

Biological control agent *Agrobacterium vitis* strain ARK-1 suppresses expression of the *virD2* and *virE2* genes in tumorigenic *A. vitis*

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Abstract A nonpathogenic strain of *Agrobacterium* (= *Rhizobium*) *vitis*, ARK-1, limits the development of crown gall of grapevine (*Vitis vinifera* L.). Co-inoculation of grapevine shoots with ARK-1 and the tumorigenic (Ti) strain VAT03-9 at a 1:1 cell ratio resulted in significantly lower expression of the virulence genes *virD2* and *virE2* of VAT03-9 1 day after inoculation (dai) compared with expression levels when shoots were inoculated only with VAT03-9. In contrast, non-pathogenic *A. vitis* strain VAR06-30, which does not limit the development of crown gall of grapevine, co-inoculated with VAT03-9 did not affect expression levels of *virD2* and *virE2* under the same conditions. ARK-1 began to suppress the VAT03-9 population by seven dai, but no such effect was observed with VAR06-30 during the nine dai study period. Thus, the biological control activity of ARK-1 is likely based on the suppression of essential virulence genes.

Keywords *Agrobacterium vitis* · Grapevine crown gall · Biological control · RT-qPCR · Gene expression

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Introduction

Grapevine (*Vitis vinifera* L.) crown gall is caused mainly by *Agrobacterium vitis* (Ti) [= *Rhizobium vitis* (Ti), *A. tumefaciens* biovar 3], where “Ti” means “tumor-inducing” or “tumorigenic”. This is one of the most important diseases of grapevine around the world (Burr et al. 1998; Burr and Otten 1999). The virulence (*vir*) genes and transfer DNA (T-DNA) are located mostly on large tumor-inducing plasmids (pTi) and also chromosomal virulence genes (*chv*) on chromosomes. Virulent *Agrobacterium* strains transfer single-strand forms of T-DNA and several Virulence effector proteins through a bacterial type IV secretion system into plant host cells (Gelvin 2012; Nester 2015). The plant molecules acetosyringone and α -hydroxyacetosyringone induce the entire *vir* regulon in *Agrobacterium* as well as the formation of T-DNA intermediate molecules (Nester 2015). These plant molecules occur specifically in the exudates from wounded and metabolically-active plant cells and probably allow *Agrobacterium* to recognize susceptible cells in nature (Nester 2015). T-DNA transfer and processing require products of the *vir* genes (*virA*, *virB*, *virC*, *virD*, *virE*, and *virG*), which are located outside of the T-DNA coding region (Gelvin 2012; Lacroix and Citovsky 2013; McCullen and Binns 2006; Nester 2015). The expression of *virB*, *virC*, *virD*, and *virE* is positively regulated at the transcriptional level by plant signal molecules (Gelvin 2012; Lacroix and Citovsky 2013; Nester 2015). Two *Agrobacterium* proteins, VirD2 and VirE2, directly associate with the T-strand; one VirD2 molecule covalently attaches to the

5'-end of the T-strand, and VirE2, a protein that binds single-stranded DNA, cooperatively coats the rest of the T-strand (Gelvin 2012; Lacroix and Citovsky 2013; Nester 2015).

Several laboratories have attempted to develop some biological measures to control grapevine crown gall. Staphorst et al. (1985) evaluated nonpathogenic *A. vitis* strain F2/5, which inhibited the growth of most Ti strains of *A. vitis* in vitro and inhibited crown gall formation in grapevine in greenhouse shoot-wounding experiments. Burr and Reid (1994) reported that F2/5 produces an unidentified agrocin. Wang et al. (2003) reported that the antibacterial compound Ar26 produced by nonpathogenic *A. vitis* strain E26 inhibited the growth of *A. vitis* (Ti) on culture plates. Chen et al. (2007) reported that *Rahnella aquatilis* strain HX2 inhibited the development of crown galls in grapevine.

Previously, we have reported that a nonpathogenic *A. vitis* strain, VAR03-1, that was isolated from grapevine nursery stock in Japan inhibited tumor formation in grapevine, rose, tomato, sunflower, and apple (Kawaguchi et al. 2008, 2012). Moreover, we identified nonpathogenic *A. vitis* strain ARK-1, which performed better than VAR03-1 at inhibiting tumor formation in grapevine, as a new antagonistic strain (Kawaguchi 2013; Kawaguchi and Inoue 2012). ARK-1 is endophytic in grapevine, induces no necrosis of the host plant, and controlled grapevine crown gall better than VAR03-1 in field trials (Kawaguchi 2013). Although ARK-1 produced a zone of inhibition against tumorigenic *Agrobacterium* spp. in in vitro assays, antibiosis was not the main mechanism of biological control, because ARK-1 suppressed tumor formation in grapevine shoots caused by a Ti strain that was insensitive to the antibiosis produced by ARK-1 (Kawaguchi 2014). Although ARK-1 reduced the pathogen population at the wound site through biological control, ARK-1 suppressed the Ti strain population significantly at 2 days after inoculation (dai) but did not suppress the Ti population at 1 and 5 dai (Kawaguchi 2014). It is most likely that transformation by *A. vitis* would occur prior to 5 days because co-cultivation of *Agrobacterium* cells and plants has been successfully tried for 2 or 3 days in a general protocol for the *Agrobacterium*-mediated transformation of plants (Jones et al. 2005). The mechanism by which ARK-1 reduces crown gall might not involve reducing populations of the Ti strains in grapevine plants at an early infection stage, so the main mechanism of ARK-1's effects remains unknown.

To provide insights into that mechanism, we tested the effect of ARK-1 to suppress the expression of two virulence genes by the Ti strain at the wound site. This report gives that suppression of expression of the *virD2* and *virE2* virulence genes of the Ti strain by ARK-1 at the grapevine wound site and in vitro; this suppression appears to be responsible for the biological control by this strain. In this paper, the nomenclature for *Agrobacterium* species proposed by Young et al. (2005) was followed.

Materials and methods

Development of antibiotic-resistant *A. vitis* strains

Table 1 lists the bacterial strains used in this study. Potato sucrose agar (PSA) medium was used to grow the bacteria in this study (Kawaguchi 2013; Kawaguchi and Inoue 2012). ARK-1sc was a streptomycin (St)- and 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 µg/ml St (minimum inhibiting concentrations (MIC) =500 µg/ml) and 250 µg/ml CuSO₄ (MIC=250 µg/mL) (Kawaguchi 2013). VAR06-30sc was also a St-CuSO₄ mutant obtained by growing on St-CuSO₄-PSA medium. VAT03-9n was a nalidixic acid (nal)-resistant mutant (nal-mutant) obtained by growing on nal-PSA medium, which is PSA amended with 50 µg/ml nal (MIC=50 µg/mL).

Tumor inhibition assay in grapevine seedlings

Grapevine seedlings (*V. vinifera* cv. 'Neo Muscat') were grown from seed. One-year-old grapevine shoots were inoculated using previously established methods (Kawaguchi and Inoue 2012). Cell suspensions of the nonpathogenic strains (ARK-1 and VAR06-30) and of the tumorigenic strain VAT03-9 (Table 1) were prepared from 48-h-old cultures on PSA medium slants, discarded a supernatant of these strains and washed with distilled water, and adjusted to OD₆₀₀=0.1, which corresponds to approximately 10⁸ cells ml⁻¹. A culture filtrate of ARK-1 by filtering with a syringe filter unit (0.2 µm diameter) as "CF of ARK-1" were prepared. Two mixed cell suspensions (ARK-1 plus VAT03-9, VAR06-30 plus VAT03-9), both at a cell ratio of 1:1, and CF of ARK-1 plus VAT03-9 and a VAT03-9 suspension were prepared. A 5-µl drop

Table 1 Bacterial strains used in this study

Bacterial strain	Pathogenicity ^a	Description
<i>Agrobacterium vitis</i> (Ti) (= <i>Rhizobium vitis</i> (Ti), <i>A. tumefaciens</i> biovar 3)		
VAT03-9 (=MAFF211676) ^a	Tumorigenic	Isolated from galled grapevines in Japan (Kawaguchi and Inoue 2012)
VAT03-9n	Tumorigenic	Nalidixic acid-resistant mutant of strain VAT03-9 (This study)
Nonpathogenic <i>A. vitis</i> (=Nonpathogenic <i>R. vitis</i> , <i>A. radiobacter</i> biovar 3)		
ARK-1	Nonpathogenic	Isolated from nursery stock of grapevine in Japan; biological control agent for crown gall (Kawaguchi and Inoue 2012)
ARK-1sc	Nonpathogenic	Streptomycin- and copper sulfate-resistant mutant of strain ARK-1 (Kawaguchi 2013)
VAR06-30	Nonpathogenic	Isolated from nursery stock of grapevine in Japan (Kawaguchi 2011)
VAR06-30sc	Nonpathogenic	Streptomycin- and copper sulfate-resistant mutant of strain VAR06-30 (This study)

^aMAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba Ibaraki, Japan

(containing approximately 5×10^5 cells) of a given cell suspension was dropped onto a needle-prick wound on the stem of a grapevine seedling. Six to seven grapevine seedlings (at one plant per pot) each received 10 inoculations (i.e., a total of 60 to 70 inoculations per treatment). Grapevines inoculated with sterile distilled water were used as the negative control. The seedlings were grown in a greenhouse at 20° to 35 °C for 3 months with natural sunlight (photoperiod; 14 h light: 10 h dark). The experiments were performed three times independently, and one experiment was defined as one replication (i. e., three independent replications). The protection rate provided by co-inoculation was defined as follows:

Protection rate (%) = $100 \times [1 - (\% \text{ of tumor formation for the mixed suspension} \times 100) / (\% \text{ of tumor formation with only VAT03-9})]$

Detection of *virD2* and *virE2* mRNA using the reverse-transcription quantitative polymerase chain reaction

Grapevine seedlings (2 years old, ‘Neo Muscat’) were inoculated with ARK-1, VAR06-30, and VAT03-9 at a cell ratio of 1:1, and CF of ARK-1 plus VAT03-9, as described above. Each grapevine seedling (one plant per pot) was inoculated once with one of the mixed cell suspensions or with only VAT03-9. The seedlings were grown in a greenhouse at 20 to 35 °C with natural sunlight as described previously. Shoot samples that included the one wound site (0.2 g fresh weight per plant, 1 sample per plant) were collected from five plants (i.e., $n=5$) at 1, 6, and 12 h after inoculation (hai), and at 1, 2, 3 and 4 dai (i.e., assessed seven times). Table 2 presents the basic information for the reverse-transcription

quantitative polymerase chain reaction (RT-qPCR) experiments, which followed the method of Bustin et al. (2009). Table 3 lists the primers used in this analysis. The A and B sets were used to detect the *virD2* and *virE2* genes of the *A. vitis* (Ti) strain, respectively (Table 3). The C set was used to amplify the *pyrG* gene of *A. vitis* (Ti) strain VAT03-9 for normalization; *pyrG* is a bacterial housekeeping gene (Table 3). The *pyrG* gene was used as an internal control after evaluation of the stability of four candidate reference genes (Table 2 and 3, Supplementary Figure S1; Vandesompele et al. 2002). Relative expression rate of *virD2* and *virE2* were described as a value based on the degree of expression of each gene at 1 dai as 100 %. These three sets (A to C) were confirmed to amplify genes only from the Ti strain VAT03-9 (Supplementary Figure S2). The amplified PCR products were analyzed using sequencing assay to confirm the specificity of the amplification (data not shown). PCR efficiency was calculated from the standard curves. Amplifications had a PCR efficiency and R^2 value of >88.9 % and 0.980 (*virD2*), respectively, and of >90.2 % and 0.999 (*virE2*). Relative quantification of the *virD2* and *virE2* mRNA concentrations was performed using the $\Delta\Delta C_t$ method (Pfaffl et al. 2002) using the *pyrG* gene’s mRNA values as a reference. Five biological replicates and three technical replicates were used for each assay. The detection assay was independently performed three times.

Detection of *virD2* and *virE2* mRNA using an in vitro RT-qPCR assay with acetosyringone

Acetosyringone (3', 5'-dimethoxy-4'-hydroxyacetophenone) (Sigma-Aldrich) was dissolved

Table 2 Experimental condition used in reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) based on MIQE requirements

Experimental design	
Control groups	Grapevine seedlings inoculated with only tumorigenic strain VAT03-9. For the negative control, inoculation with sterile distilled water was used.
Treatment groups	Grapevine seedlings inoculated with nonpathogenic strains (ARK-1 or VAR06-30) and the tumorigenic strain VAT03-9 at a cell ratio of 1:1
Sample	
Type of sample	Shoot tissue
Processing procedure	Homogenization in nitrogen liquid
Sample storage conditions	−80 °C
RNA extraction	
Reagents	NucleoSpin RNA (Macherey-Nagel, Düren, Germany)
Details of Dnase treatment	ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan)
Nucleic acid quantification	Absorbance at 260 nm
Instrument	GeneQuant Pro (GE Healthcare Japan, Tokyo, Japan)
Purity(A260/ A280)	>1.8
RNA integrity	Analyzed by agarose gel electrophoresis
Reverse transcription	
Complete reaction conditions	The reaction was performed as recommended by TOYOBO (Osaka, Japan).
Amount of RNA and reaction volume	0.1 µg of RNA, 10 µl
Priming oligonucleotide	Random primer
Reverse transcriptase	ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO)
Temp and time	15 min at 37°C followed by 5 min at 50°C
qPCR protocol	
Complete reaction conditions	95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 1 s for amplification of <i>virD2</i> and <i>virE2</i> (according to Roche Diagnostics's procedure) 95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s, 60 °C for 30 s for amplification of <i>pyrG</i> 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 53 °C for 10 s, and 72 °C for 20 s for amplification of <i>recA</i> , <i>rpoD</i> and 16S rDNA
Reaction volume and amount of cDNA	20 µl reaction, 1–10 ng cDNA
Primers, probes, and Mg and dNTP concentrations	Primers: 20 nM primers, Probes: 4nM probes Mg and dNTPs: Mixed in LightCycler® TaqMan® Master (Roche Diagnostics, Mannheim, Germany) and in LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics)
Reaction mixtures	12 µL of nuclease-free water, 4 µL of LightCycler TaqMan Master, 1 µL of forward primer (20 pmol µL ⁻¹), 1 µL of reverse primer (20 pmol µL ⁻¹), 1 µL of probe (4 pmol µL ⁻¹), and 1 µL of cDNA mixture
Polymerases	Mixed in LightCycler® TaqMan® Master (Roche Diagnostics) for amplification of <i>virD2</i> , <i>virE2</i> and <i>pyrG</i> Mixed in LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics) for amplification of <i>recA</i> , <i>rpoD</i> and 16S rDNA
Buffers	Mixed in LightCycler® TaqMan® Master (Roche Diagnostics) for amplification of <i>virD2</i> , <i>virE2</i> and <i>pyrG</i> Mixed in LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics) for amplification of <i>recA</i> , <i>rpoD</i> and 16S rDNA
qPCR instrument	LightCycler® 1.5 real-time PCR instrument (Roche Diagnostics)
qPCR validation	
Specificity	Analyzed using agarose gel electrophoresis
Method of PCR efficiency calculation	Mean PCR efficiency per amplicon calculated by Lightcycler® Software ver. 4.1 (Roche Diagnostics)
Data analysis	
qPCR analysis program	Lightcycler® Software ver. 4.1 (Roche Diagnostics)
Method of Ct determination	Lightcycler® Software ver. 4.1 (Roche Diagnostics)
Outlier identification	Lightcycler® Software ver. 4.1 (Roche Diagnostics)

Table 2 (continued)

Justification of number and choice of reference genes	Fore reference genes (<i>recA</i> , 16S rDNA, <i>rpoD</i> and <i>pyrG</i>) were tested for stability. <i>pyrG</i> was selected as the reference gene.
Description of normalization methods	$\Delta\Delta C_t$ method Pfaffl et al. (2002)
Number of technical replicates	3
Statistical methods	<i>t</i> -test and Tukey's HSD
Software	R software version 2.14.0 (http://www.r-project.org/)
Repeatability (intra-assay variation) Ct SD	0.22

in 70 % ethanol to produce a 10 mg ml⁻¹ stock solution. After filter sterilization, the stock solution was stored at -20 °C. Four cell suspensions at approximately 10⁸ cells ml⁻¹ (ARK-1 plus VAT03-9, VAR06-30 plus VAT03-9, and CF of ARK-1 plus VAT03-9 at cell ratios of 1:1, and only VAT03-9) were prepared as described above. Mixed cell suspensions (1 ml) or 0.5 ml of only VAT03-9 were added into potato sucrose (PS) medium or PS-AS medium (PS medium with 200 µg/ml acetosyringone) in an Erlenmeyer flask. Each Erlenmeyer flask with 50-mL PS or PS-AS medium (*n*=3, flasks per medium) was inoculated with one of the mixed suspensions or with a VAT03-9 cell suspension. Each flask represented one independent technical replicate. The flasks were incubated at 27 °C with shaking (approximately 2.2×*g*) for 2 days. Culture solution samples (one 1-ml sample per flask) were collected from each flask at 1 and 2 dai, bacterial RNA was extracted as described above. RT-qPCR for detection of the *virD2* and *virE2* genes and relative quantification of the *virD2* mRNA concentrations were performed as

described above. The detection assay was independently performed three times.

Population dynamics during coexistence of a nonpathogenic strain with VAT03-9n in grapevine seedlings

Grapevine seedlings (2 years old, 'Neo Muscat') were inoculated with one of three cell suspensions (ARK-1sc plus VAT03-9n and VAR06-30sc plus VAT03-9n at a 1:1 cell ratio, and VAT03-9n alone), as described above. Supplementary Figure S3 illustrates the design of this experiment. Each grapevine seedling (*n*=40 for each inoculation mixture, at one plant per pot) was inoculated once with the mixed cell suspension of ARK-1sc or VAR06-30sc plus VAT03-9n. Each grapevine seedling represented one replicate. The seedlings were grown in a greenhouse at 20 to 35 °C with natural sunlight as described previously. To determine the population dynamics of each strain, shoot samples were collected that included the wound site (0.2 g fresh weight per plant, 1

Table 3 Oligonucleotide primers and probes used in the RT-qPCR analyses

Name	Primer or probe	Primer and probe set	5'-3' nucleotide sequence	Target DNA	Reference	Accession No.	Design or supplier
Design or supplier	Primer	A	AAA TAT CGA GAT GCC GTG CT	<i>virD2</i>	This study	CP000637	ProbeFinder (Roche Diagnostics) ^a
virD2-R1P	Primer	A	GGA ATA TCT GTC CCG GAA GG	<i>virD2</i>	This study	CP000637	ProbeFinder (Roche Diagnostics) ^a
virD2-P	Probe	A	CTT GGA GC	<i>virD2</i>	This study	CP000637	Universal ProbeLibrary probe #125 cat. no. 04693604001 (Roche Diagnostics)
virE2-F1P	Primer	B	GAG TCA GTT GTT GCC GCT TT	<i>virE2</i>	This study	CP000637	ProbeFinder (Roche Diagnostics) ^a
virE2-R1P	Primer	B	ATG GCC CGA ACT TAC GTG	<i>virE2</i>	This study	CP000637	ProbeFinder (Roche Diagnostics) ^a
virE2-P	Probe	B	CTG TCC CA	<i>virE2</i>	This study	CP000637	Universal ProbeLibrary probe #123 cat. no. 04693574001 (Roche Diagnostics)
VAT03-9pyrG-F-2	Primer	C	GGT GAA GTC TTC GTC ACC GAC	<i>pyrG</i>	This study	AB272142	Licensed Dual-Labeled Probes for qPCR (Sigma-Aldrich)
VAT03-9pyrG-R-2	Primer	C	CGC CCT GTG AAG CGT TCA TAG	<i>pyrG</i>	This study	AB272142	Licensed Dual-Labeled Probes for qPCR (Sigma-Aldrich)
VAT03-9pyrG-P-2	Probe	C	CGG CGC AGA GAC CGA CCT TGA TCT TG	<i>pyrG</i>	This study	AB272142	Licensed Dual-Labeled Probes for qPCR (Sigma-Aldrich)

^a <http://roche-biochem.jp/UPL>

sample per plant) from 5 plants (i.e., $n=5$) at 1 hai (0 dai) and at 1, 2, 3, 5, 7, and 9 dai (i.e., assessed at eight times). Each shoot sample was scrubbed by hand, rinsed under tap water for 10 s, and then blotted dry with paper towels. The inoculated stems segments were washed with sterile distilled water, and then crushed in 1 mL of sterile distilled water using an autoclaved mortar and pestle. Ten-fold serial dilutions (100 μ l) of the samples were then plated and spread on St-CuSO₄-PSA and nal-PSA media, and the plates were incubated at 25 °C for 5 days. Colony growth was then observed on five plates for each dilution, and the numbers of colony-forming units (CFU) of strains ARK-1sc, VAR06-30sc, and VAT03-9n were counted on each medium. The bacterial populations in the wounded sites (CFU g⁻¹ of grapevine shoot) were log₁₀-transformed before statistical analysis. Additional experiments were carried out that each grapevine seedling was inoculated once with the each cell suspension of ARK-1sc, VAR06-30sc or VAT03-9n to confirm the population dynamics of each single strain. This assay was independently performed twice.

Data analysis

Tukey's honestly significant difference (HSD) test (after analysis of variance, ANOVA) or the *t*-test was used to compare the relative gene expression in the various treatments. Ryan's multiple-comparison test was used to compare the frequency of tumor formation among the treatments. All statistical analyses were

performed using version 2.14.0 of the R software (<http://www.r-project.org/>).

Results

Tumor inhibition assay in grapevine seedlings

To evaluate the inhibition of tumor formation by the nonpathogenic *A. vitis* strains ARK-1 and VAR06-30, grapevine seedlings were co-inoculated with a mixed cell suspension of one of these strains plus the pathogenic Ti strain VAT03-9. The combination of ARK-1 with VAT03-9 significantly suppressed tumor incidence ($P<0.01$) compared with VAT03-9 alone as the control, for a protection rate of 91.5 % (Table 4). In contrast, VAR06-30 and CF of ARK-1 did not significantly inhibit tumor formation compared with the control, with a protection rate of only 12.1 and 7.8 %, respectively (Table 4).

Suppressive effect on expression of the vir genes of the Ti strain by co-inoculation with ARK-1 in grapevine plants

To clarify whether the expression of *vir* genes of Ti strain VAT03-9 is suppressed by ARK-1 in vivo, the expression of *virD2* and *virE2* was analyzed in grapevine plants co-inoculated with ARK-1 and VAT03-9. In plants inoculated with only VAT03-9, induction of

Table 4 Effect of co-inoculation with the nonpathogenic *Agrobacterium* strains and the tumorigenic strains at a 1:1 cell ratio in grapevine seedlings

Strain	Experiment 1			Experiment 2			Experiment 3			Mean	
	No. of plants	No. of inoculations ^a	Proportion of tumor formation (%)	No. of plants	No. of inoculations ^a	Proportion of tumor formation (%)	No. of plants	No. of inoculations ^a	Proportion of tumor formation (%)	Proportion of tumor formation (%)	Protection rate (%)
ARK-1 plus the Ti strain	7	70	0.0	6	60	11.6	7	70	10.0	7.2 a	91.5
VAR06-30 plus the Ti strain	6	60	75.0	6	60	76.6	7	70	71.4	74.3 b	12.1
CF of ARK-1 plus the Ti strain	6	60	83.3	6	60	73.3	7	70	77.1	77.9 b	7.8
Only Ti strain ^b	7	70	71.4	6	60	95.0	7	70	87.1	84.5 b	

^a Ten inoculations per plant

^b Only the tumorigenic strain VAT03-9 was used as inoculum

^c Means followed by different letter differ significantly (Ryan's multiple comparison test, $P<0.01$)

bacterial *virD2* and *virE2* expression was detected at 1 dai (Fig. 1). No significant difference in the expression levels of *virD2* and *virE2* was observed upon co-inoculation with VAR06-30 or CF of ARK-1 and VAT03-9 in comparison with inoculation with only VAT03-9 (Fig. 1). On the other hand, *virD2* and *virE2* expression was significantly suppressed upon co-inoculation with ARK-1 and VAT03-9 (Fig. 1). These results suggested that treatment with ARK-1, which inhibited tumor formation, could suppress the expression of *virD2* and *virE2* by the Ti strain in grapevine plants, and that VAR06-30 and CF of ARK-1, which could not inhibit tumor formation, could not suppress expression of these genes.

To evaluate the start and end of the suppression of *vir* gene expression by ARK-1, *virD2* expression was analyzed from 1 h after inoculation (hai) to 4 dai in grapevine plants co-inoculated with ARK-1 and VAT03-9. The level of *virD2* expression upon inoculation with VAT03-9 was significantly higher at 1 dai than at any other time (Fig. 2). The levels of *virD2* expression were significantly lower upon co-inoculation with ARK-1 and VAT03-9 than upon inoculation with only VAT03-9 at 12 hai, 1 dai, and 2 dai (Fig. 2). These results

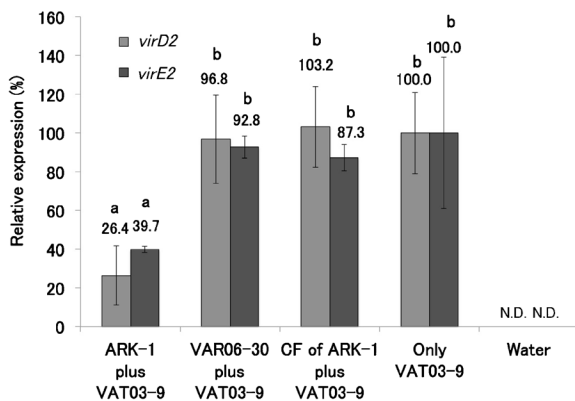


Fig. 1 Relative expression levels of the *virD2* and *virE2* genes in bacterial cells of the *Agrobacterium vitis* Ti strain in grapevine plants measured using the reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Values (%) represent the relative expression levels of the *vir* genes at 1 day after inoculation (dai) compared with the value when only Ti strain VAT03-9 was inoculated (“only VAT03-9”), which had a value of 100 %. Each cell suspension or mixture (ARK-1 plus VAT03-9, VAR06-30 plus VAT03-9, CF of ARK-1 plus VAT03-9, and only VAT03-9) at cell ratios of 1:1 was inoculated onto the stems of grapevine plants after wounding. Stem samples were harvested at 1 dai. Data are means±standard deviation for samples corresponding to individual plants. Bars labeled with different letters indicate a significant difference from the other bars ($P<0.01$, Tukey’s HSD test). CF, culture filtrate

suggest that treatment with ARK-1 could suppress *virD2* expression by the Ti strain in grapevine plants during the period from 12 hai to 2 dai.

Suppressive effect on *vir* gene expression by the Ti strain after co-incubation with ARK-1 in culture media with or without acetosyringone

To examine whether expression of the *vir* genes was suppressed by ARK-1 in culture media, with or without acetosyringone, the kinetics of *virD2* and *virE2* expression were analyzed. The levels of *virD2* and *virE2* expression by VAT03-9 incubated in PS-AS medium (which contained acetosyringone) were significantly higher than those in cells incubated in PS medium without acetosyringone (Fig. 3). In PS medium, *virD2* and *virE2* expression levels in VAT03-9 cells were not significantly affected by co-incubation with ARK-1, VAR06-30 or CF of ARK-1 (Fig. 3). In the PS-AS medium, *virD2* and *virE2* expression levels in VAT03-9 cells were not significantly affected by co-incubation with VAR06-30 or CF of ARK-1, but were significantly reduced by co-incubation with ARK-1 (Fig. 3). These results suggest that the presence of ARK-1 can suppress *virD2* and *virE2* expression by the Ti strain in culture medium that contains acetosyringone.

Population dynamics of the nonpathogenic strains and of VAT03-9n after co-inoculation onto grapevine seedlings

Antibiotic-resistant mutants of three strains (ARK-1sc, VAR06-30sc, and VAT03-9n) were used in a survival assay to differentiate the inoculated biological control agents from indigenous agrobacteria (Table 1). The three mutants grew in the St-CuSO₄-PSA medium (ARK-1sc and VAR06-30sc) and the nal-PSA medium (VAT03-9n) at rates comparable to the wild-type in PSA medium (data not shown). Populations of ARK-1sc on the plants were significantly higher than those of VAT03-9n at 7 and 9 dai (Fig. 4a). On the other hand, populations of VAR06-30sc and VAT03-9n did not differ significantly up to 9 dai (Fig. 4b), and each single population of ARK-1sc, VAR06-30sc and VAT03-9n did not differ significantly up to 9 dai (Fig. 4c). These results suggest that ARK-1sc could not reduce the pathogen population at the wound site on the grapevine plants during the early period after infection.

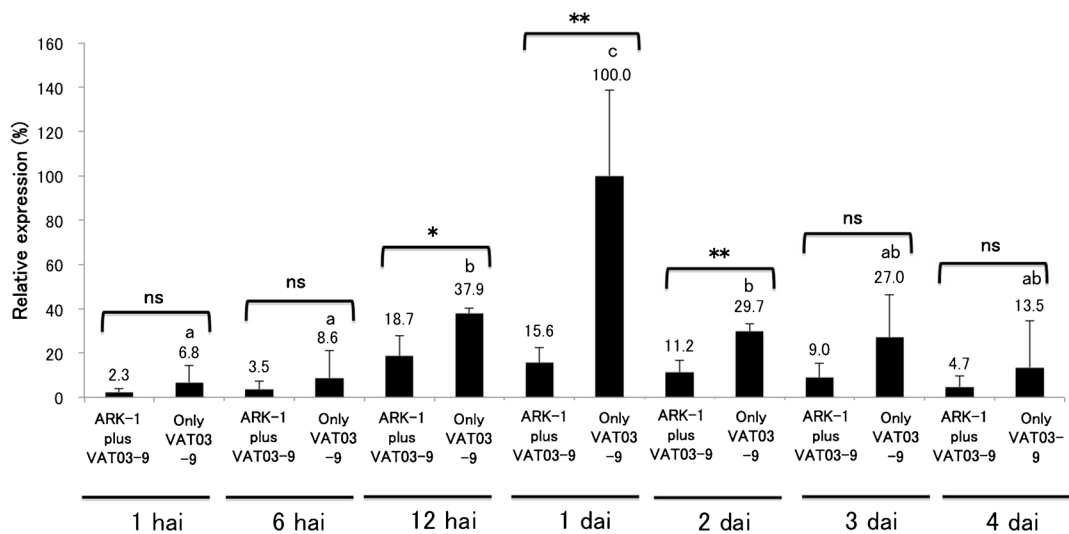
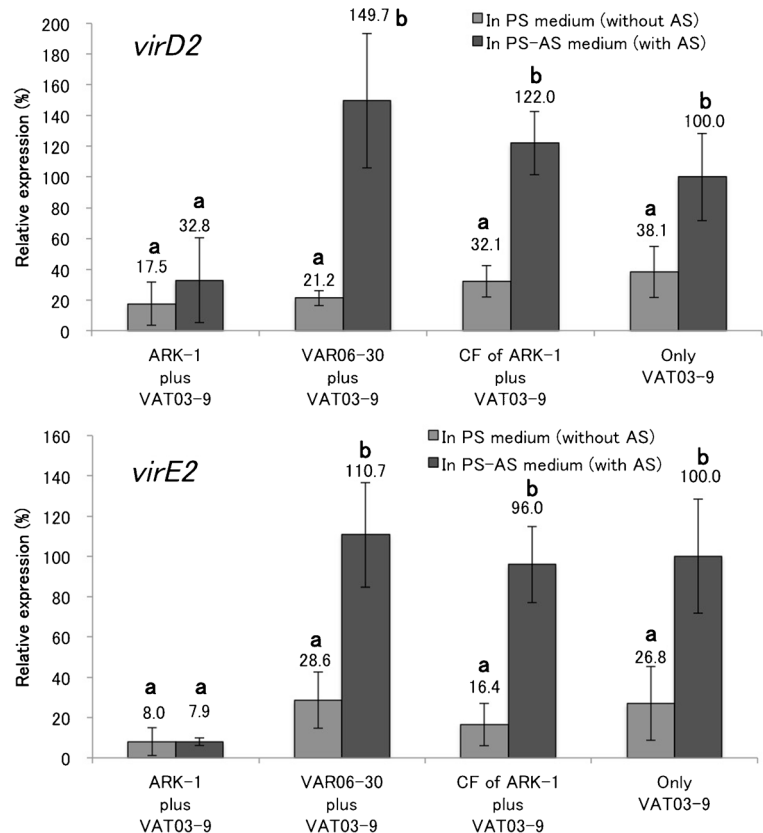


Fig. 2 Changes in the relative expression levels of the *virD2* gene in grapevine plants measured using the RT-qPCR. Values (%) represent the relative expression levels of the *vir* genes at 1 day after inoculation (dai) compared with the value when only Ti strain VAT03-9 was inoculated (“only VAT03-9”), which had a value of 100 %. A mixed cell suspension of the nonpathogenic strain ARK-1 and of the Ti strain VAT03-9 at cell ratios of 1:1 or a single cell suspension of VAT03-9 were inoculated onto the stems of wounded

grapevine plants. Stem samples were harvested at times ranging from 1 h after inoculation (hai) to 4 dai. Data are means±standard deviation for samples corresponding to individual plants. Significant differences at a given point in time between the ARK-1 plus Ti mixture and only the Ti strain are indicated by * (*t*-test, * $P < 0.05$; ** $P < 0.01$; ^{ns} $P \geq 0.05$); bars for only the Ti strain that are labeled with different letters differ significantly from other bars for only the Ti strain (Tukey’s HSD test, $P < 0.05$)

Fig. 3 Relative expression levels of the *virD2* and *virE2* genes in bacterial cells of the tumorigenic (Ti) *A. vitis* strain on potato sucrose (PS) medium and PS-AS medium, which also includes acetosyringone (AS), measured using RT-qPCR. Values (%) represent the relative expression levels of the *vir* genes at 1 day after inoculation (dai) compared with the value when only Ti strain VAT03-9 was inoculated (“only VAT03-9”), which had a value of 100 %. Data are means±standard deviation for samples corresponding to individual incubation flasks. Bars at a given date labeled with different letters differ significantly (Tukey’s HSD test, $P < 0.05$)



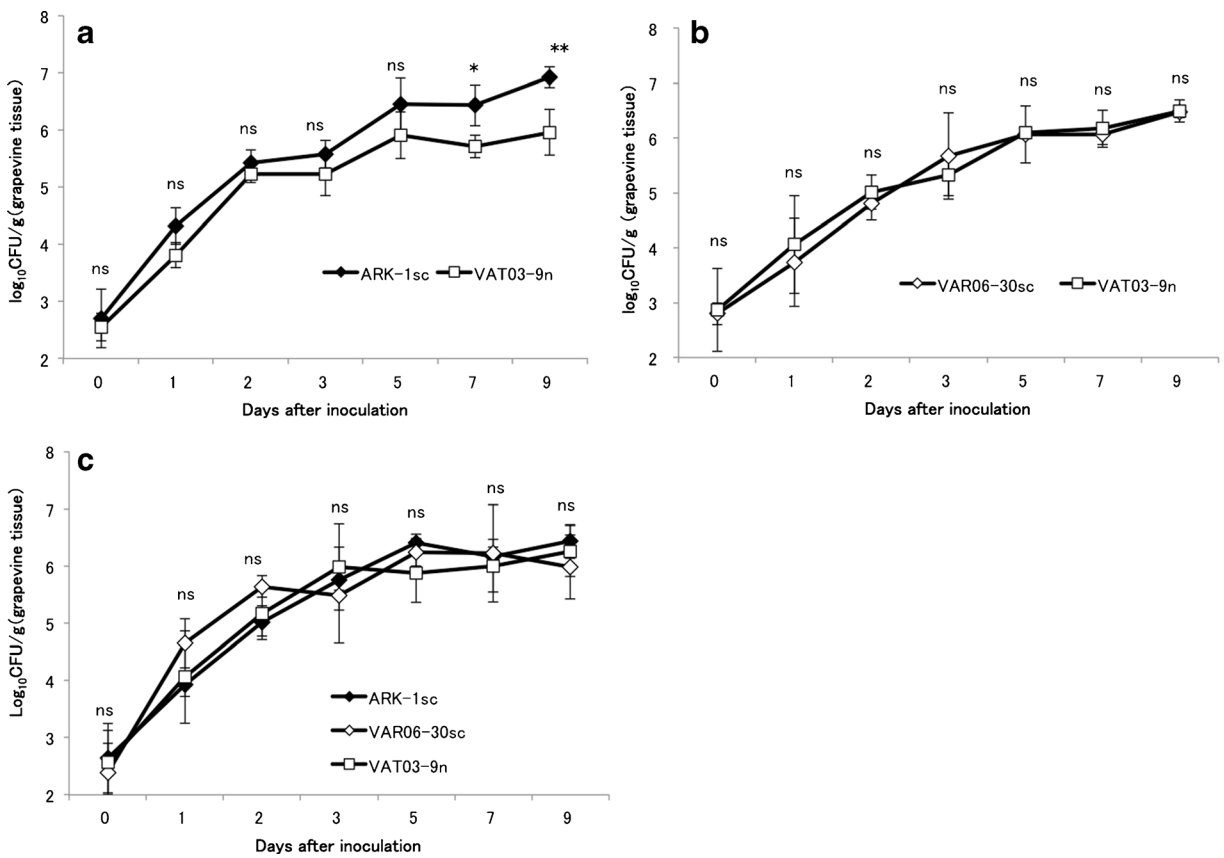


Fig. 4 Population dynamics of the nonpathogenic strains and VAT03-9n after co-inoculation onto grapevine plants. **a** Populations of the nonpathogenic strain ARK-1sc and of the tumorigenic (Ti) strain VAT03-9n after co-inoculation on wounded shoots of grapevine seedlings at a 1:1 cell ratio. **b** Populations of the nonpathogenic strain VAR06-30sc and of the Ti strain VAT03-9n after co-inoculation on wounded shoots of grapevine seedlings at a 1:1 cell ratio. **c** Populations of the nonpathogenic strain ARK-1sc, of strain

VAR06-30sc, and of the tumorigenic (Ti) strain VAT03-9n after each inoculation on wounded shoots of grapevine seedlings. Data are means±standard deviation for five plants. **(a)** and **(b)** Significant differences at a given point in time are indicated by * (*t*-test, **P*<0.05; ***P*<0.01; ^{ns} *P*≥0.05). **(c)** No significant differences at a given point in time are indicated by ns (Tukey’s HSD test, ^{ns} *P*≥0.05) dai, days after inoculation

Discussion

Two different mechanisms of biological control of plant crown gall disease using antagonistic bacteria have been reported. The first one relates to antibacterial compounds produced by nonpathogenic strains (Chen et al. 2007; Kawaguchi et al. 2008; Kerr 1980; Wang et al. 2003). The second relates to a unique mechanism associated with the quorum-sensing and caseinolytic protease (clp) systems of strain F2/5 (Kaewnum et al. 2013). In the present study, suppression of expression of two *vir* genes of the Ti strain after treatment with the nonpathogenic *A. vitis* strain ARK-1 was shown using RT-qPCR both in vitro and in vivo, and these results suggest a different mechanism from those in previous reports. In

the co-inoculation assay, ARK-1 suppressed *virD2* and *virE2* expression by the Ti strain in grapevine plants at 1 dai, even though the population of the ARK-1sc and Ti strains on the plants did not differ significantly at this time. These results suggest that the suppression of *virD2* and *virE2* expression by the Ti strain was not caused by suppression of growth of the Ti strain by ARK-1. ARK-1 significantly inhibited tumor formation in grapevine plants in this and previous reports (Kawaguchi 2013; Kawaguchi and Inoue 2012), whereas strain VAR06-30, which did not significantly inhibit tumor formation, did not suppress *virD2* and *virE2* expression or growth of the Ti strain in the plants at 1 dai. These results suggest that the suppression of *virD2* and *virE2* expression by ARK-1 inhibits tumor formation because the VirD2 and

VirE2 proteins are directly associated with transformation of plant cells by Ti strains of *Agrobacterium* (Gelvin 2012; Lacroix and Citovsky 2013; Nester 2015).

Low levels of *virD2* expression by the Ti strain were found at all dates except 1 dai. The peak level of *virD2* gene expression by the Ti strain in grapevine plants was observed at 1 dai. On the other hand, the levels of *virD2* expression in plants that were co-inoculated with ARK-1 and the Ti strain remained low for 4 dai and were significantly lower than those in plants inoculated with only the Ti strain from 12 hai to 2 dai, indicating that the suppression of *virD2* expression by ARK-1 persists for at least 2 dai but becomes unclear at 3 and 4 dai because *virD2* expression in plants inoculated with only the Ti strain was also low during this period.

The expression of *vir* genes by the *Agrobacterium* Ti strain is strongly activated by the plant molecule acetosyringone, which the bacteria recognize as one of several wound-derived compounds (Nester 2015). In the in vitro study, the levels of *virD2* and *virE2* expression in Ti cells incubated in PS-AS medium were significantly higher than those in PS medium, which agrees with previous reports (Nester 2015). Moreover, *virD2* and *virE2* expression levels in the Ti strain co-incubated with ARK-1 were significantly lower than those with only the Ti strain in PS-AS medium, suggesting that similar results were obtained in vitro and in vivo. This evidence supports the hypothesis that ARK-1 suppresses the expression of *vir* genes in the Ti strain.

Previous research suggested that ARK-1sc suppressed growth of the Ti strain in grapevine plants at 2 dai, but not at 1 and 5 dai (Kawaguchi 2014). In the present study, populations of ARK-1sc on the plants did not differ significantly from those of Ti strain VAT03-9n before 7 dai. Taken together, the present results suggest that ARK-1 cannot strongly suppress growth of the Ti strain in grapevine plants before 7 dai. Kawaguchi (2014) reported that ARK-1 suppressed growth of the Ti strain in grapevines from 9 to 88 dai. In the present study, the ARK-1sc population was significantly higher than that of the Ti strain at 7, 9, and 11 dai. These results suggest that the suppression of growth of the Ti strain by ARK-1 that occurs at 7 or 9 dai may result from suppression of *vir* gene expression by the Ti strain. This suggests a hypothesis that may explain the relationship between suppression of *vir* gene expression and growth of the Ti strain: suppression of *vir* gene expression causes failure of the transformation of the host plant's

cells, thereby preventing the Ti strain from taking up and catabolizing the opines that transformed plant cells should produce before 7 dai, leading to suppression of growth of the Ti strain and a lower population than the ARK-1 population in grapevine plants starting by 7 d. This hypothesis should be tested in future research.

In conclusion, ARK-1 suppressed expression of the virulence genes of the Ti strain at the wound site and suppressed development of the crown gall disease via what appears to be a previously unreported mechanism. Although it remains unclear how ARK-1 suppressed expression of the *vir* genes by the Ti strain in plants, the CF of ARK-1, which does not inhibit tumor formation, did not suppress *virD2* and *virE2* expression both in vitro and in vivo, suggesting that ARK-1 might catabolize acetosyringone or produce a very small amount of unknown substances that suppress expression of the *vir* genes without killing the Ti strain. Moreover, there still remains a possibility of quorum sensing system might have some relation with expression and suppression of *vir* genes and ARK-1 might affect the quorum sensing system. Additional research will be required to determine whether either of these mechanisms is correct. We are now pursuing these aspects of the bacteria and will report our results in a forthcoming paper.

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