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Expression of a crown gall biological control phenotype in an avirulent strain of *Agrobacterium vitis* by addition of the trifolitoxin production and resistance genes

Thomas C Herlache and Eric W Triplett*

Address: Department of Agronomy, University of Wisconsin-Madison, 1575 Linden Drive, Madison, WI 53706 USA

E-mail: Thomas C Herlache - tcherlache@facstaff.wisc.edu; Eric W Triplett* - triplett@facstaff.wisc.edu

*Corresponding author

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Abstract

Background: *Agrobacterium vitis* is a causal agent of crown-gall disease. Trifolitoxin (TFX) is a peptide antibiotic active only against members of a specific group of α-proteobacteria that includes *Agrobacterium* and its close relatives. The ability of TFX production by an avirulent strain of *Agrobacterium* to reduce crown gall disease is examined here.

Results: TFX was shown to be inhibitory *in vitro* against several *A. vitis* strains. TFX production, expressed from the stable plasmid pT2TFXK, conferred biological control activity to an avirulent strain of *A. vitis.* F2/5, against three virulent, TFX-sensitive strains of *A. vitis* tested on *Nicotiana glauca.* F2/5(pT2TFXK) is significantly reduces number and size of galls when co-inoculated with tumorigenic strain CG78 at a 10:1 ratio, but is ineffective at 1:1 or 1:10 ratios. F2/5(pT2TFXK) is effective when co-inoculated with tumorigenic strain CG435 at 10:1 and 1:1 ratios, but not at a 1:10 ratio. When F2/5(pT2TFXK) is co-inoculated with CG49 at a 10:1 ratio, the incidence of gall formation does not decline but gall size decreases by more than 70%. A 24 h pre-inoculation with F2/5(pT2TFXK) does not improve biological control at the 1:10 ratio.

Conclusions: TFX production by an avirulent strain of *Agrobacterium* does confer in that strain the ability to control crown gall disease on *Nicotiana glauca.* This is the first demonstration that the production of a ribosomally synthesized, post-translationally modified peptide antibiotic can confer reduction in plant disease incidence from a bacterial pathogen.

Background

Agrobacterium vitis strains are causative agents of crown gall, an economically important disease [1,2]. *A. vitis* F2/5 is an effective biological control agent against many *A. vitis* tumorigenic strains [3]. Strain F2/5 produces an antibiotic toxic to many *A. vitis* strains *in vitro.* However, two lines of evidence suggest that this antibiotic plays a minor role in disease suppression. Strains that are susceptible to the antibiotic *in vitro,* such as *A. vitis* strain CG78, are able to infect the plant in the presence of F2/5 [3], and Tn5 mutants of F2/5 lacking F2/5 antibiotic production appear to be unaffected in crown gall biological control [4]. Biological control by F2/5 is grape-specific, as F2/5 is not effective on non-grapevine host plants such as *Nicotiana glauca.* Furthermore, F2/5 is not effective against all *A. vitis* strains [3]. Thus, enhancing the F2/5 biological control phenotype and extending the host range of the efficacy of F2/5

beyond grape would be beneficial for disease control in field applications.

Agrobacterium rhizogenes strain K84 is the most studied crown gall biological control strain and is commercially utilized for crown gall disease control worldwide. Strain K84 biological control is primarily due to production of two plasmid-encoded antibiotics, agrocins 84 and 484, encoded by genes on pAgK84 and pAgK434 respectively [5]. Agrocin 84, an adenosine analog [6], is effective against tumorigenic strains carrying nopaline/agrocinopine tumor-inducing plasmids, and requires the *acc* system in the target strain for activity [7]. Agrocin 434, a di-substituted cytidine analog, is effective against, and specific for, a broad range of *A. rhizogenes* strains [8]. Curing of either agrocin-encoding plasmid results in reduction of biological control activity [9]. Thus, K84 demonstrates the efficacy of antibiosis for crown gall biological control. However, the commercial application of the K84 biological control system is limited where *Agrobacterium* strains of certain crops are not inhibited by K84. As a result, alternative biological control systems for crown gall disease are needed.

Trifolitoxin (TFX) is a peptide antibiotic produced by *Rhizobium leguminosarum* bv. trifolii T24. Antibiotic production and resistance functions are encoded by the seven-gene *tfx* operon and the unlinked *tfuA* gene [10,11]. TFX is derived from post-translational cleavage and modification of the *tfxA* gene product. TFX effectively inhibits growth of members of the α-proteobacteria including strains of *Brucella, Ochrobactrum, Rhodobacter, Rhodospeudomonas, Rhizobium,* and the etiological agent of crown gall disease, *Agrobacterium*[12]. TFX is highly specific for this group, as demonstrated by a lack of observable effect on the majority of the bacterial population in the bean rhizosphere [13]. This suggests that TFX could be used to control crown gall diseases of various plants with limited effects on non-target bacteria outside of that very specific group of TFX-sensitive α-proteobacteria. TFX is rapidly degraded in nonsterile soil but is readily extractable from sterile soil inoculated with a TFX-producing strain, suggesting that TFX is sensitive to *in situ* proteolysis [14]. Despite this rapid turnover, TFX production confers enhanced nodulation efficiency upon TFX-producing *Rhizobium* strains under field conditions [15].

Degradation of antimicrobial peptides such as cecropin B and attacin E also has been observed in plant apoplastic fluids [5,16,17]. This is likely due to apoplastic proteinases [5,16]. Expression of antimicrobial peptides in plants has had mixed results for enhancing disease resistance. For example, cecropin expression in transgenic tobacco did not confer resistance to *P. syringae* pv *tabaci*[18], likely due to low apoplastic peptide concentrations due to proteolysis [19]. Therefore, prior to this work it was not clear whether a peptide antibiotic could play a role in the inhibition of galling by tumorigenic *Agrobacterium in planta.*

Agrobacterium rhizogenes is capable of producing TFX by addition of the stable plasmid, pT2TFXK, which contains the *tfx* operon but not *tfuA*[11,12,20]. This suggests that TFX production by crown gall biological control strains of *Agrobacterium,* such as *A. rhizogenes* K84 and *A. vitis* F2/5, may be enhanced by TFX production. These strains would be excellent delivery vehicles for TFX to the infection court.

Here we present experiments demonstrating the TFX-sensitivity of a range of *A. vitis* strains and the effect of the TFX production and resistance phenotypes on the expression of biological control of crown gall using the model plant *Nicotiana glauca.* Strain F2/5 was chosen as the recipient of the TFX genes in this work because it is an avirulent strain that expresses a biological control phenotype on only one host. As a result, F2/5 is an ideal strain in which to test the ability of the TFX system to confer biological control of crown gall disease and to broaden the host-range efficacy of a known biological control strain.

Results

In vitro TFX antibiosis against A. vitis

Agrobacterium vitis strains (Table 1) were tested for sensitivity to trifolitoxin. As expected based on previous results [12], the tested *Agrobacterium vitis* strains were sensitive to TFX-producing strains. However, the level of susceptibility was lower than predicted based upon previous TFX sensitivity measurements with CG48 and CG74 [12]. No zones of *A. vitis* growth inhibition were observed around *R. leguminosarum* T24 colonies, and only relatively small zones were observed around *R. etli* CE3(pT2TFXK), which produces more TFX than T24. Furthermore, one *A. vitis* strain, F2/5, was TFX-resistant.

Because *A. vitis* F2/5 produces an antibiotic to which most of the tumorigenic strains are sensitive [3], the effect of TFX on *A. vitis* was assessed against TFX-producing and non-producing *Rhizobium* strains. None of the tested strains were sensitive to *R. leguminosarum* T24, which produces relatively low amounts of TFX. All of the *A. vitis* strains except for F2/5 were sensitive to *R. etli* CE3(pT2TFXK) as evidenced by zones of growth inhibition around the CE3(pT2TFXK) colonies. *A. vitis* growth was not inhibited by a non-TFX metabolite or nutrient competition by CE3(pT2TFXK) as evidenced by the lack of a zone around the near-isogenic *tfxA* mutant CE3(pT2TX3K) colony (Fig. 1). Wild type F2/5 produces a zone of inhibition versus the other agrobacteria used in this work. This occurred because of the antibiotic production previously observed by F2/5. These zones of inhibi-

Table 1: Bacterial Strains used in this work and their TFX phenotype (production, resistance, and sensitivity). Overproduction of TFX occurs when the TFX production is conferred using a multi-copy, broad host range plasmid such as pT2TFXK.

Strain	Characteristics	Reference
Rhizobium		
T24	*R. leguminosarum* bv. trifolii, TFX producing strain	28
T24::Tn5-1	TFX non-producing mutant, Tn5 insertion in *tfxB*	10
CE3(pT2TFXK)	*R. etli.* Contains TFX-encoding plasmid, overproduces TFX	20
CE3(pT2TX3K)	Plasmid contains *tfxA* deletion, non-TFX producing strain	20
ANU794	*R. leguminosarum* bv. trifolii, TFX-sensitive	29
ANU794(pT2TX3K)	TFX-resistant	This work
Agrobacterium vitis		
F2/5	Biological control on grapevine, no control on other host-plant species, TFX-resistant	30
F2/5(pT2TFXK)	Contains TFX-encoding plasmid, produces TFX, TFX-resistant	This work
F2/5(pT2TX3K)	Plasmid contains *tfxA* deletion, non-TFX producing strain, TFX-resistant	This work
CG49	Tumorigenic, nopaline-type Ti plasmid, controlled by F2/5, sensitive to TFX overproduction	31
CG78	Tumorigenic, vitopine-type Ti plasmid, not controlled by F2/5 co-inoculation, sensitive to TFX overproduction	from TJ Burr
K306	Tumorigenic, octopine-type Ti plasmid, controlled by F2/5, sensitive to TFX overproduction	from TJ Burr
CG107	Tumorigenic, sensitive to TFX overproduction	from TJ Burr
CG113	Tumorigenic, TFX-sensitive	from TJ Burr
CG435	Tumorigenic, TFX-sensitive	from TJ Burr

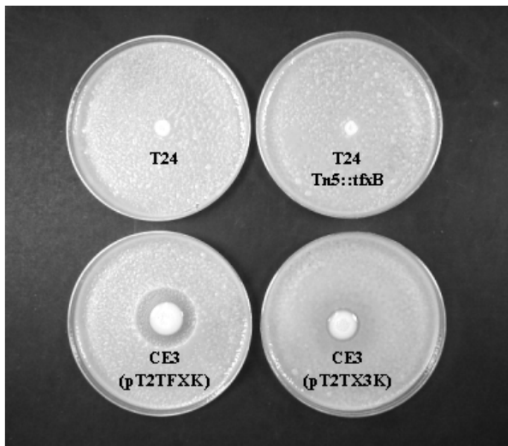

Figure 1
In vitro TFX antibiosis assay against A *vitis* CG78. Legend: The indicated TFX producing strains and their non-producing mutant derivatives cultured as a single colony near the centers of the plates and allowed to grow 4 days at 27°C. The plates were then misted with a dilute suspension of *A. vitis* CG78. The plates were photographed two days following misting. A zone of growth inhibition is present around the *R. etli* CE3(pT2TFXK) colony, but not around any of the other colonies.

tion were much larger when pT2TFXK was added to F2/5 and unchanged with the addition of pT2TX3K.

Evidence for TFX production by A. vitis strain F2/5(pT2TFXK)

Strain F2/5(pT2TFXK) inhibited TFX-sensitive R. leguminosarum bv. trifolii ANU794 but had no effect on ANU794 following addition of the TFX resistance genes provided by pT2TX3K (Fig. 2). Plasmids pT2TFXK and pT2TX3K confer resistance to TFX, tetracycline, and kanamycin with pT2TFXK also providing TFX production to a host strain [20]. Since strains F2/5 and F2/5(pT2TX3K) produced no zones of inhibition against ANU794 or ANU794(pT2TX3K) (data not shown), F2/5(pT2TFXK) is producing TFX. In addition, this shows that the agrocin-like molecule produced by F2/5 does not inhibit ANU794.

In planta biological control of crown gall conferred by TFX production

As expected, F2/5 did not inhibit galling by tumorigenic A. vitis strains on N. glauca. However, a 10:1 ratio of A. vitis F2/5(pT2TFXK):pathogen caused a statistically significant reduction in mean gall size relative to the TFX non-producing controls on N. glauca stems for all three tested tumorigenic strains (Figs. 3 and 4). High concentrations of F2/5(pT2TFXK) also reduced gall incidence for CG435 and CG78, but not for CG49 (Table 2). A 1:1 ratio of F2/5(pT2TFXK):CG435 also resulted in a significant reduction in gall size and in gall incidence compared to controls. A 1:1 ratio of F2/5(pT2TFXK):CG49 or F2/5

Table 2: Proportion of inoculations that resulted in gall formation by the tumorigenic *A. vitis* strains (CC49, CG78, and CG435) when co-inoculated with the TFX-producing strain F2/5(pT2TFXK) or the non-producing strains, F2/5 or F2/5(pT2TX3K).

	Tumorigenic Strain[a].	Proportion of inoculations that resulted in gall formation.	
Biological control	CG49	CG78	CG435
Strain			
F2/5	12/12	6/6	12/12
F2/5(pT2TFXK)	10/14	1/8	0/14
F2/5(pT2TX3K)	ND[b]	8/8	ND

a. Inoculations performed at 10:1 ratio of biological control:pathogenic strain. Presence of galls was scored visually by comparison to an uninoculated negative control one month post-inoculation. b. ND, not done.

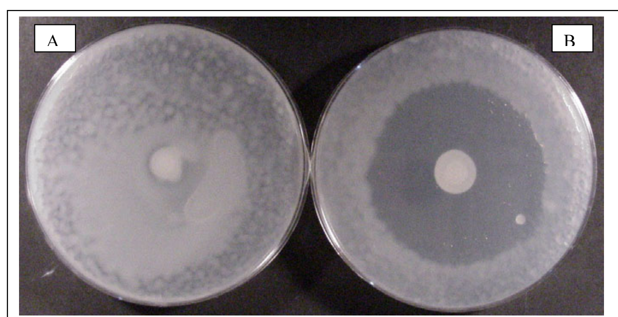

Figure 2
Ability of *Agrobacterium* to produce TFX with addition of pT2TFXK. Legend: Ability of F2/5(pT2TFXK) to inhibit ANU794. This inhibition is reversed by the addition of the TFX resistance genes to ANU794. Assay was performed as described in Figure 1 with F2/5(pT2TFXK) cultured in a single colony in the center of the plate. One day after growth at 28°C, the plates were sprayed with a dilute suspension of ANU794(pT2TX3K) (A) or ANU794 (B).

(pT2TFXK): CG78 did not affect either incidence of galls or reduce gall size. Similarly, an excess (a 1:10 ratio) of any of the virulent strains to F2/5(pT2TFXK) resulted in a high incidence of disease and large gall size.

Discussion

As all strains of *A. vitis* tested were sensitive to TFX both in this work and in a previous study [12], experiments were conducted to determine the effectiveness of a TFX-producing, avirulent strain of *A. vitis* in the prevention of crown gall caused a three strains of *A. vitis.* Although *A. vitis* F2/5 is an effective crown-gall biological control agent against most tumorigenic *A. vitis* strains when co-inoculated with a tumorigenic strain, it is not an effective against all strains of *A. vitis*.

Where *A. vitis* F2/5 is effective as a biological control agent, its control is only effective when numbers of F2/5 are equal to, or greater than, the number of cells of the virulent strain [3]. Strains that are resistant to F2/5 biological control are known. For example, strain CG78 is not controlled when co-inoculated with F2/5 [1].

Two principle benefits of TFX production by F2/5 are demonstrated here. Biological control is extended to a new host, *N. glauca*. Furthermore, biological control is extended to a strain that F2/5 fails to control (CG78). These effects are due to TFX production as demonstrated by the lack of efficacy of F2/5 against CG49, CG435, and CG78, and by the lack of efficacy of the near-isogenic TFX-non-producing F2/5(pT2TX3K) against CG78. TFX confers biological control when the TFX-producing strain is present in excess of the tumorigenic strain. Thus, F2/5(pT2TFXK) effectively inhibited gall incidence by two of the three tested tumorigenic strains when co-inoculated in approximately 10-fold excess. F2/5(pT2TFXK) inhibited gall size by more than 70% with third tumorigenic strain tested. At 1:1 or 1:10 inoculum ratios of F2/5(pT2TFXK):pathogen biological control was reduced or lost. High ratios of biological control: pathogen strain should be easily achieved in field situations by dipping the roots of planting stock in suspensions of the biological control strain, or by directly applying the bacterial suspension to the planting bed. Furthermore, TFX is inhibitory towards all tested species of *Agrobacterium*[12]. These results suggest that TFX production would enhance crown gall biological control for other biological control strains, such as *A. rhizogenes* K84, and on other host plants, especially where a mixed inoculum of different tumorigenic *Agrobacterium* species occurs.

K84 biological control is thought to be primarily due to production of two plasmid-encoded antibiotics, each of which accounts for a portion of the observed disease control [9]. This naturally occurring example suggests that

Figure 3
Effect of TFX production and inoculum ratio on gall formation. Legend: *Nicotiana glauca* stems were wounded with a dissecting needle, and 5 ul of inoculum (mixtures noted at left) was placed on the wounds. The top stem was inoculated with CG435 as a positive control. Stems 2 and 3 were inoculated with mixtures of biological control test strains and CG435 at either a 1:1 (left three inoculation sites on each stem) or 10:1 (right three inoculation sites) biological control:pathogen ratios. The photograph was taken approximately 2 months post-inoculation. pT2TFXK confers biological control on F2/5 at the 10:1 ratio, but not at the 1:1 ratio.

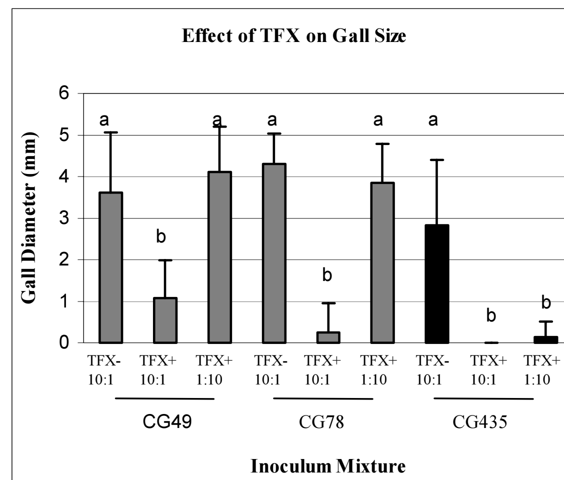

Figure 4
Effect of TFX production and inoculum ratio on *Nicotiana glauca* gall size. Legend: Gall diameter (mm) perpendicular to the stem was measured 1 month post-inoculation. Wound sites were inoculated with 5 μl of mixed bacterial suspensions. Mixtures were made immediately prior to inoculation. Each inoculum mixture was inoculated into three or four wounds on each of two plants, for a total of six to eight inoculations per treatment. Results that are significantly different at $\alpha = 0.05$ are indicated with different letters within a group of inoculum mixtures. High ratios of F2/5 (pT2TFXK) :tumorigenic strain result in significant disease suppression for all three tested tumorigenic strains.

pyramiding biological control mechanisms within one strain can enhance disease control. Strain F2/5 also produces an *A. vitis*-specific antibiotic *in vitro*, but this antibiotic is not involved in disease control [4]. Thus, the addition of TFX to F2/5 is a successful example of pyramiding unrelated disease control mechanisms and clearly demonstrates that individual antibiotics can be effectively developed as a mechanism of disease control.

Although plasmid-borne traits are frequently unstable, pT2TFXK contains the RK2 plasmid-partitioning locus that confers a high degree of stability both *in vitro* and under field conditions [15]. Stable TFX expression would be beneficial under field conditions when the biological control agent is inoculated on seeds or on roots dipped in a bacterial suspension prior to planting.

Trifolitoxin production enhances rhizosphere competitiveness of *Rhizobium etli* CE3 (pT2TFXK) in sterile soil and enhances nodulation efficiency in non-sterile soil when compared to a TFX-sensitive strain [20]. Trifolitoxin production also increases bean nodulation competitiveness of *Rhizobium etli* CE3 in the presence of indigenous rhizobia under agricultural conditions [15]. Field application of *R. etli* CE3 (pT2TFXK) dramatically reduced the diversity of indigenous α-proteobacteria in the bean rhizosphere without affecting unrelated species [13]. Thus, TFX production by *A. vitis* F2/5 should enhance the competitiveness and aid in establishment of this beneficial strain in the rhizosphere. The ability to displace indigenous tumorigenic *Agrobacterium* strains would be beneficial in those areas already infested with the pathogen.

Compared to other treatments available for pathogenic agrobacteria, the effects of TFX on non-target species is very low. Copper, a broad spectrum bacteriocide, is commonly used as a treatment of bacterial diseases. Although no comprehensive studies have been published on the taxonomic range of bacteria inhibited by K84, it is known that K84 produces multiple antibiotics and that K84 can inhibit strains of *Erwinia* and *Pseudomonas* that are unaffected by TFX [23–25]. TFX-producing strains inhibit only a very specific group within the α-proteobacteria in culture and in the field [13,26]. These observations suggest that a K84-producing strain may have a more serious im-

pact on non-target organisms than does a TFX-producing strain. However, there has been no study of the effects of K84 on non-target soil bacteria by culture-independent means to compare the effects of K84 versus the TFX system in a natural system.

Conclusions

TFX production effectively enhances crown gall biological control by *Agrobacterium vitis* F2/5. The host-plant range and range of tumorigenic *A. vitis* strains controlled by F2/5 are both broadened by TFX production. In addition, virulent strains of *A. vitis* not previously controlled by F2/5 are controlled following the addition of the trifolitoxin production and resistance genes to F2/5. The plasmid used to confer trifolitoxin production and resistance, pT2TFXK, is stably maintained in the absence of selection pressure [20]. This plasmid is not self-transmissible but is mobilizable. With the TFX system, the biological control of *Agrobacterium* pathogens may be extended to many crops far beyond what is possible today with currently available commercial products.

Materials and Methods

Bacterial and plant growth conditions, strain construction

Bacterial strains are listed in Table 1. The *A. vitis* strains (without pT2TFXK or pT2TX3K) were obtained from Dr. T.J. Burr, Cornell University. Bacteria were grown on BSM agar [21] media at 27°C. *A. vitis* F2/5(pT2TFXK) and F2/5(pT2TX3K) were constructed by triparental mating using standard procedures. Transconjugants were selected on BSM media amended with 50 μg/ml kanamycin. Trimethoprim (10 μg/ml) was added to counterselect the *E. coli* DH5α donor and helper strains. The helper strain was *E. coli* DH5a pRK2013 [27]. Strains containing the plasmids pT2TFXK and pT2TX3K were grown for routine propagation on BSM amended with 50 μg/ml kanamycin. Prior to use in making inoculum suspensions for biological control assays these strains were grown overnight on BSM agar without kanamycin.

Plants (*Nicotiana glauca*) were grown in the greenhouse with supplemental illumination and fertilized as needed with a nutrient solution called CNS containing (2 mM $CaCl_2.2H_2O$, 0.5 mM $MgSO_4.7H_2O$, 2 mM KCl, 0.4 mM KH_2PO_4, 2.5 mM NH_4NO_3, 0.065 mM $FeSO_4.7H_2O$, 2.3 μM H_3BO_3, 0.9 μM $MnSO_4.H_2O$, 0.6 μM $ZnSO_4.7H_2O$, 0.1 μM $NaMoO_4.2H_2O$, 0.11 μM $NiCl_2.6H_2O$, 0.01 μM $CoCl_2.6H_2O$, 0.15 μM $CuSO_4.5H_2O$).

In vitro antibiosis assay

TFX antibiosis assays were performed as described previously [11]. The effect of TFX on various *Agrobacterium vitis* strains (Table 1) was assessed using *Rhizobium leguminosarum* bv. trifolii strain T24 and *R. etli* strain CE3 (pT2TFXK) as producing strains. *Rhizobium leguminosarum* bv. trifolii T24::Tn5-1 and *R. etli* CE3 (pT2TX3K) were used as TFX non-producing negative controls.

In planta biological control of crown gall by TFX-producing strains

Agrobacterium vitis strains were suspended in sterile distilled water prior to the determination of colony forming units (CFU) per ml. These suspensions were adjusted to OD_{650} 0.10 (approximately 10^8 CFU/ml) using a Shimadzu UV-160 spectrophotometer and sterile distilled water, and stored until inoculation at 4°C. Actual inoculum viability and cell density were measured by dilution plating on BSM agar medium on the day that plants were inoculated [22]. Prior to inoculation, tumorigenic strains were diluted 10-fold with sterile distilled water to approximately 10^7 CFU/ml. Strain F2/5 and its derivatives were left undiluted, or diluted 10-fold (for CG49 and CG435 experiments) or 100-fold (for CG78 experiments). Thus, the CFU ratios were approximately 10:1, and 1:1 or 1:10 avirulent:tumorigenic strain. Immediately prior to plant inoculation tumorigenic strains were mixed 1:1 (vol:vol) with the appropriate biological control test strain. Positive controls were diluted 1:1 (vol:vol) with water. Thus, all plant inoculum contained approximately 5×10^6 CFU/ml of the tumorigenic strains. Plants (*Nicotiana glauca*) were inoculated by wounding the stem with a dissecting needle. Three or four inoculations were made per inoculum mixture on each of two plants. Thus, each of the two experiments included 6 to 8 repetitions per treatment. A 5 μl drop of bacterial suspension was placed on the wound and allowed to air dry. Inoculation sites were wrapped loosely with Parafilm (American National Can) for 1 week post-inoculation. Gall diameter perpendicular to the long axis of the stem was measured 4 to 7 weeks post-inoculation using a caliper, and all measurements were included for statistical analysis. Results were analyzed using ANOVA at the $\alpha = 0.05$ level of significance.

Abbreviations

TFX, trifolitoxin.

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