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Research Note

Further Evidence that the Vitopine-type pTi's of *Agrobacterium vitis* Represent a Novel Group of Ti Plasmids

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To study the incompatibility properties of the vitopine Ti plasmids of *Agrobacterium vitis*, pPM739 containing the cloned *ori* region of pTiS4 was introduced by triparental mating into seven *Agrobacterium tumefaciens* strains carrying *inc*Rh-1, *inc*Rh-2, and *inc*Ag-1 plasmids and into eight *A. vitis* strains. All strains containing pPM739 retained their original plasmids and virulence or the ability to grow on tartrate, except for the three vitopine strains S4, Sz1, and NW11. Furthermore, pTiS4 was stably maintained in S4 cells following introduction of the *ori/inc* clones of the *inc*Rh-1, *inc*Rh-2, and *inc*Rh-3 plasmids. These results show that vitopine Ti plasmids represent a novel incompatibility group for which we propose the name *incRh-4*.

Additional keywords: crown gall, grapevine, opines, plasmid incompatibility.

Strains of the genus *Agrobacterium* may contain one to several plasmids of which the large Tumor-inducing (Ti) or Root-inducing (Ri) plasmids are characteristic for phytopathogenic strains. During crown gall tumorigenesis or hairy root induction a segment of these plasmids, called T-region, is transferred from the bacterium to the plant cell and becomes stably integrated into the plant chromosome as T-DNA (Zambryski et al. 1989; Kado 1991; Hooykaas and Schilperoort 1992; Zambryski 1992; Zupan and Zambryski 1995). The transformed cells of the tumors or hairy roots produce and secrete specific amino acid derivatives called opines. Both the oncogenes and the opine biosynthetic genes are encoded by the T-DNA. Outside the transferred region, Ti and Ri plasmids contain genes for the bacterial utilization of opines. Opine synthesis and utilization form a basis for the classification of Ti and Ri plasmids (Petit and Tempé 1985; Dessaux et al. 1992). Ti and Ri plasmids have also been classified by their incompatibility properties. Plasmid incompatibility has been defined as the inability to coexist stably in the same bacterial cell (Novick and Hoppensteadt 1978; Austin and Nordström 1990). The incompatibility (*inc*) region of a given plasmid is closely linked with the replication origin (*ori*) and partition (*par*) regions (Novick and Hoppensteadt 1978; Austin and Nordström 1990) and is determined by 20 to 24 bp direct repeats (Stalker and Helinski 1985; Persson and Nordström 1986; Lin et al. 1987; Newnham and Taylor 1994). The *ori* and *par* regions have also been cloned from several plasmids of *Agrobacterium* and their use in incompatibility studies is well established (Knauf and Nester 1982; Gallie et al. 1984; Jouanin et al. 1985; Komari et al. 1986). The octopine, nopaline, and D,L-succinamopine Ti plasmids of *Agrobacterium tumefaciens* as well as the octopine Ti plasmids of *Agrobacterium vitis*, formerly called biotype 3 (Ophel and Kerr 1990), belong to *inc*Rh-1 group. The L,L-succinamopine Ti plasmids (e.g., pTiBo542, also called leucinopine-type) of *A. tumefaciens* belong to the *inc*Rh-2 group while the agropine Ri plasmids of *Agrobacterium rhizogenes* are classified into the *inc*Rh-3 group (Hooykaas et al. 1980; Nester and Kosuge 1981; Melchers and Hooykaas 1987). The tartrate-utilization (pTAR) plasmid of the *A. tumefaciens* strain 1D1422 and plasmid pAg119 belong to a fourth group designated *inc*Ag-1 (Gallie et al. 1984). Several other plasmids are partially characterized with respect to their incompatibility properties. For example, the 410-kbp cryptic pAtC58 plasmid of *A. tumefaciens* strain C58 is compatible with several Ti and Ri plasmids (White and Nester 1980; Jouanin et al. 1985), but incompatible with the *Rhizobium meliloti* plasmid pRme41a (Rosenberg and Huguet 1984; Hynes et al. 1985). The L,L-succinamopine-type *inc*Rh-2 pTiBo542 plasmid was found to be compatible with the L,L-succinamopine-type Ti plasmid of strain Chry5 (Kovács and Pueppke 1994) and the large tartrate-utilization (pTr) plasmids of *A. vitis* are also compatible with various Ti plasmids (Szegedi et al. 1992). Previous studies have shown that *A. vitis* strains can be subdivided into octopine, nopaline, and vitopine groups on the basis of the Ti plasmid encoded opine markers. Of these the vitopine Ti plasmids have been described recently (Szegedi et al. 1988; Paulus et al. 1989). One vitopine Ti plasmid, pTiS4, has already been mapped and its T-DNA has been characterized in

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detail. These studies have shown that the vitopine Ti plasmid has an unusual structure with three T-DNAs. These T-DNAs carry oncogenes and opine genes that are only distantly related to T-DNA genes from other Ti plasmids (Gérard et al. 1992; Canaday et al. 1992). Here we show evidence that vitopine pTi's belong to a new incompatibility group.

Strains and plasmids used for experiments are listed in Table 1. Two-day-old pre-cultures were prepared for all experiments on mannitol-glutamate Luria broth (MGLB) medium (Lichtenstein and Draper 1986) supplemented with the appropriate antibiotics. Plasmid pPM739 containing the *ori* region of pTiS4 cloned into the pBR322 derivative pKC7 (Gérard et al. 1992) was mobilized to various wild-type *Agrobacterium* strains by triparental mating with the helper plasmid pGJ28 as described (van Haute et al. 1983; Gérard et al. 1992). Plasmids pVK210, pVTK17, and pCH1546 were mobilized by pRK2013 (Ditta et al. 1980). Plasmid pLJ46 was introduced into S4 by transformation (Höfgen and Willmitzer 1988). Strain UBAPF2 (pTAR1D1422) was obtained by mating *A. tumefaciens* UBAPF2 and 1D1422 strains on sterile grapevine tumor tissue induced by *A. vitis* AB3 strain as described (Szegedi et al. 1992). Two to six colonies per mating combination were isolated and repeatedly purified on AB minimal medium (Lichtenstein and Draper 1986) supplemented with 1% glucose and 100 mg of kanamycin per liter or 20 mg of tetracycline per liter and characterized (see below).

For plasmid miniscreens, the transconjugants and transformants were grown in liquid MGLB medium supplemented with the appropriate antibiotics for 16 to 20 h. Cell lysis and electrophoresis were carried out as described by Kado and Liu (1981). For electrophoretic separation of plasmid DNA, 0.5 to 0.8% (wt/vol) agarose gel was used. Plasmid DNA was blotted onto Hybond C-extra membranes (Amersham, UK) according to the instructions of the supplier. Southern hybridizations were carried out according to standard conditions (Sambrook et al. 1989).

The virulence of strains was tested on wounded stems of *Kalanchoe tubiflora* or *Nicotiana tabacum* var. Petite Havana SR1 (Maliga et al. 1973). Plants were kept in the greenhouse for 4 to 6 weeks following inoculation. The plasmid-free nonpathogenic UBAPF2 (Table 1), the UBAPF2(pPM739), and the pathogenic recipient strains were used for control experiments.

To study the incompatibility properties of the well-characterized vitopine Ti plasmid pTiS4 we introduced its cloned *ori* region (Gérard et al. 1992) into 14 *Agrobacterium* strains representing four opine types of *A. tumefaciens*, three opine types of *A. vitis,* and into UBAPF2(pTAR1D1422), which contains a tartrate utilization plasmid. This set of agrobacteria included strains with *inc*Rh-1, *inc*Rh-2, and *inc*Ag-1 plasmids (Table 1) as well as several uncharacterized Ti plasmids. Except for the vitopine strains S4, Sz1, and NW11, all transconjugant colonies contained the same plasmids as the wild-type recipients plus an additional band corresponding to the 25.4-kbp plasmid pPM739 following selection and growth on kanamycin to promote maintenance of pPM739. The pPM739 band was only weakly detectable in all recipient *Agrobacterium* strains as shown for vitopine strains on Figure 1A, indicating that the pPM739 plasmid replicates at low copy numbers in *Agrobacterium.*

The vitopine strains S4, Sz1, and NW11 became nonvirulent following introduction of pPM739. The loss of their Ti plasmids was confirmed by Southern hybridization experiments. Probe pKC7 (the 5.9-kbp vector part of pPM739) hybridized to the 25.4-kbp band in *Agrobacterium* transconjugants as expected (Fig. 1B). The 1.0-kbp Ti-derived *Bam*HI fragment of the pTiS4 clone pPM628 (Gérard et al. 1992) hy-

Table 1. Bacterial strains and plasmids

Strain/Host	Characteristics (*inc* group)	Reference
Agrobacterium tumefaciens		
UBAPF2	Biotype 1, RifR^a, plasmid cured C58	Hynes et al. 1985
Ach5	Biotype 1, octopine pTi, incRh-1	Petit and Tempé 1985; Knauf and Nester 1982
C58	Biotype 1, nopaline pTi, incRh-1	Petit and Tempé 1985; Hooykaas et al. 1980
T37	Biotype 1, nopaline pTi, incRh-1	Petit and Tempé 1985; Hooykaas et al. 1980
A281	Biotype 1, L,L-succinamopine pTi, incRh-2	Petit and Tempé 1985; Komari et al. 1986
Eu6	Biotype 2, D,L-succinamopine pTi	Sciaky et al. 1978; Chilton et al. 1984
UBAPF2	Biotype 1, pTAR1D1422, incAg-1	Gallie et al. 1984; and this work
Agrobacterium vitis		
Tm4	Biotype 3, octopine pTi	Szegedi et al. 1988
B10/7	Biotype 3, octopine pTi, incRh-1[b]	Szegedi et al. 1988
AT1	Biotype 3, nopaline pTi	Szegedi et al. 1988
AT66	Biotype 3, nopaline pTi, incRh-1 [b]	Szegedi et al. 1988
Ni1	Biotype 3, nopaline pTi	Szegedi et al. 1988
S4	Biotype 3, vitopine pTi	Szegedi et al. 1988
Sz1	Biotype 3, vitopine pTi	Szegedi et al. 1988
NW11	Biotype 3, vitopine pTi	Bien et al. 1990
Escherichia coli strains and plasmids		
NM522	pPM739, ori pTiS4, KmR^a, 25.4 kbp	Gérard et al. 1992
HB101	pVK210, ori pTiA6, KmR, incRh-1, 47.1 kbp	Knauf and Nester 1982
HB101	pTVK17, ori pTiBo542, KmR, incRh-2, 49.3 kbp	Komari et al. 1986
HB101	pLJ46, ori pRiHRI, KmR, incRh-3, 42 kbp	Jouanin 1984; Jouanin et al. 1985
HB101	pCH1546, TcR^a, ori pTiChry5, 44.4 kbp	Kovács and Pueppke 1994
JC2926	pGJ28, pR64drd11, KmR, TcR, SmR^a	van Haute et al. 1983
HB101	pRK2013, KmR	Ditta et al. 1980

[a] KmR: kanamycin resistant (100 mg/l); SmR: streptomycin resistant (100 mg/l); TcR: tetracycline resistant (20 mg/l); and RifR: rifamycin resistant (60 mg/l).

[b] The incompatibility properties of pTiB10/7 and pTiAT66 were determined during these studies using plasmid pVK210.

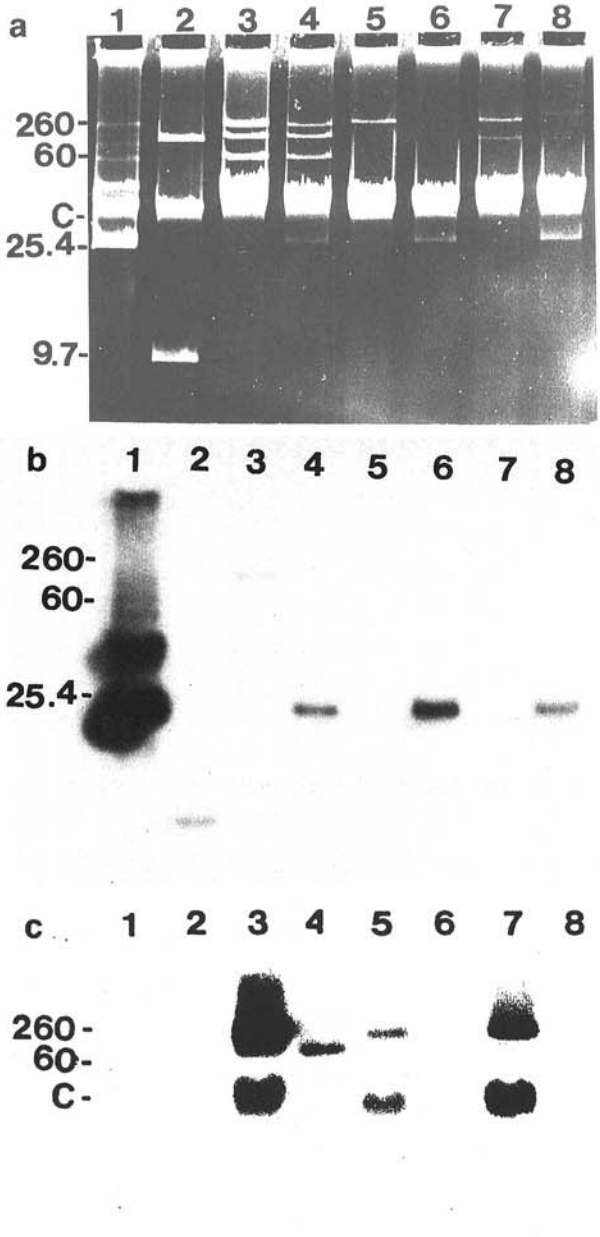

Fig. 1. A, Lane 1, Plasmid profiles of *Escherichia coli* strains NM522 (pPM739); lane 2, JC2926(pGJ28); lane 3, *Agrobacterium vitis* strains S4; lane 4, S4(pPM739) of which the 260-kbp pTi has been cured by pPM739; lane 5, Sz1; lane 6, Sz1(pPM739); lane 7, NW11 (7); and lane 8, NW11(pPM739). Strain S4 contains four plasmids of which pTiS4 and pTrS4 are of nearly equal sizes (260 and 250 kbp, respectively; Gerard et al. 1992), therefore were not properly separated. Strains Sz1 and NW11, besides the pTi, also contain a larger plasmid that becomes visible after curing of pTi's (lanes 6 and 8). The smaller band present in NW11, but not in NW11(pPM739) (lanes 7 and 8) is a plasmid that is not consistently detected. Samples were separated in 0.7% (wt/vol) agarose gel. **B,** Hybridization of identical plasmid blots using the cloning vector pKC7 and **(C)** the Ti plasmid specific *Bam*HI fragment of pPM628 (Gérard et al. 1992) as probe. The 260-kbp bands in lanes 3, 5, and 7 correspond to Ti plasmids. Plasmid sizes are given in kbp, c: sheared chromosomal and plasmid DNA.

bridized to the Ti plasmids of wild-type vitopine strains S4, Sz1, and NW11, and to the 80-kbp non-Ti plasmid of S4 (Fig. 1C). On the other hand, in S4(pPM739), Sz1(pPM739), and NW11(pPM739) strains Ti plasmids could not be detected by hybridization experiments, confirming that they were cured by pPM739 (Fig 1C). All other *Agrobacterium* (pPM739) transconjugant strains, except the negative control strain UBAPF2(pPM739), were as virulent on *Kalanchoe* or tobacco plants as the wild-type recipients. Similarly, pTAR1D422 was stably maintained in UBAPF2 following introduction of pPM739 since the transconjugants (six colonies were isolated) contained both pTAR1D1422 and pPM739 when they were grown on tartrate-free medium with kanamycin (to promote the maintenance of pPM739).

To verify these data, *ori/inc* clones of the *inc*Rh-1 plasmid pTiA6 (pVK210), the *inc*Rh-2 plasmid pTiBo542 (pTVK17),

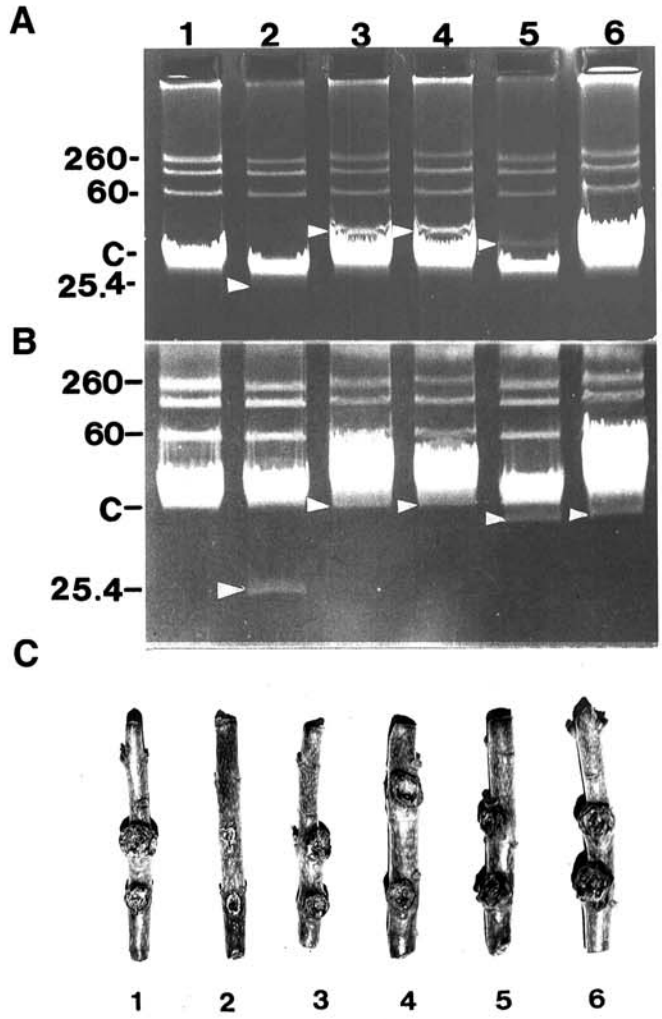

Fig. 2. A, Lane 1, Plasmid profiles of *Agrobacterium vitis* strain S4 wild-type; lane 2, S4(pPM739); lane 3, S4(pVK210); lane 4, S4(pTVK17); lane 5, S4(pLJ46); and lane 6, S4(pCH1546), separated in 0.8% (wt/vol) agarose gel. **B,** The same samples separated in 0.5% (wt/vol) agarose gel. The *ori/inc* clones are indicated by arrowheads. Sizes are given in kbp, c: sheared chromosomal and plasmid DNA. **C,** Virulence of *A. vitis* strain S4 and its transconjugant derivatives containing various *ori/inc* clones on *Kalanchoe tubiflora* stems (order of strains as listed above).

the *inc*Rh-3 plasmid pRiHRI (pLJ46), and the partially characterized *ori/inc* clone of pTiChry5 (pCH1546) were introduced into the vitopine strain S4 by triparental mating or transformation. All colonies isolated on kanamycin or tetracycline plates showed the appropriate additive plasmid profile, i.e., the introduced *ori/inc* clone and the resident S4 plasmids, and they all were also pathogenic (Fig. 2).

The above data show that pPM739 is incompatible with the vitopine Ti plasmids of *A. vitis,* but compatible with the Ti plasmids and/or with the *ori/inc* clones representing the known incompatibility groups *inc*Rh-1, *inc*Rh-2, *inc*Rh-3, and *inc*Ag-1 (Nester and Kosuge 1981; Gallie et al. 1984; Melchers and Hooykaas 1987), and with octopine and nopaline Ti plasmids of *A. vitis*. We propose to establish a novel group, *inc*Rh-4, for these pTi's. Vitopine Ti plasmids have only been detected in grapevine isolates (*A. vitis*), while other opine types (octopine, nopaline, agropine) are more common in the genus *Agrobacterium* (Petit and Tempé 1985; Szegedi et al. 1988; Paulus et al. 1989; Dessaux et al. 1992). The distinct incompatibility property is another evidence, besides the unique opine biosynthetic and catabolic genes and complex T-DNA organization, that the vitopine-type pTi's of *A. vitis* represent a novel group of Ti plasmids.

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