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Agrobacterium vitis Strain AB3 Harbors Two Independent Tartrate Utilization Systems, One of Which Is Encoded by the Ti Plasmid

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Several strains of the grapevine-associated bacterial pathogen *Agrobacterium vitis* contain large plasmids encoding tartrate utilization. These plasmids can be transferred to the cured *Agrobacterium tumefaciens* strain UBAPF2 by conjugation in grapevine tissues. Among *A. vitis* strains, AB3 is exceptional since the tartrate-positive UBAPF2 strains which can be obtained by AB3xUBAPF2 conjugation lack a detectable plasmid. When UBAPF2 was transformed with AB3 plasmid DNA, tar$^+$ transformants were found which harbored a large plasmid, called pTrAB3. The 245-kb pTrAB3 plasmid was isolated, subcloned, and mapped. Hybridization of total DNA from UBAPF2xAB3 transconjugants with pTrAB3 subclones revealed that in most transconjugants pTrAB3 is linked to a 60-kb AB3 plasmid (called pAB3b) or part of it. Recombination occurs within a 2.65-kb *Pst*I fragment in pTrAB3, and in most cases in a 9.0-kb *Pst*I fragment of pAB3b. The pTrAB3::pAB3b cointegrate structure is itself integrated into a larger DNA molecule, presumably the UBAPF2 chromosome. The tartrate utilization (TAR) region of pTrAB3 was cloned on a 9.5-kb DNA fragment. In addition to the pTrAB3 TAR region, the analysis of a rare tar$^+$ UBAPF2xAB3 transconjugant which lacked pTrAB3 sequences and carried a non-integrated pTiAB3 plasmid revealed an additional TAR region. The pTiAB3 TAR region was cloned and located on the earlier established map of pTiAB3. The TAR regions of pTrAB3 and pTiAB3 do not cross-hybridize and have different functional properties. The strain with the integrated pTrAB3 plasmid differs in its utilization of tartrate from the strain with the nonintegrated pTrAB3 plasmid. Tartrate utilization by AB3 is therefore a complex and redundant property.

Additional keywords: grapevine, crown gall, host range, plasmid transfer, bacterial conjugation.

The *Agrobacterium* species *Agrobacterium vitis* (Ophel and Kerr 1990), previously called *Agrobacterium tumefaciens* biotype III (Kerr and Panagopoulos 1977; Süle 1978) has been isolated almost exclusively from grapevine (Panagopoulos *et al.* 1978; Perry and Kado 1982; Burr and Katz 1983; Burr *et al.* 1987; Ma *et al.* 1987; Bien *et al.* 1990; Sawada *et al.* 1990). Part of this host specificity has been attributed to an *A. vitis*-specific polygalacturonidase gene which is required for grapevine root necrosis (McGuire *et al.* 1991; Rodriguez-Palenzuela *et al.* 1991). Szegedi (1985) has shown that *A. vitis* isolates can be distinguished from other *Agrobacterium* strains by their tartrate utilization properties: They prefer tartrate to glucose, whereas other strains prefer glucose to tartrate or do not utilize tartrate (Kerr and Panagopoulos 1977; Süle 1978). Tartrate utilization most probably represents an adaptation to growth in grapevine tissues which contain considerable amounts of tartaric acid (Ruffner 1982).

Early work has demonstrated that the tartrate-utilizing capacity of an unusual *A. tumefaciens* strain isolated from grapevine is encoded by a plasmid (Gallie *et al.* 1984). Recently, we have found that several *A. vitis* strains contain large plasmids encoding tartrate utilization, as shown by transfer to the *A. tumefaciens* recipient strain UBAPF2 by conjugation *in planta*. In the course of these studies it was noted that conjugation between UBAPF2 and the *A. vitis* donor strain AB3 yielded tartrate utilizing (tar$^+$) transconjugants which lacked free plasmid DNA molecules (Szegedi *et al.* 1992). Here we present the molecular analysis of these transconjugants and report the discovery and analysis of a second type of tar$^+$ transconjugant.

RESULTS

Localization of tartrate utilization genes on pTrAB3.

Strain AB3 belongs to the so called "small TA" or limited host range strains within the o/c subgroup of *A. vitis* (Paulus *et al.* 1989a). AB3 is virulent on grapevine and contains four plasmids: the 234-kb Ti plasmid which has been extensively studied, and three other plasmids (approximate sizes: 245, 160, and 60 kb). The present work shows that the 245 kb plasmid encodes tartrate utilization; it has therefore been called pTrAB3, the functions of the other two plasmids are unknown; we have called them pAB3a (160 kb plasmid) and pAB3b (60 kb plasmid). Earlier studies (Szegedi 1985) showed that strain AB3 prefers tartrate to glucose like most *A. vitis* strains. This property can be transferred to the non-tartrate utilizing rifamycin-resistant plasmidless *A. tumefaci-*

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ens strain UBAPF2, by infecting sterile grapevine tissues with a mixture of AB3 and UBAPF2; transconjugants are selected on AB minimal medium supplemented with rifamycin and tartrate. The frequency of tartrate utilization is low: Only 1–10 colonies are obtained per mixed infection experiment. Stability tests with the transconjugants described in this paper showed that in most cases tartrate utilization is stable under non-selective conditions, only in the case of A595 (UBAPF2[pTiAB3], see below) did 1% of the colonies lose their tartrate utilization capacity. However, this instability did not prevent plasmid isolation or growth analysis of this strain (see below). Contrary to other UBAPF2x *A. vitis* transconjugants, tar$^+$ transconjugants from UBAPF2xAB3 mixtures did not contain free plasmid DNA (Szegedi *et al.* 1992). We nevertheless assumed that the tar$^+$ phenotype was due to the transfer of tartrate utilization genes from AB3 to UBAPF2. This transfer could have occurred in different ways. In a first approach we tested the possibility that the tartrate utilization genes were located on a plasmid as in the case of the other *A. vitis* strains which yielded tar$^+$ transconjugants. To this end, UBAPF2 was transformed with purified total plasmid DNA from AB3 and transformants were selected on ABT and rifamycin. Two tartrate-utilizing UBAPF2 transformants (A530 and A531) were obtained (transconjugants and transformants used in this article are listed in Table 1). A530 contained two plasmids of about 240 and 250 kb. Restriction analysis of purified plasmid DNA of A530 showed the presence of several bands in addition to the pattern obtained with purified AB3 plasmid DNA (results not shown). This indicated possible rearrangements or amplifications; A530 was therefore not further investigated. A531 only contained the 245-kb plasmid. This plasmid was isolated and digested with *Eco*RI, *Pst*I, and *Hin*dIII; its restriction pattern differed from that of pTiAB3 (van Nuenen *et al.* 1993, not shown). The A531 plasmid most probably carried the tartrate utilization gene(s) permitting growth of A531 on ABT. We therefore named it pTrAB3 for tartrate utilization plasmid. Since the tartrate-selected transconjugants did not contain a detectable plasmid, we assumed that part or all of pTrAB3 was integrated into the UBAPF2 genome. To detect the presence of pTrAB3 sequences in the transconjugants, we first subcloned pTrAB3 and determined its restriction map.

Table 1. Tar$^+$ UBAPF2 transconjugants from UBAPF2xAB3 conjugation experiments and tar$^+$ UBAPF2 transformants[a]

Strain	Conj. experiment	*Pst*I fragments	Conjug. conditions
A499-A500	–	3.6/11	Tm4 tumor
A501-A502	–	4.3/10	Tm4 tumor
A503	–	10	Tm4 tumor
A505	–	3.9/12	Tm4 tumor
A506	–	19/25	Tm4 tumor
A590-A591-A592	AI	1.45*	AB3 tumor
A593	AIII	20.0	AB3 tumor
A594	AIV	3.6/9.5	AB3 tumor
A595-A596	AV	1.45*	AB3 tumor
A597-A598-A599	BI	4.9/8.0	AB3 tumor
A600-A601-A602	BII	4.9/8.0	AB3 tumor
A603	BIII	4.9/8.0	AB3 tumor
A604	BIV	4.9/8.0	AB3 tumor
A605	BIV	2.2/12.5	AB3 tumor
A606	BV	4.5/8.3	AB3 tumor
A607	BV	4.9/8.0	AB3 tumor
A608	BV	4.9/8.0	AB3 tumor
A609-A610	BVI	4.9/8.0	AB3 tumor
A611-A612-A613	BVII	4.9/8.0	AB3 tumor
A530	(transformant)	1.45*	–
A531	(transformant)	1.45*	–
A656	(recipient UBAPF2)	–	–

[a] Asterisks indicate fragments belonging to pTiAB3.

Cloning and mapping of pTrAB3.

Partial *Pst*I fragments of pTrAB3 from A531 were cloned in pUC18 and mapped with *Pst*I, *Eco*RI, and *Hin*dIII. The pTrAB3 plasmid (Fig. 1) has a size of 245 kb. The left site of the 4.6-kb *Hin*dIII fragment was arbitrarily chosen as 0 coordinate. Ten representative subclones (labeled pTrX, Fig. 1) covering the plasmid were used to analyze the UBAPF2xAB3 transconjugants.

DNA analysis of transconjugants.

Southern blots with *Pst*I-digested total DNA of one of the UBAPF2xAB3 transconjugants (A499) were probed with each of the 10 pTrAB3 subclones (Fig. 1). All except pTr133 revealed the *Pst*I bands expected from the pTrAB3 map (not shown). In the hybridization pattern obtained with probe pTr133 the 2.65 kb *Pst*I fragment (P2.65, coordinates 110.5–113.0) was replaced by two fragments of 4.9 and 8.0 kb. These probably represent junction fragments consisting of pTrAB3 and target DNA sequences. Thirty additional transconjugants (500–503, 505, 506, 590–613) were analyzed by hybridization to pTr133. In all cases, P2.65 was absent and two new fragments, variable in their sizes, were found (Table 1, Fig. 2). Strains derived from the same conjugation mixture

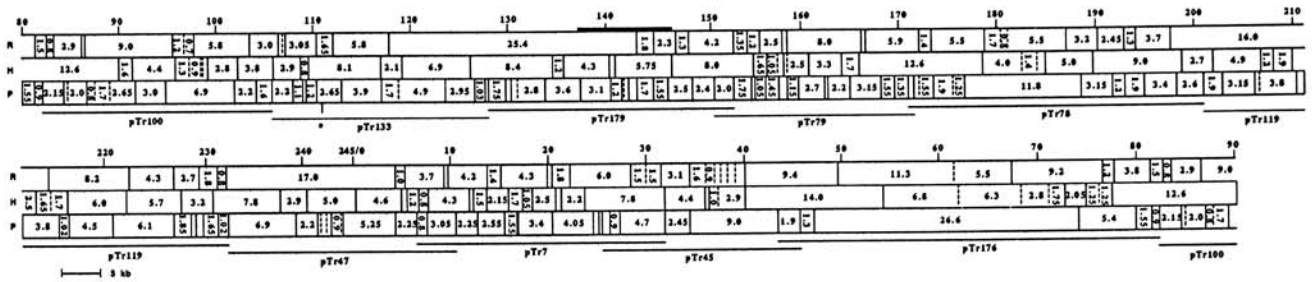

Fig. 1. Map of pTrAB3. Partial *Pst*I clones are indicated with numbers below the map. The 9.5-kb fragment carrying the tartrate utilization genes is shown above the map. The recombination site in P2.65 is indicated by an asterisk.

sometimes showed different patterns (conjugation experiments BIV and BV), whereas strains from different conjugations sometimes showed the same pattern, i.e., the 4.9/8.0-kb pattern (BI–BVII). Thus, pTrAB3 integrates at a preferential site both within P2.65 and within the target DNA. In transconjugant A503 the P2.65, P2.2, P1.1, and P1.2 fragments were lacking, indicating a deletion to the left of the P2.65 insertion site. This strain was not investigated further.

One of the transconjugants with the common 4.9/8.0-kb pattern (A597, BI) was used to clone a junction fragment in order to identify the target DNA in the different transconjugants (see below).

Five transconjugant strains (A590, A591, A592, A595, and A596) from two different matings (AI and AV) hybridized only weakly to pTr133, showing a single 1.45-kb *Pst*I band.

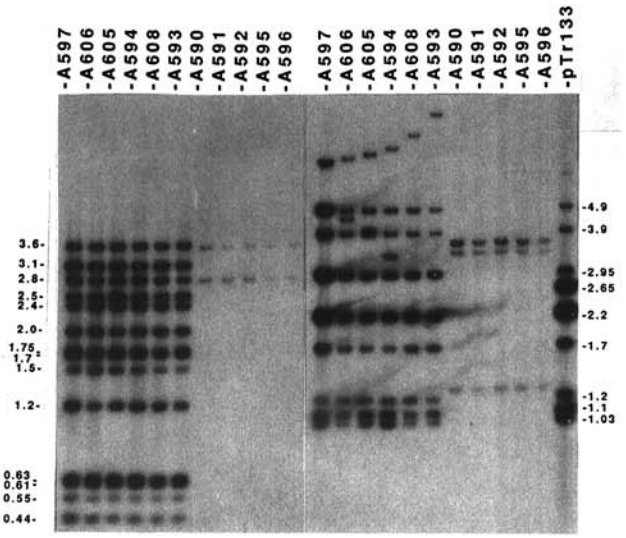

Fig. 2. Southern analysis of *Pst*I-digested, total DNA from representative UBAPF2xAB3 transconjugants. pTrAB3 subclones pTr133 and pTr179 were used as probes. pTr133: *Pst*I-digested pTr133 DNA. Sizes of clone-internal fragments are indicated in kb. Left: Hybridization to pTr179, which carries the tartrate utilization gene(s). Right: Hybridization to pTr133, which carries the integration site.

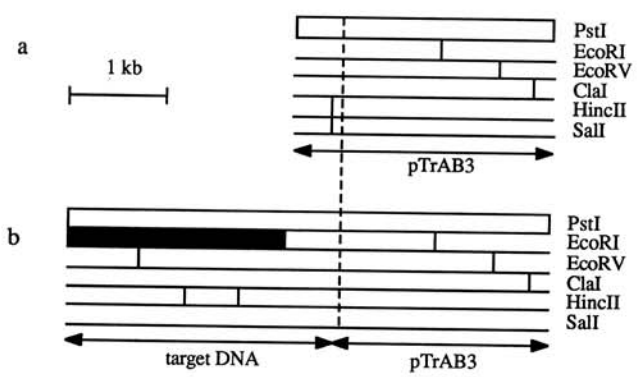

Fig. 3. Integration region on pTrAB3. **A,** Map of the unmodified 2.65-kb *Pst*I fragment from pTrAB3. **B,** Map of junction fragment isolated from A597 containing the right part of the 2.65-kb *Pst*I fragment from pTrAB3 and 2.2 kb of unknown target DNA. The dashed line represents the junction site as determined by sequencing. Black: fragment used for identification of target DNA in AB3.

Plasmid analysis of A595 and A596 showed a single plasmid band of the size of the AB3 Ti plasmid (234 kb, see below), this band was lacking in A590–592. A595 and A596 were virulent on *Kalanchoe* and induced the synthesis of octopine, contrary to A590–A592 and the other transconjugants (not shown). The analysis of A595 is described below, A590–592 were not investigated further.

Identification of pTrAB3 integration site.

*Pst*I fragments of total DNA of transconjugant A597 were cloned into pUC18. Colony hybridization with P2.65 identified a plasmid (pPM1093) with a 4.9-kb *Pst*I insert. The right end of the restriction map of this insert (Fig. 3B) can be aligned with the right end of P2.65 (Fig. 3A), the left part represents the target DNA sequence. Sequencing of P2.65 kb and part of the 4.9-kb junction fragment showed the junction site to be located 492 bp from the left end of P2.65 (not shown). pPM1093 did not hybridize to Southern blots of UBAPF2 DNA. We therefore supposed that pTrAB3 had inserted into another AB3 DNA sequence before transfer to UBAPF2. Hybridization of the 2.2-kb *Eco*RI fragment of pPM1093 (entirely composed of target sequences) to Southern blots with plasmid preparations of several *A. vitis* strains (limited host range o/c strains AT6, AB3, Zw2, B10/7, wide host range o/c strains Hm1, Tm4, K305, NW180, nopaline strains AT1, AT66, EK2, Ni1, Rr4, IS1.1, AB4, and vitopine strains S4, Sz1, Sz2, NW11, NW221, and 2681, control: UBAPF2) only showed hybridization to the 60-kb pAB3b plasmid of AB3 (not shown). Thus, in the transconjugants, pTrAB3 is linked to part or all of pAB3b. Since such a cointegrate would be about 300 kb in size, it should have been detectable in plasmid screens. We therefore assume that the pTrAB3-pAB3b cointegrate has integrated into the UBAPF2 chromosomal DNA. Southern blots with restricted total plasmid DNA from AB3 showed *Pst*I and *Eco*RI bands of 9.0 and 13.0 kb, respectively (not shown).

Cloning of tartrate utilization gene(s) from pTrAB3.

To confirm that pTrAB3 contains the tartrate utilization gene(s) and to locate these genes on the pTrAB3 restriction map, partial *Bam*HI fragments of pTrAB3 (isolated from A531) were cloned into the broad host range vector pBin19 and introduced into UBAPF2 by transformation. Several clones conferring tartrate utilization were identified on ABT medium, the smallest one (pPM4600, not shown) contained a single *Bam*HI fragment of 10 kb. A 9.5-kb DNA fragment from pPM4600 was recloned into the broad host range vector pMP2733 (yielding pPM405) as indicated in Materials and Methods. pPM405 confers tartrate utilization upon UBAPF2 and therefore contains a complete tartrate utilization (TAR) system. Restriction analysis of this fragment (not shown) and

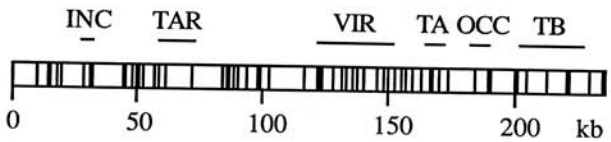

Fig. 4. Simplified map of pTiAB3, redrawn from van Nuenen *et al.* 1993. Indicated are: *Hin*dIII sites, virulence region (VIR), TA and TB region (TA, TB), octopine catabolism region (OCC), incompatibility region (INC) and tartrate utilization region (TAR). Coordinates are in kb.

comparison with the pTrAB3 map showed it to be located between pTrAB3 coordinates 137.0 and 146.5, about 30 kb to the right of the recombination site in P2.65 (Fig. 1). This region is part of pTrAB3 subclone pTr179.

Analysis of transconjugants without pTrAB3 sequences.

Contrary to other UBAPF2xAB3 transconjugants, A595 and A596 were found to contain a plasmid of about 240 kb and were virulent. Southern analysis of restricted total A595 and A596 DNA using the pTrAB3 subclone pTr179 (carrying the TAR region as a probe) did not reveal the expected pTrAB3 bands but did produce a weakly hybridizing signal with a 1.45-kb *Pst*I fragment (Fig. 2B). Strains A590–592 showed the same hybridization pattern, but lack free plasmid DNA and are avirulent. We concluded that the tar$^+$ phenotype of A590–592 and A595–596 was not due to pTrAB3 sequences but to another TAR system located on the Ti plasmid. Restriction analysis of purified A595 plasmid DNA with *Eco*RI, *Hin*dIII, and *Pst*I (not shown) identified the 240-kb plasmid as pTiAB3 (van Nuenen *et al.* 1993). The weakly hybridizing bands observed with the pTr179 (and pTr133) probe may be due to homology between pTrAB3 and pTiAB3 and were not further investigated. Transfer of pTiAB3 to UBAPF2 is less frequent than transfer of pTrAB3: In 15 conjugation experiments yielding over 30 pTrAB3 transconjugants, only two independent pTiAB3 transconjugants were obtained.

Localization of tartrate utilization gene(s) on pTiAB3.

The pTiAB3 TAR system was isolated by cloning partially digested *Hin*dIII fragments of pTiAB3 (purified from A595) into pMP2733, transformation of UBAPF2 with the ligation mixture, and selection on ABT. One tar$^+$ pMP2733 derivative (pPM1105) was obtained which contained a 12.5-kb DNA fragment between pTiAB3 coordinates 59 and 71.5 (*Hin*dIII fragments of 2.1 and 10.4 kb, van Nuenen *et al.* 1993). The position of the TAR region with respect to earlier determined pTiAB3 functions is shown in Figure 4. The pTiAB3 TAR fragment did not hybridize to the TAR region of pTrAB3 (not shown).

Functional analysis of TAR regions.

In the course of these studies, three different types of tar$^+$ UBAPF2 derivatives were obtained: transconjugants with an integrated pTrAB3 plasmid (representative strain A499), transconjugants carrying pTiAB3 (representative strain A595), and a UBAPF2 transformant containing a free pTrAB3 plasmid (A531). This provided us with an opportunity to compare the efficiency of the different TAR systems within the same chromosomal background. In a first experiment, bacterial growth and tartrate utilization of A499, A531, A595, and the control strain A656 (UBAPF2) were measured in liquid minimal medium with 0.5% tartrate (ABT medium, Fig. 5A and B).

During the exponential growth phase, the three tar$^+$ strains have very similar doubling times: 6, 7, and 7 hr for A595, A499, and A531, respectively, the tar$^-$ strain does not grow. However, the lag phases differ considerably: Whereas strain A499 with the integrated pTrAB3 plasmid starts its exponential growth phase after about 5 hr, strain A595 with pTiAB3 does so after about 10 hr and strain A531 with the free pTrAB3 plasmid after 25 hr (Fig. 5A). The utilization of tartrate by the three strains closely parallels their growth (Fig. 5B). Three independent experiments yielded the same result.

Szegedi (1985) reported that AB3 prefers tartrate to glucose in AB minimal medium with 0.5% tartrate and 0.5% glucose (ABTG). In a second experiment we determined the kinetics of tartrate and glucose utilization in ABTG for each of the three tartrate utilizing UBAPF2 derivatives. The results (Fig. 6B–E) show that each tar$^+$ UBAPF2 derivative prefers tartrate to glucose, although the strain with the free pTrAB3 plasmid starts to use glucose before the tartrate in the medium is exhausted (Fig. 6D). In none of the cases, diauxic growth is observed (Fig. 6A): Other conditions may allow a better separation of the two different growth components. As expected, the final density of the control strain UBAPF2 which does not utilize tartrate is lower than that of the three tar$^+$ strains. Al-

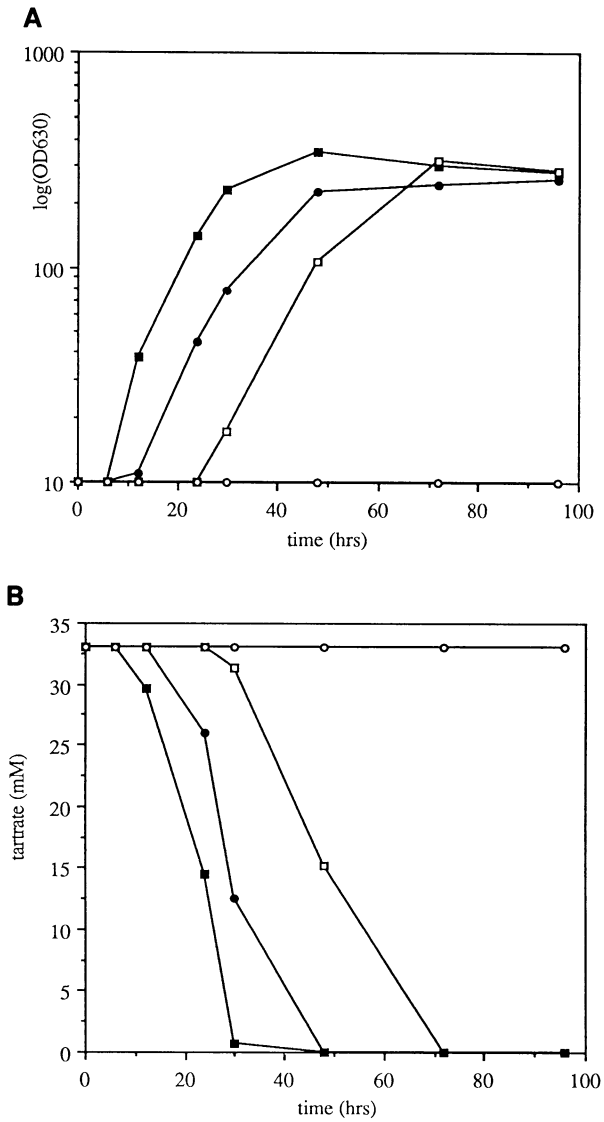

Fig. 5. Growth and tartrate utilization of UBAPF2 and tar$^+$ derivatives in ABT medium (minimal medium with 0.5% tartrate). **A,** Growth in ABT (OD_{630} values). **B,** Tartrate utilization in ABT (tartrate concentration in mM). Open circles: UBAPF2; closed circles: UBAPF2(pTiAB3); open squares: UBAPF2(pTrAB3); closed squares: UBAPF2(pTrAB3 integrated). Values are the means of three independent bacterial cultures.

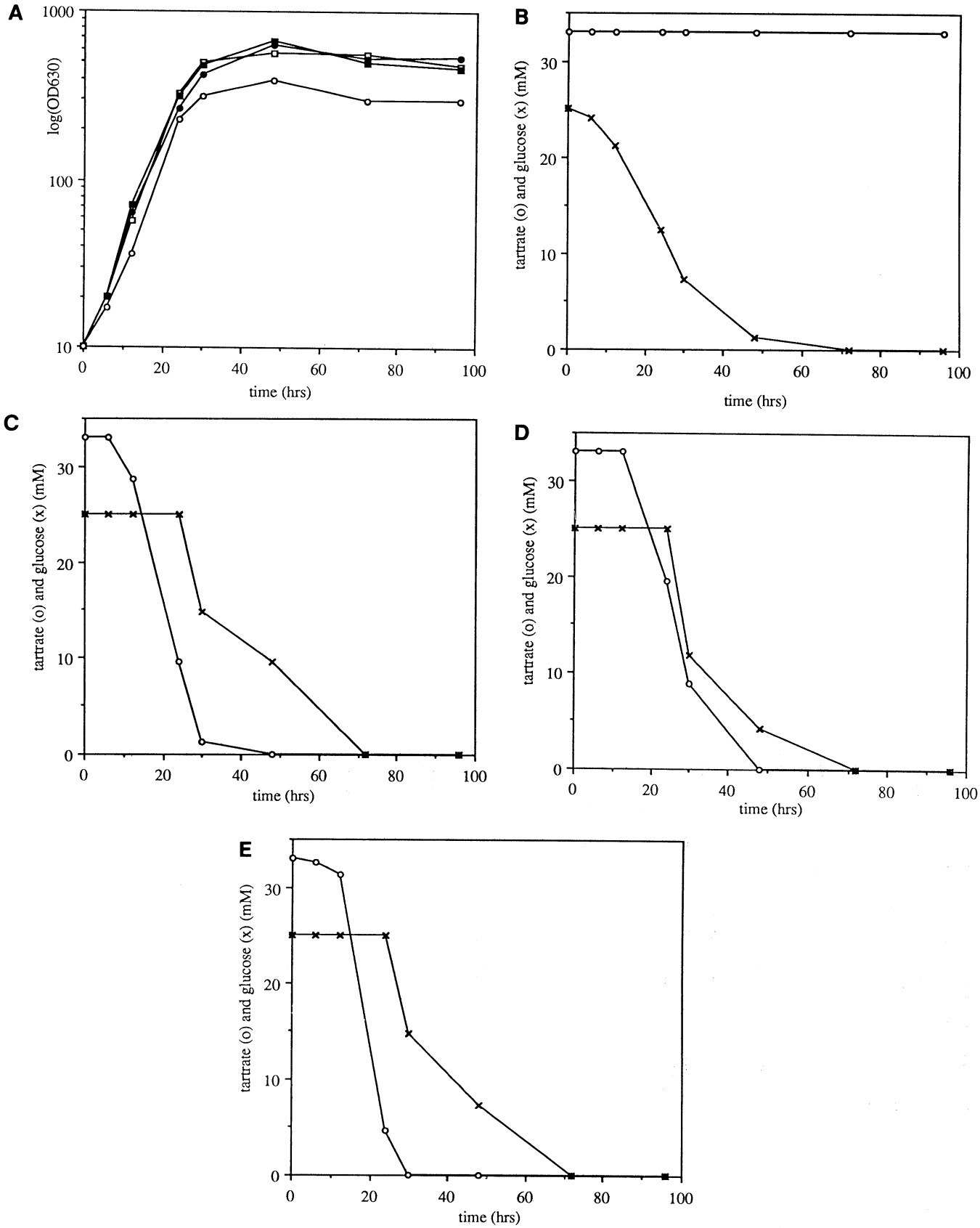

Fig. 6. Growth and tartrate utilization of UBAPF2 and tar$^+$ derivatives in ABTG medium (minimal medium with 0.5% tartrate and 0.5% glucose). Open circles: UBAPF2; closed circles: UBAPF2(pTiAB3); open squares: UBAPF2(pTrAB3); closed squares: UBAPF2(pTrAB3 integrated). **A,** Growth in ABTG (OD_{630} values). **B–E,** show tartrate (o) and glucose (x) utilization (in mM) of the following strains. **B,** UBAPF2. **C,** UBAPF2(pTrAB3 integrated). **D,** UBAPF2(pTrAB3). **E,** UBAPF2(pTiAB3). Values are the means of three independent bacterial cultures.

though the OD_{630} values fluctuate during the stationary growth phase, it can be estimated from these data that the molar yield coefficient $Y_{tartrate}$ is about 50% of the $Y_{glucose}$ value.

Occurrence of tartrate utilization genes on plasmids of different *Agrobacterium* strains.

The TAR regions of pTrAB3 and pTiAB3 were used as probes against blots with plasmid profiles of different *Agrobacterium* strains (listed in Table 2). Hybridization patterns are shown in Figure 7. Whereas the TAR region of pTrAB3 hybridized only to plasmids of the *A. vitis* o/c isolates AB3 and Zw2, the TAR region of pTiAB3 hybridized to plasmids of the *A. vitis* isolates AB3, Zw2, and SF93 and to plasmids of the *A. tumefaciens* biotype I grapevine isolates 2654 and 2655. None of the plasmids of the other strains hybridized to the AB3 pTAR region probes.

Transfer of tartrate utilization to another *Agrobacterium* strain.

AB3 was also mated with *Agrobacterium* strain Chry5Rif^R on sterile AB3-induced grapevine tumor tissues. Chry5Rif^R is a wild-type biotype I *A. tumefaciens* isolate from chrysanthemum and harbors a Ti plasmid and a cryptic plasmid (Bush and Pueppke 1991). The Ti plasmid encodes L,L-succinamopine catabolism. Six independent mating experiments yielded 10 independent tar$^+$ colonies derived from Chry5Rif^R. These colonies were tested for virulence on *Kalanchoe tubiflora*. They induced tumors which were octopine negative. We could not detect the presence of free molecules of pTiAB3 or pTrAB3 in these transconjugants (results not shown). We therefore concluded that the transconjugants probably contained an integrated pTrAB3 plasmid as in the case of the AB3xUBAPF2 transconjugants. This should be further investigated. The transfer of tartrate utilization does not seem to be specific for UBAPF2, but may be a more general phenomenon.

DISCUSSION

Mixed infection of sterile grapevine tissues with the *A. vitis* strain AB3 and the rifamycin-resistant, plasmid-cured C58 derivative UBAPF2 yielded tartrate-utilizing UBAPF2 transconjugants (Szegedi *et al.* 1992). Although we originally assumed that transfer would be favored in AB3-induced tumor tissues, transfer occurs with the same frequency in non-transformed grapevine callus. Attempts to obtain transconjugants on plates containing tartrate have so far failed.

Analysis of the transconjugant strains revealed that tartrate utilization by AB3 is a redundant feature. Two large AB3 plasmids, pTrAB3 and pTiAB3, each encode an independent TAR system. Both systems have been cloned and can now be investigated by transposon mutagenesis. Under our experimental conditions, transfer of pTrAB3 to the *A. tumefaciens* strain UBAPF2 *in planta* is more frequent than transfer of pTiAB3. pTrAB3 transfer involves recombination of pTrAB3 with some or all of the AB3 plasmid pAB3b and most likely, integration of the cointegrate into the UBAPF2 chromosomal DNA. Chromosomal integration of plasmids has been described for several bacterial species (for examples in *Pseudomonas* see Curiale and Mills 1982; Mucha and Farrand 1986). The stability of free pTrAB3 molecules in UBAPF2 (as shown by transformation) suggests that pTrAB3 is not self-conjugative and requires the conjugative plasmid pAB3b for its transfer. The systematic integration of the cointegrate into the UBAPF2 genome is remarkable. Possibly, the pAB3b sequences become integrated as a consequence of the transfer mechanism. It may be interesting therefore to study the conjugational behavior of pAB3b. From conjugation experiments with another biotype I strain, Chry5, it seems likely that pTrAB3 transfer does not depend on some particular feature of UBAPF2. The transfer of pTiAB3 and pTrAB3 plasmids to other *Agrobacterium* strains and the regulation of such processes under natural conditions remain to be investigated (see below).

When grown in ABT medium, the UBAPF2 strain with the integrated pTrAB3 plasmid shows a significantly shorter lag phase than the strain with the free plasmid. Possibly, the difference is due to pAB3b sequences, to the interruption of the pTrAB3 P2.65 fragment, or to the modification of the UBAPF2 target sequence. UBAPF2(pTiAB3) shows an intermediate growth behavior. Utilization of tartrate by each of the three types of tar$^+$ strains seems efficient since each prefers tartrate over glucose. It may be interesting to test tartrate utilization in the presence of succinate, which is the preferred carbon source of *A. tumefaciens* biotype I strains like UBAPF2 (Nautiyal *et al.* 1992; Hong *et al.* 1993).

Two groups of independently obtained UBAPF2xAB3 transconjugants (A590–592 and A595–596) lack the pTrAB3 TAR region but carry pTiAB3. A 12.5-kb region from pTiAB3 was shown to confer tartrate utilization. The same region is found on the Ti plasmid of the limited host range strain Ag57 (Panagopoulos *et al.* 1978; Hoekema *et al.* 1984) as shown by the *Eco*RI, *Pst*I, and *Hin*dIII maps of this plasmid (van Nuenen *et al.* 1993) as well as on other limited host range Ti plasmids (L. Otten, unpublished). The wide host range Ti plasmids pTiTm4 (Otten *et al.* 1993), pTiHm1 (van Nuenen *et al.* 1993), and pTi2608 (Fournier *et al.* 1994) lack homology to this region. Although transconjugants A590–

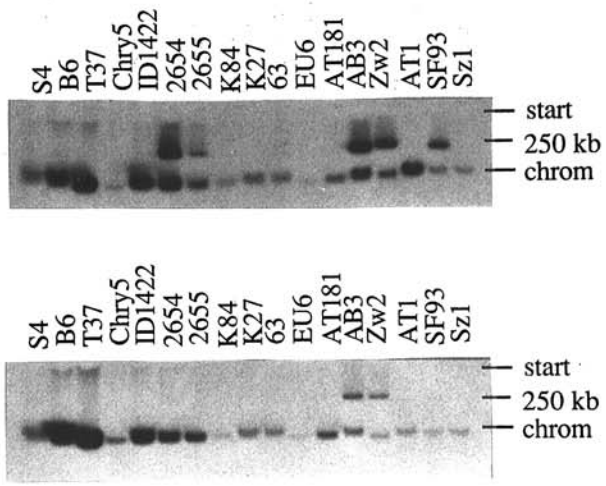

Fig. 7. Southern hybridization of plasmid profiles of different *Agrobacterium* strains (Table 2) to the two different tartrate regions of AB3. Top: probe pPM1105 (pTiAB3 TAR region). Bottom: probe pPM405 (pTrAB3 TAR region). The plasmids of strain S4 (260, 250, 80 and 60 kb) were used as size markers (Gérard *et al.* 1992).

592 carry the pTiAB3 TAR region, as shown by hybridization, they do not contain free Ti plasmid molecules, nor are they virulent. The pTiAB3 TAR region may have been inserted into the UBAPF2 chromosome and the Ti plasmid could have lost its virulence properties by loss of essential Ti sequences. Transconjugant strains A590–A592 may be further investigated by hybridization to different pTiAB3 probes.

The recovery of UBAPF2 transconjugants with an intact pTiAB3 plasmid shows that this Ti plasmid can be transferred to *A. tumefaciens* (biotype I) strains in AB3-induced tumor tissues. We have earlier calculated a pTrAB3 transfer frequency of about 10^{-9} (Szegedi *et al.* 1992), transfer of pTiAB3 is about 5–10 times lower (this work). Therefore, if transfer of pTiAB3 is induced by an opine, it is considerably less efficient than usual for opine-induced pTi transfer (Petit *et al.* 1978). Interestingly, a few natural grapevine isolates have been found to belong to biotype I and these strains contain pTiAB3-like plasmids as determined from hybridization patterns obtained with T-DNA probes (Paulus *et al.* 1989a). Two such strains, 2654 and 2655, carry a plasmid with homology to the pTiAB3 TAR region but not to the pTrAB3 TAR region, suggesting that the tartrate utilization properties of these strains (and possibly their occurrence on grapevine) are due to acquisition of a pTiAB3-like Ti plasmid.

All of the earlier identified functions of *A. vitis* Ti plasmids have been related to plant tumor induction. This suggests that the novel function described here could also play a role in crown gall formation, possibly by favoring bacterial growth in the grapevine plant before and after tumor induction. This hypothesis can be tested with tar$^-$ mutants. The clones described in this study should enable us to define the tartrate utilization gene(s), elucidate the pathway of tartrate utilization, construct tar$^-$ mutants and compare their growth in grapevine with that of wild-type strains.

Table 2. *Agrobacterium* strains tested for homology to pTrAB3 and pTiAB3 TAR regions

Strain	Type	Reference
UBAPF2	*A. tumefaciens*, cured	Hynes *et al.* 1985
B6	*A. tumefaciens*, octopine	Petit and Tempé 1985
T37	*A. tumefaciens*, nopaline	Petit and Tempé 1985
Chry5	*A. tumefaciens*, L,L-succinamopine	Bush and Pueppke 1991
Chry5Rif^R	Rifampicin-resistant Chry5 derivative	This work
1D1422	*A. radiobacter*, non-virulent	Gallie *et al.* 1984
K84	*A. radiobacter*, non-virulent	Sciaky *et al.* 1978
K27	*A. tumefaciens*, nopaline	Sciaky *et al.* 1978
63	*A. tumefaciens*, nopaline	Süle 1978; Szegedi, unpublished
EU6, AT181	*A. tumefaciens*, succinamopine	Chilton *et al.* 1984
2654, 2655	*A. tumefaciens*, o/c[a]	Paulus *et al.* 1989a
AT6, B10/7, AB3, Zw2	*A. vitis*, o/c, limited host range	Szegedi *et al.* 1988
Tm4, Hm1	*A. vitis*, o/c, wide host range	Szegedi *et al.* 1988
K305, NW180	*A. vitis*, o/c, wide host range	Paulus *et al.* 1989b
AT1, AT66, Ni1, Rr4, IS1.1, AB4, EK2	*A. vitis*, nopaline	Szegedi *et al.* 1988
S4, Sz1, Sz2	*A. vitis*, vitopine	Szegedi *et al.* 1988
NW11, NW221	*A. vitis*, vitopine	Bien *et al.* 1990
2681	*A. vitis*, vitopine	Paulus *et al.* 1989a
SF93[b]	*A. vitis*, vitopine	Szegedi, unpublished

[a] o/c: octopine/cucumopine.
[b] SF93 was isolated from grapevine in Hungary.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Various *Agrobacterium* wild-type strains are listed in Table 2. pMP2733 is a spectinomycin-resistant broad host range IncW vector with a single *Hin*dIII cloning site derived from pRI40 (Innes *et al.* 1988) by removal of the *Bam*HI and *Eco*RI sites (Spaink *et al.*, unpublished). pBin19 is a broad host range IncP vector with a M13mp19 polylinker (Bevan 1985).

Conjugation *in planta*.

Matings were carried out on sterile grapevine (cv. Narancsizü) tumor tissues induced by the octopine/cucumopine (o/c) *A. vitis* strains Tm4 and AB3 as described previously (Szegedi *et al.* 1992). As recipients, the rifamycin resistant biotype I (tartrate negative) strains UBAPF2 and Chry5Rif^R (selected in our laboratory) were used. Transconjugants were selected on AB minimal medium containing 0.5% tartrate as carbon source (ABT, Szegedi *et al.* 1992) and 100 mg/L rifamycin. Single colonies were streaked out twice on the same medium. Colonies were tested for 3-ketolactose production (Bernaerts and De Ley 1963) with Benedict's reagent as modified by Kerr and Panagopoulos (1977) to distinguish real transconjugants from spontaneous rifamycin resistant donors.

Transformation of UBAPF2.

Plasmids were isolated from strain AB3 according to Kado and Liu (1981), ethanol precipitated, and introduced into strain UBAPF2 using a modified freeze-thaw transformation method (Gallie *et al.* 1984). Transformants were selected on ABT medium and 100 mg/L rifamycin. pBin19 and pMP2733 derivatives were introduced into UBAPF2 by electroporation of competent cells (Mattanovich *et al.* 1989).

Plasmid analysis and stability tests.

Strains were grown overnight in liquid LBMG medium (Lichtenstein and Draper 1986) and analyzed for plasmids by a miniscreen method (Kado and Liu 1981). Plasmids were separated on 0.5–0.7% agarose gels in TAE buffer. To test the stability of tartrate utilization, dilutions of bacteria grown on LBMG plates were replated on LBMG medium. Individual colonies were then tested on ABT medium. Under these conditions, tar$^-$ bacteria form small colonies which can be easily distinguished from the larger tar$^+$ colonies. Alternatively, stability was tested after two cycles of growth on YEB (Leemans *et al.* 1983) by plating bacterial dilutions on ABT and ABT medium containing 0.5% glucose and bromothymolblue (ABTG-BBT, Szegedi 1985).

Bacterial growth.

Bacterial growth was determined by culturing bacteria in liquid YEB medium until stationary phase was reached. Bacteria were pelleted, washed in 10 mM $MgSO_4$, and resuspended in ABT or ABTG medium to a final concentration of 4×10^4 cfu/ml (five independent cultures per strain). Bacteria were grown at 28° C. Samples were frozen at –80° C at dif-

ferent times of culture. OD_{630} values of thawed samples were determined in microtiter plates with a Dynatech MR700 Microplate Reader.

Tartrate and glucose utilization.

Frozen samples of bacterial cultures were thawed and centrifuged for 10 min at 15,000 *g*. The tartrate concentrations in the supernatants were determined according to Rebelein as cited in Szegedi (1985). Reactions were carried out in microtiter plates, and absorbance at 550 nm was determined. Concentrations of glucose in the supernatants were measured using an enzymatic method (Trinder 1969) in microtiter plates read at 550 nm.

Preparative isolation of large plasmids.

Large plasmids (pTrAB3 and pTiAB3) were isolated from 400-ml LBMG cultures and purified by CsCl gradient centrifugation according to Currier and Nester (1976).

Virulence assays.

Kalanchoe tubiflora stems were punctured and inoculated with bacterial suspensions. Results were scored after 4 wk; opines were tested according to Otten and Schilperoort (1978).

Cloning and other molecular biology techniques.

Cloning and mapping of partial restriction fragments in pUC18 (Yanish-Perron *et al.* 1985) was as described (van Nuenen *et al.* 1993). The TAR region of pTrAB3 was cloned as a 10-kb *Bam*HI fragment in pBin19, recloned into pUC18, and from there as a partial 9.5-kb *Hin*dIII fragment into pMP2733. The TAR region of pTiAB3 was cloned as a 12.5-kb partial *Hin*dIII fragment into pMP2733. For general molecular biology techniques see Sambrook *et al.* (1989).

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LITERATURE CITED

Bernaerts, M. I., and De Ley, J. 1963. A biochemical test for crown gall bacteria. Nature 197:406-407.

Bevan, M. 1985. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 84:8711-8721.

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.

Bush, A. L., and Pueppke, S. G. 1991. Characteristics of an unusual new *Agrobacterium tumefaciens* strain from *Chrysanthemum morifolium* Ram. Appl. Environ. Microbiol. 57:2468-2472.

Chilton, W. S., Tempé, J., Matzke, M., and Chilton, M.-D. 1984. Succinamopine, a new crown gall opine. J. Bacteriol. 157:357-362.

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.

Currier, T. C., and Nester, E. W. 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. 66:431-441.

Fournier, P., de Ruffray, P., and Otten, L. 1994. Natural instability of *Agrobacterium vitis* Ti plasmid due to unusual duplication of a 2.3-kb DNA fragment. Mol. Plant-Microbe Interact. 7:164-172.

Gallie, D. R., 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.

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.

Hoekema, A., de Pater, B. S., Fellinger, A. J., Hooykaas, P. J. J., and Schilperoort, R. A. 1984. The limited host range of an *Agrobacterium tumefaciens* strain extended by a cytokinin gene from a wide host range T-region. EMBO J. 3:3043-3047.

Hong, S-B., Dessaux, Y., Chilton, W. S., and Farrand, S. 1993. Organization and regulation of the mannopine cyclase-associated opine catabolism genes in *Agrobacterium tumefaciens* 15955. J. Bacteriol. 175:401-410.

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.

Innes, R. W., Hirose, M. A., and Kuempel, P. L. 1988. Induction of nitrogen-fixing nodules on clover requires only 32 kilobase pairs of DNA from the *Rhizobium trifolii* symbiosis plasmid. J. Bacteriol. 170:3793-3802.

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.

Leemans, J., Hernalsteens, J., Deblaere, J. P., de Greeve, H., Thia-Toong, L., van Montagu, M., and Schell, J. 1983. Genetic analysis of T-DNA and regeneration of transformed plants. Pages 322-330 in: Molecular Genetics of Bacteria-Plant Interaction. A. Pühler, ed. Springer Verlag, Berlin.

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.

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. Environ. Microbiol. 523:1338-1343.

Mattanovich, D., Rüke, F., da Camara Machado, A., Laimer, M., Regner, F., Steinkellner, H., Himmler, G., and Katinger, H. 1989. Efficient transformation of *Agrobacterium* spp. by electroporation. Nucleic Acids Res. 17:6747.

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.

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.

Nautiyal, C. S., Dion, P., and Chilton, W. S. 1992. Diauxic growth of *Agrobacterium tumefaciens* 15955 on succinate and mannopine. J. Bacteriol. 174:2215-2221.

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

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.

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., Tempé, 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.

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.

Petit, A., Tempé, J., Kerr, A., Holsters, M., van Montagu, M., and Schell, J. 1978. Substrate induction of conjugative activity of *Agrobacterium tumefaciens*. Nature 271:570-572.

Petit, A., and Tempé, J. 1985. The function of T-DNA in nature. Pages 625-636 in: Molecular Form and Function of the Plant Genome. L. van Vloten-Doting, G. S. P. de Groot, and T. C. Hall, eds. Plenum Press, New York,.

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

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

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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

Sciaky, D., Montoya, A. L., and Chilton, M.-D. 1978. Fingerprints of *Agrobacterium* Ti plasmids. Plasmid 1:238-253.

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.

Szegedi, E., Otten, L., and Czako, M. 1992. Diverse types of tartrate plasmids in *Agrobacterium tumefaciens* biotype III strains. Mol. Plant-Microbe Interact. 5:435-438.

Trinder, P. 1969. Determination of blood glucose using 4-amino-phenazone as oxygen acceptor. J. Clin. Pathol. 22:246.

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.

Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119.