not restore to the level of native enzyme. This may result from overexpression of the cloned *F-avi3342*, which causes improper protein processing, such as peptide misfolding and eventually results in losing part of the enzyme activity.

Fig. 2. **A**, Organization of *F-avi3342* and flanking genes and **B**, their association with grape tumor inhibition, **C**, comparison of average gall (tumor) size in grapevine, **D**, tobacco hypersensitive response, and **E**, grape necrosis caused by F2/5, mutant $\Delta F\text{-}avi3342$, and complemented mutant $\Delta F\text{-}avi3342^+$. The vertical arrow indicates the location of disruption by a vector sequence at *F-avi3342*. An asterisk (*) represents statistically significant difference ($P < 0.05$) on a Student's *t* test.

NRPS and PKS synthesize a vast array of secondary metabolites with structural complexities and diverse biological activities in bacteria. The compounds synthesized by NRPS or PKS (or both) associated with plant disease control usually function as antibiotics that either directly suppress pathogens, act as elicitors that induce plant defense, or both. For example, WAP-8294A2 synthesized by NRPS and PKS in *Lysobacter enzymogenes* possesses antibacterial activity (Zhang et al. 2014), and surfactins synthesized by NRPS in *Bacillus* strains have not only antibacterial properties, but also, plant resistance-inducing activity (Ongena and Jacques 2008). In contrast, for *A. vitis* F2/5, the putative GTI-required compound synthesized by NRPS and PKS acts neither as an antibiotic nor as a defense elicitor. It does not inhibit growth of tumorigenic *A. vitis* on grape wounds but still blocks agro-transformation of grape (Kaewnum et al. 2013). The behavior of F2/5 toward GTI appears similar to how the plant signal molecules, salicylic acid (SA) and indoleacetic acid (IAA), affect tumorigenesis of *A. tumefaciens*. SA was reported to shut down expression of the *vir* regulon in *A. tumefaciens* but did not inhibit bacterial growth (Yuan et al. 2007). Similarly, IAA inhibited *vir* gene expression of *A. tumefaciens* and also did

not inhibit bacterial growth (Liu and Nester 2006). Further research to determine whether GTI by F2/5 results from shutting down expression of *vir* genes is warranted.

Agrobacterium rhizogenes K84 has been used successfully as a commercial biological control agent of crown gall disease caused by *A. tumefaciens*. K84 produces a bacteriocin, agrocin 84, which is a primary factor associated with crown gall inhibition. Biosynthesis of the antibiotic agrocin 84 is governed by genes present in plasmid pAgK84. Agrocin 84 specifically targets leucyl-tRNA synthetase in *A. tumefaciens* (Reader et al. 2005). K84 also harbors the siderophore vicibactin synthesis locus whose product is a hydroxamate siderophore (ALS84) (Penyalver et al. 2001). *A. vitis* also has the vicibactin locus, whereas *A. tumefaciens* does not. It was shown that ALS84 is an antibiotic-like substance that inhibits *A. tumefaciens* and other phytopathogenic *Erwinia* and *Pseudomonas* spp. regardless of their sensitivity to agrocin 84 (Peñalver et al. 1994). K84 does not control grape crown gall caused by *A. vitis*, which is not sensitive to agrocin 84 or ALS84. We determined that the only gene in the *F-vbs* locus to be required for GTI was *F-avi5730*, indicating that siderophore vicibactin production was not

Fig. 3. Comparison of *F-avi5730* and flanking genes in *Agrobacterium vitis* F2/5 with *vbs* locus in *Rhizobium etli* CFN42 in **A**, gene organization, **B**, signature of NRPS, and **C**, gene annotation and sequence similarity.

involved. It will be interesting to determine the effect siderophore production by *F-vbs* may be having benefits on microbial communities on grapevines and, especially, those associated with crown gall infections.

Genes *F-avi3342*, *F-avi4330*, and *F-avi5730* required for GTI are also present in other tumorigenic and nontumorigenic *A. vitis* strains, based on PCR analyses and sequence comparison from DNA databases. The nontumorigenic strains examined included CG561, which has no GTI activity (Burr et al. 1999). Therefore, in addition to genes *F-avi3342*, *F-avi4330*, and *F-avi5730*, which appear to be common among *A. vitis* strains, there are likely one or more unique genes in F2/5 that are required for GTI. Identification and characterization of such genes associated with the mechanism of GTI will be critical for better understanding the mechanism of associated mechanism.

Intracellular proteolysis is required for quality control of proteins and regulation of various biological processes (Porankiewicz et al. 1999). The ClpAP protease complex is one of the conserved proteolytic machines in bacteria. It consists of ATP-binding subunit (ClpA), proteolytic subunit (ClpP) and, often, also includes an adapter protein (ClpS). In *A. vitis* F2/5, *clpA* (*F-avi2537*) or *clpP1* (*F-avi1696*) were shown to be required GTI (Kaewnum et al. 2013). Here, we sought to determine whether the expression of *F-avi3342*, *F-avi4330*, and *F-avi5730* is regulated by *clpAP* by using promoter-*gfp* fusion. The results indicated that transcription of these three genes is not regulated by *clpAP* system. It suggests that either these three genes are not

regulated by the *clpAP* system or they are regulated at post-translational level.

MATERIALS AND METHODS

Bacterial strains, media and plasmids.

Bacterial strains used in this study are listed in Table 3. *A. vitis* F2/5 and the mutants were propagated on potato dextrose agar (PDA) or in potato dextrose broth (Difco Laboratories) at 28°C. Appropriate antibiotics were added to the media for culturing

Fig. 5. Cell populations of grape tumor inhibition (GTI)-negative mutants ΔF-avi4330, ΔF-avi3342, ΔF-avi5730, and GTI-positive complemented mutant ΔF-avi5730⁺, recovered from inoculated sites of grapevine. NS indicates not significantly different based on a Student's *t* test.

Fig. 4. A, Organization of *F-avi5730* and flanking genes and B, the effect of F2/5, mutants ΔF-avi5730, ΔF-avi5731, and ΔF-avi5732 on siderophore production and C, on grape tumor inhibition. Vertical arrows indicate the genes knocked down. An asterisk (*) represents statistically significant difference (*P* < 0.05) compared with F2/5, based on a Student's *t* test. ΔF-avi5730⁺ is the complemented ΔF-avi5730.

mutants. *Escherichia coli* were cultured on Luria-Bertani (LB) medium with appropriate antibiotics at 37°C.

Development of knockout mutants.

Mutants of specific target genes were generated by PCR amplifying internal fragments of target genes from F2/5 genomic DNA, 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* S17-1/ λ pir and

were selected on LB agar plus kanamycin (50 µg/ml). Constructs were transferred to F2/5 via conjugal mating, resulting in target gene disruption by insertion of the vector pVIK165, following a single homologous recombination. The resulting mutants contain 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 µg/ml) and 10% mannitol. Mutations were verified by PCR using primers derived from sequences of the F2/5 and from the pVIK165 vector (pVIK-R) (Table 4). They were tested for GTI activity on grape as described below.

Fig. 6. The proposed pathway in F2/5 that expression of *F-avi4330*, *F-avi3342*, and *F-avi5730* lead to biosynthesis of the compounds required for induction of tobacco hypersensitive response, grape necrosis, and grape tumor inhibition on a Student's *t* test.

Complementation of GTI mutants.

Complementation of ΔF -avi3342 and ΔF -avi5730 was performed using methods previously described (Hao et al. 2005). Complementation of ΔF -avi4330 was not attempted, due to its large size (approximately 7.6 Kb in length). Full-length sequences of ΔF -avi3342 and ΔF -avi5730, including promoter regions, were amplified from F2/5 genomic DNA with LongAmp *Taq* DNA polymerase (New England BioLab Inc.), using the primer pairs listed in Table 3. The PCR products were individually cloned into vector pPZP201 (Hajdukiewicz et al. 1994) and plasmid constructs were transformed into JM109 competent cells (Promega Inc.). Positive colonies were selected on LB agar containing spectinomycin (100 µg/ml). The recombinant plasmids, designated as pPZP-3342 and pPZP-5730, were verified by analyses of restriction digests and sequencing. Then pPZP-3342 and pPZP-5730 were transformed into ΔF -avi3342 and ΔF -avi5730, respectively, by electroporation. Transformants were selected on AB (plus 10% mannitol) minimal medium containing spectinomycin (400 µg/ml) and kanamycin (50 µg/ml) and were verified by PCR. Complemented ΔF -avi3342 (ΔF -avi3342⁺) and ΔF -avi5730 (ΔF -avi5730⁺) were evaluated for GTI against CG49 on grape wound sites as described above. ΔF -avi3342⁺ was also tested for its ability to cause tobacco HR and grape necrosis.

Assaying GTI.

All of the mutants were tested for their ability to inhibit crown gall on 1- to 2-month-old *V. vinifera* cv. Cabernet Franc in the greenhouse. F2/5 and mutant cells from overnight PDA cultures were suspended in water and were adjusted to an optical density at 600 nm (OD₆₀₀) = 0.1 (about 10⁸ CFU per milliliter). F2/5 or individual mutants were mixed 1:1 with tumorigenic *A. vitis* CG49, and then, 50 µl of the mixed suspensions was inoculated into slice wounds made with a scalpel on woody and green shoot tissues of the grapevines. Wounds were made just below an actively growing shoot on woody tissue or below a bud

when green shoots were inoculated. After inoculum suspensions dried, the wound sites were wrapped with Parafilm. Sterile distilled water (SDW) was applied as a negative control and CG49 mixed with an equal volume of SDW was applied as positive control. Eight plants were inoculated for each treatment. Gall diameters were measured by venire caliper at the largest point of vertical and horizontal sides of inoculation sites at 2 months postinoculation. The experiments were repeated at least three times.

HR and grape necrosis assays.

Grape necrosis and tobacco HR assays were carried out as previously described (Herlache et al. 2001). To test for necrosis, young grape shoot explants were cut into 0.5-cm sections and were supported on end in water agar (2%), with basal end up. Bacteria that were grown in PDA plates overnight were suspended in SDW and were adjusted to an OD₆₀₀ = 0.1. Cell suspensions (5 µl) of F2/5 and mutants were placed on the basal end of each explant, and necrosis was observed 3 to 5 days after inoculation. HR was determined by infiltration of tobacco leaf panels with *A. vitis* cell suspensions, as previously described (Herlache et al. 2001). Bacteria grown overnight on PDA were suspended in SDW and concentrations were adjusted to an OD₆₀₀ = 1.5.

Assay of siderophore production.

The chrome azurol S (CAS) assay was used to estimate siderophore production by F2/5 and *F*-avi5730, *F*-avi5731, and *F*-avi5732 mutants (Hibbing and Fuqua 2011), with slight modification. CAS solution was prepared by dissolving 605 mg in 500 ml of water and 100 ml of 1 mM FeCl₃ in 10 mM HCl was added. The resulting mixture was added slowly to 729 mg of hexadecyltrimethylammonium bromide (HDTMA) in 400 ml of water for final concentrations of 1 mM CAS, 2 mM HDTMA, 100 µM FeCl₃, and 1 mM HCl. Cultures of F2/5 and mutants were grown in AB medium without Fe for 2 days at 28°C. Cells

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference
<i>Agrobacterium vitis</i>		
F2/5	Wild type	Staphorst et al. 1985
ΔF -avi3342	<i>F</i> -avi3342::pPVIK165, Km ^R	Zheng and Burr 2013
ΔF -avi3342 ⁺	ΔF -avi3342 carrying pPZP-3342, Km ^R , Sp ^R	This work
ΔF -avi4329	<i>F</i> -avi4329::pPVIK165, Km ^R	Zheng and Burr 2013
ΔF -avi4330	<i>F</i> -avi4330::pPVIK165, Km ^R	Zheng and Burr 2013
ΔF -avi5730	<i>F</i> -avi5730::pPVIK165, Km ^R	Zheng and Burr 2013
ΔF -avi5731	<i>F</i> -avi5731::pPVIK165, Km ^R	This work
ΔF -avi5732	<i>F</i> -avi5732::pPVIK165, Km ^R	This work
ΔF -avi5730 ⁺	ΔF -avi5730 carrying pPZP-5730, Km ^R , Sp ^R	This work
ΔF -avi7306	<i>F</i> -avi7306::pPVIK165, Km ^R	This work
ΔF -avi7309	<i>F</i> -avi7309::pPVIK165, Km ^R	This work
$\Delta P2476$	<i>F</i> -aviP2476::pPVIK165, Km ^R	This work
$\Delta P2477$	<i>F</i> -aviP2477::pPVIK165, Km ^R	This work
$\Delta clpA$	<i>F</i> -avi2537::pPVIK165, Km ^R	Kaewnum et al. 2013
$\Delta clpP1$	<i>F</i> -avi1696::pPVIK165, Km ^R	Kaewnum et al. 2013
<i>Escherichia coli</i>		
JM109	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> , <i>supE44</i> , <i>relA1</i> , (<i>lac</i> - <i>proAB</i>)/F' [<i>traD36</i> , <i>proAB</i> +, <i>lacIq</i> , <i>lacZM15</i>]	Promega
S17-1/ λ pir	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
pAT-P3342	pPROBE-AT carrying <i>F</i> -avi3342 promoter, Cb ^R	Zheng and Burr 2013
pAT-P4330	pPROBE-AT carrying <i>F</i> -avi4330 promoter, Cb ^R	Zheng and Burr 2013
pAT-P5730	pPROBE-AT carrying <i>F</i> -avi5730 promoter, Cb ^R	This work
pVIK165	Suicide-vector, Km ^R	Kalogeraki and Winans 1997
pPZP201	Broad-host range cloning vector, Sp ^R	Hajdukiewicz et al. 1994
pPZP-3342	pPZP201 carrying <i>F</i> -avi3342, Sp ^R	This work
pPZP-5730	pPZP201 carrying <i>F</i> -avi5730, Sp ^R	This work

^a Km^R = kanamycin resistance, Sp^R = spectinomycin resistance, and Cb^R = carbenicillin resistance.

were removed by centrifugation, and 500 µl of the culture supernatant was mixed with 100 µl of CAS solution and 10 µl of 0.2 M 5-sulfosalicylic acid shuttle solution (CAS solution containing 5-sulfosalicylic acid) and was incubated for 30 min at room temperature. Siderophore production was measured by a decrease in the adsorbance at 630 nm (A_{630}) of the sample, and the amount of siderophore produced was calculated using the following formula: $[(A_{630}^{\text{reference}} - A_{630}^{\text{sample}})/A_{630}^{\text{reference}}] \times 100 = \text{siderophore units}$. The $A_{630}^{\text{reference}}$ was measured from the CAS assay solution mixed with AB medium and shuttle solution. Data are the means and standard errors of five replicates.

Populations of F2/5-mutants on inoculated wound sites of grapevine.

To determine the effect of gene disruption on survival and growth of F2/5-mutants on grape wounds, $\Delta F\text{-}avi4330$, $\Delta F\text{-}avi3342$, $\Delta F\text{-}avi5730$, and $\Delta F\text{-}avi5730^+$ were individually coinoculated with CG49 on wound sites of grape shoots as described above. $\Delta F\text{-}avi5730^+$ was used as a positive control because it is GTI positive and provided a second antibiotic resistance marker to facilitate plating on culture media. Each inoculation was done on four different plants. Five days after inoculation, the tissue encompassing the inoculated wound site (about 0.1 g of tissue) was collected and triturated in 1 ml of SDW. Tenfold serial dilutions were made and were plated in on PDA plus kanamycin (50 µg/ml). For $\Delta F\text{-}avi5730^+$, the dilutions were plated on PDA plus kanamycin (50 µg/ml) and spectinomycin (400 µg/ml). Plates were incubated at 28°C for 48 h and colonies were counted. Populations of $\Delta F\text{-}avi4330$, $\Delta F\text{-}avi3342$, $\Delta F\text{-}avi5730$, and $\Delta F\text{-}avi5730^+$ are represented as colonies per milliliter of dilution. The difference between populations was determined by Student's *t* test, using Salstat2 (Salstat Statistics Package 2). The experiment was repeated twice.

Determining potential ClpAP regulation of GTI-associated PKS and NRPS genes.

We previously reported that mutants of *clpA* (*F-avi2537*) and *clpP1* (*F-avi1696*) included in a Clp system were GTI-negative (Kaewnum et al. 2013). To determine if the Clp system is associated with transcriptional regulation of GTI-required PKS and NRPS genes, the promoter region of *F-avi5730* was amplified from F2/5 genomic DNA with primers *F-avi5730P-F*

and *F-avi5730P-R* (Table 4) and was cloned into the pPROBE-AT vector (Miller et al. 2000) to create an *F-avi5730* promoter-*gfp* fusion (pAT-P5730-*gfp*). Previously developed promoter-*gfp* fusion constructs pAT-P3342-*gfp* and pAT-P4330-*gfp* (Zheng and Burr 2013) as well as pAT-P5730-*gfp* were individually introduced into F2/5 and $\Delta clpA$ and $\Delta clpP1$, respectively. Bacterial cells containing promoter-*gfp* fusion constructs were assayed for fluorescence levels by a multimode microplate reader Synergy 2 (Biotek Instruments Inc.) as described previously (Zheng et al. 2012).

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Table 4. Primers used in this study^a

Primer	Sequence
For mutagenesis of target genes	
F-avi5731-F	5'-ACGAGCTCAGATCTGGTCACAGGCACCAAC-3'
F-avi5731-R	5'-AGTCTAGAAGGGATGGTTTCTTGCCGATAC-3'
F-avi5732-F	5'-ACGAGCTCAGACATCGTTACGGAAGTGC-3'
F-avi5732-R	5'-AGTCTAGATAGCCTCGATGGTGCAGCAG-3'
F-avi5730P-F	5'-AGTCTAGATGAAGGAATAACTGCGGAAGC-3'
F-avi5730P-R	5'-ATGGTACCAGAGCCTCGCGCTTTTGGAG-3'
F-avi7306-F	5'-ACGAGCTCTGCTGCTGACGCTGCACCAC-3'
F-avi7306-R	5'-AGTCTAGAACAGCGTGGCGAACAGGGTG-3'
F-avi7309-F	5'-ACGAGCTCGCAAGGGAGAAAGGACGAG-3'
F-avi7309-R	5'-AGTCTAGAATTGGCCTGTATCTGGCTGA-3'
F-P2476-F	5'-ACGAGCTCGCTGCTGGCTTATGGATTGT-3'
F-P2476-R	5'-AGTCTAGATATGCAACACGGCAGAAATG-3'
F-P2477-F	5'-ACGAGCTC CAGCGGTTTCACTTCTGGAAGT-3'
F-P2477-R	5'-AGTCTAGA CTTGAGGTACCGGATTTGGA-3'
pVIK165-R	5'-TACTCATCTTTGTTTCTCC-3'
For complementation of $\Delta F\text{-}avi3342$ and $\Delta F\text{-}avi5730$	
F-avi3342FF	5'-AGTCTAGAACGGGTTATTGCAAGTCGGTC-3'
F-avi3342RR	5'-ACGAGCTCTGTGATGCTGGAACCTGTGC 3'
F-avi5730FF	5'-AGTCTAGAATGAAGGAATAACTGCGGAAGC-3'
F-avi5730RR	5'-ACGAGCTCCCGGATTACGGCTTGACTT-3'

^a Restriction sites are underlined. *sacI*: GAGCTC, *xbaI*: TCTAGA, and *kpnI*: GGTACC.

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