

Reduction in Pathogen Populations at Grapevine Wound Sites is Associated with the Mechanism Underlying the Biological Control of Crown Gall by *Rhizobium vitis* Strain ARK-1

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A nonpathogenic strain of *Rhizobium* (= *Agrobacterium*) *vitis*, ARK-1, limited the development of grapevine crown gall. A co-inoculation with ARK-1 and the tumorigenic strain VAT07-1 at a 1:1 cell ratio resulted in a higher population of ARK-1 than VAT07-1 in shoots without tumors, but a significantly lower population of ARK-1 than VAT07-1 in grapevine shoots with tumors. ARK-1 began to significantly suppress the VAT07-1 population 2 d after the inoculation. This result indicated that ARK-1 reduced the pathogen population at the wound site through biological control. Although ARK-1 produced a zone of inhibition against other tumorigenic *Rhizobium* spp. in *in vitro* assays, antibiosis depended on the culture medium. ARK-1 did not inhibit the growth of tumorigenic *R. radiobacter* strain AtC1 in the antibiosis assay, but suppressed the AtC1-induced formation of tumors on grapevine shoots, suggesting that antibiosis by ARK-1 may not be the main mechanism responsible for biological control.

Key words: *Rhizobium vitis*, grapevine crown gall, biological control, pathogen populations, antibiosis assay

Grapevine (*Vitis vinifera* L.) crown gall, which is mainly caused by *Rhizobium vitis* (Ti) (40) [= *Agrobacterium vitis* (Ti), *A. tumefaciens* biovar 3, where “Ti” means “tumor-inducing” or “tumorigenic”], is one of the most important diseases of grapevines worldwide (7, 8, 36). The pathogenicity genes are mostly located on large tumor-inducing plasmids (pTi). During infection, part of the plasmid (T-DNA) is transferred to the host plant and inserted into its nuclear DNA (11). The main symptom of crown gall is the fleshy galls that are produced in response to infection (4). These galls are mostly found on the lower trunk of grapevines near the soil surface (4). Crown gall represents a serious economic problem in viticultural regions of northern Japan, in which *Vitis vinifera* and interspecific hybrids are grown and climatic conditions favor freeze injury. The incidence of crown gall was previously reported to be high at the basal ends and belowground disbudded nodes of some cultivars and rootstocks in nurseries in Japan. Shoot growth by galled vines is frequently inferior, and portions of the vine above these galls can die (4). Thus, grapevine crown gall causes substantial economic losses, and there is currently no effective strategy for managing this disease.

The nonpathogenic *R. rhizogenes* strain K84 has successfully been used to control crown gall in many plant species (14, 28, 30, 33). An agrocin produced by K84 (agrocin 84) is thought to be the primary factor responsible for this control (28). However, K84 does not control grapevine crown gall caused by *R. vitis* (Ti), which is insensitive to agrocin 84 (3, 5, 18, 19, 21). Recombinant DNA techniques have been used to construct the new biological control strain, K1026, which is identical to K84 except for a 5.9-kb deletion that overlaps

the transfer (Tra) region of pAgK84 (16). K1026 is unable to transfer its mutant agrocin 84 plasmid, designated pAgK1026, to other agrobacteria, but remains inhibitory to strains that are sensitive to agrocin 84 (16, 33, 34).

Several laboratories have attempted to identify other biological measures to control grapevine crown gall (10, 13, 38, 39). Staphorst *et al.* (38) evaluated nonpathogenic *R. vitis* strain F2/5, which inhibited the growth of most tumor-inducing strains of *R. vitis in vitro* and greatly inhibited crown gall formation on grapevines in greenhouse shoot-wounding experiments. Burr and Reid (6) reported that F2/5 produced agrocin, which inhibits most *R. vitis* (Ti) strains *in vitro*, and effectively inhibited tumor formation at wound sites on grapevine shoots artificially inoculated with one of several *R. vitis* (Ti) strains. However, F2/5 did not inhibit tumor formation caused by other strains of *R. vitis* (Ti) (8), and F2/5 caused necrosis on grapevine shoot explants (15). Wang *et al.* (39) reported that the antibacterial compound Ar26 produced by nonpathogenic *R. vitis* strain E26 inhibited the growth of *R. vitis* (Ti) strain MI3-2 and *R. radiobacter* (Ti) strain CY4 on culture plates. Chen *et al.* (10) also demonstrated that *Rahnella aquatilis* strain HX2 inhibited the development of crown galls on grapevines. Although these antagonistic strains have been described in the literature, they have not yet been implemented.

We previously reported that the nonpathogenic *R. vitis* strain, VAR03-1, which was isolated from a grapevine nursery stock in Japan, inhibited tumor formation caused by several *R. radiobacter* (Ti), *R. rhizogenes* (Ti), and *R. vitis* (Ti) strains isolated from different plants in Japan on grapevines, roses, tomatoes, sunflowers, and apples (18, 19, 21, 22, 23, 25). Repetitive sequence-based polymerase chain reaction (rep-PCR) DNA fingerprint analysis and an analysis of the sequences of three housekeeping genes revealed that

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VAR03-1 differed genetically from the tumorigenic and non-antagonistic *R. vitis* strains (21, 26). Moreover, non-pathogenic *R. vitis* strain ARK-1, which was found to be more effective than VAR03-1 at inhibiting tumor formation on grapevines, was identified as a new antagonistic strain (24). ARK-1 is endophytic in grapevines, and treatments with ARK-1 controlled grapevine crown gall better than VAR03-1 in field trials (27). We have been developing a new bactericide of ARK-1 to control grapevine crown gall together with a private enterprise. ARK-1 did not produce a zone of inhibition around the *R. vitis* (Ti) strain on yeast-mannitol agar (YMA) medium, and did not reduce the incidence of tumors in grapevine shoots when it was killed by autoclaving or when the culture filtrate alone was used, indicating the absence of some bacteriocins or antibacterial materials in the dead-cell suspension and culture filtrate of ARK-1 (24). These findings indicated that ARK-1 inhibited grapevine crown gall in plants by a different mechanism from that of VAR03-1 (24).

Thus, we hypothesized that ARK-1 could not inhibit the growth of tumorigenic strains on culture plates, but could on grapevines. Moreover, we attempted to confirm whether ARK-1 did not produce bacteriocins or antibacterial materials on any kinds of culture plates. We herein demonstrated that a reduction in the pathogen population at the grapevine wound site by ARK-1 was responsible for the biological control achieved by this strain, and also that antibiotic activity was not the main control mechanism. In the present study, we followed the nomenclature for *Rhizobium* species adopted by Young *et al.* (40) to avoid confusion, although other valid naming systems have been proposed (1, 9, 29, 31, 32, 37, 41).

Materials and Methods

Development of antibiotic resistant *R. vitis* strains

The antibiotic-resistant mutants of two strains, ARK-1sc and VAT07-1n, were used in a survival assay to differentiate the inoculated biological control agents from indigenous agrobacteria. Potato sucrose agar (PSA: 300 g potato, 0.5 g Ca[NO₃]₂·4H₂O, 2 g Na₂HPO₄·12H₂O, 5 g peptone, 20 g sucrose, 15 g agar, 1 L distilled water, pH 6.8–7.0) medium was used to grow the bacteria in this study. ARK-1sc was a streptomycin (St)-copper sulfate (CuSO₄)-resistant mutant (St-CuSO₄-mutant) obtained by growing strain ARK-1 on St-CuSO₄-PSA medium, which is PSA amended with 500 ppm St and 250 ppm CuSO₄ (24, 27). VAT07-1n was a nalidixic acid (Nal)-resistant mutant (Nal-mutant) obtained by growing strain VAT07-1 on Nal-PSA medium, which is PSA amended with 50 ppm Nal. VAT07-1 was grown on Nal-PSA by selecting the colonies that had wild-type growth rates on PSA medium with no antibiotics. This method was repeated twice to confirm the stability of Nal resistance. The cell suspensions of ARK-1sc and VAT07-1n were prepared from 48-h-old cultures on PSA slants and adjusted to OD₆₀₀ = 0.1 (corresponding to approximately 10⁸ cells mL⁻¹) and mixed at cell ratios of 1:1.

Survival of ARK-1sc and VAT07-1n on grapevine seedlings

Grapevine seedlings (*V. vinifera* cv. 'Neo Muscat') were grown from seeds. One-year-old grapevine shoots were inoculated using previously established methods (18, 19). An outline of this experiment is shown in Fig. S1. A 5-μL drop of a cell suspension (ARK-1sc, VAT07-1n, or mixed) was dropped onto a needle-prick wound on the grapevine shoot. Each grapevine seedling ($n = 30$, one plant per pot) was inoculated once with ARK-1sc or VAT07-1n in each treatment, and each grapevine seedling ($n = 60$, one plant per pot)

was inoculated once with the mixed cell suspension of ARK-1sc and VAT07-1n in each treatment. Each grapevine seedling represented one biological replicate. The seedlings were grown in a greenhouse at 20 to 35°C for 3 months with natural sunlight. Tumors developed in 30 plants inoculated with VAT07-1n and in 10 plants co-inoculated with the mixed cell suspension of ARK-1sc and VAT07-1n, whereas no tumors developed in 30 plants inoculated with ARK-1sc or in 50 plants co-inoculated with the mixed cell suspension. To determine the populations of each strain, 10 shoots were randomly sampled at the inoculation wound site (0.1 g fresh weight per plant, 1 sample per plant) 3 months after the inoculation from 30 plants inoculated with ARK-1sc and 60 plants co-inoculated with ARK-1sc and VAT07-1n. One tumor per plant (0.5 to 1.0 g fresh weight per plant) was randomly collected from 30 plants inoculated with VAT07-1n and from 60 plants co-inoculated with ARK-1sc and VAT07-1n. Each tumor was scrubbed by hand, rinsed under tap water for 10 s, and then blotted dry with paper towels. The surface of the tumor was washed with sterile distilled water, and then crushed in 1 mL of sterile distilled water with an autoclaved mortar and pestle. Ten-fold serial dilutions (100 μL) of the samples were then plated on St-CuSO₄-PSA and Nal-PSA, and the plates were incubated at 25°C for 5 d. Colony growth was then observed on five plates for each dilution, and the numbers of colony-forming units (CFU) of strains ARK-1sc and VAT07-1n were counted on each medium. The bacterial populations in the wounded shoots and tumors (CFU g⁻¹ of the grapevine shoot) were log₁₀-transformed before statistical analysis. This assay was performed twice.

Population dynamics of coexistence of ARK-1sc and VAT07-1n on grapevine seedlings

Grapevine seedlings (2 years old, 'Neo Muscat') were inoculated with ARK-1sc and VAT07-1n at a cell ratio of 1:1 as described above. An outline of this experiment is shown in Fig. S2. Each grapevine seedling ($n = 65$, one plant per pot) was inoculated once with the mixed cell suspension of ARK-1sc and VAT07-1n. Each grapevine seedling represented one replication. The seedlings were grown in a greenhouse at 20 to 35°C with natural sunlight. To determine the population dynamics of each strain, shoot samples including the wound site (0.2 g fresh weight per plant, 1 sample per plant) were collected from 5 plants (*i.e.*, $n = 5$) at 1, 2, 5, 9, 11, 14, 18, 23, 30, 37, 46, 63, and 88 d after the inoculation (assessed on 13 dates). After washing and crushing of the samples as described above, ten-fold serial dilutions (100 μL) were plated on St-CuSO₄-PSA and Nal-PSA media. The colonies were incubated and counted as described above. This assay was performed twice.

Dependence of tumor formation on the bacterial titer

The modeling experiments were performed to demonstrate the dependence of the incidence of tumors on variations in the titers of the pathogenic strain between 10⁷ and 10⁶ cells mL⁻¹, and also to determine whether these differences significantly affected the rate of tumor induction. Concentrations of VAT07-1n were adjusted to 10⁸, 10⁷, 10⁶, and 10⁵ cells mL⁻¹ based on OD₆₀₀ values. Grapevine seedlings (2 years old, 'Neo Muscat') were inoculated with various concentrations of VAT07-1n as described above. Six pots of grapevine seedlings (one plant per pot) each received 10 inoculations (*i.e.*, a total of 60 inoculations). The seedlings were grown in a greenhouse at 20 to 35°C for 6 months with natural sunlight. The formation of tumors on the roots and stems of grapevines was assessed six months after the inoculation. This experiment was performed three times.

In vitro antibiotics assay

The antibiotics assay was based on a previously described method (19, 20, 21, 25). A sterile paper disk (8 mm in diameter) with 50-μL of the ARK-1 cell suspension as the test strain (approximately 10⁸ cells mL⁻¹) was placed on culture plates containing PSA medium; King's B medium (10 g peptone, 1.5 g anhydrous K₂HPO₄, 15 g

Table 1. Bacterial strains used in this study

Bacterial strain	Pathogenicity ^a	Opine type	Description (supplier)
<i>Rhizobium radiobacter</i> (Ti) (= <i>Agrobacterium tumefaciens</i> (Ti), <i>A. tumefaciens</i> biovar 1)			
ARAT001	Ti	Nopaline	Isolated from galled apple trees in Japan (K. Suzaki) (25)
CH3	Ti	Unknown	Isolated from galled chrysanthemums in Japan (T. Takikawa) (20)
CH5	Ti	Unknown	Isolated from galled chrysanthemums in Japan (T. Takikawa) (20)
AtC1	Ti	Agrosinopine, Mannopine, Octopine	Isolated by M. Ohta from galled chrysanthemums in Japan (T. Takikawa) (18)
<i>R. rhizogenes</i> (Ti) (= <i>A. rhizogenes</i> (Ti), <i>A. tumefaciens</i> biovar 2)			
ARAT002	Ti	Nopaline	Isolated from galled apple trees in Japan (K. Suzaki) (25)
NEAR8	Ti	Unknown	Isolated from galled apple trees in Japan (K. Kondo) (25)
NEAR11	Ti	Unknown	Isolated from galled apple trees in Japan (K. Kondo) (25)
<i>R. vitis</i> (Ti) (= <i>A. vitis</i> (Ti), <i>A. tumefaciens</i> biovar 3)			
G-Ag-27	Ti	Vitopine	Isolated from galled grapevine trees in Japan (H. Sawada) (24)
MAFF211674	Ti	Unknown	Isolated from galled grapevine trees in Japan (MAFF ^b) (24)
VAT07-1	Ti	Nopaline	Isolated from galled grapevine trees in Japan (24)
UK-2	Ti	Octopine	Isolated from galled apple trees in Japan (T. Misawa) (24)
9-3-4	Ti	Unknown	Isolated from galled grapevine trees in Japan (This study)
VAT07-1n	Ti	Nopaline	Nalidixic acid-resistant mutant of strain VAT07-1 (This study)
Nonpathogenic <i>R. vitis</i> (=Nonpathogenic <i>A. vitis</i> , <i>A. radiobacter</i> biovar 3)			
ARK-1	N	...	Isolated from a nursery stock of grapevines in Japan; biological control agent for crown gall (24, 27)
ARK-1sc	N	...	Streptomycin- and copper sulfate-resistant mutant of strain ARK-1 (24, 27)

^a Ti: Tumorigenic. N: Nonpathogenic. ^b MAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba Ibaraki, Japan.

glycerol, 1.5 g MgSO₄, 15 g agar, and 1 L distilled water, pH 7.0); YMA medium (0.4 g yeast extract, 10 g mannitol, 0.1 g NaCl, 0.2 g MgSO₄, 0.5 g K₂HPO₄, 15 g agar, and 1 L distilled water, pH 7.0); nutrient agar (NA) medium (5 g peptone, 3 g yeast extract, 15 g agar, and 1 L distilled water, pH 7.0); D-1 medium, which is semi-selective for *Agrobacterium* spp. (30); Roy and Sasser's medium, which is semi-selective for *R. vitis* (= *Agrobacterium* biovar 3) (35); 3DG medium, which is selective for *R. vitis* (= *Agrobacterium* biovar 3) (2); or grapevine-peptone (GP) medium (100 g macerated green shoot explants of grapevine 'Pione', 10 g peptone, 15 g agar, and 1 L distilled water). The plates were then incubated for 2 d at 27°C. ARK-1 was spotted onto five plates (one spot per plate) of each medium. To release antibiotics or plasmids from the bacterial cells (*i.e.*, to provide an insight into the antibiosis mechanism), the plates were then placed face down on a filter paper soaked with chloroform for 20 min. After chloroform had been evaporated, plates were inverted for 60 min to allow residual chloroform to escape. Five plates for each of the Ti strains, except VAT07-1n (Table 1), were misted with a cell suspension (about 10⁷ cells mL⁻¹) of the strain as an indicator (sensitive) strain. The zone of inhibition was assessed 2 d after misting (Table 3). This assay was performed twice.

In planta tumor inhibition assays

Tumor inhibition assays were carried out using methods we previously established (18, 19, 24). Grapevine seedlings (1-yr-old, *V. vinifera* L. cv. Neo Muscat) that were grown from seeds were prepared. The cell suspensions of tumorigenic strains VAT07-1 and AtC1 and nonpathogenic strain ARK-1 (Table 1) were prepared from 48-h-old cultures on PSA medium slants and adjusted to OD₆₀₀ = 0.1 (corresponding to approximately 10⁸ cells mL⁻¹), respectively. A cell suspension of tumorigenic strains VAT07-1 or AtC1 and strain ARK-1 was mixed in various combinations at cell ratios of 1:1. A 5-μL drop of a mixed cell suspension was dropped onto a needle-prick wound on the stem of a grapevine seedling. Five pots of grapevine seedlings (one plant per pot) each received 10 inoculations (*i.e.*, a total of 50 inoculations per treatment). The seedlings were grown in a greenhouse at 20 to 35°C with natural sunlight, and tumor formation was assessed two months later. This experiment was performed three times. The protective rate was defined as: Protection rate = 100% - [(% of tumor formation in mixed strain ARK-1 × 100) / (% of tumor formation by the pathogen only)].

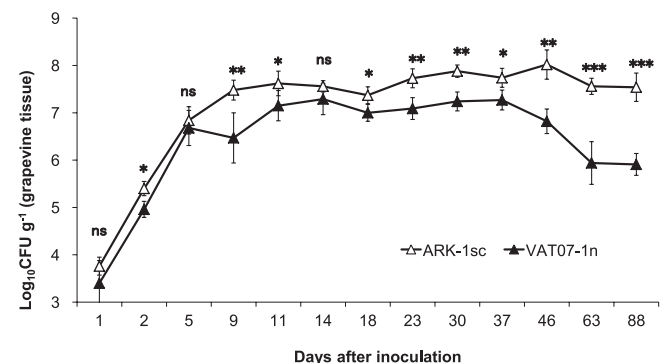
Table 2. Populations of nonpathogenic strain ARK-1sc and tumorigenic strain VAT07-1n after inoculation at a 1:1 cell ratio on grapevine seedlings^a

Strain	Tumor formation	Log ₁₀ CFU g ⁻¹ (fresh weight) grapevine shoot ^b	
		ARK-1sc	VAT07-1n
ARK-1sc	–	7.0 ± 0.1	nd
VAT07-1n	+	nd	7.1 ± 0.1
ARK-1sc + VAT07-1n	–	6.7 ± 0.1***	6.0 ± 0.5
ARK-1sc + VAT07-1n	+	6.7 ± 0.1*	7.0 ± 0.1

^a Data are means of 10 samples of grape shoots. Values are means (after log₁₀ transformation) ± standard error.

^b Significantly different from the VAT07-1n population (*t*-test; ****P* < 0.001, **P* < 0.05).

^c nd; no detection.

**Fig. 1.** Populations of nonpathogenic strain ARK-1sc and tumorigenic strain VAT07-1n after inoculation on the shoots of grapevine seedlings at a 1:1 cell ratio. Data are means ± standard deviation for five seedlings. Significant differences at a given point in time were determined by the *t*-test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns *P* > 0.05).

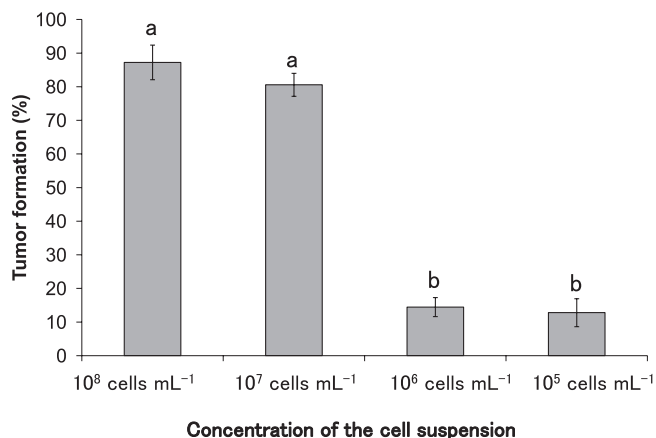


Fig. 2. Dependence of tumor formation on each concentration of the cell suspension of tumorigenic strain VAT07-1n. Data are means ± standard deviation for three experiments. The different letters indicate a significant difference ($P < 0.01$) according to Tukey’s honestly significant difference (HSD) test of arcsine-transformed data.

Data analysis

Based on the description in Table 2 and Fig. 1, the *t*-test was used to compare the bacterial densities of ARK-1sc and VAT07-1n after the inoculation at a 1:1 cell ratio and also to compare population growth during the 88-d post-inoculation growth period. Based on the description in Fig. 2, Tukey’s honestly significant difference (HSD) test was used to compare each treatment. The *t*-test and Tukey’s HSD was performed in R software (The R Foundation for Statistical Computing, version 2.14.0). The Cochran-Mantel-Haenszel test was performed in XLSTAT software (Addinsoft Inc., Brooklyn, NY, USA) to compare the number of plants with tumors treated with ARK-1 with the number of the plants with tumors treated with the pathogen only (Table 4).

Results

Survival of ARK-1sc and VAT07-1n after the inoculation at a 1:1 cell ratio

Both mutants grew in St-CuSO₄-PSA and NaI-PSA at wild-type rates in PSA medium (data not shown). The populations of ARK-1sc and VAT07-1n were similar, at approximately 10⁷ CFU g⁻¹ (fresh weight) of the grapevine

shoot, three months after the inoculation (Table 2). In the co-inoculation treatment, the population of ARK-1sc was approximately 5 × 10⁶ CFU g⁻¹ (≈ 10^{6.7}) of the grapevine shoot regardless of whether tumors formed. In contrast, the VAT07-1n population was significantly higher in shoots with tumors (10⁷ CFU g⁻¹) than in shoots without tumors (10⁶ CFU g⁻¹).

Population dynamics of ARK-1sc and VAT07-1n after the co-inoculation onto grapevine seedlings

The co-inoculation of ARK-1sc and VAT07-1n at a 1:1 cell ratio resulted in no crown gall formation over the 88-d study period (data not shown). The populations of ARK-1sc on the plants were significantly higher than those of VAT07-1n from 2 to 88 d after the inoculation (except at 5 and 14 d), and the magnitude of the difference increased after 37 d (Fig. 1). Colonization by ARK-1sc remained roughly constant at 4 × 10⁷ CFU g⁻¹ (fresh weight) of the grapevine shoot for up to 88 d after the inoculation. In contrast, colonization by VAT07-1n never exceeded 1 × 10⁷ CFU g⁻¹ of the grapevine shoot, and decreased to 1 × 10⁶ CFU g⁻¹ of the grapevine shoot after 88 d.

Dependence of tumor formation on the concentration of the pathogenic strain

Modeling experiments were performed to demonstrate the dependence of the incidence of tumors on variations in the titers of the pathogenic strain between 10⁷ and 10⁶ cells mL⁻¹ to confirm that the decrease provided by ARK-1 was the main reason for the observed effect. The proportions of tumors that formed on the grapevine stems, which were inoculated with cell suspensions of VAT07-1n at 10⁶ and 10⁵ cells mL⁻¹, were significantly lower than those inoculated with 10⁸ and 10⁷ cells mL⁻¹ (Fig. 2). The mean proportion of tumors that formed on the grapevine stems inoculated with 10⁶ cells/mL was under 15%, while that of the grapevine stems inoculated with 10⁷ cells mL⁻¹ was over 80% (Fig. 2).

In vitro antibiosis assay

Although all combinations of ARK-1 and indicator strains, except for *R. radiobacter* (Ti) strain AtC1, resulted in zones of inhibition on PSA and King’s B medium, ARK-1 did not inhibit any of the indicator strains on YMA, NA, D-1, Roy

Table 3. *In vitro* antibiosis assay of nonpathogenic *Rhizobium vitis* strain ARK-1 against *Rhizobium* Ti strains

Indicator strain	Formation of an inhibition zone ^a on the medium plate							
	PSA	King’s B	YMA	NA	D-1	Roy and Sasser	3DG	GP
<i>R. radiobacter</i> (Ti)								
ARAT001	++	++	–	–	–	ng	ng	ng
CH3	+	+	–	–	–	ng	ng	ng
CH5	+	+	–	–	–	ng	ng	ng
AtC1	–	–	–	–	–	–	–	–
<i>R. rhizogenes</i> (Ti)								
ARAT002	+	+	–	–	–	ng	ng	ng
NEAR8	+	+	–	–	–	ng	ng	ng
NEAR11	+	+	–	–	–	ng	ng	ng
<i>R. vitis</i> (Ti)								
G-Ag-27	++	++	–	–	–	–	–	–
MAFF211674	++	++	–	–	–	–	–	–
VAT07-1	++	++	–	–	–	–	–	–
UK-2	++	++	–	–	–	–	–	–

^a Relative size of the inhibition zone (mm): – = no inhibition, 10 < + ≤ 15, 15 < ++ ≤ 20, ng = indicator strains did not grow.

Table 4. Effects of the co-inoculation with nonpathogenic *Rhizobium vitis* and tumorigenic strains at a 1:1 cell ratio on grapevine seedlings. Strain VAT07-1 was sensitive to antibiosis by ARK-1; strain AtC1 was insensitive

Strain	Experiment	Against Ti strain sensitive to antibiosis activity				Against Ti strain insensitive to antibiosis activity			
		No. of plants	No. of inoculations ^a	Proportion of tumor formation (%) ^b	Protection rate (%)	No. of plants	No. of inoculations ^a	Proportion of tumor formation (%) ^b	Protection rate (%)
ARK-1	1	5	50	8.0		5	50	2.0	
	2	5	50	18.0		5	50	0.0	
	3	5	50	16.0		5	50	12.0	
	Mean			14.0***	83.9			4.7***	93.4
Only pathogen	1	5	50	92.0		5	50	66.0	
	2	5	50	82.0		5	50	68.0	
	3	5	50	86.0		5	50	80.0	
	Mean			86.7				71.3	

^a Ten inoculations per plant.

^b Means followed by asterisks differ significantly between the ARK-1 treatment and sterile water treatment (Cochran-Mantel-Haenszel test, *** $P < 0.001$).

and Sasser, 3DG, or GP media (Table 3). These results were identical in both repetitions of this experiment.

In planta tumor inhibition assays

Tumors were observed on the shoots of the grapevine seedlings in all three independent experiments. On grapevine seedlings, a 1:1 cell ratio of strain ARK-1 to Ti strain VAT07-1, which was sensitive to the antibiotic activity of ARK-1, significantly suppressed the incidence of tumors ($P < 0.001$) in stems relative to the only VAT07-1 control, and the protection rate was 83.9% (Table 4). A 1:1 cell ratio of strain ARK-1 to Ti strain AtC1, which was not sensitive to the antibiotic activity of ARK-1, significantly suppressed the incidence of tumors ($P < 0.001$) in stems relative to the only AtC1 control, and the protection rate was 93.7% (Table 4).

Discussion

In the co-inoculation assay, the populations of VAT07-1n were approximately 10^7 cells g^{-1} (fresh weight) in shoots with tumors and 10^6 cells g^{-1} in shoots without. These results demonstrated that the tumorigenic bacterial population co-inoculated with ARK-1sc was ten-fold less than the population without this ARK-1sc, and this difference was significant; thus, ARK-1sc significantly inhibited the growth of the Ti strain.

These results confirmed the expected inhibition of tumor formation by ARK-1. However, ARK-1 did not completely eliminate the formation of tumors on grapevines, as shown in previous greenhouse and field trials in which the incidence of crown gall disease during treatments with ARK-1 decreased by 15% (20, 27). When ARK-1 does not inhibit the growth of tumorigenic bacteria in plants, Ti strains may reach population densities of up to 10^7 cells g^{-1} of the grapevine shoot, leading to the development of the symptoms of crown gall. In the present study, the population of VAT07-1n after the co-inoculation with ARK-1sc reached 10^7 cells g^{-1} in shoots that formed tumors. At grapevine wound sites co-inoculated with ARK-1sc and VAT07-1n, the population of ARK-1sc remained roughly constant at approximately 10^7 CFU g^{-1} of the grapevine shoot up to 88 d after the inoculation, whereas the population of VAT07-1n never exceeded the population of ARK-1sc, and decreased to $<10^6$ CFU g^{-1} (4×10^5 CFU

g^{-1}). Moreover, the inoculation with 10^6 cells mL^{-1} of VAT07-1n induced significantly fewer tumors on the grapevine stems than that with 10^7 cells mL^{-1} . These results suggest that the mechanism underlying inhibition by ARK-1 may be bacteriostatic rather than bactericidal. ARK-1 began to suppress the growth of the Ti strain significantly by 2 d after the inoculation. This result indicated that ARK-1 controlled the pathogen population at the grapevine wound site. The degree of inhibition of the growth of the Ti strain by ARK-1 changed over time. In contrast, F2/5 did not inhibit the survival or growth of *R. vitis* (Ti) strains at grapevine wound sites (22).

Wang *et al.* (39) reported that an antibacterial compound named Ar26 produced by nonpathogenic *R. vitis* strain E26 inhibited the growth of some Ti strains of *Rhizobium* on culture plates. A previous study (20) showed that the antibiotic activity of nonpathogenic *R. vitis* VAR03-1 may be one of the factors responsible for the control of apple crown gall. On the other hand, Burr and Reid (6) reported that the biological control of grapevine crown gall by nonpathogenic *R. vitis* F2/5 was not associated with the production of agrocin or competition for attachment sites on grapevine cells. The antibiosis assay in the present study showed that the antibiotic activity of ARK-1 inhibited three strains of *R. radiobacter* (Ti), three strains of *R. rhizogenes* (Ti), and four strains of *R. vitis* (Ti) on PSA and King's B media, but did not inhibit these strains on the other media (Table 2), suggesting that antibiotic activity depended on the medium. The composition of the GP medium, which contained macerated grapevine shoot explants and peptone, was the closest to that of natural grapevines. ARK-1 did not inhibit the growth of *R. vitis* (Ti) strains on GP medium (Table 3), which suggests that ARK-1 may not have antibiotic effects on grapevines. Moreover, if antibiotic activity blocks the bacterial synthesis of some nutrients, which can be present in the culture medium, bacteria may use that nutrient to continue to grow.

R. radiobacter (Ti) strain AtC1 was insensitive to the antibiotic activity of ARK-1, and ARK-1 did not inhibit its growth on PSA or King's B media. Nevertheless, ARK-1 suppressed crown gall formation on grapevines caused by AtC1, indicating that the control mechanism of ARK-1 may depend on factors other than antibiotic activity. In addition, ARK-1 did not reduce the incidence of tumors on the grape-

vine shoots when ARK-1 cells were dead or when only the filtrate of the PS broth culture was used (24), which supports this result and suggests that ARK-1 antibiotic activity may not be the main control factor *in planta*.

Creasap *et al.* (12) showed that at least one of the *luxR* homologs, *aviR*, as well as the *clpA* homolog, was involved in biological control by strain F2/5 and that one or more necrosis mechanisms (*e.g.*, the hypersensitivity reaction) may be related to biological control. Kaewnum *et al.* (17) demonstrated that two regulatory systems, the quorum-sensing and caseinolytic protease (*clp*) systems, were associated with the biological control mechanism of strain F2/5. Although F2/5 induces necrosis and controls crown gall on grapevines, but not on other plant species, ARK-1 did not induce necrosis on grapevines (27). ARK-1 is a different type of antagonistic strain from F2/5. Thus, the expression of the *luxR*, *aviR*, and *clpA* genes needs to be investigated in ARK-1.

Conclusion

ARK-1 reduced the pathogen population at grapevine wound sites, but antibiotic activity was not the main mechanism. These results provide an insight into the mechanism responsible for biological control, and suggest that ARK-1 may have a unique, previously unreported mechanism responsible for its control of crown gall. Further studies are warranted to elucidate this mechanism in more detail.

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References

- Bouzar, H., W.S. Chilton, X. Nesme, Y. Dessaux, V. Vaudequin, A. Petit, J.B. Jones, and N.C. Hodge. 1995. A new *Agrobacterium* strain isolated from aerial tumors on *Ficus benjamina* L. *Appl. Environ. Microbiol.* 61:65–73.
- Brisbane, P.G., and A. Kerr. 1983. Selective media for three biovars of *Agrobacterium*. *J. Appl. Bacteriol.* 54:425–431.
- Bull, C.T., S.H. De Boer, T.P. Denny, G. Firrao, M. Ficher-Le Saux, G.S. Saddler, M. Scortichini, D.E. Stead, and Y. Takikawa. 2010. Comprehensive list of names of plant pathogenic bacteria, 1980–2007. *J. Plant Pathol.* 92:551–592.
- Burr, T.J., and B.H. Katz. 1983. Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap, and from vineyard soil. *Phytopathology* 73:163–165.
- Burr, T.J. 1988. Crown gall. p. 41–42. *In* C.P. Pearson, and A.C. Goheen, (ed.), *Compendium of Grape Diseases*. APS Press, Minnesota.
- Burr, T.J., and C.L. Reid. 1994. Biological control of grape crown gall with nontumorigenic *Agrobacterium vitis* F2/5. *Amer. J. Enol. Vitic.* 45:213–219.
- Burr, T.J., C.L. Reid, E. Taglietti, C. Bazzi, and S. Süle. 1997. Biological control of grape crown gall by strain F2/5 is not associated with agrocin production or competition for attachment site on grape cells. *Phytopathology* 87:706–711.
- Burr, T.J., C. Bazzi, S. Süle, and L. Otten. 1998. Crown gall of grape: Biology of *Agrobacterium vitis* and the development of disease control strategies. *Plant Dis.* 82:1288–1297.
- Burr, T.J., and L. Otten. 1999. Crown gall of grape: Biology and disease management. *Annu. Rev. Phytopathol.* 37:53–80.
- Chen, F., Y.B. Guo, J.H. Wang, J.Y. Li, and H.M. Wang. 2007. Biological control of grape crown gall by *Rahnella aquatilis* HX2. *Plant Dis.* 91:957–963.
- Chilton, M.D., M.H. Drummond, D.J. Merlo, D. Sciaky, A.L. Montoya, M.P. Gordon, and E.W. Nester. 1977. Stable incorporation of plasmid DNA into higher plant cells: The molecular basis of crown gall tumorigenesis. *Cell* 11:263–271.
- Creasap, J.E., C.L. Reid, M.C. Goffinet, R. Aloni, C. Ullrich, and T.J. Burr. 2005. Effect of wound position, auxin, and *Agrobacterium vitis* strain F2/5 on wound-healing and crown gall in grapevine. *Phytopathology* 95:362–367.
- Eastwell, K.C., P.L. Sholberg, and R.J. Sayler. 2006. Characterizing potential bacterial biocontrol agents for suppression of *Rhizobium vitis*, causal agent of crown gall disease in grapevines. *Crop Prot.* 25:1191–1200.
- Hayman, G.T., and S.K. Farrand. 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. *J. Bacteriol.* 170:1759–1767.
- Herlache, T.C., H.S. Zhang, C.L. Ried, S.A. Carle, D. Zheng, P. Basaran, M. Thaker, A.T. Burr, and T.J. Burr. 2001. Mutations that affect *Agrobacterium vitis*-induced grape necrosis also alter its ability to cause a hypersensitive response on tobacco. *Phytopathology* 91:966–972.
- Jones, D.A., and A. Kerr. 1989. *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. *Plant Dis.* 73:15–18.
- Kaewnum, S., D. Zheng, C.L. Reid, K.L. Johnson, J.C. Gee, and T.J. Burr. 2013. A host-specific biological control of grape crown gall by *Agrobacterium vitis* strain F2/5; its regulation and population dynamics. *Phytopathology* 103:427–435.
- Kawaguchi, A., K. Inoue, and H. Nasu. 2005. Inhibition of crown gall formation by *Agrobacterium radiobacter* biovar 3 strains isolated from grapevine. *J. Gen. Plant Pathol.* 71:422–430.
- Kawaguchi, A., K. Inoue, and H. Nasu. 2007. Biological control of grapevine crown gall by nonpathogenic *Agrobacterium vitis* strain VAR03-1. *J. Gen. Plant Pathol.* 73:133–138.
- Kawaguchi, A., K. Inoue, and Y. Ichinose. 2008a. Biological control of crown gall of grapevine, rose, and tomato by nonpathogenic *Agrobacterium vitis* strain VAR03-1. *Phytopathology* 98:1218–1225.
- Kawaguchi, A., H. Sawada, and Y. Ichinose. 2008b. Phylogenetic and serological analyses reveal genetic diversity of *Agrobacterium vitis* strains in Japan. *Plant Pathol.* 57:747–753.
- Kawaguchi, A. 2009. Studies on the diagnosis and biological control of grapevine crown gall and phylogenetic analysis of tumorigenic *Rhizobium vitis*. *J. Gen. Plant Pathol.* 75:462–463.
- Kawaguchi, A., and K. Inoue. 2009. Grapevine crown gall caused by *Rhizobium radiobacter* (Ti) in Japan. *J. Gen. Plant Pathol.* 75:205–212.
- Kawaguchi, A., and K. Inoue. 2012. New antagonistic strains of non-pathogenic *Agrobacterium vitis* to control grapevine crown gall. *J. Phytopathol.* 160:509–518.
- Kawaguchi, A., K. Kondo, and K. Inoue. 2012. Biological control of apple crown gall by nonpathogenic *Rhizobium vitis* strain VAR03-1. *J. Gen. Plant Pathol.* 78:287–293.
- Kawaguchi, A. 2011. Genetic diversity of *Rhizobium vitis* strains in Japan based on multilocus sequence analysis using the sequences of *pyrG*, *recA* and *rpoD*. *J. Gen. Plant Pathol.* 77:299–303.
- Kawaguchi, A. 2013. Biological control of crown gall on grapevine and root colonization by nonpathogenic *Rhizobium vitis* strain ARK-1. *Microbes Environ.* 28:306–311.
- Kerr, A. 1980. Biological control of crown gall through production of agrocin 84. *Plant Dis.* 64:25–30.
- Kerstens, K., and J. De Ley. 1984. Genus III. *Agrobacterium* Conn 1942, p. 244–254. *In* N.R. Kring, and J.G. Holt (ed.), *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams & Wilkins Co., Baltimore.

30. Moore, L.W., and G. Warren. 1979. *Agrobacterium radiobacter* strain 84 and biological control of crown gall. *Ann. Rev. Phytopathol.* 17:163–179.
31. Moore, L.W., C.L. Kado, and H. Bouzar. 1988. Gram-negative bacteria. A. *Agrobacterium*, p. 16–36. In N.W. Schaad (ed.), *A Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 2nd ed. APS Press, Minnesota.
32. Ophel, K., and A. Kerr. 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. *Int. J. Syst. Bacteriol.* 40:236–241.
33. Penyalver, R., and M.M. López. 1999. Cocolonization of the rhizosphere by pathogenic *Agrobacterium* strains and nonpathogenic strains K84 and K1026, used for crown gall biocontrol. *Appl. Environ. Microbiol.* 65:1936–1940.
34. Penyalver, R., B. Vicedo, and M.M. López. 2000. Use of the genetically engineered *Agrobacterium* strain K1026 for biological control of crown gall. *Eur. J. Plant Pathol.* 10:801–810.
35. Roy, M.A., and M. Sasser. 1983. A medium selective for *Agrobacterium tumefaciens* biotype 3. *Phytopathology* 73:810 (abstract).
36. Sawada, H., H. Ieki, and Y. Takikawa. 1990. Identification of grapevine crown gall bacteria isolated in Japan. *Ann. Phytopathol. Soc. Japan* 56:199–206.
37. Sawada, H., H. Ieki, H. Oyaizu, and S. Matsumoto. 1993. Proposal for rejection of *Agrobacterium tumefaciens* and for revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. *Int. J. Syst. Bacteriol.* 43:694–702.
38. Staphorst, J.L., F.G.H. van Zyl, B.W. Strijdom, and Z.E. Groenewold. 1985. Agrocin-producing pathogenic and nonpathogenic biotype-3 strains of *Agrobacterium tumefaciens* active against biotype-3 pathogens. *Curr. Microbiol.* 12:45–52.
39. Wang, H.M., H.X. Wang, T.B. Ng, and J.Y. Li. 2003. Purification and characterization of an antibacterial compound produced by *Agrobacterium vitis* strain E26 with activity against *A. tumefaciens*. *Plant Pathol.* 52:134–143.
40. Young, J.M., L.D. Kuykendall, E. Martínez-Romero, A. Kerr, and H. Sawada. 2001. A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al.* 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *Int. J. Syst. Evol. Microbiol.* 51:89–103.
41. Young, J.M., A. Kerr, and H. Sawada. 2005. Genus II. *Agrobacterium*, p. 340–345. In G.M. Garrity (ed.), *Bergey's Manual of Systematic Bacteriology*, 2nd ed. vol. 2. Springer Verlag, New York.