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Biological control of grapevine crown gall by nonpathogenic *Agrobacterium vitis* strain VAR03-1

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Abstract A nonpathogenic strain of *Agrobacterium vitis* VAR03-1 was tested as a biological control agent against crown gall of grapevine (*Vitis vinifera* L.). A mixture of the nonpathogenic strain VAR03-1 and a tumorigenic strain G-Ag-27 of *A. vitis* at cell ratios of 1:1, 3:1, 9:1, and 99:1 significantly inhibited gall formation and size on stems of tomato (*Lycopersicon esculentum* Mill.). Strain VAR03-1 also inhibited gall formation on stems of both tomato and grapevine at a 1:1 cell ratio with several tumorigenic *A. vitis* strains isolated from different fields of grapevine in Japan. In biological control tests, when roots of grapevine and tomato seedlings were soaked in a cell suspension of strain VAR03-1 for 24 h before a 1-h soaking in a cell suspension of the pathogen and subsequent planting in pots of infested soil, strain VAR03-1 significantly reduced the incidence of gall formation on both plants.

Key words *Agrobacterium vitis* · Crown gall · Grapevine · Biological control · Strain VAR03-1

Introduction

Grapevine crown gall caused by *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 (Burr et al. 1998; Burr and Otten 1999; Sawada et al. 1990).

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 (Moore and Warren 1979; Shim et al. 1987). An agrocin produced by K84 (agrocin 84) is thought to be the primary factor in the control. However, K84 cannot prevent the initial infection of grapevine by tumorigenic *A. vitis* (Burr et al. 1998).

Several laboratories have attempted to identify other biological control measures for grape crown gall (Liang et al. 1990; Staphorst et al. 1985; Webster and Thomson 1986; Xiaoying and Wangnian 1986). Staphorst et al. (1985) evaluated 16 strains, including nonpathogenic *A. vitis* strain F2/5, which inhibited growth of most tumorigenic strains of *A. vitis* in vitro and greatly inhibited crown gall on grapevine in stem-wounding experiments in the greenhouse. Burr and Reid (1993) reported that F2/5 produces agrocin, which is inhibitory to most tumorigenic *A. vitis* strains in vitro, and effectively inhibits gall formation at wound sites on grapevine stems artificially inoculated with one of several tumorigenic *A. vitis* strains. However, F2/5 did not inhibit tumor formation caused by other strains of tumorigenic *A. vitis* (Burr and Otten 1999).

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 gall formation on tomato (the model plant) and grapevine seedlings caused by tumorigenic *A. vitis* strain G-Ag-27 (Kawaguchi et al. 2005). In this article, we report that strain VAR03-1 can inhibit gall formation caused by several tumorigenic *A. vitis* strains and reduce crown gall on roots and stems of grapevine after the roots were soaked in cell suspensions of the antagonist.

We follow the nomenclature for *Agrobacterium* species adopted in *Bergey's manual of systematic bacteriology* (Young et al. 2005) and as reported by Ophel and Kerr (1990) to avoid confusion, although other valid naming systems have been proposed (Bouzar et al. 1995; Kersters and De Ley 1984; Sawada et al. 1993; Young et al. 2001).

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Materials and methods

Gall inhibition assays

Gall inhibition assays were carried out using methods we established earlier (Kawaguchi et al. 2005). Seedlings (1–2 months old) of sunflower (*Helianthus annuus* L. cv. Mammoth), tomato (*Lycopersicon esculentum* Mill. cv. Ponderosa), and grapevine (*Vitis vinifera* L. cv. Neo Muscat), grown from seed, were prepared. Respective cell suspensions of nonpathogenic *A. vitis* strain VAR03-1 and tumorigenic *A. vitis* strains (Table 1) were prepared from 48-h-old cultures on potato semisynthetic agar medium [PSA; 300 g potato, 0.5 g $Ca(NO_3)_2 \cdot 4H_2O$, 2 g $Na_2HPO_4 \cdot 12H_2O$, 5 g peptone, 20 g sucrose, 15 g agar, 1 l distilled water, pH 6.8–7.0] slants and adjusted to an optical density at 600 nm (OD_{600}) of 0.1 (corresponding to about 10^8 CFU/ml), respectively. A cell suspension of strain VAR03-1 and of the tumorigenic strain were mixed in various combinations at cell ratios of 1:9, 1:3, 1:1, 3:1, 9:1, and 99:1. A 5-µl drop of either one strain or of the mixture was dropped onto a needle-prick wound on stems of a tomato or grapevine. Each of ten plants of tomato and sunflower received four inoculations (i.e., a total of 40 inoculations per treatment). Grapevines in ten pots each received six inoculations (i.e., a total of 60 inoculations per treatment). Gall formation was assessed 4 weeks later for tomato and sunflower and 12 weeks later for grapevine in a greenhouse at 25° to 28°C.

Biological control of crown gall

Biological control tests were carried out using seedlings (1–2 months old) of tomato cv. Ponderosa and grapevine cv. Neo Muscat, grown from seed using methods from a previous report (Cooksey and Moore 1980). Cell suspensions of VAR03-1 and G-Ag-27 were prepared from 48-h-old cultures on PSA slants and adjusted to about 10^8 cells/ml. Young plants were pulled from the soil and the root rinsed in water until clean. Roots were then pruned and soaked for 24 h in a cell suspension of strain VAR03-1 at 27°C, and then immersed in a cell suspension of strain G-Ag-27 for 1 h to induce a gall effectively. After these treatments, 15 tomatoes and 15 grapevines were each planted in a pot (12 cm deep × 15 cm in diameter) in soil infested with a cell suspension of strain G-Ag-27 at a final concentration 10^8 cells/g soil in soil 10 cm deep. Plants were then grown in a greenhouse at 25°–28°C, and gall formation was assessed after 8 weeks for tomato and 12 weeks for grapevine. The experiment was repeated three times.

Table 1. Bacterial strains used in this study

Bacterial strain[a]	Source and location (supplier)[b]
Tumorigenic *Agrobacterium vitis* (= *A. tumefaciens* biovar 3, tumorigenic *Rhizobium vitis*)	
G-Ag-27	Grape; Nagano, Japan (H. Sawada)
At-5, At-90-23, At-90-62	Grape; Shimane, Japan (J. Yamamoto)
A5-1, A5-2, A5-4, A5-5, A5-6, A5-7, A5-9	Grape; Akita, Japan
VAT03-9, VAT3-2, VAT8-1	Grape; Okayama, Japan
9-1-5	Grape; Nagano, Japan
Nonpathogenic *Agrobacterium vitis* (= *A. radiobacter* biovar 3, nonpathogenic *R. vitis*)	
VAR03-1	Grape; Okayama, Japan

[a] Taxa of the genus *Agrobacterium* are named according to the nomenclature system proposed by Ophel and Kerr (1990) and Young et al. (2005)
[b] Unless a supplier is stated, we isolated the strain (Kawaguchi et al. 2005)

Table 2. Effect on tomato seedlings of coinoculation with nonpathogenic *Agrobacterium vitis* strain VAR03-1 and tumorigenic *A. vitis* strain G-Ag-27 at different cell ratios

Cell ratio (VAR03-1:G-Ag-27)	Experiment 1		Experiment 2		Experiment 3	
	Gall formation (%)	Gall size[a] (mm)	Gall formation (%)	Gall size (mm)	Gall formation (%)	Gall size (mm)
99:1	0	0 a	0	0 a	0	0 a
9:1	0	0 a	0	0 a	0	0 a
3:1	0	0 a	0	0 a	0	0 a
1:1	5	0.14 a	2.5	0.08 a	0	0 a
1:3	67.5	1.91 b	90	3.81 b	82.5	3.42 b
1:9	100	4.95 c	100	5.33 c	85	4.73 b
Only G-Ag-27[b]	100	6.38 c	95	5.75 c	100	5.22 b

Data are means of ten replications of four inoculations per tomato seedling Means within a column followed by the different letter differ significantly ($P < 0.01$) according to Tukey's honest significant difference (HSD) test
[a] Gall diameter perpendicular to the long axis of the stem
[b] Only a pathogenic strain (about 10^8 CFU/ml) was used as inoculum

Table 3. Effect of coinoculation on tomato seedlings with a 1:1-cell ratio mixture of nonpathogenic *Agrobacterium vitis* strain VAR03-1 and pathogenic strains of tumorigenic *A. vitis* isolated from different locations in Japan

Pathogenic strain	Experiment 1				Experiment 2				Experiment 3			
	Gall formation (%)		Gall size[a] (mm)		Gall formation (%)		Gall size (mm)		Gall formation (%)		Gall size (mm)	
	VAR03-1	Only pathogen[c]	VAR03-1	Only pathogen	VAR03-1	Only pathogen	VAR03-1	Only pathogen	VAR03-1	Only pathogen	VAR03-1	Only pathogen
G-Ag-27	0	92.5	0**	4.94	12.5	100	0.44**	7.98	0	100	0**	5.13
At-5	2.5	97.5	0.08**	5.05	7.5	92.5	0.22**	4.29	7.5	95	0.25**	5.43
At-90-23	0	100	0**	5.98	10	95	0.37**	5.95	0	95	0**	4.84
At-90-62	27.5	100	0.99**	5.78	75	77.5	3.17	3.58	2.5	85	0.06**	3.13
A5-1	7.5	100	0.15**	6.15	20	100	0.58**	8.21	7.5	97.5	0.18**	6.13
A5-2	2.5	100	0.08**	4.6	2.5	80	0.07**	3.19	0	85	0**	3.15
A5-4	10	100	0.36**	6.97	2.5	97.5	0.05**	5.54	7.5	100	0.29**	5.99
A5-5	0	32.5	0**	1.27	0	32.5	0**	1.17	0	35	0**	1.02
A5-6	2.5	100	0.07**	4.2	0	95	0**	3.07	0	45	0**	1.49
A5-7	7.5	100	0.21**	6.91	5	85	0.15**	4.08	0	100	0**	5.76
A5-9	0	70	0**	2.47	2.5	90	0.07**	3.83	0	67.5	0**	2.07
VAT03-9	0	100	0**	5.78	12.5	95	0.34**	6.86	35	100	1.11**	6.45
VAT3-2	17.5	92.5	0.51**	4.55	75	97.5	3.11	4.74	10	87.5	0.27**	3.84
VAT8-1	0	40	0**	0.89	0	20	0**	0.75	5	20	0.23*	0.57

Data are means of ten replications of four inoculations per tomato seedling
[a] Gall diameter perpendicular to the long axis of the stem
[b] Only a pathogenic strain (about 10^8 CFU/ml) was used as inoculum
* $P < 0.05$; ** $P < 0.01$; significant difference from plants inoculated with only a pathogenic strain (Student's *t*-test)

Results

Inhibition of gall formation on tomato with mixtures of strain VAR03-1 and the tumorigenic strain at various cell ratios

On tomato seedlings, VAR03-1 mixed at cell ratios of 1:1, 3:1, 9:1, and 99:1 with G-Ag-27 completely suppressed gall formation ($P < 0.01$) on the stems in three experiments (Table 2). The 1:1-cell ratio mixture completely suppressed gall formation in two of the three experiments and largely suppressed gall formation and size in the remaining experiment (Table 2). However, the 1:3 and 1:9 cell ratios of VAR03-1: G-Ag-27 did not suppress gall formation (Table 2).

Inhibition of gall formation on three plants by mixtures of strain VAR03-1 and various tumorigenic strains at 1:1 cell ratios

In tests against 14 tumorigenic *A. vitis* strains isolated from different fields of grapevine in Japan (Table 1), a 1:1 cell ratio of strain VAR03-1 to a tumorigenic strain significantly reduced gall formation and size ($P < 0.05$) on stems of tomato in three experiments, whereas VAR03-1 was not effective against two tumorigenic strains, At-90-62 and VAT3-2, in experiment 2 (Table 3). In addition, a 1:1 cell ratio of VAR03-1 with any of eight tumorigenic *A. vitis* strains (G-Ag-27, At-90-23, At-90-62, A5-1, A5-6, VAT03-9, VAT3-2, and 9-1-5) significantly reduced gall formation and size ($P < 0.01$) on stems of grapevine (Table 4). Moreover, as shown in Table 5, a 1:1 cell ratio of G-Ag-27 to VAR03-1 also completely reduced gall formation and size ($P < 0.01$) on stems of sunflower similar to the effect on tomato and grapevine (Tables 3, 4).

Table 4. Effect of coinoculation on grapevine seedlings with a 1:1-cell ratio mixtures of nonpathogenic *Agrobacterium vitis* strain VAR03-1 and each pathogenic strain of tumorigenic *A. vitis* isolated from different locations in Japan

Pathogenic strain	Gall formation (%)		Gall size[a] (mm)	
	VAR03-1	Only pathogen[b]	VAR03-1	Only pathogen
G-Ag-27	1.7	90	0.04**	4.09
At-90-23	28.3	98.3	0.56**	4.11
At-90-62	26.4	95	0.58**	2.83
A5-1	38.3	71.7	1.01**	2.72
A5-6	3.3	96.7	0.11**	3.29
VAT03-9	13.3	80	0.34**	2.44
VAT3-2	36.7	100	0.89**	3.46
9-1-5	18.3	96.7	0.45**	3.49

Data are means of ten replications of six inoculations per grapevine seedling
[a] Gall diameter perpendicular to the long axis of the stem was measured
[b] Only a pathogenic strain (about 10^8 CFU/ml) was used as inoculum
** $P < 0.01$; significant difference from plants inoculated with only a pathogenic strain (Student's *t*-test)

Biological control of crown gall by strain VAR03-1

In the biological control test using the method of planting grapevine and tomato seedlings in the soil infested with tumorigenic strain G-Ag-27, pretreatment of grapevine roots with strain VAR03-1 significantly reduced the percentage of galled plants ($P < 0.05$) and controlled crown gall of grapevine seedlings by 71.4% (Table 6). Moreover, VAR03-1 pretreatment of tomato roots also significantly reduced the percentage of galled plants ($P < 0.05$) and controlled crown gall of tomato seedlings by 73.1%, although the reduction in number of galls per plant was not statistically significant (Table 6).

Discussion

In this study, not only 1:1, but also 1:3, 1:9, and 1:99 cell ratios of G-Ag-27 to VAR03-1 greatly suppressed gall formation and size ($P < 0.01$) on stems of tomato, but the cell ratios of 9:1 and 3:1 did not (Table 2). This result indicated that high ratios of VAR03-1 to the pathogenic strain should effectively inhibit gall formation. A type of bacteriocin production by VAR03-1 was indicated by an inhibitory zone in the bacterial lawn around some test strains of tumorigenic *A. vitis* including G-Ag-27 (Kawaguchi et al. 2005). The antibiosis was suggested to be dependent on the density of pathogen because the inhibiting effect by VAR03-1 was lost when the number of pathogen cells was greater than that of VAR03-1 (Table 2). Whether only bacteriocin production

Table 5. Effect of coinoculation on sunflower seedlings with nonpathogenic *Agrobacterium vitis* strain VAR03-1 and tumorigenic *A. vitis* strain G-Ag-27 at a 1:1 cell ratio

Strain	Gall formation (%)	Gall size[a] (mm)
VAR03-1	0	0**
Only G-Ag-27[b]	47.5	2.36
Sterile distilled water	0	–

Data are means of ten replications of four inoculations per sunflower seedling
[a] Gall diameter perpendicular to the long axis of the stem was measured.
[b] Only a pathogenic strain (about 10^8 CFU/ml) was used as inoculum
** $P < 0.01$; significant difference from plants inoculated with only G-Ag-27 (Student's *t*-test)

Table 6. Effect of nonpathogenic *Agrobacterium vitis* strain VAR03-1 on crown gall of grapevine and tomato seedlings after presoaking plant roots in bacterial cell suspensions

Strain	Grapevine			Tomato		
	Plants with galls (%)	No. of galls/plant	Control[b] (%)	Plants with galls (%)	No. of galls/plant	Control(%)
VAR03-1	8.9*	0.17*	71.4	13.3*	0.13	73.1
Only G-Ag-27[c]	31.1	0.44	–	49.4	0.78	–

Data are means of three replications of 15 tomato or grapevine seedlings per treatment
[b] Control (%) = [1 – Galled plants treated with VAR03-1]/[Galled plants treated with sterile distilled water (only G-Ag-27)] × 100
[c] Young plants were soaked in sterile distilled water 24 h before dipping in a cell suspension (10^8 cells/ml) of tumorigenic *A vitis* strain G-Ag-27
* $P < 0.05$; significant difference from plants inoculated with only G-Ag-27 (Student's *t*-test)

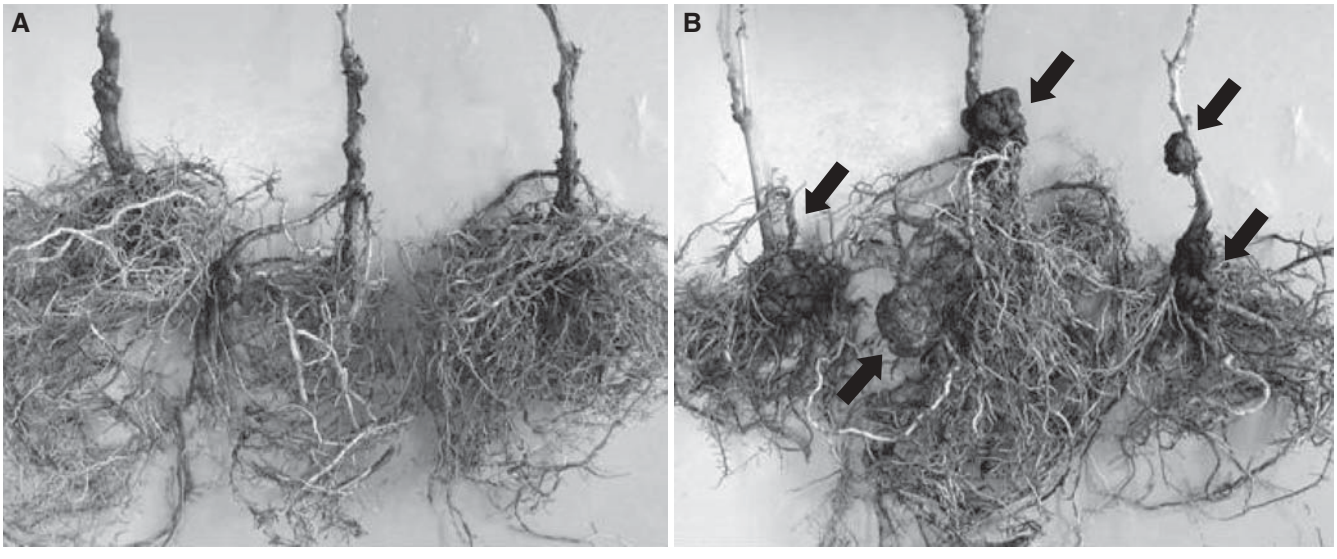

Fig. 1A, B. Biological control activity of grapevine crown gall by nonpathogenic strain VAR03-1 of *Agrobacterium vitis*. **A** The grapevines were planted after presoaking with nonpathogenic strain VAR03-1 of *A. vitis* followed by inoculation with a cell suspension of tumorigenic strain G-Ag-27. **B** The grapevines were planted after inoculation only with a cell suspension of strain G-Ag-27 as a positive control. Galls (*arrows*) developed on the roots or stems. Photographs were taken approximately 6 months after inoculation

is related to the inhibition of gall formation in planta must be investigated. We are planning to generate a bacteriocin-minus mutant of strain VAR03-1 by transposon mutagenesis, and we will then investigate whether the bacteriocin-minus mutant can inhibit gall formation by tumorigenic strains on plants at a 1:1 cell ratio.

In the gall inhibition assay using plants, a 1:1 cell ratio of any of the 14 tumorigenic *A. vitis* strains with VAR03-1 significantly reduced gall formation and size ($P < 0.05$) on stems of tomato in three experiments, whereas it did not against At-90-62 and VAT3-2 in experiment 2 (Tables 1, 3). Moreover, a 1:1 cell ratio of VAR03-1 to each of 8 tumorigenic *A. vitis* strains also significantly reduced gall formation and size ($P < 0.01$) on grapevine seedlings (Tables 1, 4). This result indicated that VAR03-1 might be effective against numerous strains of tumorigenic *A. vitis*.

As stated before, 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* (Burr and Reid 1993). However, biological control 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*) (Burr et al. 1997). In contrast, VAR03-1 seems to be able to protect more types of plants than F2/5; VAR03-1 is effective not only on grapevine and tomato, but also on sunflower (Table 5). Nonpathogenic *Agrobacterium rhizogenes* (= *Agrobacterium radiobacter* biovar 2) strain K84, which can colonize numerous species of plants, is effective against tumorigenic *Agrobacterium tumefaciens* (= *A. tumefaciens* biovar 1) and tumorigenic *A. rhizogenes* (= *A. tumefaciens* biovar 2) on peach, almond, raspberry, cherry, plum, rose, apple, euonymus, boysenberry, tomato, and chrysanthemum in greenhouse or field tests (Du Plessis et al. 1985; Makino 1986, 1993; Moore and Warren 1979). We must further investigate which species of plants other than grapevine VAR03-1 can colonize and examine the inhibitory activity of VAR03-1 against tumorigenic strains of *A. tumefaciens* and *A. rhizogenes*.

As far as we know, our report is the first on the use of a root soak to pretreat grapevine and tomato before planting in soils infested with tumorigenic strain G-Ag-27 of *A. vitis*. Pre-inoculation of grapevine roots with VAR03-1 was effective in reducing the percentage of galled plants ($P < 0.05$) (Table 6, Fig. 1.). In particular, VAR03-1 controlled crown gall of grapevine seedlings by 71.4% after the roots were presoaked in a cell suspension of the agent (Table 6). Makino (1993) reported that nonpathogenic *A. rhizogenes* strain K84 gave no control of crown gall of tomato seedling caused by tumorigenic *A. rhizogenes* strain R257 using this method at the same concentration (10^8 cells/ml), although K84 reduced the number of tomato seedlings with galls (1–3 months old) by 75.0% at a 100-fold higher concentration than that of the pathogen R257 (cell suspension of K84 = 10^9 cells/ml, cell suspension of R257 = 10^7 cells/ml). In contrast, VAR03-1 reduced the number of galled tomato seedlings by an average of 73.1% at the same concentration (10^8 cells/ml) (Table 6). Thus, the ability of VAR03-1 to control crown gall may be greater than that of K84 in the test using tomato plants. This is the first report that bacterial agent VAR03-1 can control crown gall of grapevine and tomato caused by tumorigenic *A. vitis* using this method. In the future we will test the efficacy of VAR03-1 in controlling grapevine crown gall in fields by presoaking the roots of planting stock in suspensions of VAR03-1 or by directly applying the suspension to the planting bed.

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