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Research Notes

Diverse Types of Tartrate Plasmids in *Agrobacterium tumefaciens* Biotype III Strains

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Twelve biotype III isolates of *Agrobacterium tumefaciens* that belong to three opine types were mated *in planta* with a plasmid-free biotype I recipient strain. When selection for tartrate utilization was imposed, seven of the 12 mating combinations resulted in transconjugants. In six combinations, large plasmids were transferred from the donor strains capable of tartrate utilization. In four of the six combinations, the transferred plasmids could be distinguished from the Ti plasmids and were named Tr plasmids. Tr plasmids form a diverse group; they differ in size, transfer frequency, and stability in a biotype I chromosomal background, and three of them were self-conjugal.

Isolates of *Agrobacterium tumefaciens* (Smith and Townsend) Conn are classified as three biotypes according to their chromosomal markers (Kerr and Panagopoulos 1977; Süle 1978). Biotype III strains, also called *A. vitis* (Ophel and Kerr 1990), show high specificity for grapevine; this is an example of ecological host specificity (Perry and Kado 1982). Biotype II and III strains utilize L-tartrate as a sole carbon source (Kerr and Panagopoulos 1977; Süle 1978); but biotype II strains prefer glucose to tartrate, whereas biotype III strains utilize tartrate more intensively than glucose (Szegedi 1985). Previous studies have shown that plasmid gene(s) can code for tartrate utilization in *Agrobacterium* (Gallie *et al.* 1984).

Biparental mating combinations were set up for testing whether the tartrate utilization trait could be transferred *in planta* and *ex planta* from biotype III *Agrobacterium* strains (Table 1) to a biotype I recipient strain. Strain UBAPF2, which is a Ti-plasmid-cured, rifamycin-resistant derivative of the biotype I strain C58 (Hynes *et al.* 1985), was used. For *in planta* matings, sterile grapevine (*Vitis vinifera* L. 'Narancsízü') tumors induced with an octopine- (Tm4), a nopaline- (AT1), and a vitopine-type (S4) strain, respectively, were established as described by Szegedi *et al.* (1988). Two-day-old cultures of the strains, which were grown at 25° C on mannitol-glutamate Luria broth (MGLB) plates (Lichtenstein and Draper 1985), were resuspended in 10 mM $MgSO_4$ (10^8 cells per milliliter, as determined by serial dilution and plating of bacterial suspensions onto solid MGLB medium) and mixed with the recipient strain UBAPF2 (3:1, donor/recipient). A 5-μl drop of mixed suspensions was inoculated onto the appropriate tumor tissue (e.g., octopine strains were mated with UBAPF2 on a Tm4 tumor) and incubated for 4 days at 25° C. The matings were repeated three to four times. A 100-mg piece of the tumor tissue was homogenized in 500 μl of distilled water. One-hundred-microliter samples (two to four replicates) were plated onto AB minimal medium (Lichtenstein and Draper 1985) containing 0.5% (w/v) L(+)tartrate (K-Na salt of tartaric acid was used throughout the experiments) and 60 μg/ml of rifamycin. As a maximum, $5–10 \times 10^9$ cells were plated from a mating combination. After 5–6 days of incubation at 25° C, single colonies were picked and resuspended in 3 ml of 10 mM $MgSO_4$ and repeatedly purified on the same medium. Transconjugants were stored on selective medium at 4° C. *Ex planta* matings were done on MGLB medium.

Strain UBAPF2 is resistant to rifamycin and shows the characteristic properties of biotype I (3-ketolactose production, growth on melezitose, but not on tartrate; Kerr and Panagopoulos 1977; Süle 1978). Because UBAPF2 never acquired tartrate utilization spontaneously, colonies growing on minimal medium containing tartrate and rifamycin should be either transconjugants or spontaneous rifamycin-resistant biotype III colonies after the mating procedure. To distinguish between these types, we tested all of the isolated colonies for 3-ketolactose production (Kerr and Panagopoulos 1977) and for growth in AB minimal medium supplemented with 0.5% (w/v) melezitose. Seven of the 12 mating combinations resulted in transconjugant colonies, and rifamycin-resistant biotype III colonies also occasionally occurred. Matings with Tm4, AB3, AT66, and NW221 as donors yielded between one and eight transconjugant colonies. In contrast, conjugations with strains AT6, AB4, and S4 resulted in $10^3–10^4$ colonies. From each mating combination, three to eight colonies were chosen for further characterization with the exception of UBAPF2

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and Tm4, from which only one transconjugant colony was obtained, and UBAPF2 and AT66, from which only two transconjugant colonies were obtained. In the case of strains Hm1, AT1, Ni1, Sz1, and NW11 (all tartrate-utilizing), tartrate utilization was not transferred under these conditions. *Ex planta* matings on MGLB plates only sporadically resulted in transconjugant colonies (one to two colonies, irregularly), suggesting that the plasmid transfer is induced by plant- or tumor-specific factor(s).

We purified plasmid DNA from each of the representative tartrate-utilizing transconjugants by using the high-temperature alkaline method of Kado and Liu (1981). In each case, electrophoresis in 0.5% agarose gel showed the presence of a large plasmid (Fig. 1, top panel), which was also present in the donor strains, except in the case of AB3 (lanes 7–8). Because these plasmids are associated with tartrate degradation, we call them Tr plasmids, and we add the name of the strain from which each was derived (e.g., pTrAB4 in AB4). Plasmids in colonies from the UBAPF2 and Tm4 and the UBAPF2 and AT66 matings were only detected if these strains were grown under selective pressure. In AB3, with the Ti plasmid and an additional large plasmid (Fig. 1, lane 7), there is an approximately 50-kbp plasmid detectable only when cell lysis at 95° C, which completely removes chromosomal DNA, is carried out (Kado and Liu 1981). Although all eight UBAPF2 × AB3 transconjugants grew well on tartrate, none of those plasmids could be detected in these strains as is shown for one exconjugant in Figure 1 (top panel, lane 8).

Biotype III strains prefer tartrate to glucose when tartrate and glucose are both present in minimal medium, and these strains cause an alkaline pH change in the medium. In contrast, biotype II strains, which prefer glucose to tartrate, and biotype I strains, which can metabolize only the glucose, cause an acidic pH change in the medium (Szegedi 1985). Glucose and tartrate were added in equal (i.e., 0.5%:0.5%, w/v) amounts to AB minimal medium. The tartrate-utilizing biotype I strains from matings between UBAPF2 and AT6, AB3, AB4, S4, or NW221 caused an alkaline pH change (blue coloration of bromothymol blue), indicating that they metabolize tartrate as efficiently as the biotype III donor strains. But biotype I strains from the UBAPF2 and Tm4 and the UBAPF2 and AT66 matings caused an acidic pH change in the medium, indicating glucose preference.

Pathogenicity of all of the colonies was tested on the stems of greenhouse-grown sunflower, *Kalanchoë tubiflora* Hamet, and grapevine (Rhine riesling) plants (Szegedi 1985). Wild-type donor strains were pathogenic on all three species. Among the transconjugants, only colonies from the UBAPF2 and Tm4 and the UBAPF2 and AT66 matings were tumorigenic. Tumors initiated by these strains contained the characteristic opine compounds (Fig. 2) when assayed according to Otten and Schilperoort (1978). Because all colonies from a given mating combination had the same properties with respect to all traits studied so far, only one colony per mating combination was used for further analysis.

The demonstration of opine utilization by the transconjugants showing virulence substantiated the presence of the Ti plasmid. Octopine or nopaline was provided as the sole carbon source supplied at 0.2% (w/v) in AB minimal medium (Szegedi 1985). Vitopine utilization was assayed according to Szegedi *et al.* 1988. The virulent transconjugant from the UBAPF2 and Tm4 mating grew on octopine, whereas the virulent transconjugant from the UBAPF2 and AT66 mating grew on nopaline. No other transconjugant showed opine utilization.

We tested the stability of the tartrate-utilizing plasmids by using the method of Gallie and Kado (1987). Bacteria were grown under nonselective conditions in 523 medium

Table 1. Donor bacterial strains used in this study

Strain	Opine marker	Date of isolation	Source[a]	References[b]
AT6	Octopine	1976	J. Lehoczky	1,2
Hm1	Octopine	1981	E. Szegedi	1,2
Tm4	Octopine	1980	E. Szegedi	1,2
AB3	Octopine	1982	E. Szegedi	1,2
AT1	Nopaline	1967	J. Lehoczky	1,2
AT66	Nopaline	1977	J. Lehoczky	1,2
Ni1	Nopaline	1980	E. Szegedi	1,2
AB4	Nopaline	1982	E. Szegedi	1,2
S4	Vitopine	1981	E. Szegedi	1,2
Sz1	Vitopine	1981	E. Szegedi	1,2
NW11	Vitopine	1984	E. Bien	3
NW221	Vitopine	1985	E. Bien	3

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[b] 1, Szegedi 1985; 2, Szegedi *et al.* 1988; 3, Bien *et al.* 1990 and E. Szegedi, unpublished.

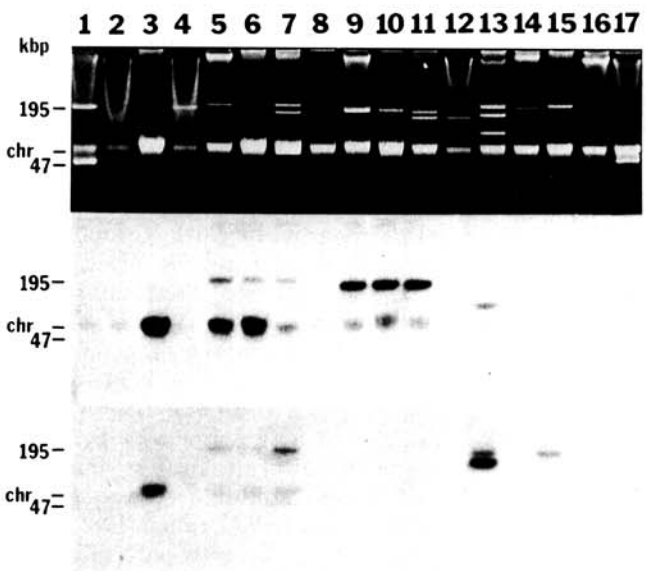

Fig. 1. Plasmid screen (top panel) and hybridization analysis with TL-Ach5 (middle panel) and IS866-Tm4 (lower panel) probes of wild-type strains and their tartrate-utilizing transconjugants. Lane 1, *Agrobacterium radiobacter* K84 containing a 47-kbp and a 195-kbp plasmid (Merlo and Nester 1977); 2, UBAPF2; 3, AT6; 4, UBAPF2(pTrAT6), 192 kbp; 5, Tm4; 6, tartrate-utilizing transconjugant from UBAPF2 × Tm4, 200 kbp; 7, AB3; 8, tartrate-utilizing transconjugant from UBAPF2 × AB3; 9, AT66; 10, tartrate-utilizing transconjugant from UBAPF2 × AT66, 192 kbp; 11, AB4; 12, UBAPF2(pTrAB4), 169 kbp; 13, S4; 14, UBAPF2(pTrS4), 192 kbp; 15, NW221; 16, UBAPF2(pTrNW221), 200 kbp; 17, 1D1422 (Gallie *et al.* 1984), 44 kbp. Chr, chromosomal DNA. Tartrate-utilizing transconjugants from UBAPF2 × Tm4 and from UBAPF2 × AT66 were grown in AB minimal medium with 1% (w/v) K-Na tartrate; the others were grown in MGLB medium.

(Kado *et al.* 1972) for at least 50 cell generations. Subsequently, a 10-μl vol of the culture was diluted in 1 ml of sterile distilled water, and a loopful was streaked onto solid 523 medium. Twenty colonies were tested for growth on tartrate. The stability tests showed that the loss was greater than or equal to 95% for plasmids in colonies from the UBAPF2 and Tm4 and the UBAPF2 and AT66 matings after 50 generations of growth in 523 medium, because none of the 20 colonies isolated from each transconjugant grew on tartrate. None of the eight tartrate-nonutilizing colonies picked from both transconjugants for screening contained the plasmid(s) initially acquired from Tm4 or AT66, and they all became nonvirulent. The plasmid loss, also observed on glucose-tartrate medium, was responsible for the inefficient tartrate utilization and consequent acidic reaction caused by these transconjugants. pTrS4 was partially stable (loss was ≥15%). The remaining plasmids (pTrAT6, pTrAB4, and pTrNW221) were stable in UBAPF2 (loss was <5%), as shown by growth on tartrate and by plasmid miniscreens of three to five randomly chosen colonies per transconjugant. Similarly, the tartrate utilization gene(s) proved to be stable in all the eight transconjugants from the UBAPF2 and AB3 mating (160 colonies tested).

To test if the Tr plasmids are self-conjugal, we mated transconjugants with UBAPF2NR as the recipient on the appropriate tumors. Strain UBAPF2NR is a spontaneous nalidixic-acid-resistant derivative of UBAPF2 selected in our laboratory. Only UBAPF2(pTrAT6), UBAPF2(pTrAB4), and UBAPF2(pTrS4) were used because of the relatively high initial transfer frequency and the stability of these Tr plasmids in UBAPF2. Colonies were selected for resistance to nalidixic acid (50 μg/ml of nalidixic acid, Na salt) and tartrate utilization. These matings resulted in similar numbers of colonies acquiring the Tr plasmids, as in the case of UBAPF2NR and wild-type (AT6, AB4, or S4) combinations, which showed that these Tr plasmids are self-conjugal. On control plates, only one spontaneous nalidixic-acid-resistant colony was obtained.

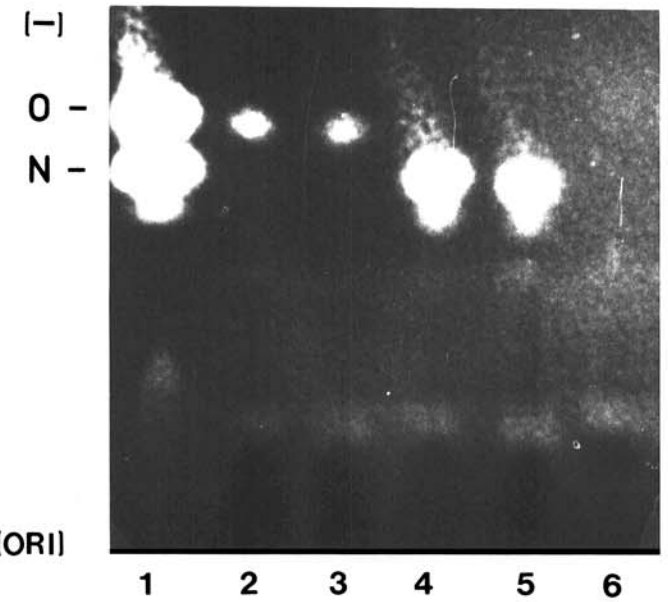

Fig. 2. Paper electrophoretic detection of opine compounds in extracts of grapevine tumors induced by Tm4 (lane 2), a tartrate-utilizing transconjugant from UBAPF2 × Tm4 (lane 3), AT66 (lane 4), and a tartrate-utilizing transconjugant from UBAPF2 × AT66 (lane 5). Lane 1, 2.5 μg of each of octopine (O) and nopaline (N). Lane 6, wound callus extract of a plant inoculated with UBAPF2. The origin of electrophoretic migration (ORI) and the direction of the cathode (−) are indicated.

We carried out DNA-DNA hybridization experiments to examine the relatedness of the Tr and Ti plasmids. The following probes were used: TL-Ach5, the TL-region of pTiAch5 (combined clones pGV153 and pGV201 covering 12.5 kbp; De Vos *et al.* 1981), which hybridizes with the T-region of the octopine strains Tm4 and AB3, with the T-region of the nopaline strains AT66 and AB4, and with the 100-kbp non-Ti plasmid of S4 (Paulus *et al.* 1989a; this study); IS866-Tm4, an internal 1.2-kbp *Hin*dIII-*Bam*HI fragment of the insertion element IS866, isolated from pTiTm4, which is specific for biotype III octopine and vitopine Ti plasmids, as well as for the 153-kbp non-Ti plasmid of S4 (Bonnard *et al.* 1989; Paulus *et al.* 1989b). All DNA manipulations were done according to standard protocols (Maniatis *et al.* 1982).

In the cases of AB4 (Fig. 1, middle and lower panels, lane 11) and S4 (lane 13), the Ti plasmids could be clearly distinguished from the Tr plasmids by their size and by their T-region-specific hybridization. In strain AT6, the probes did not hybridize to the plasmid bands but to the region of the chromosomal band in the donor (lane 3). In NW221 (lane 15), Ti and Tr plasmids were the same size. The Tr plasmids of the above four strains did not show homology either with TL-Ach5 (Fig. 1, middle panel) or IS866-Tm4, or both (Fig. 1, lower panel, lanes 3–4, 11–12, 13–14, and 15–16, respectively).

In the cases of Tm4 and AT66, the two T-DNA-specific probes hybridized with plasmids of wild-type strains and with plasmids of their transconjugant derivatives (Fig. 1, lanes 5–6 and 9–10). In Tm4 (lane 5), the intensity of the hybridization to the chromosomal DNA debris varied considerably, indicating the presence of sheared plasmid DNA. These data show that these transconjugants are virulent and able to utilize the respective opine (see above). Whether the genes responsible for tartrate utilization and tumor induction are located on the same plasmid in these strains or whether the Tr and Ti plasmids of Tm4 and AT66 have very similar molecular sizes and are co-transferred into UBAPF2 is not known. The tartrate-utilizing transconjugants from matings between UBAPF2 and AB3 did not contain any detectable plasmid. Total DNA from these transconjugants showed a high level of homology with AB3 total plasmid DNA (data not shown), raising the possibility that chromosomal integration (see examples in *Pseudomonas*; Curiale and Mills 1982; Mucha and Farrand 1986) of the tartrate utilization gene(s) took place in this particular mating combination.

The Tr plasmids identified in this study represent a different group of tartrate plasmids than those previously described (Gallie *et al.* 1984), because they are much larger and do not cross-hybridize with pTAR (data not shown), which encodes tartrate utilization in the 1D1422 biotype I strain (Gallie *et al.* 1984). The Tr plasmids form a diverse group with respect to several characteristics in the biotype

I recipient strain used. How common the plasmids carrying gene(s) for catabolism of tartrate are in a much larger sample of biotype III strains isolated from grapevine is not known. Grapevine contains significant amounts of tartrate (Ruffner 1982), which may be a factor in host specialization (Gallie and Kado 1988). Determining if the Tr plasmids contribute in the grapevine specialization of biotype III and how the conjugative activity of certain Tr plasmids might promote their distribution in nature is of interest.

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LITERATURE CITED

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

Bonnard, G., Vincent, F., and Otten, L. 1989. Sequence and distribution of IS866, a novel T region-associated insertion sequence from *Agrobacterium tumefaciens*. Plasmid 22:70-81.

Curiale, M. C., and Mills, D. 1982. Integration and partial excision of a cryptic plasmid in *Pseudomonas syringae* pv. *phaseolicola*. J. Bacteriol. 152:797-802.

De Vos, G., De Beuckeleer, M., van Montagu, M., and Schell, J. 1981. Restriction endonuclease mapping of the octopine tumor-inducing plasmid pTiAch5 of *Agrobacterium tumefaciens*. Plasmid 6:249-253.

Gallie, D., Zaitlin, D., Perry, K. L., and Kado, C. I. 1984. Characterization of the replication and stability regions of *Agrobacterium tumefaciens* pTAR. J. Bacteriol. 157:739-745.

Gallie, D., and Kado, C. I. 1987. *Agrobacterium tumefaciens* pTAR promoter region involved in autoregulation, incompatibility and plasmid partitioning. J. Mol. Biol. 193:465-478.

Gallie, D., and Kado, C. I. 1988. Minimal region necessary for autonomous replication of pTAR. J. Bacteriol. 170:3170-3176.

Hynes, M. F., Simon, R., and Pühler, A. 1985. The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pAtC58. Plasmid 13:99-105.

Kado, C. I., Heskett, M. G., and Langley, R. A. 1972. Studies on *Agrobacterium tumefaciens*: Characterization of strains 1D135 and B6, and analysis of the bacterial chromosome, transfer RNA and ribosomes for tumor inducing ability. Physiol. Plant Pathol. 2:47-57.

Kado, C. I., and Liu, S. T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.

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. 1985. Genetic engineering of plants. Pages 67-119 in: DNA Cloning. Vol. 2. A Practical Approach. D. M. Glover, ed. IRL Press, Washington, DC.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Merlo, D. J., and Nester, E. 1977. Plasmids in avirulent strains of *Agrobacterium*. J. Bacteriol. 129:76-80.

Mucha, D. K., and Farrand, S. K. 1986. Diversity of determinants encoding carbenicillin, gentamycin and tobramycin resistance in nosocomial *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 30:281-289.

Ophel, H., 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. A. B. M., and Schilperoort, R. A. 1978. A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activities. Biochim. Biophys. Acta 527:497-500.

Paulus, F., Huss, B., Bonnard, G., Ride, 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., Ride, 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.

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.

Ruffner, H. P. 1982. Metabolism of tartaric and malic acids in Vitis: A review. Part A. Vitis 21:247-259.

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