Research Note

Tartrate Utilization Genes Promote Growth of *Agrobacterium* spp. on Grapevine

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Crown gall on grapevine is mainly caused by *Agrobacterium vitis*, which metabolizes tartrate. Competition experiments between a tartrate-utilizing strain and its non-utilizing derivative showed that tartrate utilization confers a selective advantage on grapevine.

Agrobacterium vitis induces crown gall on grapevine (*Vitis vinifera*) (Panagopoulos and Psallidas 1973). Its narrow natural host range has been attributed to a chromosomally encoded polygalacturonase responsible for grapevine root necrosis (Burr et al. 1987b, Rodriguez-Palenzuela et al. 1991, McGuire et al. 1991). L(+)-tartrate degradation may be an additional host range factor since *A. vitis* and a few *A. tumefaciens* grapevine isolates utilize L(+)-tartrate in preference to glucose, whereas *A. tumefaciens* isolates from other hosts do not (Szegedi 1985; Gallie and Kado 1988). Tartaric acid is an abundant compound in grapevine (Ruffner 1982).

Three tartrate utilization (TAR) regions were cloned and partially sequenced. They carry the genes *ttuA-E* and *ttuC'* (required for tartrate utilization) and four open reading frames (ORFs) of unknown function (ORFX, ORFY, ORFZ1, and ORFZ2) (Crouzet and Otten 1995; Otten et al. 1995; Salomone et al. 1996).

To test the importance of tartrate utilization on grapevine, we constructed a TAR⁻ mutant from a natural TAR⁺ *A. tumefaciens* isolate from grapevine and compared its growth on grapevine with that of the parent strain, 2655. Strain 2655 carries a pTi plasmid with a TAR region that was probably acquired from an AB3-like *A. vitis* strain by natural plasmid transfer (Salomone et al. 1996).

The *ttuC*-ORFY region of pTi2655 was deleted by double recombination with the suicide plasmid pKC7 (a pBR322 derivative; Rao and Rogers 1979) containing two fragments from the pTiAB3 TAR region, yielding pPM420 (Fig. 1). pPM420 was introduced into 2655 by triparental mating with the mobilizing strain GJ23, *A. tumefaciens* 2655, and NM522(pPM420). An *A. tumefaciens* exconjugant with a pTi2655::pPM420 cointegrate (AJ19) was selected on mini-

mal medium containing neomycin (400 µg/ml). A double recombinant (AJ16) was isolated by screening AJ19 for the loss of tartrate utilization. Ti plasmid structures of AJ16 and AJ19 were checked by Southern analysis. Growth of 2655, AJ16, and AJ19 was the same in liquid yeast extract broth (YEB) medium with or without tartrate (data not shown). AJ19 was used as a TAR⁺ control strain because it allowed selection on kanamycin after extraction of bacteria from nonsterile plant material.

Strains AJ16 and AJ19 were grown on YEB medium and washed, diluted, and mixed in various proportions shortly before infection of *Vitis vinifera* cv. Gamay plants. Shoot fragments (2 to 3 cm long) were sterilized and put apical side down on Murashige and Skoog medium (Murashige and Skoog 1962) with 0.7% agar. The basal end was infected with 2 µl of a bacterial suspension.

AJ16 and AJ19 induced tumors of similar size. After 4 weeks, tumors were excised, crushed, and resuspended in 1 ml of 10 mM MgSO₄. After dilution, bacteria were plated on minimal AB medium containing 0.5% tartrate (ABT medium; Szegedi 1985) and kanamycin (100 µg/ml). On this medium, TAR⁻ colonies are smaller than TAR⁺ colonies. A relative increase of the TAR⁺ population was found in 10 out of 14 cases, without a clear correlation with absolute numbers or initial ratios (Table 1). Thus, tartrate utilization genes favor growth of *Agrobacterium* spp. in grapevine tumors.

To exclude artefacts due to in vitro conditions, we also infected intact grapevine plants in the greenhouse. *Nicotiana rustica* plants were used as a control. Stems were wounded by a small incision with a scalpel at different positions and infected with 2 µl of a bacterial suspension. The small volume assured that most bacteria were absorbed at the wound site. AJ19 and AJ16 formed tumors on grapevine after 6 weeks and on *N. rustica* after 4 weeks (not shown). On grapevine, the percentage of TAR⁺ strains in the mixed population increased by 3, 13, and 14%, in *N. rustica* the proportion of TAR⁻ and TAR⁺ bacteria varied by less than 2% (Table 1). Bacterial multiplication occurred in each case (about 12 generations on grapevine stem fragments and about five generations on *N. rustica* and grapevine plants), showing that changes in proportion did not result from differential survival.

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Growth was also tested in grapevine bleeding sap. Bleeding sap is an exudate of the xylem and rich in organic and inorganic nutrients. Besides tartrate, it contains amino acids (mainly glutamine), other organic acids (malate and citrate), and sugars (Andersen and Brodbeck 1989a, 1989b; Glad et al. 1992). Equal amounts of 2-day-old cultures of AJ16 and AJ19 were suspended in M9 salt solution (Sambrook et al. 1989) and mixed 1:1, yielding a 5.10^6 cells per ml suspension. Aliquots of 5 ml of autoclaved bleeding sap from *V. vinifera* cv. Bluefrankish (obtained from wounded canes during spring) were inoculated in three replicates with 20 μ l of bacterial suspension. Samples (5 μ l) were removed each 24 h and counted after dilution and plating on ABT medium. Strong bacterial growth was observed after 24 h. The results (Table 2) show a relative increase of the TAR⁺ population with time. Although the TAR⁻ mutant does not grow on tartrate as a sole carbon source, it multiplies on grapevine in vitro and in vivo, albeit less efficiently. Since 2655-induced tumors contain octopine and cucumopine (Paulus et al. 1989), tartrate is not expected to constitute the sole carbon source.

Tartrate may confer a selective advantage under noninfectious conditions, as suggested by the bleeding sap experiments. It is remarkable that even with high levels of organic acids, sugars, and amino acids in bleeding sap, growth can be increased through the use of tartrate. Nonsymptomatic, systemic infection by *Agrobacterium* spp. is common on grapevine (Burr et al. 1987a). Induction of tumors and use of opines may serve as a second selection step favoring growth of *A. vitis*.

Opine-producing plants favor growth of opine-utilizing *Agrobacterium* spp. (Guyon et al. 1993; Oger et al. 1997; Savka and Farrand 1997), demonstrating the importance of opines for the growth of this pathogen. Our studies show for the first time that *Agrobacterium* spp. can also take advantage of an organic compound made by nontransformed host cells.

Table 1. Competition experiments between TAR⁺ and TAR⁻ strains in plant tissues

Host	No. of bacteria in inoculum (CFU)	TAR ⁺ bacteria in inoculum (%)	TAR ⁺ bacteria in tumor extract (%)
Grapevine stem fragments (in vitro)	5.10 ²	6.9	11.8 $\pm$ 4.6
		16.6	42.5 $\pm$ 16.0
		24.9	31.0 $\pm$ 9.9
	3.10 ³	4.3	15.8 $\pm$ 8.8
		10.4	27.6 $\pm$ 8.4
		13.3	33.0 $\pm$ 9.9
	3.10 ⁴	4.9	10.7 $\pm$ 4.9
		8.8	16.7 $\pm$ 1.5
		19.4	19.7 $\pm$ 4.3
	3.10 ⁵	26.6	28.0 $\pm$ 0.9
		46.5	51.9 $\pm$ 3.0
		7.0	19.2 $\pm$ 5.5
<i>N. rustica</i> plants (greenhouse)	3.10 ³	42.5	45.1 $\pm$ 9.4
	3.10 ⁴	47.5	46.5 $\pm$ 7.6
	3.10 ⁵	47.4	48.8 $\pm$ 4.0
Grapevine plants (greenhouse)	3.10 ³	40.6	54.8 $\pm$ 6.1
	3.10 ⁴	40.9	53.7 $\pm$ 15.8
	3.10 ⁵	46.7	50.5 $\pm$ 4.8

Table 2. Competition experiments between TAR⁺ and TAR⁻ strains in grapevine bleeding sap

Days	TAR ⁺ bacteria (%)
0	42.3 $\pm$ 3.2
1	46.3 $\pm$ 7.1
2	48.6 $\pm$ 6.5
3	62.3 $\pm$ 4.2
4	70.0 $\pm$ 7.0

Fig. 1. Construction of the TAR⁻ derivative of strain 2655. The first recombination between pPM420 and pTi2655 over *ttuB* (A) yields AJ19 (B). AJ16 (C) results from a second, intramolecular recombination. White boxes represent the kanamycin resistance gene. Hatched boxes correspond to the common regions between pPM420 and pTi2655.

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