A Host-Specific Biological Control of Grape Crown Gall by *Agrobacterium vitis* Strain F2/5: Its Regulation and Population Dynamics

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ABSTRACT

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Nontumorigenic *Agrobacterium vitis* strain F2/5 is able to prevent crown gall caused by tumorigenic *A. vitis* on grape but not on other plant species such as tobacco. Mutations in a quorum-sensing transcription factor, *aviR*, and in caseinolytic protease (*clp*) component genes *clpA* and *clpP1* resulted in reduced or loss of biological control. All mutants were

complemented; however, restoration of biological control by complemented *clpA* and *clpP1* mutants was dependent on the copy number of vector that was used as well as timing of application of the complemented mutants to grape wounds in relation to inoculation with pathogen. Mutations in other quorum-sensing and *clp* genes and in a gene associated with polyketide synthesis did not affect biological control. It was determined that, although F2/5 inhibits transformation by tumorigenic *A. vitis* strains on grape, it does not affect growth of the pathogen in wounded grape tissue over time.

Crown gall disease of grapevine, caused by *Agrobacterium vitis*, is a limiting factor to grapevine production in several regions of the world (6). *A. vitis* survives systemically in grapevines and, therefore, is disseminated in propagation material (4,18,37). Like crown gall on other plant species, tumorigenesis results following expression of a set of oncogenes called the T-DNA that are carried on a bacterial Ti plasmid. The T-DNA is transferred to and expressed in the plant cell chromosome (14). *A. vitis* strains can be grouped based on the type of Ti plasmid they carry and are designated by opine catabolic genes that reside on their Ti plasmid. Examination of a geographically diverse collection of *A. vitis* strains revealed that Ti plasmid type is correlated with chromosomal background (6,30).

Biological control of crown gall on specific crops such as stone fruit has been successfully implemented in commercial agriculture using *A. rhizogenes* strain K84 (27). K84 is not effective against *A. vitis* strains on grapevine and, therefore, several research groups have investigated other candidate biological control strains for their effectiveness against grape crown gall. Some of the more recent research on biological control of grape crown gall included nontumorigenic *A. vitis* strains E26 (28) and VAR03-1 (24). Other strains, including *Rahnella aquatilis* strain HX2 (12), *Serratia plymuthica* strain IC1270, *Pseudomonas fluorescens* strain Q8r1-96, and others, were shown to provide various levels of grape crown gall inhibition under experimental conditions (16).

Our laboratory has studied biological control of grape crown gall by nontumorigenic *A. vitis* strain F2/5 (7). We previously determined that control activity is dependent on timing of appli-

cation of F2/5 to grape wounds in relation to pathogen inoculation and that gall formation by some strains is inhibited more than for others (7). We also demonstrated that antibiosis-minus mutants of F2/5 retained biological control activity whereas heat-killed or sonicated cell preparations were ineffective for biological control (9). If tumorigenic *A. vitis* initiates infection of susceptible plant cells prior to treatment with F2/5, biological control is also ineffective. In addition, it was shown that F2/5 does not appear to inhibit attachment of tumorigenic *A. vitis* strains at grape wounds (9). A variation of F2/5 that carries a trifolitoxin gene has been generated (22) and is predicted to enhance control against a wider range of *Agrobacterium* spp.

We have reported that all *A. vitis* strains, including F2/5, cause a necrosis of grape roots and a hypersensitive response (HR) following infiltration into tobacco leaves (21). Initial screening of Tn5 mutants for HR and necrosis phenotypes revealed the presence of a quorum-sensing *luxR* homolog, *aviR* (43), and a caseinolytic protease (*clpA*) (ATP-binding subunit of ATP-dependent Clp protease) homolog associated with both phenotypes (21). Several other HR and necrosis mutants were generated by knocking out additional quorum-sensing-associated genes (19,20) and genes that make up a large operon homologous to polyketide synthase and long-chain fatty acid operons (11).

Initial evidence suggested that at least one of the *luxR* homologs, *aviR* (42), as well as the *clpA* homolog is involved in the mechanism of F2/5-induced biological control, and that mechanisms of necrosis and biological control may be related (15). In this article, we provide further evidence to show that the two regulatory systems, quorum sensing and the *clp* system, are associated with the mechanism of biological control. We also demonstrated that F2/5 prevents tumorigenic *A. vitis* strains from causing infection (i.e., transformation of grape cells) but not cells of other plant species, such as tobacco. It was also determined that, although F2/5 prevents infection of grape, it does not inhibit survival and growth of tumorigenic *A. vitis* at grape wound sites.

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MATERIALS AND METHODS

Strains, plasmids, and culture media. The bacterial strains and plasmids used in this study and their relevant characteristics are shown in Table 1. *A. vitis* strains were grown on potato dextrose agar (PDA), in yeast extract-mannitol broth (YMB), or on AB minimal medium supplemented with 0.5% mannitol (13) at 28°C. *Escherichia coli* strains were grown on Luria-Bertani (LB) agar or broth at 37°C. Kanamycin (50 µg/ml) and carbenicillin (100 µg/ml) were added to media for mutant selection.

Generation of mutants. To further explore the role of the *clp* regulatory system in biological control by F2/5, targeted mutations were made in genes *clpA*, *clpS* (ATP-dependent Clp protease, adaptor protein), *clpP1*, and *clpP2* (putative proteases), corresponding to Avi2537, Avi2538, Avi1696, and Avi3244 in *A. vitis* strain S4 (33). Sequences of primers used to polymerase chain reaction (PCR) amplify internal fragments of the genes from F2/5 genomic DNA are listed in Table 2. *XbaI* or *SacI* restriction sites were incorporated into primers. The PCR products were purified, digested, and cloned into suicide vector pVIK165

(23). Resulting constructs were transformed into *E. coli* strain S17-1/λpir and selected on LB agar plus kanamycin. Constructs were transferred to F2/5 by conjugation and the targeted gene disruptions occurred following single homologous recombination and insertion of pVIK165 into the F2/5 genome. Mutants were verified by PCR with primers that flank the insertion site and from within pVIK165 (pVIK-R) (Table 2).

Complementation of F2/5Δ*clpA* and F2/5Δ*clpP1*. The *clpA* and *clpP1* mutants were found to be biological control negative. They were complemented by PCR amplifying a 3.1-kb DNA fragment containing *clpA* and its promoter region and a 1.1-kb DNA fragment containing *clpP1* and its promoter region, digesting with *XbaI* and *SacI*, and ligating into vector pProbe-AT (29). *clpP1* expressed in this vector did not complement F2/5Δ*clpP1* for biological control regardless of timing of application to wounds in relation to inoculation with tumorigenic *A. vitis* CG49. We suspected that the failure to complement may be associated with the level of protein (ClpP1) that is required for appropriate regulation. Therefore *clpP1* was ligated into a low-copy vector, pWSK29 (40). Resulting constructs, designated as pAT-*clpA* and

TABLE 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics ^z	Source or reference
<i>Agrobacterium vitis</i>		
CG49	Tumorigenic strain, nopaline Ti plasmid	30
CG49(GUS)	CG49 carrying p35SGUSINT	36
F2/5	Nontumorigenic, biological control	34
F2/5(GUS)	F2/5 carrying p35SGUSINT	This study
M1154	F2/5 <i>aviR</i> ::Tn5, Km ^R	43
F2/5Δ <i>clpA</i>	F2/5 <i>clpA</i> ::pVIK165, Km ^R	This study
F2/5Δ <i>clpP1</i>	F2/5 <i>clpP1</i> ::pVIK165, Km ^R	This study
F2/5Δ <i>clpP2</i>	F2/5 <i>clpP2</i> ::pVIK165, Km ^R	This study
F2/5Δ <i>clpS</i>	F2/5 <i>clpS</i> ::pVIK165, Km ^R	This study
F2/5Δ <i>avhE</i>	F2/5 <i>avhE</i> ::pVIK165, Km ^R	11
F2/5Δ <i>avsR</i>	F2/5 <i>avsR</i> ::pVIK165, Km ^R	20
F2/5Δ <i>avhR</i>	F2/5 <i>avhR</i> ::pVIK165, Km ^R	20
F2/5Δ <i>avsI</i>	F2/5 <i>avsI</i> ::pVIK165, Km ^R	20
CmF2/5Δ <i>clpA</i>	Complemented F2/5Δ <i>clpA</i> , Cb ^R , Km ^R	This study
CmF2/5Δ <i>clpP1</i>	Complemented F2/5Δ <i>clpP1</i> , Cb ^R , Km ^R	This study
CmF2/5Δ <i>aviR</i>	Complemented M1154, Gm ^R , Km ^R	43
<i>Escherichia coli</i>		
S17-1/λpir	RK2 <i>tra</i> regulon, <i>pir</i> , host for <i>pir</i> -dependent plasmid p35SGUSINT	32
Plasmid		
pVIK165	Suicide vector, Km ^R	23
pProbe-AT	<i>gfp</i> Broad-host-range promoter-probe (vector, Amp ^R	29
pWSK29	low-copy-number expression vector, lacZ, Amp ^R , Cb ^R	40
pAT- <i>clpA</i>	pProbe-AT carrying <i>clpA</i> , Amp ^R	This study
pWSK- <i>clpP1</i>	pProbe-AT carrying <i>clpP1</i> , Amp ^R	This study

^z Km^R, Cb^R, Gm^R, and Amp^R = resistant to kanamycin, carbenicillin, gentamicin, and ampicillin, respectively.

TABLE 2. Primers used in this study

Primer	Sequences (5'–3') ^z
Primers for amplifying sequences used in site-directed mutagenesis	
ClpA_F	TCCGAGCTCCGTGCTGTTCATCGA
ClpA_R	AGATCTAGAGAAGCGCAGCATTTCCA
ClpS-F	ATCGAGCTCGCAAGCGCAGGGTAACA
ClpS-R	ATCTCTAGATGGTTTCGGCGACTTCATA
ClpP1_F	ACGAGCTCACGACATCTACTCGCGTCTG
ClpP1_R	AGTCTAGACACGGCTGATGCACCATGATG
ClpP2_F	ACGAGCTCAGAAGGTCTGCACACAGCTG
ClpP2_R	AGTCTAGAAGATCTTGATCAGGCGCTG
Primers for complementation	
ClpA-FF	TCTCTAGACGTGCTGCGATTGCTGGTCTG
ClpA-RR	TCCGAGCTCACCGTTCTTCCAAAAGAATTACTTC
ClpP1_FF	AGTCTAGATCGTCACATTCTGGTTTATGCTTC
ClpP1_RR	ACGAGCTCTCTGAGAATATTCCAGGCTATGTC
AVR-F	ACCTGCAGCGCGAAGATATTGC
AVR-R	ATGGATCCAGGCAAGTCAGCAGAACG
Primers to verify insertion of pVIK165 in F2/5 genome	
pVIK165_R	AACCTTCGGGCATGGCACTCTTG

^z The underlined GAGCTC was added to create a *SacI* site for subcloning and the underlined TCTAGA was added to create an *XbaI* site for subcloning.

pWSK-clpP1, were verified by PCR. The pAT-clpA construct was transformed into *E. coli* Topo-10 competent cells (Invitrogen, Inc., Grand Island, NY) and pWSK-clpP1 was transformed into *E. coli* S17-1/λpir. Construct pAT-clpA was introduced into F2/5ΔclpA by electroporation and pWSK-clpP1 into F2/5ΔclpP1 by conjugation. Complemented mutants were selected on AB minimal medium containing kanamycin and carbenicillin. Transformants were verified by restriction analysis and PCR. Complementation of the *aviR* mutant was previously reported (43). The mutants and complemented mutants were tested for their ability to inhibit crown gall on grape, as described below.

Biological control activity on grape and tobacco. F2/5 mutants were tested for their ability to inhibit crown gall on 1- to 2-month-old *Vitis vinifera* 'Chardonnay' in the greenhouse. In addition to the *clpA*, *clpP1*, and *clpP2* mutants, previously generated mutants *clpS*, *aviR* (43), *avhR*, *avsR*, and *avsl* associated with quorum-sensing regulation (19,20) and *avhE* associated with polyketide synthesis (11) were also tested for biological control.

F2/5, mutants, and tumorigenic *A. vitis* strain CG49 were grown overnight on PDA or PDA plus kanamycin and cells were suspended in sterile distilled water (SDW) to an optical density at 600 nm (OD₆₀₀) of 0.1. F2/5 or individual mutants were mixed 1:1 with CG49 and then 50 μl of the mixed suspensions were 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. SDW was applied as a negative control and strain 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 side of inoculation sites at 2 months postinoculation. Statistical analysis was conducted (Duncan's new multiple range test [MRT]) using the Students *t* test (JMP statistical software; SAS Institute Inc., Cary NC) to determine the effect of treatment on gall size.

Because, in initial experiments, the complemented *clpA* and *clpP1* mutants did not restore biological control when they were applied together in cell suspensions with CG49, further experiments were done to determine whether the timing of application of complemented mutants in relation to CG49 affected gall inhibition. The complemented *clpA* and *clpP1* mutants were inoculated on wounds 24, 48, or 72 h prior to inoculation with CG49. To determine whether wound age affected crown gall susceptibility, wounds that were not treated with F2/5 or mutants were inoculated with CG49 at 24, 48, and 72 h after wounding.

To determine the effectiveness of F2/5 in controlling crown gall on plants other than grape, stems of tobacco plants (*Nicotiana tabacum* 'Xanthi') and sunflower were inoculated with CG49 and with mixed F2/5 and CG49 cell suspensions as described for grapevines. The tobacco plants were ≈25 cm in height, with four to six leaves. The sunflower plants had just formed first true leaves. All experiments on grape, tobacco, and sunflower were repeated at least twice.

Effect of F2/5 on transformation of grape and tobacco by CG49. Direct evidence for the effect of F2/5 on T-DNA transfer by CG49 was obtained using *A. vitis* strain CG49 (p35SGUSINT), referred from here forward as CG49(GUS) (36). p35SGUSINT is a derivative of pBIN19 that carries the 35S promoter, a eukaryotic intron, and the *uidA*(GUS) gene bracketed by T-DNA borders (39). The vector also confers resistance to kanamycin. CG49(GUS) was inoculated together with F2/5 on grape and tobacco wounds, as described above. Seven days after inoculation, thin sections of inoculated tissues were made with a sterile scalpel blade and were incubated at 37°C for 24 h in a solution of 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide cyclohexyl ammonium salt (1 mg/ml dissolved in N,N-dimethyl formamide), 5 mM potassium ferro-

cyanide, 5 mM potassium ferricyanide, and 0.1 M phosphate buffer. Tissues were observed for the presence of blue staining of cells, indicative of transformation (15).

Populations of F2/5 and CG49 in inoculated grape wounds.

To determine the effect of F2/5 on survival and growth of CG49 in grape wounds, F2/5, CG49, F2/5(GUS), and CG49(GUS) were inoculated in different combinations on wounded grape shoots, as described above. In this case, the presence of the binary vector p35SGUSINT was utilized to confer kanamycin resistance, thereby allowing differentiation between populations of CG49 and F2/5 over time. F2/5(GUS), like CG49(GUS), was generated via conjugal transfer of p35SGUSINT. Inoculated plants were maintained in the greenhouse for the duration of the experiment. At 24, 48, and 72 h after inoculation, the tissue encompassing the wound was collected and triturated in 1 ml of SDW. Serial dilutions were made and plated in triplicate on PDA or PDA plus kanamycin. Plates were incubated for 72 h at 28°C at which time colonies were counted to determine populations of CG49, CG49(GUS), F2/5, or F2/5(GUS). Experiments were repeated at least three times. The results were statistically analyzed using Duncan's MRT with Statistical Package for the Social Sciences (SPSS), version 15 (SPSS Inc., Chicago, IL). The analyses were initially done on replicates of the experiment to ensure that differences did not exist between them. Because F2/5 did not prevent gall formation by CG49 on tobacco, populations of both strains at tobacco wound sites were determined 24 and 72 h postinoculation. Inoculations were done as on grape and colony counts on PDA were made 48 h after dilution plating.

Growth comparisons of F2/5, F2/5ΔclpA, and F2/5ΔclpP1 and complemented mutants.

It was necessary to inoculate grape wounds with complemented *clpA* and *clpP1* mutants at least 2 to 3 days prior to inoculation with CG49 for biological control to be effective. To determine whether this phenomenon was related to mutant growth rate compared with F2/5, strains were grown on PDA, PDA amended with kanamycin, or PDA amended with kanamycin and carbenicillin (100 μg/ml) for complemented mutants at 28°C for 24 h. Cells were suspended in SDW to OD₆₀₀ = 0.1 (≈10⁸ CFU/ml) and 1.0 ml added to 100 ml of YMB in sterile flasks. The flasks were incubated at 28°C with shaking (200 rpm) for the duration of the experiment. OD₆₀₀ of each culture was recorded over a 4-day period, and cell counts were determined by plating serial dilutions in triplicate on PDA or PDA amended with kanamycin. Colonies were counted after culturing at 28°C for 48 h. The experiment was repeated twice.

In addition the populations of F2/5, complemented F2/5ΔclpA and F2/5ΔclpP1 and CG49 were compared in grape wounds 24 and 48 h postinoculation. The populations were determined using methods described above to compare F2/5 and CG49. The results were statistically analyzed using Duncan's MRT with SPSS, version 15 (SPSS Inc.).

RESULTS

F2/5 prevents specific transformation of grape but does not inhibit survival and growth of CG49 in wounds. The ability of F2/5 to inhibit development of crown gall by CG49 was tested on grapevines, sunflower, and tobacco. It was determined that F2/5 only inhibited crown gall (Fig. 1) on grape whereas galls formed on tobacco (Fig. 2) and sunflower (not shown). Galls on tobacco developed within 2 weeks of inoculation and gall size and number were not affected by the presence of F2/5. Inhibition of transformation on grape was then directly assessed via β-glucuronidase (GUS) staining of inoculated grape and tobacco tissues. Grape wounds inoculated with F2/5 together with CG49(GUS) resulted in the absence of detectable GUS expression and development of crown gall at the inoculated sites. In contrast, blue spots indicative of transformation were observed in grape wounds inoculated only with CG49(GUS) (Fig. 3). For tobacco inoculated with F2/5

combined with CG49(GUS) and CG49(GUS) alone, blue spots were abundant in the tissues. Observations were consistent for all inoculated wounds from each of the three replications of each experiment.

To further determine the mechanism of control and determine the effect of F2/5 on survival and growth of CG49 in wounds, populations of CG49 or CG49(GUS) and F2/5 or F2/5(GUS) were measured at time intervals following inoculation. It was determined that the presence of F2/5 or F2/5(GUS) did not affect survival and growth of CG49 or CG49(GUS) over 72 h (Table 3). There were no significant differences in the populations of CG49 or CG49(GUS) alone or in the presence of F2/5 or F2/5(GUS) in inoculated grape wound tissues at 24, 48, or 72 h postinoculation.

Fig. 1. Biological control activity of F2/5 and mutants on grapevine 2 months postinoculation. Wound sites were inoculated with **A**, CG49; **B**, equal concentrations of CG49 and F2/5 (arrow shows inoculation site); **C**, CG49 and F2/5 Δ *clpA*; and **D**, CG49 and F2/5 Δ *clpP1*.

The observation that F2/5 inhibits transformation of CG49 on grape but not survival or growth of the pathogen is particularly interesting and important to further research on the mechanism associated with biological control.

Regarding inoculations on tobacco stems, at 24 h postinoculation, F2/5 populations were 6.5×10^6 CFU/ml compared with 1.2×10^7 CFU/ml for CG49. At 72 h, populations were 2.1×10^9 and 6.6×10^8 CFU/ml for F2/5 and CG49, respectively. Therefore, failure of F2/5 to control infection on tobacco is not related to its inability to grow in tobacco wound sites.

Grape crown gall inhibition by F2/5 is associated with quorum sensing and *clp* regulation. It was determined that mutations in *avsR*, *avhR*, *avsI*, *clpP2*, and *avhE* did not affect the ability of F2/5 to inhibit crown gall on grapevines, observed as a lack of tumor development on vines inoculated with mixtures of the mutants and CG49 (Table 4). Conversely, crown gall induction by CG49 was not inhibited when grape wounds were treated with F2/5 Δ *clpA* or F2/5 Δ *clpP1*. The development of crown gall was reduced when wounds were treated with F2/5 Δ *aviR* (Tables 4 and 5). These results indicate that at least two regulatory systems, quorum sensing and proteolytic regulation, are associated with the underlying control mechanism. It also shows that not all quorum-sensing genes in F2/5 are involved.

Association of *aviR*, *clpA*, and *clpP1* with crown gall inhibition on grape was further substantiated through complementation of the mutants. Whereas complementation of F2/5 Δ *aviR* restored biological control when challenged on grape wounds with CG49 at 0 h, (Table 5), a longer incubation period on grape wounds was required before complemented F2/5 Δ *clpA* and F2/5 Δ *clpP1* were effective in providing gall inhibition. In these cases, it was necessary to apply the complemented mutants to wounds 2 to 3 days prior to inoculation with CG49 (Table 5; Fig. 4). The susceptibility of wounded tissue to infection by CG49 was also verified at 24, 48, and 72 h after wounding (Table 6).

Gall production on grapevines treated with F2/5 Δ *clpA*, F2/5 Δ *clpP1*, and F2/5 Δ *aviR* and challenged with CG49 were not significantly different from those produced on vines treated with CG49 alone (Table 5). Grapevines treated with complemented F2/5 Δ *clpA* and F2/5 Δ *clpP1* 0 and 24 h before being challenged with CG49 also produced galls that were not significantly different from those produced by CG49 alone. There was no significant difference in crown gall inhibition by F2/5 and complemented F2/5 Δ *clpA* and F2/5 Δ *clpP1* when applied 72 h prior to inoculation with CG49 (i.e., the treatments prevented gall production on vines and all three strains were significantly different from

Fig. 2. Tobacco inoculated with **A**, CG49 and **B**, CG49 in combination with F2/5.

vines inoculated with CG49 alone). Grape wounds treated with complemented F2/5 Δ clpA and F2/5 Δ clpP1 and then inoculated with CG49 48 h later produced galls that were significantly smaller than those produced by CG49 alone.

Growth rates of F2/5 Δ clpA and F2/5 Δ clpP1 and complemented mutants were also compared with F2/5 to determine whether reduced growth might be associated with biological control. No significant differences in growth rates in vitro were discovered. Populations were also measured in grape wounds. After 24 h, CG49 averaged 5.5×10^4 CFU/ml, F2/5 averaged 5.0×10^4 CFU/ml, F2/5 Δ clpA averaged 1.0×10^3 CFU/ml, and F2/5 Δ clpP1 averaged 1.6×10^4 CFU/ml. Therefore, after 24 h, there were significantly fewer cells of F2/5 Δ clpA and F2/5 Δ clpP1. At 72 h, CG49 averaged 5.3×10^6 , F2/5 averaged 7.1×10^6 , F2/5 Δ clpA averaged 4.5×10^6 , and F2/5 Δ clpP1 averaged 4.2×10^6 , showing no significant differences in populations at this time. These results suggest that gall inhibition may not have been effective at 0 and 24 h because the mutants did not colonize the wounds to the level of CG49.

DISCUSSION

Crown gall disease is a limiting factor affecting vineyard production, particularly throughout cold-climate grape-growing regions of the world. The disease is primarily managed by proper site and cultivar selection and by using other cultural methods such as establishment of multiple trunks per vine and hilling soil over lower trunks in the autumn (3). Obtaining *A. vitis*-free vines for planting can be difficult because the pathogen persists in symptomless cuttings and, therefore, is spread in propagation material (4,18,37). Hot-water treatment of dormant canes reduces but does not eradicate systemic populations of *A. vitis* (2,8) whereas initiating propagation of grapes via tissue culture allows exclusion of *A. vitis* from the plant (5).

A. vitis is able to persist in grape root debris in soil for years (10) and, therefore, even planting clean vines into such soils may result in development of crown gall. Therefore, an effective treatment of clean vines prior to planting, especially in nurseries or in soils previously planted to vineyards, will be an important compo-

Fig. 3. Blue spots indicating β -glucuronidase (GUS) expression in grape and tobacco tissues inoculated with strain CG49(GUS) alone and in combination with F2/5. **A**, Grape and **C**, tobacco tissues inoculated with CG49(GUS) alone. **B**, Grape and **D**, tobacco tissues inoculated with CG49(GUS) in combination with F2/5.

TABLE 3. Average populations of CG49 and F2/5 in individual wound sites on grape shoots^z

Treatment	24 h		48 h		72 h	
	PDA	PDA+Km	PDA	PDA+Km	PDA	PDA+Km
F2/5	8.96×10^5 a	...	2.48×10^6 a	...	3.13×10^6 ab	...
CG49	4.97×10^5 a	...	1.75×10^6 ab	...	2.62×10^6 ab	...
F2/5(GUS)	9.98×10^5 a	8.57×10^5 a	8.81×10^5 b	6.44×10^5 ab	5.39×10^6 a	3.07×10^6 a
CG49(GUS)	6.34×10^5 a	5.20×10^5 ab	1.56×10^6 ab	1.31×10^6 a	3.30×10^6 ab	2.28×10^6 a
CG49 + F2/5 (GUS)	8.60×10^5 a	3.24×10^5 bc	1.53×10^6 ab	5.84×10^5 ab	3.14×10^6 ab	9.67×10^5 b
CG49(GUS)+F2/5	3.31×10^5 a	1.31×10^5 bc	1.18×10^6 b	7.03×10^5 ab	1.88×10^6 b	9.22×10^5 b

^z Cell counts on potato dextrose agar (PDA) represent total population size of CG49 and F2/5 whereas the PDA + kanamycin (Km) counts represent the population size of the β -glucuronidase (GUS)-marked strain. Time shown is hours postinoculation. Eight grapevines plants were inoculated for each treatment. Serial dilutions were plated and colony counts made after 48 to 72 h. Results were statistically analyzed using Duncan's MRT with Statistical Package for the Social Sciences, version 15 (SPSS Inc.). For each treatment, at least three replications were evaluated. Numbers in columns followed by different letters are significantly different, $P \leq 0.01$.

ment of a disease management program. We and others have explored this need through determining the potential of controlling grape crown gall by biological control (6,7,12,25,28). Although gall formation is reduced or prevented by treatment of grape wound tissues with certain bacterial strains, the treatments have not been developed for use in commercial nurseries and vineyards, and associated underlying mechanisms are generally unknown.

TABLE 4. Effect of F2/5 and mutants on crown gall inhibition on grapevine^z

Treatment	Biological control
CG49+ F2/5	+
CG49 + F2/5Δ <i>clpA</i>	–
CG49 + F2/5Δ <i>clpP1</i>	–
CG49 + F2/5Δ <i>clpP2</i>	+
CG49 + F2/5Δ <i>clpS</i>	+
CG49 + F2/5Δ <i>clpS</i>	+
CG49 + F2/5Δ <i>aviR</i>	±
CG49 + F2/5Δ <i>avhR</i>	+
CG49 + F2/5Δ <i>avsI</i>	+
CG49 + F2/5Δ <i>avhE</i>	+

^z Eight grapevines plants were inoculated for each treatment. Gall development was recorded 8 weeks postinoculation: + = no galls developed at any of the inoculation sites, – = galls formed at all inoculation sites, ± = a reduced level of biological control described in Table 4. All experiments were repeated at least twice.

TABLE 5. Effect of F2/5 and mutants on crown gall of grapevine^y

Treatment ^z	Average gall size (mm ²)
Experiment A	
CG49	86.2 a
CG49 + F2/5	0.0 d
CG49 + F2/5Δ <i>clpA</i>	89.3 a
CG49 + cmF2/5Δ <i>clpA</i> (0 h)	76.3 ab
CG49 + cmF2/5Δ <i>clpA</i> (24 h)	70.4 abc
CG49 + cmF2/5Δ <i>clpA</i> (48 h)	43.4 bc
CG49 + cmF2/5Δ <i>clpA</i> (72 h)	0.0 d
CG49 + F2/5Δ <i>clpP1</i>	93.1 a
CG49 + cmF2/5Δ <i>clpP1</i> (0 h)	65.0 abc
CG49 + cmF2/5Δ <i>clpP1</i> (24 h)	67.9 abc
CG49 + cmF2/5Δ <i>clpP1</i> (48 h)	35.1 c
CG49 + cmF2/5Δ <i>clpP1</i> (72 h)	0.0 d
Experiment B	
CG49	138.4 a
CG49 + F2/5	0.0 c
CG49 + F2/5Δ <i>aviR</i>	57.0 b
CG49 + cmF2/5Δ <i>aviR</i>	0.0 c

^y Inoculations were made to eight grapevines for each treatment, as described. Gall diameters were measured with a venire caliper at the widest vertical and horizontal sides. Experiments were replicated three times. Results were statistically analyzed using Duncan’s MRT with Statistical Package for the Social Sciences, version 15 (SPSS Inc.). Numbers followed by different letters are significantly different, *P* ≤ 0.05.

^z Abbreviation: cm = complemented strains.

Fig. 4. Biological control activity of complemented F2/5 *clpA* and *clpP1* mutants applied to wound sites at different time intervals prior to inoculation with CG49. Inoculation with **A**, CG49 alone; **B**, CG49 plus cmF2/5Δ*clpA*; and **C**, CG49 plus cmF2/5Δ*clpP1*. CG49 was applied to the wound sites at 0 h (1), 24 h (2), 48 h (3), and 72 h (4) after wounding and treatment with water or complemented mutants. Arrows indicate wound sites on vines where galls were small or did not develop.

Our previous work on *A. vitis*-induced grape necrosis and HR revealed the presence of quorum-sensing-associated *luxR* genes being involved in regulation of these phenotypes. Analyses of the *A. vitis* S4 genome (NC_011989.1) revealed at least five transcription factors belonging to the LuxR family and a subset of these (*aviR*, *avsR*, and *avhR*) and a single *luxI* (autoinducer synthase) homolog (*avsl*) were previously shown to be associated with the ability of F2/5 to cause an HR on tobacco and induce necrosis of grape shoot explant tissues (20,43). Preliminary work has also indicated that an *aviR* mutant is reduced in biological control activity (42). In this article, we further confirm the role of *aviR* via mutant complementation to specifically show that quorum sensing is involved in the F2/5 mechanism of biological control. Quorum-sensing regulation is common among prokaryotes and regulates several phenomena, including virulence, motility, biofilm formation, biological control, and bacterial colonization of plant roots. For example, in *P. fluorescence* strain 2P24, quorum sensing was found to play a role in suppression of wheat take-all disease. Although previously the control of take-all was found to be associated with production of the antifungal compound 2,4-diacetylphloroglucinol, the knockout of the auto-inducer synthase gene in 2P24 did not affect production of the antifungal metabolite but significantly reduced biological control. It was determined that the reduced biological control resulted from reduction in biofilm formation and subsequent colonization of wheat roots (41). Another example involves *P. aeruginosa* strain PUPa3 that expresses antifungal and plant-growth-promoting activities, the latter of which is dependent on rice root colonization (35). PUPa3 possesses two nonhierarchical quorum-sensing systems characterized by RhlR and LasR transcription factors, respectively, which are both required for full growth promotion and antifungal activity. Thus, each system regulates independent cellular functions required for components of rice plant growth enhancement. For F2/5, we also characterized a set of LuxR-type transcription factors and determined that, whereas all are required for HR and grape necrosis, only one, *AviR*, is associated with biological control. This was not surprising because previous work revealed that *luxR* homologs in F2/5 are expressed in a nonhierarchical fashion (19). It will now be important to identify target genes regulated by *AviR* as a means of further understanding the mechanism of control.

In this article, we also show that biological control by F2/5 is dependent on the *clp* system, which is also common in prokaryotes and functions by recruiting, unfolding, and degrading selected native intercellular proteins (1). The *clp* system selectively recruits proteins for degradation based on the presence of sequence tags that are often located at the N or C ends of proteins targeted for degradation. Therefore, this widely present protease system in prokaryotes helps to maintain a proper balance of intercellular proteins under various environmental conditions, thereby regulating different bacterial responses. For example, in *Streptococcus pneumoniae*, mutations in *clpP* resulted in reduced growth at elevated temperatures and increased sensitivity to oxidative stress and attenuation of virulence (31). In *Salmonella enterica*, it was discovered that ClpXP is involved in regulation of flagella synthesis. Mutants were hyperflagellated, overproduced flagellin, and increased expression of *flhC*, suggesting that ClpXP may be responsible for suppressing expression of the flagella regulon (38). In our work, we initially discovered that a Tn5 mutant of F2/5 with an insertion in a *clpA* homolog was HR minus and reduced in grape necrosis (21). In this article, we show *clpA* is also required for biological control. ClpA is an ATP-dependent chaperone that binds selected proteins and delivers them to protease ClpP for degradation. There are two *clpP* homologs in the S4 genome and we subsequently identified their presence in F2/5. One, *clpP1* (Avi1696), is required for biological control and the other, *clpP2* (Avi 3244), is not. Further research will be essential to demonstrate how the *clp* system is specifically

involved in biological control. One approach may be to identify specific proteins that interact with the F2/5 ClpAP complex and how mutations affect their concentrations within F2/5.

It was necessary to apply complemented F2/5 Δ *clpA* and F2/5 Δ *clpP1* to wounds 2–3 days prior to CG49 for gall inhibition to be similar to F2/5. It was determined that tissues remained susceptible to infection by CG49 3 days after wounding; therefore, we are confident that the complemented strains were able to restore biological control. When a high-copy vector, pPZP201, was used in the complementation of *clpA* and *clpP1* mutants, biological control was not restored whereas, when low-copy-number vectors were used, biological control was restored. Therefore, we suspected that the level of protein expression is associated with the mechanism of gall inhibition. The copy number influences the plasmid stability and the maintenance of the plasmid within the cells during cell division. A positive effect of a high copy number is the greater stability of the plasmid when the random partitioning occurs at cell division. On the other hand, a high number of plasmids generally decrease the growth rate, thus possibly allowing for cells with few plasmids to dominate the culture (26). We suspect that maintenance of proper ClpA and ClpP1 protein levels within the cells is critical to expression of biological control by F2/5. This phenomenon, related to complementation of the F2/5 *clp* genes, was not surprising because other attempts to complement *clpA* mutants in *Brucella suis* resulted in the complemented mutant growing more slowly than the mutant and wild type. In this case, it was concluded that *clpA* was involved in temperature-dependent growth regulation and in bacterial clearance from mice (17). Similarly, the F2/5-complemented *clpA* and *clpP1* mutants initially established lower populations in grape wounds than the wild-type strain but populations were not different after 72 h. The results indicate that the *clp* system is essential for maintenance of proteins at specific levels to carry out various functions in bacteria.

A key feature of biological control by F2/5 is that it prevents transformation by tumorigenic *A. vitis* strains on grape but not on other plant species. For example, whereas co-inoculations of F2/5 and CG49 on grape wounds resulted in no crown gall, disease development on tobacco stems did not differ whether F2/5 was present or not. F2/5 did not inhibit CG49 T-DNA transfer in tobacco, as shown by the development of galls and the presence of GUS staining following inoculation with tumorigenic strain CG49 carrying a binary Ti-plasmid vector expressing a *uidA* gene driven by the 35S promoter with a eukaryotic intron. It is also intriguing that the mechanism of control is not associated with a reduction of pathogen population or growth at the grape wound site. Thus, the mechanism for this highly specific biological control activity suggests different possibilities, including an essential role of grape chemistry or a specific effect of F2/5 on grape tissue, thereby making it resistant to infection. Alternatively, F2/5

TABLE 6. Effect of wound age prior to inoculation on crown gall development by CG49^z

Treatment	Average gall size (mm ²)
CG49 (0 h)	148.52 a
CG49 (24 h)	110.25 ab
CG49 (48 h)	118.33 ab
CG49 (72 h)	112.28 ab
CG49 + F2/5 (0 h)	0.00 c
Water	0.00 c

^z Single inoculations were made to six grapevines for each treatment, as described. Gall diameters were measured by venire caliper at the largest point of vertical and horizontal side of inoculation site. Each treatment was repeated at least once. The results were statistically analyzed using Duncan's MRT with Statistical Package for the Social Sciences, version 15 (SPSS Inc.). There were at least three replications for each treatment in individual experiments. Numbers followed by different letters are significantly different, $P \leq 0.01$.

could be acting directly on the pathogen in a manner that affects a specific function required for transformation of grape but not for tobacco.

A previous report published by members of our laboratory indicated that F2/5 was able to prevent *A. vitis* crown gall induction on *Ricinus communis* (42). We have employed this system repeatedly for studies on F2/5-induced biological control, with mixed results. Whereas sometimes gall formation is prevented, this was not universally the case. We do not understand the variability observed but are confident that F2/5 does not always prevent crown gall on *R. communis*. One possible explanation is that Zauner et al. (42) utilized a different strain of *A. vitis*, K306, in their experiments. We plan to investigate this discrepancy further because *R. communis* may provide clues to identification of specific plant factors that are associated with the biological control.

Whereas our findings have revealed important information associated with biological control of grape crown gall, further research will be essential to elucidate the mechanism involved. All associated genes identified thus far that are essential for biological control are present in the strain S4 genome as well as in F2/5. Therefore, to further understand the mechanism of control, it will be necessary to compare genome sequences of F2/5 with S4 to identify unique regions in F2/5 and determine their possible roles in biological control. Our laboratory is currently initiating this approach.

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