Biological Control of Crown Gall of Grapevine, Rose, and Tomato by Nonpathogenic *Agrobacterium vitis* Strain VAR03-1

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ABSTRACT

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A nonpathogenic strain of *Agrobacterium vitis* VAR03-1 was tested as a biological control agent for crown gall of grapevine (*Vitis vinifera*). When roots of grapevine, rose (*Rosa multiflora*), and tomato (*Lycopersicon esculentum*) were soaked in a cell suspension of antagonists before planting in soil infested with tumorigenic *A. vitis*, *A. rhizogenes*, and *A. tumefaciens*, respectively, treatment with VAR03-1 significantly reduced the number of plants with tumors and disease severity in the three plant species. The inhibitory effects of

treatment with VAR03-1 and the nonpathogenic *A. rhizogenes* strain K84 on crown gall of rose and tomato were almost identical, and the inhibitory effect of VAR03-1 on grapevine was superior to that of K84. Moreover, VAR03-1 greatly controlled crown gall of grapevine due to tumorigenic *A. vitis* in the field. VAR03-1 established populations averaging 10^6 colony forming units (CFU)/g of root in the rhizosphere of grapevine and persisted on roots for 2 years. VAR03-1 was bacteriocinogenic, producing a halo of inhibition against those three species of *Agrobacterium*. This is the first report that a nonpathogenic strain, VAR03-1, can effectively control crown gall caused by tumorigenic *A. vitis*, *A. rhizogenes*, and *A. tumefaciens*.

Grapevine crown gall due to *Agrobacterium vitis* Ophel and Kerr 1990 (= *Agrobacterium tumefaciens* biovar 3 [Smith and Townsend 1907] Conn 1942, *Rhizobium vitis* Young et al. 2001) is the most important bacterial disease of grapevine throughout the world (3,6,31). The nonpathogenic *Agrobacterium rhizogenes* (Riker, Banfield, Wright, Keitt, and Sagen, 1930) Conn 1942 (= *Agrobacterium radiobacter* biovar 2 [Beijerinck and van Delden 1902] Conn 1942, *Rhizobium rhizogenes* Young et al. 2001) strain K84 has been used successfully to control crown gall in many plant species (24,27). An agrocin produced by K84 (agrocin 84), which is thought to be the primary factor in the control (14), targets a tRNA synthetase in the tumorigenic *Agrobacterium* strains (28). Recombinant DNA techniques have been used to construct a new biological control strain, K1026, that is identical to K84 apart from 5.9-kb deletion overlapping the Tra region of pAgK84 (13). K1026 is unable to transfer its mutant agrocin 84 plasmid, designated pAgK1026, to other agrobacteria, but remains inhibitory to strains sensitive to agrocin 84 (13,26). However, K84 does not prevent the initial infection of grapevine by tumorigenic *A. vitis* (3,4,16,17).

Several laboratories have attempted to identify other biological control agents for grapevine crown gall (5,7,8,32,36,37). Staphorst et al. (32) evaluated 16 strains, including the nonpathogenic *A. vitis* strain F2/5, that inhibited growth of most tumorigenic strains of *A. vitis* in vitro and inhibited crown gall on grapevine in stem-wounding experiments in the greenhouse. Burr and Reid (5) reported that F2/5 produced a

bacteriocin and effectively inhibited tumor formation at wound sites on grapevine stems inoculated with one of the tumorigenic *A. vitis* strains. However, F2/5 had very low antibacterial activity against tumorigenic *A. tumefaciens* (= *A. tumefaciens* biovar 1) and *A. rhizogenes* (= *A. tumefaciens* biovar 2) (5) and did not inhibit tumor formation caused by other strains of tumorigenic *A. vitis* (7). Wang et al. (36) reported that an antibacterial compound named "Ar26" produced by nonpathogenic *A. vitis* strain E26 inhibited the growth of tumorigenic *A. vitis* strain MI3-2 and *A. tumefaciens* strain CY4 on culture plates.

Previously, we reported that a nonpathogenic *A. vitis* strain, VAR03-1, isolated from nursery stock of grapevine in Japan produced a bacteriocin and greatly inhibited tumor formation on the stems of tomato and grapevine seedlings caused by several tumorigenic *A. vitis* strains isolated from different grapevine fields in Japan (16,17). In greenhouse tests, when roots of grapevine seedlings were soaked in a cell suspension of strain VAR03-1 for 24 h before a 1-h soak in a cell suspension of tumorigenic *A. vitis* and subsequent planting in pots of infested soil, strain VAR03-1 significantly reduced the incidence of tumor formation (17).

Today, crown gall diseases on rose and grapevine occur in many countries around the world (3,23). Rose crown gall is caused by mainly tumorigenic *A. rhizogenes* in Japan (23). Further, tomato crown gall caused by tumorigenic *A. tumefaciens* was recognized in the United Kingdom in 2005 (38), indicating the possibility that tomato crown gall will be found in other countries. In the present paper, we report that soaking the roots in cells suspensions of strain VAR03-1 reduced the frequency of crown gall of grapevine, rose, and tomato caused by several tumorigenic strains of *A. vitis*, *A. rhizogenes*, and *A. tumefaciens*, respectively, in the greenhouse test and controlled crown gall of grapevine by several tumorigenic *A. vitis* strains in the field.

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We follow the nomenclature for *Agrobacterium* species adopted in Bergey's Manual of Systematic Bacteriology (40) and as reported by Ophel and Kerr (25) to avoid confusion, although other valid naming systems have been proposed (1,11,20,30,39).

MATERIALS AND METHODS

Biological control in greenhouse experiment. Biological control assays based on the method we established (17) were carried out using seedlings and nursery stocks. Seedlings of tomato (*Lycopersicon esculentum* Mill. cv. Ponderosa) (1 to 2 months old) and grapevine (*Vitis vinifera* L. cv. Neo Muscat) (1 year old) were grown from seeds using methods previously reported (9). Nursery stocks of rose (scion cultivar: *Rose hybrida* L. cv. Rote Rose, rootstock: *Rose multiflora* Thunb. cv. Multiflora Rose) grown from cuttings for 1 year were prepared.

Cell suspensions of nonpathogenic strains VAR03-1 and K84 were prepared from 48-h slant cultures grown on potato semi-synthetic agar (PSA) medium (300 g of potato, 0.5 g of Ca (NO₃)₂ 4H₂O, 2 g of Na₂HPO₄ 12H₂O, 5 g of peptone, 20 g of sucrose, 15 g of agar, and 1 liter of distilled water, pH 6.8 to 7.0) and adjusted to OD₆₀₀ = 1.0 (corresponding to about 10⁹ cells/ml) for inoculation, and 10 tumorigenic strains (*A. tumefaciens* strains CH3, CH5, and C054; *A. rhizogenes* strains AtR1, Ch-Ag-2, and P-Ag-1; *A. vitis* strains At-90-23, G-Ag-27, MAFF211674, and MAFF211676) (Table 1) that were adjusted to about 10⁸ cells/ml (OD₆₀₀ = 0.1). The soil was inoculated with each *Agrobacterium* pathogen by pouring 10⁸ cells/ml cell suspensions onto the soil (500 ml/kg of soil) and to obtain a final concentration about 5 × 10⁷ cells/g of soil. Each young plant was pulled from the soil and the root rinsed in water until clean. Roots were then pruned and soaked for 1 h in a cell suspension of strain VAR03-1, K84, or sterile distilled water (as a control) at 25°C. After soaking, 15 tomato seedlings per treatment were planted in pots (10 cm depth × 12 cm diameter, one plant per pot) with soil infested with a mixture of three tumorigenic *A. tumefaciens* strains at a final concentration 5 × 10⁷ cells/g of soil in soil 8 cm deep. Correspondingly, 15 rose nursery stocks were planted in pots (12 cm depth × 15 cm diameter) with soil infested with a

mixture of cell suspension of three tumorigenic *A. rhizogenes* strains at a final concentration of 10⁸ cells/g of soil in soil 10 cm deep, and 15 grapevine seedlings were planted in pots (12 cm depth × 15 cm diameter) with soil infested with a mixture of four tumorigenic *A. vitis* strains at a final concentration 5 × 10⁷ cells/g of soil in soil 10 cm deep. Plants were then grown in a temperature-controlled greenhouse at 25 to 28°C, and tumor formation was assessed after 2 months for tomato, 5 months for rose, and 3 months for grapevine. The tomato and rose experiment were repeated three times, and the grapevine experiment was repeated four times.

Protective value was defined in terms of previous report by Penyalver and López (26) as follows: Protective value = 100% - ([% of plants with tumors in treatment × 100]/[% of plants with tumors in sterile distilled water treatment]).

Biological control in field experiment. In 2007, two experiments on biological control for grapevine crown gall were carried out in the field at the experimental farm of the Okayama Prefectural General Agriculture Center in Akaiwa City, Okayama, Japan.

In experiment 1, the field trial was conducted using concrete-frame plots (1.5 m × 1.6 m/plot, 1.0 m tall) filled in the soil (pH = 6.0, NO₃-N = 7.2 mg/100 g of soil, P₂O₅ = 119 mg/100 g of soil, K₂O = 67 mg/100 g of soil, CaO = 255 mg/100 g of soil, the cation exchange capacity = 14.2 meq/100 g of soil, organic matter content = 1.7%). One month before the experiment, a commercial organic fertilizer (Temporon, including ligno-cellulose, humic acid, Ca, Mg, Mn, and B; Mitsubishi-Shouji, Tokyo, Japan) was applied at a rate of 4.5-kg per plot and thoroughly incorporated. Two weeks before the experiment, 20 liters per plot of a mixed cell suspension (about 10⁸ cells/ml) of tumorigenic *A. vitis* strains At-90-23, G-Ag-27, MAFF211674, and MAFF211676 was poured on. Cell suspensions of nonpathogenic strains VAR03-1 and K84 were adjusted to about 10⁹ cells/ml, respectively. The roots of grapevine seedlings cv. Neo Muscat (1 year old) were pruned and soaked for 1 h in a cell suspension of strain VAR03-1, K84, or sterile distilled water at 25°C in each treatment and then these 14 grapevine seedlings per treatment were planted in the center area of each plot in six rows (two rows per treatment; VAR03-1, K84, and sterile distilled water, respectively) spaced 15 cm apart with a 15 cm space between

TABLE 1. Bacterial strains used in this study

Bacterial strain	Source and location (supplier) ^y
Tumorigenic <i>Agrobacterium tumefaciens</i> (= <i>A. tumefaciens</i> biovar 1, tumorigenic <i>Rhizobium radiobacter</i>)	
CH3	Chrysanthemum; Shizuoka, Japan (Y. Takikawa)
CH5	Chrysanthemum; Shizuoka, Japan (Y. Takikawa)
C054	Cosmos; Shizuoka, Japan (Y. Takikawa)
Tumorigenic <i>A. rhizogenes</i> (= <i>A. tumefaciens</i> biovar 2, tumorigenic <i>R. rhizogenes</i>)	
AtR1	Rose; Shizuoka, Japan (Y. Takikawa)
Ch-Ag-2	Cherry; Yamagata, Japan (H. Sawada)
P-Ag-1	Pear; Mie, Japan (H. Sawada)
Nonpathogenic <i>A. rhizogenes</i> (= <i>A. radiobacter</i> biovar 2, nonpathogenic <i>R. rhizogenes</i>)	
K84	Soil; Australia (T. Makino, isolated by A. Kerr [11,12])
Tumorigenic <i>A. vitis</i> (= <i>A. tumefaciens</i> biovar 3, tumorigenic <i>R. vitis</i>)	
At-90-23	Grapevine; Shimane, Japan (J. Yamamoto)
G-Ag-27	Grapevine; Nagano, Japan (H. Sawada)
MAFF 211674 ^z	Grapevine; Akita, Japan
MAFF 211676 ^z	Grapevine; Okayama, Japan
Nonpathogenic <i>A. vitis</i> (= <i>A. radiobacter</i> biovar 3, nonpathogenic <i>R. vitis</i>)	
VAR03-1	Grapevine; Okayama, Japan

^y Unless a supplier is stated, we isolated the strain.

^z MAFF; Ministry of Agriculture, Forestry and Fisheries, Tsukuba Ibaraki, Japan.

each plant on 13 February 2007. The topsoil in each concrete-frame plot was covered by a sheet of nonwoven fabric as covering material to weed after planting. Tumor formation was investigated on 12 October 2007. Three plots were used for this experiment.

In experiment 2, the field trial was carried out using grapevine nursery stocks *V. vinifera* × *V. labrusca* cv. Pione grown from cuttings (2 years old). The field experiment was conducted using nonframe plots (8.0 m × 3.0 m/plot). One month before the experiment, the commercial organic fertilizer described previously was applied at a rate of 45 kg per plot and thoroughly incorporated. Two weeks before the experiment, 90 liters of a mixed cell suspension (about 5×10^7 cells/ml) of tumorigenic *A. vitis* strains At-90-23, G-Ag-27, MAFF211674, and MAFF211676 per plot was poured on, and then soil was broken up in depth 16 cm by the cultivator Punch-X F402-J (Honda Motor, Tokyo, Japan) to disperse the inoculum. Cell suspensions of nonpathogenic strain VAR03-1 were adjusted to about 10^9 cells/ml. Fifteen grapevine nursery stocks per treatment were inoculated as described previously and planted in the center area of each plot in two rows (one row per treatment; VAR03-1 and sterile distilled water, respectively) spaced 150 cm apart with a 50 cm spacing between plants on 19 April 2007. The ground in each plot was covered by a sheet of nonwoven fabric as covering material to weed after planting. Tumor formation was investigated on 27 November 2007. Three plots were used in this experiment.

The rainy season in Okayama, Japan, was from June to July. The temperature ranged from 10 to 37°C, and no severe damage by weather or insects was observed during the cultivation. The severity of crown gall disease was assayed as described previously for the greenhouse experiment.

In vitro antibiosis assay. The antibiosis assay, based on previous reports (16), consisted of placing a sterile paper disk (8-mm diameter) with a 50-μl drop of the test strain (about 10^8 cells/ml) on yeast-mannitol agar (YMA) medium (0.4 g of yeast extract, 10 g of mannitol, 0.1 g of NaCl, 0.2 g of $MgSO_4$, 0.5 g of K_2HPO_4 , 15 g of agar, and 1 liter of distilled water, pH 7.0), and incubating it for 2 days at 27°C. Each test strain was spotted onto four plates. After the test strains K84 and VAR03-1 were killed with chloroform on each plate, the plates were misted with a dilute suspension of *Agrobacterium* strains (*A. tumefaciens* strains CH3, CH5, and C054; *A. rhizogenes* strains AtR1, Ch-Ag-2, and P-Ag-1; *A. vitis* strains At-90-23, G-Ag-27, MAFF211674, and MAFF211676) (Table 1) as an indicator (sensitive) strain. The plates were photographed 2 days after misting, and the diameter of the halo of inhibition indicative of antibiosis, was measured.

Survival assay. The survival rate of nonpathogenic *A. vitis* strain VAR03-1 and *A. rhizogenes* strain K84 in the grapevine seedling root was determined. Thirty seedlings of grapevine cv. Neo Muscat grown for 1 year were prepared. Cell suspensions of nonpathogenic strain VAR03-1 and K84 contained 10^8 cells/ml, respectively. Grapevine seedlings were root inoculated as described for the biological control in the greenhouse experiment: grapevine seedlings were soaked for 24 h in a cell suspension (10^8 cells/ml) or for 1 h in a cell suspension (10^9 cells/ml), and planted in pots (12 cm depth × 15 cm diameter) with soil. Nontreatment grapevine seedlings with the test strain were prepared as negative control. The temperature ranged from 20 to 30°C. To determine the populations of each strain, roots (1 g of fresh weight per plant) were collected from 3 or 10 randomly selected plants. Each piece was scrubbed and rinsed under tap water and dried with paper towels. To avoid an influence of the nonhomogeneous colonization by the other bacteria in the roots, the surface was treated with 70% ethanol for 1 min and washed with sterile distilled water, and then mashed with an autoclaved mortar

and pestle in 1 ml of sterile distilled water. Serial dilutions of grapevine treated with VAR03-1 were plated on 3DG medium, which is selective for *A. vitis* (2), and serial dilutions of grapevine treated with K84 were plated on 2E medium for *A. rhizogenes* (2). The plates were incubated at 28°C for 5 days. The observations were based on three plates of each dilution, and colony forming units (CFU) of *A. vitis* and *A. rhizogenes* were counted on each selective medium. The bacterial population on the root was transformed as a logarithm (base 10) of CFUs per gram of root for analysis. Characteristic colonies of *A. vitis* on 3DG medium and *A. rhizogenes* on 2E medium were counted, but similar colonies on 3DG were determined by a colony-direct multiplex polymerase chain reaction (PCR) method for diagnosis of *A. vitis* (19). After multiplex PCR, a total of 10 to 20 *A. vitis* colonies per plate were isolated and analyzed. The rep-PCR DNA fingerprint analysis with BOX and ERIC primer sets (18) was performed to confirm that the isolates from these colonies showed the same fingerprint pattern of strain VAR03-1. Furthermore, the antibiosis activity of the isolates were confirmed against G-Ag-27 as an indicator strain described previously. As for strain K84 on 2E medium, a total of 10 to 20 *A. rhizogenes* colonies per plate were isolated and analyzed agrocin 84 production according to the Stonier procedure (21,34,35) to confirm strain K84.

Data analysis. In this study, statistical analyses were performed using the software STATISTICA 03J (StatSoft Inc., Tulsa, OK). The data on plants with tumors (%) and disease severity in the greenhouse experiments and field experiment 1 were subjected to analysis of variance (ANOVA), and the mean values in each treatment were compared with Fisher's protected least significant difference (LSD) test at $P = 0.05$. The data on greenhouse experiments were pooled and analyzed. In field experiment 2, Student's *t* test was used to compare the number of plants with tumors treated with strain VAR03-1 to those treated with sterile distilled water. Differences between the radius of the halo of inhibition against each tumorigenic *Agrobacterium* strain of VAR03-1 and K84 were compared using the Mann-Whitney *U*-test. The data on root colonization of strain VAR03-1 and K84 in the roots of grapevine were subjected to ANOVA, and the mean values in each treatment were compared with Scheffe's test.

RESULTS

Biological control in greenhouse experiment. The biological control assays in the greenhouse using the method of soaking the roots in a cell suspension of the antagonist were performed before planting grapevine, tomato seedlings, and rose nursery stocks in soil infested with tumorigenic strains of *A. vitis*, *A. tumefaciens*, and *A. rhizogenes*. Tumors formed on plants in these experiments were observed on roots and on a stem. Treatment of tomato roots with nonpathogenic *A. vitis* strain VAR03-1 or *A. rhizogenes* strain K84 significantly reduced the percentage of tomato seedlings with tumors caused by the three tumorigenic *A. tumefaciens* strains ($P < 0.05$); the protective values of VAR03-1 and K84 against crown gall of tomato were 63.6 and 69.7%, respectively (Table 2). VAR03-1 or K84 treatment of rose roots significantly reduced the percentage of plants with tumors caused by the three tumorigenic *A. rhizogenes* strains ($P < 0.05$), and the protective values of VAR03-1 and K84 against crown gall of rose were 73.0 and 82.2%, respectively (Table 2). Moreover, VAR03-1 treatment of grapevine roots also significantly reduced the percentage of plants with tumors caused by four tumorigenic *A. vitis* strains ($P < 0.05$); the protective value of VAR03-1 against crown gall of grapevine was 91.8%, although K84 treatment of grapevine roots scarcely inhibited the outbreak of crown gall disease because the protective value of K84 was 12.0% (Table 2).

Biological control in field experiment. Biological control assays using the method of planting grapevine seedlings cv. Neo Muscat and nursery stocks cv. Pione were performed in the field in 2007. The grapevine was infested in the soil with four tumorigenic *A. vitis* strains isolated from different fields in Japan (Table 1). In experiment 1, as shown in Table 3, VAR03-1 treatment of grapevine roots inhibited crown gall of grapevine completely ($P < 0.05$), and thus the protective value of VAR03-1 was 100%. On the other hand, K84 treatment of grapevine roots hardly reduced the percentage of plants with tumors, and the result was not significantly different from that of the treatment with sterile distilled water as the control. In experiment 2, treatment of grapevine roots with strain

VAR03-1 significantly reduced the percentage of plants with tumors ($P < 0.05$), and the protective value against crown gall of grapevine was 92.4% (Table 3).

In vitro antibiosis assay. Nonpathogenic *Agrobacterium* strains VAR03-1 and K84 were used as test strains for an in vitro antibiosis assay against various strains of three species of tumorigenic *Agrobacterium*. Some combinations of test and indicator strains resulted in halos of inhibition on YMA medium (Fig. 1 and Table 4). In addition, VAR03-1 inhibited all indicator strains including three species of agrobacteria (10 of 10 strain combinations), and the inhibition halo of VAR03-1 was significantly wider than that of K84 against eight indicator strains ($P < 0.05$). K84 did not inhibit tumorigenic

TABLE 2. Effect of nonpathogenic *Agrobacterium* strains VAR03-1 and K84 on crown gall of tomato and rose after soaking plant roots in bacterial cell suspensions in the greenhouse experiments

Treatment	No. of experiment	Tomato ^v		Rose ^w		Grapevine ^x	
		Plants with tumors (%) ^y	Protective value (%) ^z	Plants with tumors (%)	Protective value (%)	Plants with tumors (%)	Protective value (%)
VAR03-1	1	20.0		10.0		6.7	
	2	26.7		0.0		0.0	
	3	33.3		5.0		0.0	
	4	NT		NT		6.7	
	Mean	26.7 a	63.6	5.0 a	73.0	3.4 a	91.8
K84	1	46.7		5.0		66.7	
	2	13.3		5.0		20.0	
	3	6.7		0.0		13.3	
	4	NT		NT		46.7	
	Mean	22.2 a	69.7	3.3 a	82.2	36.7 b	12.0
Sterile distilled water	1	40.0		20.0		53.3	
	2	80.0		25.0		40.0	
	3	100.0		10.0		33.3	
	4	NT		NT		40.0	
	Mean	73.3 b	...	18.3 b	...	41.7 b	...

^v Data are means of three experiments of 15 tomato seedlings per treatment. Plants were soaked in cell suspension of antagonist (10^9 cells/ml) or sterile distilled water 1 h before being planted in a pot in soil infected with the mixture of cell suspension of tumorigenic *A. tumefaciens* strain CH3, CH5, and C054 at a final concentration 5×10^7 cells/g of soil.

^w Data are means of three experiments of 20 rose nursery stocks per treatment. Plants were soaked in cell suspension of antagonist (10^9 cells/ml) or sterile distilled water 1 h before being planted in a pot in soil infected with the mixture of cell suspension of tumorigenic *A. rhizogenes* strain AtR1, Ch-Ag-2, and P-Ag-1 at a final concentration 5×10^7 cells/g of soil.

^x Data are means of four experiments of 15 grapevine seedlings per treatment. Plants were soaked in cell suspension of antagonist (10^9 cells/ml) or sterile distilled water 1 h before being planted in a pot in soil infected with the mixture of cell suspension of tumorigenic *A. vitis* strain At-90-23, G-Ag-27, MAFF 211674, and MAFF 211676 at a final concentration 5×10^7 cells/g of soil.

^y Means followed by the same letter are not significantly different according to Fisher's protected least significant difference test on the arcsine transformation of the data at $P < 0.05$. NT; not tested.

^z Protective value = $100\% - ([\% \text{ of plants with tumors in treatment} \times 100] / [\% \text{ of plants with tumors in sterile distilled water treatment}])$.

TABLE 3. Effect of nonpathogenic *Agrobacterium* strains VAR03-1 and K84 on crown gall of grapevine seedlings after soaking plant roots in bacterial cell suspensions^w

Treatment	No. of replication	Experiment 1		Experiment 2	
		Plants with tumors (%) ^x	Protective value (%) ^y	Plants with tumors (%) ^z	Protective value (%)
VAR03-1	1	0.0		6.7	
	2	0.0		0.0	
	3	0.0		0.0	
	Mean	0.0 a	100.0	2.2*	92.4
K84	1	21.4		NT	
	2	7.1		NT	
	3	7.1		NT	
	Mean	11.9 b	15.0	...	...
Sterile distilled water	1	20.0		46.7	
	2	14.3		20.0	
	3	7.7		20.0	
	Mean	14.0 b	...	28.9	...

^w Data are means of three replications of 14 grapevine seedlings per treatment in experiment 1 and three replications of 15 grapevine nursery stocks per treatment in experiment 2. Plants were soaked in a cell suspension of antagonist (10^9 cells/ml) or sterile distilled water 1 h before being planted in the field. NT; not tested.

^x Means followed by the same letter are not significantly different according to Fisher's protected least significant difference test on the arcsine transformation of the data at $P < 0.05$.

^y Protective value = $100\% - ([\% \text{ of plants with tumors in treatment} \times 100] / [\% \text{ of plants with tumors in sterile distilled water treatment}])$.

^z Significantly different according to Student's *t* test on the arcsine transformation of the data at $P < 0.05$.

A. tumefaciens C054 or any of tumorigenic *A. vitis* strains examined in this study (Table 4).

Population dynamics of strain VAR03-1 and K84 on root of grapevine. One month after grapevine roots were pruned and soaked for 24 h in a cell suspension of nonpathogenic *A. vitis* strain VAR03-1 (10^8 cells/ml), the bacterial population was about 10^7 CFU/g (fresh weight) of grapevine root (Table 5). Colonization of VAR03-1 remained at 10^6 to 10^7 CFU/g of root for up to 7 to 14 months, then dropped to about 10^4 to 10^5 CFU/g of root after 22 months (Table 5). VAR03-1 established populations averaging 10^6 CFU/g of root in the rhizosphere of grapevine and persisted in roots for 2 years. The rep-PCR analysis confirmed the identity of the isolates as VAR03-1 (Fig. 2), and all isolates showed in vitro antibiosis activity as well as VAR03-1 (Fig. 3). In contrast, characteristic colonies of *A. vitis* on 3DG medium isolated from nontreatment plants with VAR03-1 were not formed (Table 5).

One month after grapevine roots were soaked for 1 and 24 h in a cell suspension of 10^9 and 10^8 cells/ml of VAR03-1 and 10^9 and 10^8 cells/ml of K84, respectively, the bacterial popu-

lations with all treatments were about or over 10^7 CFU/g of root. Three months after, those with treatments at 10^9 and 10^8 cells/ml of VAR03-1 were about 10^7 CFU/g of root, whereas those with both treatments at 10^9 and 10^8 cells/ml of K84 were about 10^5 CFU/g of root. And the bacterial population treated with VAR03-1 was significantly higher than that treated with K84 ($P < 0.05$). Eighteen months after grapevine roots were soaked for 24 h in a cell suspension (10^8 cells/ml) of VAR03-1 and K84, though the bacterial population of VAR03-1 was about 10^6 CFU/g of root, the population of K84 has already reached an undetectable level 18 months after application (Table 6).

DISCUSSION

The nonpathogenic *A. vitis* strain F2/5 has been studied as a biological control agent of grapevine crown gall and is effective against tumorigenic strains of *A. vitis* (5). However, the biological efficacy of F2/5 is specific to grapevine; F2/5 is not effective on other host plants such as tomato, sunflower, and devil's backbone (*Kalanchoe daigremontiana*) (7). In contrast, VAR03-1 seems to protect a wider variety of plant species than F2/5 does; VAR03-1 is effective not only on grapevine but also on tomato, rose (Table 2), and sunflower, as we have already reported (17). Moreover, VAR03-1 reduced the number of crown galls caused by several tumorigenic strains of *A. vitis*, *A. rhizogenes*, and *A. tumefaciens* when the plant roots were soaked in cell suspensions of VAR03-1 before planting in soil infested with pathogens (Table 2), indicating that VAR03-1 covers a wide range of host plants and tumorigenic *Agrobacterium* species. Strain K84, which can colonize numerous species of plants, is effective in controlling tumorigenic strains of *A. tumefaciens* and *A. rhizogenes* on peach, almond, raspberry, cherry, plum, rose, apple, euonymus, boysenberry, tomato, and chrysanthemum in greenhouse or field tests (10,22,24). We will investigate further which species of plants VAR03-1 can colonize and control in field experiments.

In the two field experiments of biological control, soaking the roots of grapevine in a cell suspension of strain VAR03-1 before planting in soil infested with tumorigenic *A. vitis* significantly reduced the percentage of plants with tumors ($P < 0.05$) (Table 3). The results obtained in the field experiment strongly indicated that soaking for 1 h in a 10^9 cells/ml of cell suspension of VAR03-1 is suitable for practical use. More

Fig. 1. In vitro antibiosis assay against tumorigenic *Agrobacterium* spp. Paper disks with a suspension of either nonpathogenic *A. vitis* strain VAR03-1 (A, C, and E) or nonpathogenic *A. rhizogenes* strain K84 (B, D, and F) were placed on yeast mannitol agar (YMA) medium plates, which were then misted with a dilute suspension of tumorigenic *A. tumefaciens* strain CH5 (A and B), tumorigenic *A. rhizogenes* strain Ch-Ag-2 (C and D), or tumorigenic *A. vitis* strain G-Ag-27 (E and F). Two days after misting, a halo of growth inhibition was seen around the paper disk with the suspension of VAR03-1 (A, C, and E) against each tumorigenic *Agrobacterium* spp. and of K84 (B and D) against both tumorigenic *A. tumefaciens* and *A. rhizogenes*, but not around the one with K84 (F) against tumorigenic *A. vitis*.

TABLE 4. In vitro antibiosis assay of nonpathogenic *Agrobacterium* strains against tumorigenic *Agrobacterium* strains

Indicator strain	Formation of inhibition zone ^z on YMA medium (mm)	
	K84	VAR03-1
Tumorigenic <i>A. tumefaciens</i>		
CH3	11.8 ± 4.3	19.6 ± 0.7
CH5	6.1 ± 3.5	17.2 ± 1.6*
C054	0.0 ± 0.0	16.4 ± 0.8*
Tumorigenic <i>A. rhizogenes</i>		
AtR1	5.3 ± 1.3	18.9 ± 0.3
Ch-Ag-2	2.1 ± 0.6	20.0 ± 1.9*
P-Ag-1	1.5 ± 0.1	18.3 ± 1.4*
Tumorigenic <i>A. vitis</i>		
At-90-23	0.0 ± 0.0	20.8 ± 0.7*
G-Ag-27	0.0 ± 0.0	21.7 ± 2.8*
MAFF 211674	0.0 ± 0.0	20.0 ± 1.3*
MAFF 211676	0.0 ± 0.0	14.8 ± 0.3*

^z Data are mean (± standard error) radiuses of the inhibition zone of four replications per treatment. Strains with an inhibition zone wider than 1 mm were evaluated as sensitive. Means followed by an asterisk differ significantly ($P < 0.05$) between VAR03-1 and K84 according to the Mann-Whitney *U* test on the square root transformation ($X + 0.5$) data.

field experiments should be performed under different environmental conditions in the future to confirm the stability of the inhibitory effect.

Penyalver et al. (27) has suggested that the ability of strain K84 to colonize and persist on roots is important in the biological control process. Stockwell et al. (33) reported that K84 established populations averaging 10^5 CFU/g (fresh weight) on galled root tissue in the rhizosphere of cherry after inoculation with a cell suspension (10^8 CFU/ml), but the populations dropped to about 10^2 CFU/g of galled root tissue after 22 months. Our study demonstrated that a number of viable cells of K84 on grapevine roots after inoculation with 10^8 or 10^9 cells/ml of cell suspensions were significantly smaller than that of VAR03-1 after 3 months and colonization of K84 has already been less than 10^2 CFU/g of root after 18 months (Table 6). Furthermore, colonization of grapevine roots by VAR03-1 remained at 10^4 to 10^5 CFU/g of root after 22 months (Table 5) and that VAR03-1 established populations averaging 10^6 CFU/g of root in the rhizosphere of grapevine, indicating that VAR03-1 persists on grapevine roots for up to 2 years. These results of comparison with survival of K84 and VAR03-1 on grapevine roots demonstrate that the ability of VAR03-1 to colonize and persist on grapevine roots is significantly superior to that of K84. Correspondingly, the ability of VAR03-1 to control grapevine crown gall was significantly superior to that of K84 in greenhouse and field experiments, and K84 could hardly control grapevine crown gall in greenhouse after 3 months and field experiments after 7 to 8 months (Tables 2 and 3). As Penyalver et al. (27) suggested, our results indicated that the population level of K84 on grapevine rhizosphere is important to control grapevine crown gall.

A number of viable cells of VAR03-1 on grapevine roots after soaking for 1 and 24 h in a cell suspension of 10^9 and 10^8 cells/ml of VAR03-1 were not significantly different but the efficiency of root colonization after treatment with 10^9 cells/ml seems to be better than that with 10^8 cells/ml after 1 and 3 months (Table 6). In the biological control test, the protective value of treatment with 10^9 cells/ml of VAR03-1 soaking for 1 h against crown gall of grapevine was 91.8 to 100% in greenhouse and field experiments (Tables 2 and 3), but that of treatment with 10^8 cells/ml of VAR03-1 for 24 h was 71.4% in greenhouse experiments that we had already reported (17). Thus, the abilities of VAR03-1 to colonize on grapevine roots treated with a cell suspension of 10^9 cells/ml of VAR03-1 for 1 h and to control crown gall were superior to that of treatment with 10^8 cells/ml for 24 h, indicating the possibility that population level of VAR03-1 on grapevine rhizosphere have an effect on ability to control grapevine crown gall. Treatment condition of VAR03-1 with grapevine roots, which a cell suspension is 10^9 cells/ml and soaking time is 1 h, is more suitable for practical use than a cell suspension of 10^8 cells/ml and soaking time for 24 h because short at soaking time, colonization ability on grapevine roots, and control ability of the former is better than those of latter.

The antibiotic production by strains VAR03-1 and K84 was indicated by a halo of inhibition in the bacterial lawn around the test strain (Fig. 2; Table 4). K84 produces an antibiotic

named “agrocin 84” that selectively inhibits pathogenic agrobacteria (15), but *A. vitis* is insensitive to agrocin 84 (3,4, 14,16). In this study, it was confirmed that K84 did not inhibit tumorigenic *A. vitis* strains by production of agrocin 84 on YMA medium (Fig. 1; Table 4). Additionally, in vitro anti-

Fig. 2. DNA fingerprint patterns of 11 strains of *Agrobacterium vitis* revealed by rep-polymerase chain reaction DNA fingerprint analysis with BOX (A) and ERIC (B) primer sets. Lanes: M, 100-bp marker; V, VAR03-1; 1 to 10, each isolate obtained from grapevine roots 18 months after treatment for 24 h in a cell suspension (10^8 cells/ml) of VAR03-1 in survival experiment of Table 6.

TABLE 5. Population dynamics of nonpathogenic *Agrobacterium vitis* strain VAR03-1 in the roots of grapevine cv. Neo Muscat after inoculation

Treatment	Log ₁₀ CFU per g (fresh weight) grapevine root ^y				Average
	Months after inoculation				
	1	7	14	22	
VAR03-1	7.24 ± 0.15	6.91 ± 0.23	6.18 ± 0.39	4.67 ± 0.84	6.25 ± 0.40
Nontreatment	<2 ^z	<2	<2	<2	–

^y Data are means of three grapevines. Mean log₁₀ ± standard error.

^z The approximate detection limit was 10^2 colony forming units (CFU).

biosis assay was preformed not only on YMA medium but also on Stonier medium by Stonier's method (34,35). Though K84 inhibited indicator strains including tumorigenic *A. tumefaciens* except C054 and tumorigenic *A. rhizogenes* in the same as on YMA medium, *A. vitis* VAR03-1 as a test strain and tumorigenic strains as an indicator could not grow and produce a halo of inhibition (data not shown). We have already reported that VAR03-1 produced a type of bacteriocin and inhibited tumorigenic *A. vitis* on YMA medium (16). In this study, we found that VAR03-1 produced a halo of inhibition against not only tumorigenic *A. vitis* but also *A. tumefaciens* and *A. rhizogenes*, clarifying that the bacteriocin produced by VAR03-1 also could inhibit *A. tumefaciens* and *A. rhizogenes*. In the biological control test, VAR03-1 actually controlled crown gall of tomato and rose caused by tumorigenic *A. tumefaciens* and *A. rhizogenes*, respectively (Table 2), indicating the possibility that the bacteriocin of VAR03-1 might be one of the factors in the control of crown gall disease.

However, whether only the bacteriocin production of VAR03-1 is related to the inhibition of tumor formation in planta must be investigated because Burr et al. (7) reported that biological control of grapevine crown gall by nonpathogenic *A. vitis* strain F2/5 was neither associated with agrocin production nor competition for attachment sites on grapevine cells. Though the inhibition halo of VAR03-1 was significantly wider than that of K84 against eight indicator strains ($P < 0.05$) (Table 4), VAR03-1 treatment reduced the percent-

Fig. 3. In vitro antibiosis assay against tumorigenic *Agrobacterium vitis* strain G-Ag-27 as indicator strain. Paper disk no. 1 to 3 with a suspension of each isolate obtained from grapevine roots 18 months after treatment for 24 h in a cell suspension (10^8 cells/ml) of VAR03-1 in survival experiment of Table 6 were placed on yeast mannitol agar medium plates, which were then misted with a dilute suspension of G-Ag-27.

age of plants with tumors in tomato and rose experiments as well as K84 treatment (Table 2), indicating that the width of the inhibition halo was not involved in the degree of the inhibitory effect of strains VAR03-1 and K84, respectively. We plan to generate a mutant strain of VAR03-1 that lacks bacteriocin by transposon mutagenesis, and we will then investigate whether the bacteriocin-minus mutant inhibit tumors formation to reveal the main factor of VAR03-1 to control crown gall disease.

K84 did not inhibit growth of tumorigenic *A. tumefaciens* strain C054 in this antibiosis assay (Table 4) but did control crown gall of tomato caused by a mixture of tumorigenic strains containing C054 (Table 2). Kerr (14) reported that the biological control of K84 depended on production of agrocin 84, but Makino (23) found that K84 could control crown gall of rose caused by tumorigenic *A. rhizogenes* strain R73, which was insensitive to agrocin 84, indicating that the control activity of K84 depends on factors besides agrocin 84. K84 may compete for nutrients or attachment sites on plant cells against other agrobacteria in the rhizosphere. Our result that K84 inhibited tumor formation caused by tumorigenic strains, including C054 in planta, supports Makino's report (23), though there is a possibility that the effect of biological control was achieved by the antibiotic activity against agrocin-sensitive strains CH3 and CH5, which were the majority in the cell suspension mixture. Whether VAR03-1 has a competitive action like K84 is a very interesting question; thus, study of the control mechanism of VAR03-1 should be continued.

Some opines can be catabolized by the inciting bacteria by using functions encoded by the Ti-plasmid, and K84 is effective against only the nopaline/agrocinopine A strains of agrobacteria (12). Tumorigenic *A. vitis* vitopine strains are broadly distributed in Japan, and strain G-Ag-27 induces vitopine (29). Thus, it was verified that VAR03-1 was effective against at least the vitopine strain (Fig. 1; Tables 2, 3, and 4). What kinds of opine-type strains VAR03-1 is applicable against should be investigated in the future.

As far as we know, there is no effective antagonist to control crown gall of not only grapevine caused by tumorigenic *A. vitis* but also in the other plants caused by *Agrobacterium* spp. anywhere in the world. This study is the first report that one nonpathogenic strain, VAR03-1, can effectively reduce tumor formation of crown gall in grapevine, rose, and tomato due to tumorigenic *A. vitis*, *A. rhizogenes*, and *A. tumefaciens*. Furthermore, in the field tests, strain VAR03-1 similarly controlled crown gall of grapevine. Strain VAR03-1 is a promising agent for the control of crown gall of not only of grapevine but also of other plants, and the applicability of VAR03-1 for many kind of plants in the field should be further investigated.

TABLE 6. Root colonization of nonpathogenic *Agrobacterium vitis* strain VAR03-1 and *A. rhizogenes* strain K84 in the roots of grapevine cv. Neo Muscat after inoculation

Strain	Treatment		Log ₁₀ CFU per g (fresh weight) grapevine root ^v		
	Cell suspension (cells/ml)	Soaking time (h)	Months after inoculation		
			1 ^w	3 ^w	18 ^x
VAR03-1	10 ⁹	1	7.87 ± 0.12 a	7.26 ± 0.13 a	NT ^y
	10 ⁸	24	7.47 ± 0.10 ab	6.90 ± 0.14 a	6.19 ± 0.09
K84	10 ⁹	1	6.99 ± 0.04 b	5.71 ± 0.17 b	NT
	10 ⁸	24	7.44 ± 0.20 ab	5.57 ± 0.20 b	<2 ^z

^v Data are means of 10 grapevines. Mean log₁₀ ± standard error.

^w Treated grapevines were planted in pots with soil on 1 March 2008. Means followed by the same letter are not significantly different according to Scheffe's test at $P < 0.05$.

^x Treated grapevines were planted in pots with soil on 6 June 2006.

^y NT; not tested.

^z The approximate detection limit was 10² colony forming units (CFU).

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