Characterization of *Agrobacterium vitis* Strains Isolated from Feral *Vitis riparia*

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ABSTRACT

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Agrobacterium vitis was isolated from roots of 41 of 66 feral *Vitis riparia* vines collected in three different regions of New York State. Two of the regions were more than 150 km from commercial vineyards. The strains were highly diverse as determined by DNA fingerprinting of the chromosomal region lying between the 16S and 23S rRNA genes. Of 24 strains examined, 15 different fingerprints were generated, and none was identical to fingerprints generated by previously identified groups of tumorigenic *A. vitis* strains. Results of physiological tests that were done to characterize strains from *V. riparia* conformed closely to those expected for *A. vitis,* except that 23 of 26 strains did not utilize tartrate. All strains were nontumorigenic, did not hybridize with a probe consisting of T-DNA genes, did not utilize octopine or nopaline, and carried zero to three plasmids. Of 26 strains, 7 inhibited *A. vitis* strain K306 from causing galls at wound sites on grape as well as or better than a previously studied nontumorigenic *A. vitis* strain, F2/5, that is known to have biological control activity.

Agrobacterium vitis, the primary bacterial species that causes crown gall of grape, survives systemically in grape (15) and therefore is disseminated in vegetative propagation material. A successful procedure for eliminating *A. vitis* from sources of propagation material is to initiate plants by in vitro propagation from shoot tips (6). *A. vitis* has not been detected in plant genera other than *Vitis* nor from soils other than those sampled from vineyards (4). Therefore, shoot tip-initiated vines are likely to remain free of crown gall if planted at sites where they will not contact *A. vitis*-infested grape plants or debris from such plants that may persist in soil (10). Because several *Vitis* spp. grow in the wild, often in close proximity to vineyards, we questioned whether they may harbor *A. vitis*. *V. riparia* is the most common native *Vitis* sp. found in New York. In this paper, we report the isolation and characterization of *A. vitis* strains from feral *V. riparia.*

Diversity within a large collection of tumorigenic *A. vitis* strains has been recently described by determining the structural type of Ti plasmid carried by them and by comparing genetic fingerprints that were generated by restriction analysis of specific regions of the bacterial chromosome (16,23) and by random amplified polymorphic DNA (RAPD; 17). It was determined that the type of Ti plasmid carried by a strain is highly correlated with the chromosomal markers. In the current study, we investigated the genetic diversity of strains from *V. riparia* by generating DNA fingerprints from an intergenic chromosomal region that lies between the 16S and 23S rRNA genes and comparing them to those previously generated from tumorigenic strains.

Some nontumorigenic *A. vitis* strains (such as strain F2/5) are able to prevent crown gall at wound sites on grape (8,27). Therefore, we also investigated the ability of nontumorigenic *A. vitis* strains from *V. riparia* to inhibit crown gall development on grape.

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MATERIALS AND METHODS

Bacterial isolations and characterization. *V. riparia* vines were sampled from different regions in New York, including the Finger Lakes, where many commercial vineyards exist. Samples were also collected in regions where vineyards are not present, including northern New York (near the St. Lawrence River) and along the New York-Vermont border (near Lake Champlain; Table 1). In the Finger Lakes region, none of the *V. riparia* samples were taken from within or bordering vineyards but were from apple orchards, vacant fields, state parks, and from the Montezuma Wildlife Refuge. Vines were identified as *V. riparia*, the primary native American species growing in this region of New York, by type of leaf morphology. A total of 66 vines were sampled during the summer and fall seasons. Each location was recorded, and vines were sampled by excavating roots and cutting off at least six root pieces (each at least 15 cm long) per vine. Roots varied in thickness from fibrous up to approximately 1.5 cm in diameter. Roots with some associated soil were placed in plastic bags and kept in an ice chest, transported to the laboratory, and refrigerated until processed. None of the vines that were sampled had any apparent galls on their roots or stems.

To isolate *A. vitis*, approximately 5 g of root tissue from each sample was cut into small pieces (approximately 1 cm in length) using disinfected pruning shears. Root pieces were placed in a blender jar with 250 ml of the liquid medium of Roy and Sasser, RS (17), which is semiselective for *A. vitis*. Roots were blended for 1 min; then the suspension of chopped root tissue was transferred to a 500-ml flask and allowed to shake on a rotary shaker at 150 rpm. Serial dilutions of the root mixture were made at 2 h and 5 days and were plated on RS solidified by adding 10 g of agar per liter. RS plates were incubated at 28°C for 5 to 7 days, after which colonies resembling *A. vitis* were transferred to potato dextrose agar (PDA). Isolates that exhibited typical growth of *A. vitis* on PDA after 48 h at 28°C were tested with an immunoblot procedure for their reaction to an *A. vitis*-specific monoclonal antibody, as previously described (3). All strains that reacted positively were saved for further studies by suspending a loopful of bacteria from PDA in microcentrifuge tubes containing 1 ml of sterile water and storing at 4°C. Subsequently, strains of interest were saved by cryogenic storage at –80°C.

A total of 26 strains, each originating from a different location within the three regions sampled, were characterized further using the general testing scheme of Kerr and Panagopoulos (14). Tests as described previously (17) included growth at 36°C, production of 3-ketolactose, growth in 2 and 5% NaCl, alkaline reaction in litmus milk, acid production from erythri-

tol and melezitose, and alkali production from L-tartaric acid and malonic acid. Other tests included the ability of strains to produce polygalacturonase (24) and endoglucanase (20) and the ability to utilize the opines, octopine and nopaline (15). *A. vitis* strains CG49 and K306 were included as positive controls; negative control strains were K84 (*A. rhizogenes*) and CG628 (*A. tumefaciens*). All tests were repeated at least once. Strains were tested for ability to cause galls on sunflower (*Helianthus annuus* L.), tomato (*Lycopersicon esculentum* Mill.), and *Kalanchoe daigremontiana* Hamet & E. Perrier by applying 48-h-old bacterial growth from PDA to puncture wounds on the stems of plants as previously described (5).

The plasmid profiles of strains, including known tumorigenic strains CG49 and K306, were determined using previously described methods (10). Southern blots of plasmid preparations were probed as described previously (7) with pTHE17, which contains all T-DNA from strain C58.

Biological control. Because all strains that were isolated from *V. riparia* were found to be nontumorigenic, we wished to determine if some of them might also be effective as biological controls for grape crown gall. The 26 strains were screened for their ability to inhibit growth of *A. vitis* in vitro and to prevent gall formation on grape. In vitro antibiosis was measured against tumorigenic *A. vitis* strain K306 by using a method reported previously (8). Briefly, candidate strains were spotted in the center of Mannitol-glutamate (MG) agar plates (17), grown for 48 h at 28°C, and killed with chloroform vapor. After airing plates with lids removed in a fume hood, they were sprayed with a suspension of strain K306 containing approximately 10^8 CFU/ml, optical density 0.1 at 600 nm. The presence or absence of growth inhibition zones around the candidate strains was recorded within 48 h.

Assays for crown gall inhibition on potted 1- to 2-month-old grapevines (*V. vinifera* cv. Chardonnay) were done following reported methods (8). Strain F2/5, a nontumorigenic *A. vitis* with known biocontrol activity (27), was compared to the strains from *V. riparia*. Growth of strains from PDA were suspended in sterile distilled water (SDW) to about 10^8 CFU/ml. Wounds that were made with an electric drill (6-mm-diameter bit) were inoculated with 50 µl of the bacterial cell suspensions containing approximately equal numbers of biological control candidate and strain K306. SDW was applied as a negative control and strain K306 mixed with an equal volume of SDW was applied as positive control. For each experiment, three inoculations were made to each of three plants and inoculation sites were wrapped with Parafilm. The numbers of inoculation sites at which galls developed and the cross-sectional area (at widest point) of each gall were recorded 8 weeks after inoculation. Gall suppression was quantified by dividing the average gall cross-sectional area by the number of inoculation sites. Gall ratings were as follows: 1 = cross-sectional surface area of 1 to 25 mm^2, 2 = 26 to 50 mm^2, 3 = 51 to 75 mm^2, 4 = 76 to 100 mm^2 and 5 = area > 100 mm^2. Plants were maintained in a cooled greenhouse and the experiment was repeated once.

Genetic diversity of strains. Tumorigenic strains of *A. vitis* can be divided into four major groups based on the structures of the Ti plasmids they carry (21). The Ti structures differ in gene content, presence of insertion sequences, and number of T-DNAs found in the plasmids (13). As a way of measuring the genetic diversity of *A. vitis* strains from *V. riparia*, fingerprints of the 16S to 23S intergenic spacer (IGS) region were generated from 24 of the 26 strains that were isolated from different locations in New York and Vermont and characterized in detail. Polymerase chain reaction amplifications were carried out as previously reported (10). Amplicons were digested with *Taq*I, *Ava*I, *Rsa*I, *Cfo*I, and *Hae*III (Promega Corp., Madison, WI) and DNA fragments were resolved by agarose (3%) gel electrophoresis. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed with a UV illuminator. Relatedness of strains was determined based on the presence or absence of restriction fragments using the method of Nei and Li (18). Strain relationships were quantified based on an arithmetic means cluster analysis procedure as previously reported (25). Fingerprints of the 24 strains from *V. riparia* were compared to those of strain F2/5, 14 tumorigenic *A. vitis* strains representative of different genetic groups of the pathogen, *A. tumefaciens* strain CG628, and *A. rhizogenes* strain K-84.

RESULTS

Isolations and characterization. A total of 66 *V. riparia* vines were sampled, and *A. vitis* was isolated from 41 of these. In all, 450 isolates were recovered that resembled *A. vitis* by producing typical colonies on RS and PDA media and by reacting positively with the species-specific monoclonal antibody.

Table 1. Bacterial strains of *Agrobacterium* spp.

Strain designation	Characteristics[a]	Origin
CG511, CG515, CG517, CG518, CG523, CG526, CG529, CG531	Nontumorigenic *A. vitis* from *Vitis riparia*	Finger Lakes Region, NY
CG535, CG537, CG538, CG542, CG544, CG546, CG548, CG550, CG553, CG555	Nontumorigenic *A. vitis* from *V. riparia*	St. Lawrence River Region, NY
CG556, CG559, CG561, CG565, CG567, CG569, CG571, CG572	Nontumorigenic *A. vitis* from *V. riparia*	Lake Champlain Valley, NY-VT
F2/5	Nontumorigenic *A. vitis*, biocontrol for grape crown gall	South Africa (27)
CG47	Tumorigenic *A. vitis*, IGS group A1a, N type Ti	NY, grape gall
CG49, CG56	Tumorigenic *A. vitis*, IGS group B1a, N type Ti	MI, grape gall
CG78	Tumorigenic *A. vitis*, IGS group A1b, V type Ti	NY, grape gall
CG81	Tumorigenic *A. vitis*, IGS group B1a, V type Ti	MI, grape gall
CG98	Tumorigenic *A. vitis*, IGS group B1b, V type Ti	VA, grape gall
CG102	Tumorigenic *A. vitis*, IGS group B2a, OL type Ti	VA, grape gall
CG108	Tumorigenic *A. vitis*, IGS group A2, OL type Ti	NM, grape gall
CG415	Tumorigenic *A. vitis*, IGS group B2b, OS type Ti	NY, vineyard soil
CG474	Tumorigenic *A. vitis*, IGS group B1a, On type Ti	NM, grape root
CG475	Tumorigenic *A. vitis*, IGS group B2a, OS type Ti	NM, grape root
K306	Tumorigenic *A. vitis*, OL type Ti	Australia, grape gall
2641	Tumorigenic *A. vitis*, IGS group B2a, OS type Ti	France, grape gall
2613	Tumorigenic *A. vitis*, IGS group B2c, OS type Ti	France, grape gall
B10/7	Tumorigenic *A. vitis*, IGS group B2c, OS type Ti	Hungary, grape gall
K-84	Nontumorigenic *A. rhizogenes*, biocontrol strain	Australia
CG628	Tumorigenic *A. tumefaciens*	NY, grape gall

[a] Intergenic spacer (IGS) groups were determined by fingerprinting the intergenic spacer region between the 16S and 23S rRNA genes (23). Ti plasmid types: N= catabolizes nopaline, OL = catabolizes octopine and has large TA region, OS = catabolizes octopine and has small TA region, V = catabolizes vitopine, and On = octopine type with undefined structure.

The reactions to the tests used to characterize 26 strains that originated from the different sampling locations are shown in Table 2. Although some variation among strains was observed, all strains that produced typical colony morphologies in culture and that reacted with the antibody conformed closely to results expected for *A. vitis*. However, only three of the strains utilized tartrate, as evidenced by alkali production from L-tartaric acid. The three strains that did utilize tartrate (CG555, CG544, and CG561) all carried at least two relatively large plasmids (Fig. 1, lane S for CG544).

All strains from *V. riparia* were nontumorigenic on the three plant species that were inoculated and their plasmids did not hybridize to the T-DNA probe, pTHE17 (*data not shown*). Plasmid profiles showed that strains varied in number and size of plasmids they carry (Fig. 1). Some strains had no detectable plasmids (CG506, CG523, CG533, CG537, CG538, and CG542), others carried one or two large plasmids (100 to 200 kilobases) and some had smaller plasmids that were not observed in a group of previously-investigated tumorigenic *A. vitis* strains (CG511, CG515, and CG526; 10). None of the strains utilized octopine or nopaline as determined by lack of growth on media described above.

Biological control. Unlike strain F2/5, none of the *V. riparia* strains inhibited growth of strain K306 in vitro. Some of the strains however, strongly inhibited crown gall formation on grape by K306 (Table 3). Whereas 7 of the strains inhibited gall formation as well as or better than F2/5, 19 strains were not as effective as F2/5 in this assay. Strains CG542 and CG561 expressed no gall-inhibiting ability (that is, gall severity ratings on plants inoculated with these strains combined with K306 were greater than when plants were inoculated with K306 alone).

Genetic diversity of strains. Analysis of IGS fingerprints from the 24 nontumorigenic strains from *V. riparia* and from 13 tumorigenic strains from various origins resulted in the identification of three main groups (similarity coefficients <0.05; Fig. 2). The tumorigenic strains had been previously analyzed and were known to have different fingerprints and to represent unique genetic groups (23). The analysis also included outgroup strains of *A. tumefaciens* and *A. rhizogenes*.

A great level of diversity within the *V. riparia* strains is apparent by the fact that 15 different fingerprints were generated from the 24 strains analyzed. The largest group of strains with identical fingerprints (CG518, CG553, CG567, and CG542) were isolated from all three of the major collection regions (Finger Lakes, St. Lawrence, and Lake Champlain). The tumorigenic *A. vitis* strains were widely dispersed among the strains from *V. riparia* in the phenogram. None of the *V. riparia* strains generated fingerprints that were identical to any of the tumorigenic strains.

Table 2. Characterization of 26 putative *Agrobacterium* strains from *Vitis riparia*

Test	Reactions of different *Agrobacterium* spp.				Positive[a]
	CG49	K306	K84	CG628	
Growth at 36°C	–	–	–	–	5
Growth 2% NaCl	+	+	–	+	25
Growth 5% NaCl	–	–	–	–	2
3-ketolactose production	–	–	–	+	0
Alkaline on litmus milk	+	+	–	+	26
Acid from erythritol	–	–	+	–	0
Acid from melezitose	–	–	–	+	0
Alkali from malonic acid	+	+	+	–	26
Alkali from L-tartaric acid	+	+	+	–	3
Reaction with *A. vitis* antibody	+	+	–	–	26
Polygalacturonase production	+	+	–	–	26
Endoglucanase production	+	+	–	–	26
Utilization of octopine/nopaline	–/+	+/–	–/+	NT[b]	0/0
Tumorigenic	+	+	–	+	0

[a] Number of *V. riparia* strains showing a positive reaction.
[b] NT = not tested.

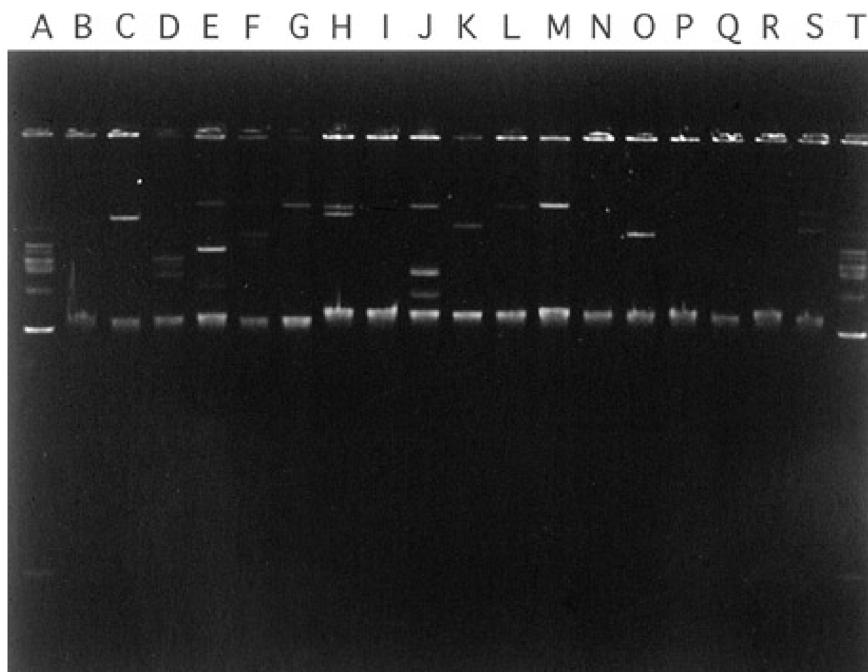

Fig. 1. Plasmids profiles of nontumorigenic *Agrobacterium vitis* strains from *Vitis riparia*. Lanes A to T: plasmids of corresponding strains SW2 (*Erwinia stewartii*), CG506, CG507, CG511, CG515, CG517, CG49 (tumorigenic), CG518, CG523, CG526, CG529, CG531, K306 (tumorigenic), CG533, CG535, CG537, CG538, CG542, CG544, and SW2. The largest plasmid bands observed (e.g., lane G) are in the size range of Ti plasmids (approximately 200 kilobases).

DISCUSSION

A. vitis was frequently isolated from roots of feral *V. riparia* vines that were

Table 3. Gall suppression rating of strain K306-induced grape crown gall by nontumorigenic *Agrobacterium vitis* strains from *Vitis riparia*

Strain	Rating[a]
CG544	0
CG556	0.1
CG546, CG565	0.3
CG523, CG526, CG531	0.4
F2/5	0.5
CG537, CG553	0.6
CG548	0.7
CG515, CG517	0.8
CG529, CG535, CG571	1.0
CG511, CG550, CG569	1.2
CG567	1.1
CG572	1.7
CG555	1.8
CG559	1.9
CG518	2.0
CG538	2.7
K306	3.6
CG542	3.9
CG561	4.0

[a] Gall suppression rating is equal to the average gall size rating/no. of inoculation sites. Gall size ratings: 1 = cross-sectional surface area of 1 to 25 mm^2, 2 = 26 to 50 mm^2, 3 = 51 to 75 mm^2, 4 = 76 to 100 mm^2, and 5 = area > 100 mm^2.

collected from a region of New York where commercial vineyards exist and from two regions that are more than 150 km from commercial vineyards. Because *A. vitis* was isolated from the majority of vines that were sampled, it appears to be a common inhabitant of *V. riparia* roots. This raises questions concerning the ecology and spread of the bacterium in nature. It is possible that *A. vitis* may be disseminated with *V. riparia* seed or that it exists at low populations in soils and multiplies to a detectable level on grapevines. Previous research, however, demonstrated that, although nontumorigenic *Agrobacterium* spp. are frequently detected in cultivated and noncultivated soils (11,26), *A. vitis* was never identified within the bacterial populations. Therefore, if, as suggested in our paper, *A. vitis* is a frequent soil inhabitant, why has it not previously been detected in association with roots of other plants? One possibility is that *A. vitis* colonizes grape roots more efficiently than it colonizes roots of other plants (2) and therefore, under natural conditions, populations of *A. vitis* may not reach detectable levels on plants other than grape. Continued research is needed to identify factors that influence grape-*A. vitis* interactions and survival and spread of the bacterium in nature.

Isolates from *V. riparia* were identified, using a standard set of biochemical and physiological tests, by their ability to produce extracellular enzymes that, within *Agrobacterium* spp., are unique to *A. vitis;* and by reaction to a species-specific antibody. The only result that was significantly different from previously-studied tumorigenic *A. vitis* strains was that few of the strains from *V. riparia* utilized tartrate. Tartrate utilization by tumorigenic strains, a trait encoded on conjugative plasmids (pTAR and Ti), is thought to provide the bacterium a competitive advantage on grape (22). At this time, however, it is unclear if tartrate utilization affects interactions between grape and nontumorigenic *A. vitis* strains, such as those recovered from *V. riparia.*

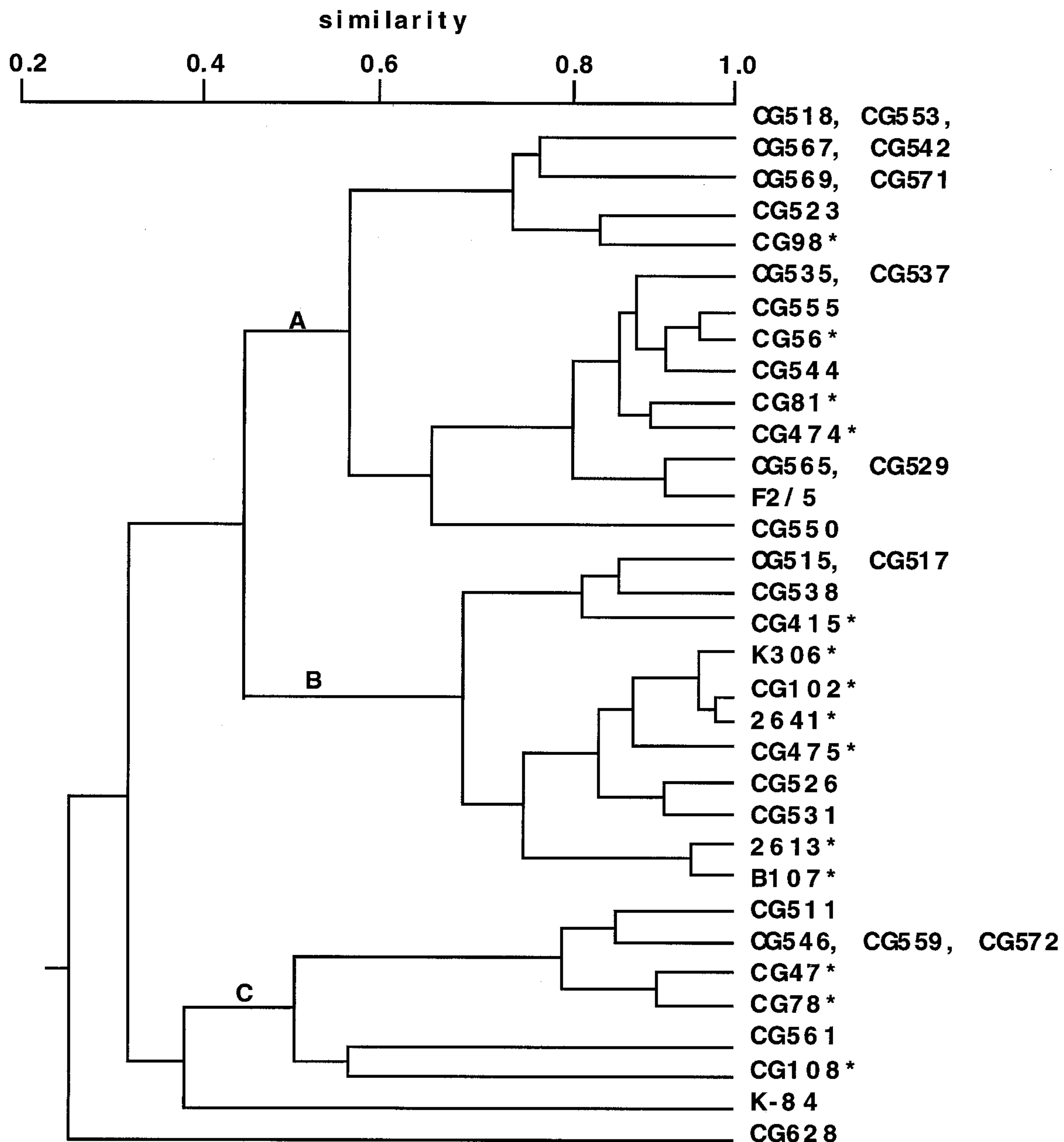

Fig. 2. Phenogram demonstrating relatedness of nontumorigenic *Agrobacterium vitis* strains from *Vitis riparia*, tumorigenic *A. vitis* strains (*), *A. rhizogenes* (K84), and *A. tumefaciens* (CG628).

Proposed strategies for the management of grape crown gall include planting vines which are free of *A. vitis* and preventing trunk and cane injuries that are frequent sites of infections. Several researchers (1,9,31,32) are developing biological controls for preventing infections (that may be caused by tumorigenic *A. vitis*) that are known to persist in vineyard soils in association with decaying grape organic matter. Therefore, it is highly significant that all strains recovered from *V. riparia* thus far have been found to be nontumorigenic. It appears that strains from *V. riparia* are not likely to serve as inoculum for crown gall unless they are able to acquire Ti plasmids, possibly by conjugal transfer from tumorigenic strains. Repeated attempts to transfer Ti plasmids to strains from *V. riparia* (by conjugation with tumorigenic strains) have been unsuccessful, implying that if transfer occurs in nature it is at a very low frequency (T. J. Burr, *unpublished*).

Fingerprinting of a specific *A. vitis* chromosomal region revealed that strains from *V. riparia* are highly diverse. They are distinct from a large collection of tumorigenic strains in that none of them have fingerprints identical to the tumorigenic strains. Chromosomal type of tumorigenic strains, as determined by fingerprinting and by RAPD, were highly correlated with the structural type of Ti plasmid they carried (16,23). For example, 29 of 30 strains carrying a nopaline-type Ti plasmid (representing strains from the United States and Hungary) had identical fingerprints. Therefore, it has been proposed that Ti transfer to *A. vitis* may represent a recent evolutionary event and that Ti transfer in nature is rare. Factors that may prevent stable transfer of Ti plasmids to the *V. riparia* strains, such as plasmid incompatibility (12) or entry exclusion mechanisms (19), need to be studied. Further evaluations of *V. riparia* should also be done to confirm the absence of tumorigenic strains and to determine if strains with genetic fingerprints identical to tumorigenic strains can be detected. It would also be interesting to index other native *Vitis* spp. for *A. vitis* and to compare strains to those from commercial vineyards and from *V. riparia*.

No crown galls were apparent on any *V. riparia* vines that we observed growing in the wild. It has been demonstrated that most *V. riparia* selections (e.g., cv. Riparia Gloire) and grape rootstocks having *V. riparia* parentage (e.g., cv. Courderc 3309 and MGT 101-14) are highly resistant to crown gall (11,29,30). It was also shown that endophytic colonization by tumorigenic *A. vitis* is greatest on crown gall-susceptible, as compared to -resistant, grape cultivars (28). Similar studies have not been done with nontumorigenic *A. vitis* strains. It would be interesting to determine whether certain *A. vitis* strains (e.g., those from *V. riparia*) are better adapted for survival on *V. riparia* and, therefore, exclude colonization by tumorigenic strains.

In this study, we found that some strains from *V. riparia* were at least as effective as strain F2/5 in inhibiting tumorigenic strain K306 from causing crown gall. These results are encouraging and should complement other research on biological control of grape crown gall. Since in vitro antibiosis was not observed with any of the *V. riparia* strains, it appears that, as with F2/5, the mechanism of control is not associated with antibiotic production (9). It is now essential to determine if the new control strains inhibit the same group of tumorigenic strains as F2/5 and if, like F2/5, they only inhibit gall formation on grape. If biocontrol strains are active against different types of tumorigenic strains, it may be advantageous to use mixtures of biocontrols to improve the spectrum of control on grape.

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