

## Grapevine crown gall caused by *Rhizobium radiobacter* (Ti) in Japan

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Received: 22 November 2008 / Accepted: 17 February 2009 / Published online: 27 March 2009  
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**Abstract** In 2005, characteristic symptoms of crown gall on grapevines (*Vitis vinifera* L. cv. Muscat of Alexandria, and cv. Seto Giants) were observed in a commercial greenhouse-orchard in Okayama Prefecture, Japan. Isolations from diseased tissues consistently yielded bacterial colonies that were white, glistening, and produced abundant polysaccharide on potato semi-synthetic agar (PSA) medium. Ten representative isolates were chosen for further characterization. A multiplex polymerase chain reaction (PCR) assay showed these strains were not *Rhizobium vitis* but did possess a Ti plasmid. The bacteriological characteristics of the isolates corresponded well with *R. radiobacter*. The almost complete 16S ribosomal DNA sequences of isolates AT06-1 and AT06-2, selected from 10 grapevine isolates, were determined and corresponded to sequences of *R. radiobacter*. The pathogenicity of the isolates was tested on young grapevine and tomato (*Lycopersicon esculentum* Mill.) plants. Gall symptoms developed on both plant species after inoculation, and bacteria with the same colony morphology as those inoculated were reisolated. Based on these results, the isolates were identified as *R. radiobacter* (Ti). This report is the first of the occurrence of *R. radiobacter* (Ti) on grapevine in Japan. Phylogenetic analyses using the partial nucleotide sequences of *virC* operon located on a Ti plasmid showed

that the isolate of *R. radiobacter* (Ti) isolated from grapevine and some strains of *R. vitis* (Ti) belonged to the same monophyletic group, which differed from the groups of *R. radiobacter* (Ti) isolated from plants other than grapevine and of the majority of *R. vitis* (Ti) strains isolated from grapevine.

**Keywords** *Rhizobium radiobacter* (Ti) · *Rhizobium vitis* (Ti) · Crown gall · Grapevine · pTi · *virC*

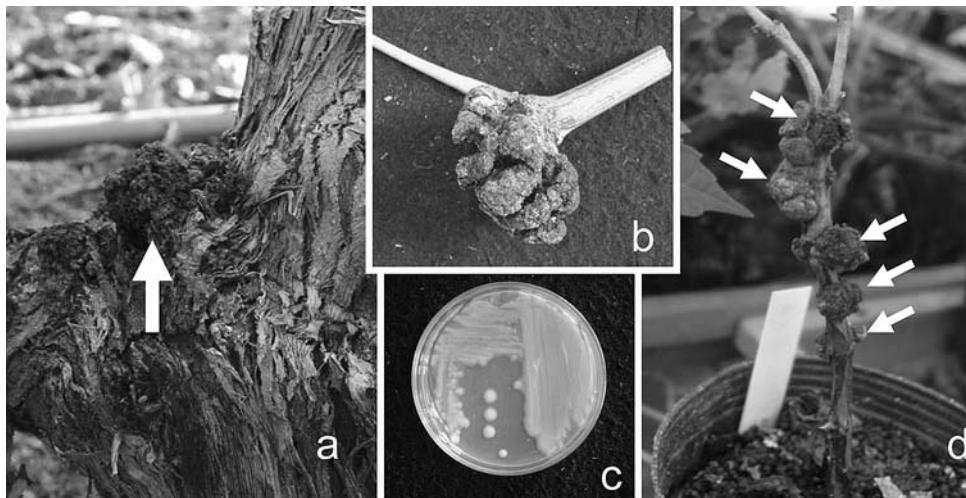
### Introduction

Grapevine crown gall, which is caused mainly by *Rhizobium vitis* (Ti) Young et al. 2001 [= *Agrobacterium vitis* Ophel and Kerr 1990, *Agrobacterium tumefaciens* biovar 3 (Smith and Townsend 1907) Conn 1942], is the most important bacterial disease of grapevine throughout the world (Burr et al. 1998; Burr and Otten 1999; Sawada et al. 1990). The pathogenicity genes are mostly located on large tumor-inducing plasmids (pTi). During infection, a part of this plasmid (T-DNA) is transferred and inserted into the nuclear DNA of the plant (Chilton et al. 1977; Schilperoot 1972).

*Rhizobium radiobacter* (Ti) Young et al. 2001 [= *Agrobacterium tumefaciens* biovar 1 (Smith and Townsend 1907) Conn 1942, *A. tumefaciens* (Ti) (Smith and Townsend 1907) Conn 1942] was isolated from grapevines in the United States that had developed tumors or were symptomless (Burr and Katz 1983, 1984; Thies et al. 1991). *Rhizobium rhizogenes* (Ti) Young et al. 2001 [= *A. tumefaciens* biovar 2, *A. rhizogenes* (Ti) (Riker et al. 1930)] was isolated from tumors that developed on grapevines in Hungary (Süle 1978) and Spain (López et al. 2008). In Japan, crown gall of grapevine due to *R. vitis* (Ti) has

The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under accessions AB306890, AB306891, and AB465432–AB465459.

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**Fig. 1** Symptoms and colony morphology of *Rhizobium radiobacter* (Ti) isolated from grapevine in Okayama, Japan. **a** Natural tumors of crown gall (arrow) on grapevine cv. Muscat of Alexandria. **b** Natural tumors of crown gall on a lateral shoot of a bearing branch of grapevine cv. Seto Giants. **c** Colony morphology of *R. radiobacter*

(Ti) isolate ST05-2 incubated on PSA medium for 5 days at 27°C. **d** Tumor formation (arrows) on grapevine seedlings after artificial inoculation with *R. radiobacter* (Ti) isolate ST05-1. The photograph was taken approximately 3 months after inoculation

been reported (Sawada et al. 1990), but other tumorigenic *Rhizobium* strains isolated from grapevine have not been reported.

In October and December 2005, tumor tissues of grapevine (*Vitis vinifera* L.) were observed in a commercial greenhouse-orchard in Okayama Prefecture, Japan. Two tumors had developed on an above-ground part of grapevine cv. Muscat of Alexandria (6 years old) and on a lateral shoot of a bearing branch of grapevine cv. Seto Giants (9 years old), respectively (Fig. 1a, b). In this article, we report that isolates from grapevine crown gall were identified as *Rhizobium radiobacter* (Ti), and we carried out a phylogenetic analysis using a partial sequence of *virC* operon located on pTi.

We follow the nomenclature for *Rhizobium* species adopted in the report of Young et al. (2001) to avoid confusion, although other valid naming systems have been proposed (Bouzar et al. 1995; Kersters and De Ley 1984; Ophel and Kerr 1990; Sawada et al. 1993; Young et al. 2005).

## Materials and methods

### Isolation of pathogen

Each tumor (ca. 1 g) was scrubbed and rinsed under tap water and dried with paper towels. They were surface sterilized with 70% ethanol for 1 min, washed with sterile distilled water, and ground with an autoclaved mortar and pestle in 5 ml of sterile distilled water. The suspension (0.1 ml) was spread onto surface-dried agar plates of 3DG

medium, which is selective for *R. vitis* (Brisbane and Kerr 1983). Inoculated plates were incubated at 30°C for 5 days, and colonies that resembled *Rhizobium* strains were picked up and purified on potato semi-synthetic agar medium [PSA, 300 g potato, 0.5 g  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 2 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 5 g peptone, 20 g sucrose, 15 g agar, and 1 l distilled water, pH 6.8–7.0].

### Multiplex polymerase chain reaction

The multiplex polymerase chain reaction (PCR) was performed using a mixture of two primer sets Ab3-F3 (5'-ATGACGGTAGTCGGAGAAGAAGCC-3')/Ab3-R4 (5'-CTG TCTCTGTGTCCCCGAAAGG-3') and VCF3 (5'-GGCGGGCGYGCYGAAAGRAARACYT-3')/VCR3 (5'-AAGAACGYGGNATGTTGCATCTYAC-3') to identify tumorigenic and nonpathogenic strains of *R. vitis* according to the procedure of Kawaguchi et al. (2005a). DNA fragments (414 bp) from a partial sequence of the *virC1* and *virC2* genes encoded in the *virC* operon are expected to be amplified from the cell lysate of tumorigenic or rhizogenic *Rhizobium* strains by PCR with VCF3 and VCR3 primers (Kawaguchi et al. 2005a; Sawada and Tsuchiya 2003), and 570 bp fragments from 16S rDNA are expected to be amplified from *R. vitis* strains by PCR with Ab3-F3 and Ab3-R4 primers (Kawaguchi et al. 2005a).

### Bacteriological properties

*R. radiobacter* (Ti) strain AtC1 isolated from chrysanthemum, *R. rhizogenes* (Ti) strain Ch-Ag-2 isolated from cherry, *R. vitis* (Ti) strain G-Ag-27 isolated from grapevine,

**Table 1** Bacterial strains used in this paper

Bacteria and strains <sup>a</sup>	Source and location (supplier) <sup>b</sup>	Genetic group of <i>Rhizobium vitis</i> <sup>c</sup>
Present bacteria		
Pathogenic strain		
ST05-1, ST05-2	Grapevine (cv. Seto Giants); Okayama, Japan	–
AT06-1, AT06-2, AT06-3, AT06-4	Grapevine (cv. Muscat of Alexandria); Okayama, Japan	–
AT06-5, AT06-6, AT06-7, AT06-8	Grapevine (cv. Muscat of Alexandria); Okayama, Japan	–
Reference bacteria		
<i>Rhizobium radiobacter</i> (Ti) [= <i>Agrobacterium tumefaciens</i> (Ti), <i>A. tumefaciens</i> biovar 1]		
AtC1, CH3	Chrysanthemum; Shizuoka, Japan (Y. Takikawa)	–
<i>Rhizobium radiobacter</i> (nonpathogenic) [= <i>A. tumefaciens</i> (nonpathogenic), <i>A. radiobacter</i> biovar 1]		
Ar-4	Grapevine; Okayama, Japan	–
<i>R. rhizogenes</i> (Ti) [= <i>A. rhizogenes</i> (Ti), <i>A. tumefaciens</i> biovar 2]		
Ch-Ag-2	Cherry; Yamagata, Japan (H. Sawada)	–
<i>R. vitis</i> (Ti) [= <i>A. vitis</i> (Ti), <i>A. tumefaciens</i> biovar 3]		
G-Ag-27	Grapevine; Nagano, Japan (H. Sawada)	A
G-Ag-52	Grapevine; Iwate, Japan (H. Sawada)	A
G-Ag-62	Grapevine; Yamagata, Japan (H. Sawada)	A
At-90-23	Grapevine; Shimane, Japan (J. Yamamoto)	A
G-Ag-4	Grapevine; Shimane, Japan (H. Sawada)	A
MAFF 211676 (=VAT03-9)	Grapevine; Okayama, Japan (MAFF, isolated by A. Kawaguchi)	A
YGA132-3	Grapevine; Yamanashi, Japan (H. Sawada)	A
9-1-5, 9-3-1, 9-3-5	Grapevine; Nagano, Japan	B
G-Ag-19, G-Ag-21, G-Ag-23	Grapevine; Nagano, Japan (H. Sawada)	B
G-Ag-66	Grapevine; Akita, Japan (H. Sawada)	B
MAFF 211674 (=A5-1)	Grapevine; Akita, Japan (MAFF, isolated by A. Kawaguchi)	B
MAFF 211675 (=A5-2)	Grapevine; Akita, Japan (MAFF, isolated by A. Kawaguchi)	B
MAFF 211677 (=A5-4)	Grapevine; Akita, Japan (MAFF, isolated by A. Kawaguchi)	B
A5-8	Grapevine; Akita, Japan	B
At-5	Grapevine; Shimane, Japan (J. Yamamoto)	B
G-Ag-60	Grapevine; Aomori, Japan (H. Sawada)	C
VAT07-1	Grapevine; Okayama, Japan	D

<sup>a</sup> Taxa of the genus *Rhizobium* are named according to nomenclature of Young et al. (2001)

<sup>b</sup> Unless a supplier is stated, we isolated the strain. MAFF, Ministry of Agriculture, Forestry and Fisheries, Tsukuba Ibaraki, Japan

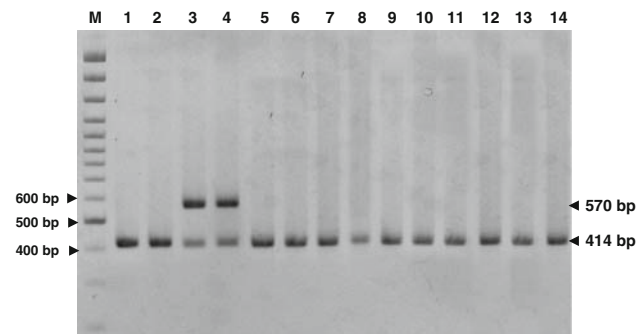
<sup>c</sup> Genetic group based on serological analyses, rep-PCR DNA fingerprinting and multilocus sequence analysis of housekeeping genes *pyrG*, *recA* and *rpoD* (Kawaguchi et al. 2008)

and *R. radiobacter* (nonpathogenic) strain Ar-4 isolated from grapevine were used as references (Kawaguchi et al. 2005b; Sawada et al. 1990) in the identification tests and compared with the nine isolates obtained from tumor tissues of grapevine. Biochemical and physiological tests were performed according to the methods of Kawaguchi et al. (2005b) and Goto and Takikawa (1984a, b) to characterize the nine isolates: growth at 30°C; arbutin hydrolysis; arginine dihydrolase; reaction on litmus milk; 3-ketolactose production; acid production from erythritol and ethanol; alkali from malonic acid, L-tartaric acid, and utilization of citrate; oxidase reaction; growth on 1A medium (Brisbane and Kerr 1983), and D-1 medium (Moore et al. 1988).

#### Analysis of 16S ribosomal DNA sequence

The DNA region coding for 16S rRNA of isolates AT06-1 and AT06-2 (Table 1) were amplified with primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') (the same sequence as positions 8–27 in the *Escherichia coli* numbering system) and 1510-1492r (5'-GGCTACCTTGTT-ACGACTT-3') (positions complementary to 1510-1492). These primers were designed based on the conserved bacterial sequences at the 5' and 3' ends of the 16S rDNA gene, which allowed amplification of almost the entire gene (Lane 1991). PCR amplification was performed in a total volume of 50 µl containing 1 µl of template (cell

lysate), 1 µl of 8-27f primer (20 pmol/µl), 1 µl of 1510-1492r primer (20 pmol/µl), 25 µl of AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA),



**Fig. 2** Amplification products obtained by multiplex PCR separated by agarose (3%) gel electrophoresis. Lanes M, 100 bp marker; 1, *Rhizobium radiobacter* (Ti) strain CH3; 2, *R. radiobacter* (Ti) strain AtC1; 3, *R. vitis* (Ti) strain MAFF 211676; 4, *R. vitis* (Ti) strain G-Ag-27; 5, ST05-1; 6, ST05-2; 7, AT06-1; 8 AT06-2; 9, AT06-3; 10, AT06-4; 11, AT06-5; 12, AT06-6; 13, AT06-7; 14, AT06-8. The expected positions of the 570 and 414 bp fragments are shown

and 22 µl of sterile distilled water. The amplification reaction was done in a TaKaRa PCR thermal cycler MP (Takara Bio, Otsu, Shiga, Japan) with initial denaturation at 95°C for 8 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and an additional extension at 72°C for 10 min. The amplified 16S rDNA was purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) according to the supplier's instructions. The purified DNA was sequenced with an ABI PRISM BigDye Terminator version 2 cycle sequencing kit (PE Biosystems, Mississauga, Ontario, Canada) and an ABI PRISM 3700 genetic analyzer using the following primers: 8-27f and 1510-1492r. Homology searches using the DDBJ (DNA Data Bank of Japan, Japan) were conducted using the FASTA program (<http://fasta.ddbj.nig.ac.jp>).

### Pathogenicity test

All isolates were tested for pathogenicity on young tomato (*Lycopersicon esculentum* Mill. cv. Ponderosa) and grapevine (*Vitis vinifera* L. cv. Neo Muscat) plants. All

**Table 2** Bacteriological characteristics and pathogenicity of grapevine isolates differing from those of the reference strains of genus *Rhizobium*

Test	Isolates <sup>a</sup>	<i>R. radiobacter</i> (Ti) strain AtC1 <sup>b</sup>	<i>R. radiobacter</i> (nonpathogenic) strain Ar-4 <sup>c</sup>	<i>R. rhizogenes</i> (Ti) strain Ch-Ag-2 <sup>d</sup>	<i>R. vitis</i> (Ti) strain G-Ag-27 <sup>e</sup>
<b>Bacteriological characteristics</b>					
Production of 3-ketolactose (2) <sup>c</sup>	+	+	+	–	–
Litmus milk reaction (20)	K, R	K, R	K, R	A	K, R
Oxidase reaction	+	+	+	–	+
Growth at 30°C (7)	+	+	+	–	+
Growth on 1A medium (7)	+	+	+	–	–
Growth on D-1 medium (10)	K, +	K, +	K, +	A, +	A, +
Acid from					
Erythritol (7)	–	–	–	+	–
Ethanol (7)	+	+	+	–	–
Alkali from					
L-Tartaric acid (7)	+	–	+	+	+
Malonic acid (7)	–	–	–	+	+
Utilization of citrate (7)	–	–	–	+	+
Arbutin hydrolysis (5)	+	+	+	+	–
Arginine dihydrolase (25)	+	+	+	–	+
<b>Pathogenicity</b>					
Grapevine (90)	+	+	–	+	+
Tomato (30)	+	+	–	+	+

<sup>a</sup> Ten isolates including (ST05-1, ST05-2, AT06-1, AT06-2, AT06-3, AT06-4, AT06-5, AT06-6, AT06-7, AT06-8)

<sup>b</sup> The strain was isolated from chrysanthemum

<sup>c</sup> The strain was isolated from grapevine

<sup>d</sup> The strain was isolated from cherry

<sup>e</sup> Duration of incubation is in parentheses

+ positive, – negative, K alkaline, A acid, R reduction of litmus

**Table 3** Pathogenicity of grapevine isolates differing from those of the reference strains of genus *Rhizobium*

Strain	Tumor size (mm) <sup>a</sup>	
	Grapevine <sup>b</sup>	Tomato <sup>c</sup>
Grapevine isolates		
ST05-1	7.1 ± 0.5 a	3.7 ± 0.9 a
AT06-1	7.5 ± 0.6 a	3.3 ± 0.8 a
<i>Rhizobium vitis</i> (Ti)		
VAT07-1	6.3 ± 0.5 a	2.2 ± 0.3 a
G-Ag-27	6.1 ± 0.6 a	6.5 ± 0.4 b
<i>R. radiobacter</i> (Ti)		
AtC1	1.1 ± 0.2 b	6.6 ± 0.5 b
<i>R. rhizogenes</i> (Ti)		
Ch-Ag-2	1.3 ± 0.2 b	6.8 ± 0.5 b
<i>R. radiobacter</i> (nonpathogenic)		
Ar-4	0.0 ± 0.0 c	0.0 ± 0.0 c

<sup>a</sup> Tumor diameter perpendicular to the long axis of the stem. Mean within a column followed by the different letter differ significantly ( $P < 0.01$ ) according to Tukey's honestly significant difference (HSD) test of square root transformed ( $X + 0.5$ ) data

<sup>b</sup> Mean ( $\pm$ SE) of 3 seedlings, each with 5 inoculations

<sup>c</sup> Mean ( $\pm$ SE) of 3 seedlings, each with 4 inoculations

plants were grown from seed. Respective cell suspensions of the 10 isolates were prepared from 48-h-old cultures on PSA medium and adjusted to  $10^8$  cells/ml. The stems of 1- to 2-months-old plants grown in an air-conditioned greenhouse between 25 and 28°C were inoculated by the needle prick method (Kawaguchi et al. 2005b; Sawada et al. 1990). Three tomato seedlings were each inoculated four times with an inoculum mixture (i.e., 12 inoculations per treatment). Grapevine seedlings in three pots received five inoculations for each pot (i.e., 15 inoculations per treatment). Water alone was used for negative controls. The experiments were repeated twice, and isolates that failed to induce tumors on all test plants were regarded as nonpathogenic.

#### Phylogenetic analysis based on sequence of *virC1–virC2* genes

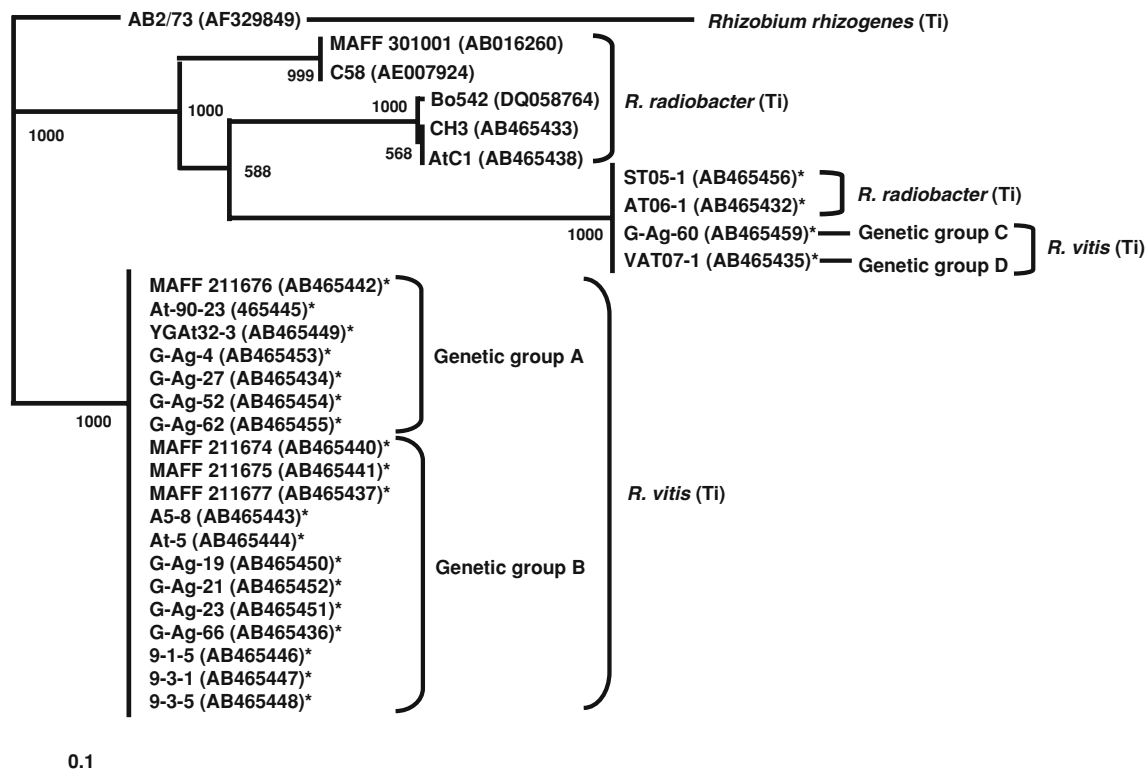
A partial sequence of the *virC1–virC2* genes encoded in the *virC* operon on pTi of tumorigenic *Rhizobium* strains was amplified with primers VCF3 and VCR3 (Sawada and Tsuchiya 2003). The PCR amplification was performed in a total volume of 50  $\mu$ l containing 1  $\mu$ l of template (cell lysate), 1  $\mu$ l of VCF3 primer (20 pmol/ $\mu$ l), 1  $\mu$ l of VCR3 primer (20 pmol/ $\mu$ l), 25  $\mu$ l of AmpliTaq Gold PCR Master Mix (Applied Biosystems), and 22  $\mu$ l of sterile distilled water. The amplification reaction was done in a TaKaRa PCR thermal cycler MP (Takara Bio) after initial

denaturation at 95°C for 8 min: followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 90 s, extension at 72°C for 90 s: and an additional extension at 72°C for 10 min. The amplified *virC1–virC2* gene was purified using the QIAquick PCR Purification kit (Qiagen) and the supplier's instructions. The purified DNA was sequenced with an ABI PRISM BigDye Terminator ver2 cycle sequencing kit (PE Biosystems) and an ABI PRISM 3700 genetic analyzer using a VCR3 primer. The data of each *virC1–virC2* sequence of *R. radiobacter* (Ti) strain C58 (AE007924), MAFF301001 (AB016260), and Bo542 (DQ058764) and *R. rhizogenes* (Ti) strain AB2/73 (AF329849) were downloaded from the DDBJ database (<http://getentry.ddbj.nig.ac.jp>). The DNA sequence (ca. 350 bp) excluding the sequences of VCF3 and VCR3 primers was manipulated using the Clustal W program of the DDBJ website (<http://clustalw.ddbj.nig.ac.jp>). A neighbor-joining (NJ) tree was constructed using distances estimated by Kimura's two parameters model, and the strength of the internal branches from the resulting tree was tested by bootstrap analysis using 1000 replications. The phylogenetic tree was drawn using Tree-view software downloaded from the DDBJ website (Page 1996).

## Results and discussion

Isolations from diseased tissues of grapevine consistently yielded bacterial colonies that were white, glistening, and covered with polysaccharide on PSA medium (Fig. 1c). Ten representative isolates (ST05-1 and ST05-2 from cv. Seto Giants and AT06-1 to AT06-8 from cv. Muscat of Alexandria) were chosen for further characterization (Table 1). The multiplex PCR assay showed that no isolate produced specific band of *R. vitis*, but that they possessed the *virC* operon (Fig. 2), indicating that not *R. vitis* (Ti) but some other pathogenic *Rhizobium* spp. is the causal agent.

As shown in Table 2, all isolates were positive for oxidase reaction, 3-ketolactose, growth at 30°C, arginine dihydrolase, and arbutin hydrolysis, produced acid from ethanol but not from erythritol and alkali from L-tartaric acid but not from malonic acid, did not utilize citrate, grew on D-1 medium, and alkalized and reduced litmus milk. All isolates grew on 1A medium, which is selective for *R. radiobacter* (= *Agrobacterium* biovar 1) (Brisbane and Kerr 1983). These bacteriological characteristics of the 10 isolates coincided perfectly with the reference strain *R. radiobacter* (nonpathogenic) Ar-4 previously isolated from grapevine (Kawaguchi et al. 2005b) (Table 2), and corresponded well with *R. radiobacter* (Ti) strain AtC1 isolated from chrysanthemum (Sawada et al. 1990) (Table 2).



**Fig. 3** Phylogenetic analysis of 21 strains of *Rhizobium vitis* (Ti), seven strains *R. radiobacter* (Ti), and one strain of *R. rhizogenes* (Ti) based on the neighbor-joining (NJ) method using partial sequence data for *virC1*–*virC2* genes (ca. 350 bp). Numbers above branches are confidence values obtained for 1000 bootstrap replicates. The bar represents a phylogenetic distance of 10%. Stars indicate strains

isolated from grapevine. Genetic groups of grapevine isolates were determined by Kawaguchi et al. (2008). The data for the *virC1*–*virC2* sequence of *R. radiobacter* (Ti) strain C58 (AE007924), MAFF301001 (AB016260), and Bo542 (DQ058764) and *R. rhizogenes* (Ti) strain AB2/73 (AF329849) were downloaded from the DDBJ database

In 16S rDNA sequencing analysis, almost the full length (ca. 1400 bp) of the 16S rDNA sequence was determined for isolates AT06-1 (accession AB306890) and AT06-2 (AB306891). Both sequences were identical to the 16S rDNA of a number of *R. radiobacter* strains and completely matched that of *R. radiobacter* strain CFBP2714 (AJ389891).

In pathogenicity tests using tomato and grapevine seedlings, crown gall symptoms developed on inoculated tomatoes within 1 month and grapevines within 3 months (Fig. 1d; Table 2). Isolates ST05-1 and AT06-1 induced a tumor on grapevine as large as that induced by *R. vitis* (Ti) strains G-Ag-27 and VAT07-1 (Table 3), as did the other eight isolates (data not shown). The bacteria with the same colony morphology as those inoculated were reisolated. No symptoms developed on control plants.

Based on these results, 10 isolates from crown gall-infected grapevines (ST05-1, ST05-2, and AT06-1–AT06-8) were identified as *R. radiobacter* (Ti). To our knowledge, this report is the first of the occurrence of *R. radiobacter* (Ti) on grapevine in Japan.

Most strains of *R. vitis* use L-tartrate as a carbon source, and L-tartrate concentrations in grapevine are much higher than those in most other plants (Ruffner 1982). L-Tartrate utilization by *R. vitis* is thought to play a role in host specificity (Szegedi 1985). On the other hand, *R. radiobacter* (Ti) isolated from host plants other than grapevine do not utilize L-tartaric acid, but some strains of *R. radiobacter* (Ti) isolated from grapevine do (Burr and Katz 1983; Salomone et al. 1996, 1998; Sawada and Tsuchiya 2003). Ten tumorigenic isolates identified as *R. radiobacter* (Ti) also utilized L-tartaric acid (Table 2). Tartrate utilization genes are located on the tartrate utilization plasmid (pTr) and pTi, which some strains of *R. vitis* (Ti) possess (Salomone et al. 1996; Szegedi et al. 1992). Thus, our result suggests that the isolates might have received the plasmids from *R. vitis* (Ti) strains. As one of the approaches to verify the exchange of the plasmids, a phylogenetic analysis based on the sequence of *virC1*–*virC2* genes located on pTi was performed.

In the phylogenetic tree constructed by the NJ method using the partial sequence data of *virC1*–*virC2* genes,

almost all *R. vitis* (Ti) strains (19 in 21) isolated from grapevine were placed in one monophyletic group (Fig. 3). *R. radiobacter* (Ti) and *R. rhizogenes* (Ti) strains isolated from plants other than grapevine were divided into two other clades, respectively (Fig. 3). *R. radiobacter* (Ti) isolates ST05-1 and AT06-1, which were isolated from grapevine, grouped into the same monophyletic group as *R. vitis* (Ti) strains G-Ag-60 and VAT07-1, which were isolated from grapevine. The sequence data of the four strains in this group coincided perfectly with each other (Fig. 3), suggesting the possibility that the strains in this group possess the same type of pTi. In pathogenicity test using grapevine seedlings, the sizes of tumors induced by *R. radiobacter* (Ti) isolates ST05-1, AT06-1, *R. vitis* (Ti) strains G-Ag-27, and VAT07-1 were equal to each other and significantly larger than *R. radiobacter* (Ti) strain AtC1 and *R. rhizogenes* (Ti) strain Ch-Ag-2 (Table 3). Additionally, on tomato seedlings, the tumor induced by grapevine isolates ST05-1, AT06-1, and strain VAT07-1 were the same size, but were significantly smaller than those caused by strains AtC1, Ch-Ag-2, and G-Ag-27 (Table 3). These results suggest that the grapevine isolates ST05-1, AT06-1, and *R. vitis* (Ti) strain VAT07-1, which grouped into the same monophyletic group, have a same type of pathogenicity that induces large tumors on grapevines and small tumors on tomato plants. In 2006, moreover, *R. vitis* (Ti) strain VAT07-1 was isolated from the grapevine tree cv. Muscat of Alexandria from which *R. radiobacter* (Ti) isolates AT06-1–AT06-8 had been isolated in 2005. Namely, plasmids might have been transferred between *R. vitis* (Ti) VAT07-1 and *R. radiobacter* (Ti) AT06-1 in the grapevine tissue.

Nineteen strains of *R. vitis* (Ti) with one group of *virC1*–*virC2* genes (Fig. 3) belong to genetic groups A and B according to repetitive sequence-based PCR (rep-PCR) and multilocus sequence analysis (MLSA) (Kawaguchi et al. 2008), suggesting that homeotypical pTi could be distributed in strains of *R. vitis* (Ti) belonging to genetic groups A and B. Moreover, strains G-Ag-60 and VAT07-1 of *R. vitis* (Ti) belonging to another group of *virC1*–*virC2* genes also belong to genetic groups C and D (Fig. 3), suggesting that both strains carry the same type of pTi. These results indicate that the grouping of *R. vitis* (Ti) constructed by the *virC1*–*virC2* genes and by MLSA using chromosomal markers are different. Thus, plasmids might have been transferred between *R. vitis* (Ti) strains belonging to genetic groups A and B, and C and D, respectively.

Our studies provide a starting point to reveal horizontal transfer Ti-plasmids between *R. vitis* and *R. radiobacter* isolated from grapevine in Japan. In the future, we will investigate the ecology of *R. radiobacter* (Ti) in grapevine tissue using primers/probes designed based on the *virC1*–*virC2* gene information obtained in this study and develop

methods for the control of grapevine crown gall caused by *R. radiobacter* (Ti).

**Acknowledgments** The authors are grateful to Drs. Y. Takikawa (Shizuoka University, Shizuoka, Japan), J. Yamamoto (Shimane Agricultural Experiment Station, Shimane, Japan), and H. Sawada (National Institute for Agrobiological Sciences, Ibaraki, Japan), who supplied some of the strains in this study.

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