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Phylogenetic Relationships Between *Agrobacterium vitis* Isolates and Their Ti Plasmids

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A collection of 76 *Agrobacterium vitis* isolates was analyzed by several molecular methods. PCR analysis of the 16S-23S intergenic spacer (IGS) region of the ribosomal DNA, restriction patterns of total DNA, and hybridization with different Ti plasmid probes enabled us to distinguish chromosomal and Ti plasmid characteristics. The various strains could be divided into several chromosomal groups based on IGS patterns. Each group was found to contain a particular type of Ti plasmid (nopaline, vitopine, and octopine/cucumopine Ti plasmids), although a few exceptions occur which may represent rare natural transconjugants. The Ti plasmid of isolate CG474 did not fit within existing *A. vitis* Ti plasmid groups.

Additional keywords: Agrobacterium ecology, crown gall, grapevine, ribosomal RNA gene.

Agrobacterium vitis (Ophel and Kerr 1990), previously called *Agrobacterium tumefaciens* biotype III (Kerr and Panagopoulos 1977; Süle 1978), has been found to be specifically associated with grapevine (Panagopoulos et al. 1978; Perry and Kado 1982; Burr and Katz 1983; Burr et al. 1987; Ma et al. 1987; Szegedi et al. 1988; Bien et al. 1990; Sawada et al. 1990). An *A. vitis*-specific polygalacturonase gene that leads to grapevine root necrosis plays an important role in host specificity (McGuire et al. 1991; Rodriguez-Palenzuela et al. 1991).

Szegedi (1985) has shown that many *A. vitis* isolates use tartrate even in the presence of glucose, whereas other *Agrobacterium* species do not (Kerr and Panagopoulos 1977; Süle 1978). Tartrate utilization is plasmid-encoded (Otten et al. 1995, Crouzet and Otten 1995, Salomone et al. 1996) and may constitute another important host range factor for *A. vitis*.

The Ti plasmids of the *A. vitis* strains have been classified into four main groups: nopaline plasmids (N plasmids), octopine/cucumopine (o/c) plasmids with a small or large TA region (OS and OL plasmids, respectively), and vitopine (V) plasmids (Paulus et al. 1989a). Within these major groups, several subgroups have been found (exemplified by pTiAB3, pTiAg57, and pTiNW233 within the OS group and pTiTm4, pTiHm1, and pTi2608 within the OL group, van Nuenen et al. 1993). The V and N plasmids (of which pTiS4 and pTiAB4 have been studied in detail, Gérard et al. 1992, Otten and de Ruffray 1994) are less variable. The OS and OL plasmids are associated with particular chromosomal backgrounds (Paulus et al. 1989b). The phylogenetic relationships between the *A. vitis* chromosomal backgrounds have not been studied earlier.

Here we describe chromosomal and Ti plasmid characteristics of 76 *A. vitis* isolates, 22 of which are reference strains chosen on the basis of earlier analyses, whereas 54 (CG numbers) are new grapevine isolates from the United States. Different molecular methods based on chromosomal and Ti plasmid characteristics were used. Analysis of 16S sequences (Sawada et al. 1993; Willems et al. 1993) and of the intergenic spacer separating the 16S and 23S rRNA genes (Barry et al. 1991) has been suggested for identification of bacterial strains. In this study, we have used restriction enzyme analysis of the PCR-amplified intergenic spacer (ribo-fingerprinting) and the *Eco*RV digestion pattern of total DNA to place the strains into chromosomal groups. Most of the Ti plasmids could be placed in the earlier defined OS, OL, N, and V groups, but one (pTiCG474) has an unusual T-DNA structure.

RESULTS

Digestion patterns of total DNA.

Total DNA of the 76 *A. vitis* isolates was digested with *Eco*RV and run on agarose gels.

Thirteen different restriction patterns were obtained (I to XIII, Fig. 1) and allowed a first grouping of the isolates.

PCR amplification of the 16S-23S intergenic spacer regions.

The 16S-23S intergenic spacer regions (IGS) from all strains were amplified by PCR and the amplicons were di-

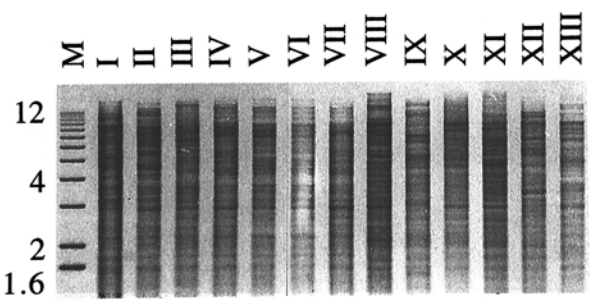

Fig. 1. *Eco*RV digestion patterns of representative *Agrobacterium vitis* isolates. CG49 (I), CG56 (II), CG78 (III), CG81 (IV), CG98 (V), CG101 (VI), CG450 (VII), CG102 (VIII), CG108 (IX), CG415 (X), CG475 (XI), 2612 (XII), CG474 (XIII). M: DNA marker (fragment size in kb).

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Table 1. *Agrobacterium vitis* isolates and their properties[a]

Strains and origin	IS*867* type	IS*869* type	Ti type	*Eco*RV type	IGS type
CG47(NY,g,C), CG49(NY,g,R), CG60(NY,g,G), CG94(NY,g,R), CG109(NY,g,-), CG110(NY,g,-), CG112(NY,g,-), CG213(NY,s,Cb), CG218(NY,s,Cb), CG406(NY,r,G), CG407(NY,r,G), CG424(NY,so), CG439(WA,r,Cb), CG440(WA,r,Cb), CG441(WA,r,Cb), CG442(WA,r,R), CG443(NY,r,Ch), CG447(NY,r,-), CG470(WA,s,Sb), CG471(WA,s,Sb), CG485(NY,r,Cd), Ni-1(H), IS1.1(H),AT1(H), EK2(H)	–	1	N	I	A1
CG111(NY,g,-)	–	1	–	I	A1
CG453(NY,s,R), CG455(NY,s,R)	–	2	N	I	A1
CG486(NY,r,Co)	–	3	N	I	A1
AB4(H)	–	4	N	I	A1
CG78(NY,g,M), CG88(NY,g,R)	–	–	V	III	A1
CG113(NY,g,-), CG114(NY,g,-), Sz2(H), NW11(G), NW113(G), NW121(G), NW161(G)	1	–	V	III	A1
CG108(NM,g,Z)	27	–	OL-like	IX	A2
CG81(MI,g,Ch)	–	5	V	IV	B1a
2681(F)	1	6	V	IV	B1a
CG448(NY,s,Ch), CG450(NY,s,Ch), CG452(NY,s,Ch)	1	6	V	VII	B1a
CG56(MI,g,Cl)	–	7	N	II	B1a
CG474(NM,r,Z)	–	8	On	XIII	B1a
CG98(VA,g,R)	–	9	V	V	B1b
CG101(VA,g,Gt), CG228(NY,s,Cs), CG229(NY,s,Cs), CG230(NY,s,Cs), CG231(NY,s,Cs)	–	10	V	VI	B1b
2618(F)	13	–	OL	VIII	B2a
2949(F)	14	–	OL	VIII	B2a
K308(A), K309(A)	15	–	OL	VIII	B2a
2686(F)	17	–	OL	VIII	B2a
2641(F)	19	–	OL	VIII	B2a
CG102(VA,g,Ch), CG106(MS,g,P), CG107(MS,g,P), CG435(VA,so), CG437(VA,so)	24	–	OL	VIII	B2a
CG412(NY,so), CG413(NY,so)	25	–	OL	VIII	B2a
CG475(NM,r,Fc), CG477(NM,r,Fc) CG478(NM,r,Fc), CG479(NM,r,Fc)	26	–	OS	XI	B2a
CG415(NY,s,Cb)	28	–	OS	X	B2b
2612(F)	3	12	OS	XII	B2c
AB3(H)	4	14	OS	XII	B2c
2607(F)	5	13	OS	XII	B2c
2613(F)	8	13	OS	XII	B2c
B10/7(H)	9	11	OS	XII	B2c

[a] CG series: new isolates from the United States, the others are reference strains described by Paulus et al. 1989a, 1989b. Strains were analyzed by hybridization to IS*867*, IS*869*, and (to determine the Ti plasmid type) to N231 (T-DNA of the nopaline pTiAB4 plasmid) and pPM649 (T-DNAs T1 and T2 of the vitopine pTiS4 plasmid), by comparison of EcoRV digests of total DNA, and by IGS ribo-fingerprinting. Origin: for the american isolates (CG series) the first two letters represent the abbreviation for the state in which the sample was collected. Isolations were made from g = galls, r = roots, s = sap or so = soil. Cultivars of grape from which isolation were made include: C = Chancellor, Ch = Chardonnay, Cb = Chenin blanc, Cl = Chelois, Co = Concord, Cs = Cabernet-Sauvignon, Fc = French Colombard, Gt = Gewurztraminer, G = Gamay beaujolais, M = Merlot, P = Pinot noir, R = Riesling, Sb = Sauvignon blanc, Z = Zinfandel, Cd = C3309. For the other isolates A, F, G, and H denote Australia, France, Germany, and Hungary, respectively. Ti types: N, V, OS, OL, and On: nopaline, vitopine, o/c small TA, o/c large TA and new octopine type Ti plasmids. IS*867* types 1-23 have been described by Paulus et al. 1989b.

gested with five different restriction endonucleases. The Nei and Li coefficients, calculated from the resulting fragments, were used in the unweighted pair group method with the arithmetic mean (UPGMA, Sneath and Sokal 1973) in the NTSYS program to generate the phenogram shown in Figure 2B. Two main groups of strains (similarity coefficients [s] < 0.50) were identified. UPGMA is commonly used, easy to understand and generates equally weighted comparisons of all of the isolates that are tested (Rohlf 1988).

Group A consists of two subgroups, A1 and A2. Within group A1, group A1a contains 30 of the 31 strains with N plasmids (all with identical IGS patterns) and group A1b contains eight strains with V plasmids. Group A2 has a single strain (CG108) with an OL plasmid. Group B consists of subgroups B1 and B2. Group B1 is divided into two subgroups comprised of B1a with a set of five identical strains with V plasmids, strain CG56 with an N plasmid and strain CG474 with an unusual octopine plasmid. Group B1b has six identical strains with V plasmids. Group B2 consists of three subgroups all containing strains with OS or OL plasmids. B2a has primarily strains with OL plasmids (13 of 17 strains), the other B2 strains carry OS plasmids. These data are summarized in Figure 2B and Table 1.

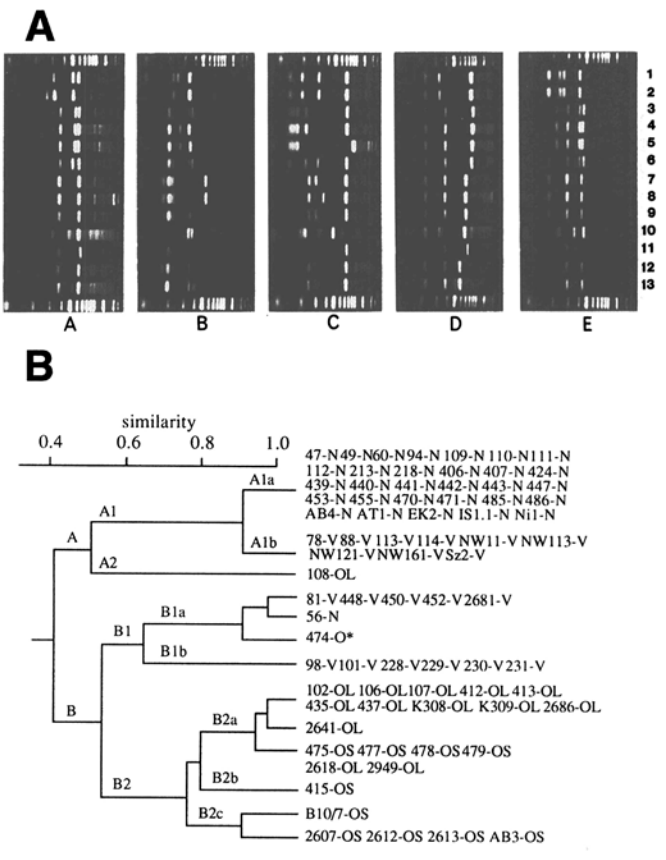

Fig. 2. A, Ribofingerprinting patterns of PCR-amplified IGS region of rDNA from representative *Agrobacterium vitis* strains with *Ava*I (A), *Cfo*I (B), *Hae*III (C), *Rsa*I (D) and *Taq*I (E) endonucleases. IGS groups are indicated within brackets. First and last lane in each gel is a 100-bp DNA marker. Lanes 1 to 13: CG47 (A1a), CG78 (A1b), CG56 (B1a), CG81 (B1a), CG474 (B1a), CG98 (B1b), CG102 (B2a), 2641 (B2a), CG475 (B2a), CG108 (A2), CG415 (B2b), B10/7 (B2c), 2613 (B2c). **B,** Phenogram from UPGMA clustering of strains based on values of Nei-Li similarity coefficients (strain designation is the same as in Table 1).

Hybridization to different probes.

Contrary to the PCR technique, Southern hybridization can reveal banding patterns of repeated elements like bacterial insertion sequences (IS elements). These patterns can then be related to each other (Paulus et al. 1989b). The *Eco*RV-digested DNAs of the 54 new *A. vitis* isolates were blotted and hybridized to different probes. The 22 reference strains were only analyzed for the occurrence of IS*869*, the other probes were tested in previous studies.

Hybridization to IS*867*.

All OS and OL plasmids studied so far contain an IS*867* copy just outside the right border of the TA region, and another one within the right part of the TB region. OL plasmids also contain a copy of the IS*867*-related IS*866* element in the TA region, which cross-hybridizes to IS*867* (Paulus et al. 1989b). Apart from the pTi-located IS*866* and IS*867* copies, strains with OS and OL plasmids contain chromosomal copies which can be identified by their particular hybridization patterns. Some strains with V plasmids have also been found to contain chromosomal IS*867* copies, while strains with N plasmids lack IS*867* and IS*866* (Paulus et al. 1989b).

Total DNA of the 54 new isolates (CG series) was digested with *Eco*RV and hybridized to the IS*867* probe F7 (Paulus et al. 1989b). Results are summarized in Table 1. The 11 patterns of the hybridizing reference strains (Table 1) have been described and defined before (Paulus et al. 1989b). Thirteen CG strains grouped into five hybridization patterns different from the 23 patterns described before; these patterns were called patterns 24-28 (Fig. 3).

Hybridization to IS*869*.

IS*869* was first detected in the TA and TB region of OS plasmids (Paulus et al. 1991). Strains with such plasmids also carry IS*869* copies in their chromosomes. The exceptional OS plasmids pTiAg57 and pTiNW233 lack IS*869*. Strains with N or V plasmids sometimes carry IS*869* copies on their chromosomes but not on the Ti plasmid (Paulus et al. 1991). An IS*869* probe (a 0.6-kb *Hin*fI-*Hin*dIII fragment from the pTiAB3 TA region, Paulus et al. 1991) was hybridized to the 76 *A. vitis* isolates; 49 strains hybridized with 14 different patterns (Table 1, Fig. 4).

Hybridization to vitopine T-DNAs.

Vitopine Ti plasmids (V plasmids) have been found to have identical *Eco*RV T region patterns (L. Otten, unpublished). The *Eco*RV-digested DNAs of the new isolates were hybridized to an 18-kb insert of the vitopine T1-T2 region from the model V plasmid pTiS4 contained in clone pPM649 (Gérard et al. 1992). Fourteen isolates hybridized, all with the characteristic pTiS4 pattern (Table 1, not shown).

Hybridization to the AB4 T-DNA.

AB4 is an *A. vitis* strain with an N-type Ti plasmid. The pTiAB4 T-DNA has been cloned (clone N231, Otten and de Ruffray 1994) and partially sequenced. The left part of N231 hybridizes to the left part of the OL TA region, while the right part is pTiAB4-specific. N231 does not hybridize to vitopine T-DNAs nor to the OS TA-DNA or OS/OL TB-DNA. Clone N231 can therefore identify strains with N plasmids and OL plasmids. It was used as a probe against *Eco*RV-digested DNA of the new *A. vitis* isolates; 25 strains hybridized with the characteristic N plasmid pattern, 8 with the OL plasmid pattern (Table 1). CG111, 415, 474, 475, 477, 478, and 479 did not hybridize and therefore did not contain V, N, or OL plasmids. They were tested for virulence on stems of *Kalanchöe tubiflora*: CG111 was avirulent and was not further investigated, the others induced undifferentiated tumors (CG415, 475, 477, 478, 479) or tumors with shoots (CG474, results not shown).

Hybridization with other probes.

CG415, 474, 475, 477, 478, and 479 were further investigated with an OL virulence region probe (P28, Otten et al. 1993), an OS TA region probe and an OS TB region probe

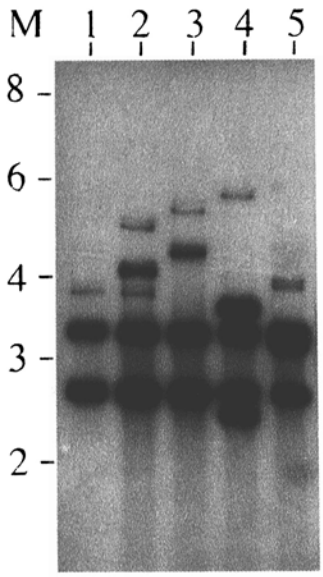

Fig. 3. Five new IS*866/867* patterns from representative *Agrobacterium vitis* strains. Lanes 1 to 5 (pattern numbers indicated in brackets): CG102 (24), CG412 (25), CG475 (26), CG108 (27), CG415 (28). M: molecular weight marker in kb. Patterns 1 to 23 have been defined in Paulus et al. 1989b.

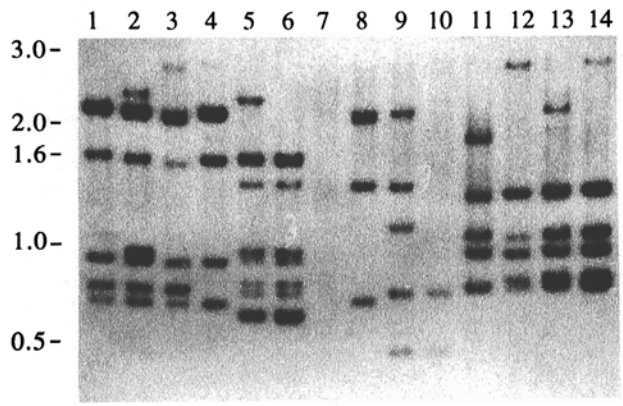

Fig. 4. IS*869* patterns from representative *Agrobacterium vitis* strains. Lanes 1 to 14 (pattern numbers indicated in brackets): CG49 (1), CG453 (2), CG486 (3), AB4 (4), CG81 (5), CG450 (6), CG56 (7), CG474 (8), CG98 (9), CG101 (10), B10/7 (11), 2612 (12), 2607 (13), and AB3 (14). M: molecular weight marker in kb.

(Bh5 and Bh6, van Nuenen et al. 1993). The hybridization patterns (not shown) demonstrated that CG415 and CG475, 477, 478, and 479 are OS strains of the Ag57/NW233 type (they have characteristic P28 and Bh6 patterns but lack IS*869*). CG474 does hybridize to the virulence region and the OS TA region but with unusual patterns (not shown). It does not hybridize to the OS TB region (found in strains with OL and OS plasmids).

DISCUSSION

Hybridization analysis of 54 new *A. vitis* isolates from the United States showed that most of their Ti plasmids could be classified into one of the groups defined earlier by Paulus et al. 1989a, confirming that *A. vitis* strains contain either o/c, nopaline, or vitopine plasmids.

Agrobacterium strain classification should be based on chromosomal characteristics, not on Ti plasmid properties like opine synthesis, opine utilization, or T-DNA structure. Although we found a strong correlation between chromosome and Ti plasmid structures, both in previous studies and in the present study, the correlation is not perfect. We therefore propose to abandon opine induction and utilization as criteria to classify and name *Agrobacterium* isolates. Practically, this means that one can no longer speak of octopine, vitopine, or nopaline strains as systematic groups, as has been usual up to now. Opines may still be used to classify Ti plasmids but further specification is clearly necessary. There are (for example) at least three different types of nopaline Ti plasmid (representatives of which are pTiC58 in an *A. tumefaciens* biotype I strain, pTi82139 in an *A. tumefaciens* biotype II strain, and pTiAB4 in an *A. vitis* strain, Otten and de Ruffray 1994) ; at the moment it is not clear how such plasmids should be named.

We propose to use the 16S-23S intergenic region of the rRNA operon to establish the phylogenetic relationships within *A. vitis* species. In this study we have defined two main IGS groups, A and B. Group B can be divided into B1 and B2, each subdivided further. The IGS data correlate very well with the *Eco*RV DNA digestion patterns, but contrary to the latter, also provide a clue to the phylogenetic relations between the chromosomes.

All isolates with N plasmids except CG56 belong to IGS group A1a and have similar IS*869* patterns and indistinguishable *Eco*RV restriction patterns (type I). This indicates that this group is of recent, clonal origin. CG111 belongs to the same group, but lacks a Ti plasmid and may therefore be a naturally cured derivative. V plasmids are found in three different IGS groups: A1b, B1a, and B1b. V plasmids may therefore have entered their bacterial "hosts" on more than one occasion. Group B1a also contains CG474 with a novel type of Ti plasmid (see below) and CG56 with an N plasmid. The latter strain may result from the transfer of an N plasmid from an A to a B1 background. The B2 group carries OL or OS plasmids but no V or N plasmids. CG108 is the only isolate outside group B2 with an OL plasmid and like CG56, may result from a Ti plasmid transfer event. The IS*869* and IS*867* patterns provide a means to distinguish between closely related strains which cannot be separated by IGS analysis.

It is clear from our studies that the different Ti plasmids are strongly associated with particular chromosomal "hosts," although exceptions do occur. Our studies provide for the first time information about the genetic relatedness between the *A. vitis* subgroups. If the close association between Ti plasmid types and chromosomes is further confirmed, it will become possible to analyze the evolutionary history of the different Ti plasmid-chromosome associations. These studies may be further refined by sequencing the IGS regions of selected strains, or even their complete *rrn* operons; the sequences of an *rrn* operon of strain S4 (which contains a V plasmid, Otten et al. 1996) and of strain K309 (the *A. vitis* type strain with an OL plasmid, Otten and de Ruffray 1996) have been obtained recently. For initial screening of large numbers of isolates, IGS ribo-fingerprinting is however more convenient.

CG474 represents a special case, although its chromosome belongs to group B1a, its T-DNA hybridization pattern is completely different from published T-DNA maps. Partial sequencing (L. Otten, unpublished data) shows that it is related to the octopine TL and TA-DNAs. Interestingly, pTiCG474 lacks the TB region and the IS*867* elements characteristic for the *A. vitis* o/c Ti plasmids. Further studies are required to establish its evolutionary origin.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Various *A. vitis* wild-type strains are listed in Table 1. All were shown to be *A. vitis* strains by classical microbiological tests (Paulus et al. 1989a; T. Burr, unpublished).

Bacterial growth and DNA isolation.

Strains were grown overnight in liquid LBMG medium (Lichtenstein and Draper 1986). Total DNA was prepared from 1.5-ml cultures according to Paulus et al. (1989a) and separated on 0.5 to 0.7% agarose gels in TAE buffer.

Virulence assays.

Kalanchöe tubiflora reacts to different *Agrobacterium vitis* strains with the formation of undifferentiated tumors, shooty or rooty tumors (Paulus et al. 1989a). Stems were punctured and inoculated with bacterial suspensions (10^8 bacteria/ml, Paulus et al. 1989a). Results were scored after 4 weeks.

PCR amplification and ribo-fingerprinting.

PCR amplifications were carried out in 100-µl volumes, the reaction mixture contained 50 ng of DNA, 2.5 mM $MgCl_2$, 5 pmole of each primer (Burr et al. 1995), 0.1 mM (each) of dNTP, 2.5 U Taq polymerase (Promega, Madison, WI), reaction buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton-X100) under 3 drops of mineral oil. Amplification was done in a PTC-100 programmable thermal controller (MJ Research, Inc., San Francisco) programmed for 2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C, and a final extension for 5 min at 72°C. Amplification products were purified using Millipore regenerated cellulose columns (Millipore Corp., Bedford, MA). Amplicons were digested with *Taq*I, *Ava*I, *Rsa*I, *Cfo*I, and *Hae*III purchased from Promega, and digests were analyzed by 3% agarose gel electrophoresis in TBE buffer. Gels were stained with 0.5 µg/ml of EtBr and photographed under a UV source.

Cluster analysis.

Similarities between strains were computed from the presence or absence of restriction fragments of amplicons by the

method of Nei and Li (1979). For each pair of strains, the Nei-Li coefficient is the number of fragments present in both strains divided by the average number of fragments present in both strains. To determine relationships between strains, these similarities were used in the unweighted pair group method using arithmetic means (UPGMA) cluster analysis procedure (Sneath and Sokal 1973), an option of the computer program NTSYS-pc (Rohlf 1988).

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LITERATURE CITED

Barry, T., Colleran, G., Glennon, M., Dunican, L. K., and Gannon, F. 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify Eubacteria. PCR Methods Appl. 1:51-56.

Bien, E., Lorenz, D., Eichhorn, K., and Plapp, R. 1990. Isolation and characterization of *Agrobacterium tumefaciens* from the German vine region Rheinpfalz. J. Plant Dis. Prot. 97:313-322.

Burr, T. J., and Katz, B. H. 1983. Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap and from vineyard soil. Phytopathology. 73:163-165.

Burr, T. J., Katz, B. H., and Bishop, A. L. 1987. Populations of *Agrobacterium* in vineyard and nonvineyard soils and grape roots in vineyards and nurseries. Plant Dis. 71:617-620.

Burr, T. J., Reid, C. L., Yoshimura, M., Momol, E. A., and Bazzi, C. 1995. Survival and tumorigenicity of *Agrobacterium vitis* in living and decaying grape roots and canes in soil. Plant Dis. 79:677-682.

Crouzet, P., and Otten, L. 1995. Sequence and mutational analysis of a tartrate utilization operon from *Agrobacterium vitis*. J. Bacteriol. 177:6518-6526.

Gérard, J.-C., Canaday, J., Szegedi, E., de la Salle, H., and Otten, L. 1992. Physical map of the vitopine Ti plasmid pTiS4. Plasmid 28:146-156.

Kerr, A., and Panagopoulos, C. G. 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Phytopathol. Z. 90:172-179.

Lichtenstein, C., and Draper, J. 1986. Genetic engineering of plants. Pages 67-119 in: DNA cloning, Vol. II: A Practical Approach. D. M. Glover, ed. IRL Press Oxford, Washington, DC.

Ma, D., Yanofsky, M. F., Gordon, M. P., and Nester, E. W. 1987. Characterization of *Agrobacterium tumefaciens* strains isolated from grapevine tumors in China. Appl. Environm. Microbiol. 523:1338-1343.

McGuire, R. G., Rodriguez-Palenzuela, P., Collmer, A., and Burr, T. J. 1991. Polygalacturonase production by *Agrobacterium tumefaciens* biovar 3 from grapevines. Int. J. Syst. Bacteriol. 40:236-241.

Nei, M., and Li, W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76:5267-5273.

Ophel, K., and Kerr, A. 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. Int. J. Syst. Bacteriol. 40:236-241.

Otten, L., Gérard, J-C., and de Ruffray, P. 1993. The Ti plasmid from the wide host range *Agrobacterium vitis* strain Tm4: Map and homology with other Ti plasmids. Plasmid 29:154-159.

Otten, L., and de Ruffray, P. 1994. *Agrobacterium vitis* nopaline Ti plasmid pTiAB4: Relationship to other Ti plasmids and T-DNA structure. Mol. Gen. Genet. 245:493-505.

Otten, L., Crouzet, P., Salomone, J-Y., de Ruffray, P., and Szegedi, E. 1995. *Agrobacterium vitis* strain AB3 harbors two independent tartrate utilization systems, one of which is encoded by the Ti plasmid. Mol. Plant-Microbe Interact. 8:138-146.

Otten, L., de Ruffray, P., de Lajudie, P., and Michot, B. 1996. Sequence and characterization of a ribosomal RNA operon from *Agrobacterium vitis*. Mol. Gen. Genet. 251:99-107.

Otten, L., and de Ruffray, P. 1996. Major differences between the *rrnA* operons of two strains of *Agrobacterium vitis*. Arch. Microbiol. 166:68-70.

Panagopoulos, C. G., Psallidas, P. G., and Alivizatos, A. S. 1978. Studies on biotype 3 of *Agrobacterium tumefaciens*. Pages 221-228: in Proc. Int. Conf. Plant Pathol. Bacteria. Angers, France.

Paulus, F., Huss, B., Bonnard, G., Ridé, M., Szegedi, E., Tempe, J., Petit, A., and Otten, L. 1989a. Molecular systematics of biotype III Ti plasmids of *Agrobacterium tumefaciens*. Mol. Plant-Microbe Interact. 2:64-74.

Paulus, F., Ridé, M., and Otten, L. 1989b. Distribution of two *Agrobacterium tumefaciens* insertion elements in natural isolates: Evidence for stable association between Ti plasmids and their bacterial hosts. Mol. Gen. Genet. 219:145-152.

Paulus, F., Canaday, J., Vincent, F., Bonnard, G., Kares, C., and Otten, L. 1991. Sequence of the *iaa* and *ipt* region of different *Agrobacterium tumefaciens* biotype III octopine strains: Reconstruction of octopine Ti plasmid evolution. Plant Mol. Biol. 16:601-614.

Perry, K. L., and Kado, C. I. 1982. Characteristics of Ti plasmids from broad-host range and ecologically specific biotype 2 and 3 strains of *Agrobacterium tumefaciens*. J. Bacteriol. 151:343-350.

Rodriguez-Palenzuela, P., Burr, T., and Collmer, A. 1991. Polygalacturonase is a virulence factor in *Agrobacterium tumefaciens* biovar 3. J. Bacteriol. 173:6547-6552.

Rohlf, F. J. 1988. NTSYS-pc. Applied Biostatistics, Setauket, New York.

Salomone, J-Y., Crouzet, P., de Ruffray, P., and Otten, L. 1996. Characterization and distribution of tartrate utilization genes in the grapevine pathogen *Agrobacterium vitis*. Mol. Plant-Microbe Interact. 9:401-408.

Sawada, H., Ieki, H., and Takikawa, Y. 1990. Identification of grapevine crown gall bacteria isolated in Japan. Ann. Phytopathol. Soc. Jpn. 56:199-206.

Sawada, H., Ieki, H., Oyaizu, H., and Matsumoto, S. 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. Int. J. Syst. Bacteriol. 43:694-702.

Sneath, P. H. A., and Sokal, R. R. 1973. Numerical Taxonomy. W. H. Freedman and Co., San Francisco, CA

Süle, S. 1978. Biotypes of *Agrobacterium tumefaciens* in Hungary. J. Appl. Bacteriol. 44:207-213.

Szegedi, E. 1985. Host range and specific L(+)-tartrate utilization of biotype 3 of *Agrobacterium tumefaciens*. Acta Phytopathol. Acad. Sci. Hung. 20:17-22.

Szegedi, E., Czako, M., Otten, L., and Koncz, C. 1988. Opines in crown gall tumours induced by biotype 3 isolates of *Agrobacterium tumefaciens*. Physiol. Mol. Plant Pathol. 32:237-247.

van Nuenen, M., de Ruffray, P., and Otten, L. 1993. Rapid divergence of *Agrobacterium vitis* octopine-cucumopine Ti plasmids from a recent common ancestor. Mol. Gen. Genet. 240:49-57.

Willems, A., and Collins, M. D. 1993. Phylogenetic analysis of Rhizobia and Agrobacteria based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. 43:305-313.