Mol Gen Genet (1994) 245:493–505

ORIGINAL PAPER

Léon Otten · Patrice De Ruffray

Agrobacterium vitis nopaline Ti plasmid pTiAB4: relationship to other Ti plasmids and T-DNA structure

Received: 23 February 1994 / Accepted: 13 May 1994

Abstract The Ti plasmid of the *Agrobacterium vitis* nopaline-type strain AB4 was subcloned and mapped. Several regions of the 157 kb Ti plasmid are similar or identical to parts of the *A. vitis* octopine/cucumopine (o/c)-type Ti plasmids, and other regions are homologous to the nopaline-type Ti plasmid pTiC58. The T-DNA of pTiAB4 is a chimaeric structure of recent origin: the left part is 99.2% homologous to the left part of the TA-DNA of the o/c-type Ti plasmids, while the right part is 97.1% homologous to the right part of an unusual nopaline T-DNA recently identified in strain 82.139, a biotype II strain from wild cherry. The 3′ non-coding regions of the *ipt* genes from pTiAB4 and pTi82.139 are different from those of other *ipt* genes and contain a 62 bp fragment derived from the coding sequence of an *ipt* gene of unknown origin. A comparison of different *ipt* gene sequences indicates that the corresponding 62 bp sequence within the coding region of the AB4 *ipt* gene has been modified during the course of its evolution, apparently by sequence transfer from the 62 bp sequence in the 3′ non-coding region. In pTi82.139 the original coding region of the *ipt* gene has remained largely unmodified. The pTiAB4 6b gene differs from its pTi82.139 counterpart by the lack of a 12 bp repeat in the 3′ part of the coding sequence. This leads to the loss of four glutamic acid residues from a series of ten. In spite of these differences, the *ipt* and 6b genes of pTiAB4 are functional. Our results provide new insight into the evolution of *Agrobacterium* Ti plasmids and confirm the remarkable plasticity of these genetic elements. Possible implications for the study of bacterial phylogeny are discussed.

Communicated by A. Kondorosi

L. Otten (✉) · P. De Ruffray
C.N.R.S. Institute of Plant Molecular Biology,
Rue du Général Zimmer 12, 67084 Strasbourg, Cedex,
France

Key words *Agrobacterium* · Bacterial evolution
Nopaline strains · Ti plasmids · Grapevine

Introduction

Agrobacterium is well known for its ability to induce plant tumours by the transfer of discrete Ti plasmid fragments (T-DNAs, located on tumour-inducing or Ti plasmids) into plant cells. Expression of T-DNA genes in the plant cell nucleus leads to the production of plant hormones and low molecular weight compounds called opines. Opines are metabolized by the tumour-inducing strains via Ti plasmid-encoded functions (for recent reviews see Kado 1991; Hooykaas and Schilperoort 1992; Winans 1992; Dessaux et al. 1992; Zambryski 1992). Ti plasmids and the strains containing them are generally classified according to the nature of the opines produced by the tumours they induce.

Most Ti plasmid studies have been carried out with pTiA6 (octopine type, very similar to pTi15955, pTiAch5 and pTiB6S3) and pTiC58 (nopaline type, very similar to pTiT37); other Ti plasmid types, e.g. the leucinopine/agropine-type Ti plasmids (Strabala et al. 1989) or the succinamopine-type Ti plasmids (Blundy et al. 1986) are less well known. We previously subcloned and mapped six Ti plasmids from *Agrobacterium vitis*. This *Agrobacterium* species is mainly found on grapevine (Panagopoulos and Psallidas 1973; Kerr and Panagopoulos 1977; Perry and Kado 1982; Burr and Katz 1983; Burr et al. 1987; Ophel and Kerr 1990) and can be subdivided into octopine/cucumopine- (o/c), nopaline- and vitopine-type strains with characteristic Ti plasmids. Each group of Ti plasmids is associated with a particular chromosomal background characterized by the presence of specific bacterial insertion elements (Szegedi et al. 1988; Bonnard et al. 1989a; Paulus et al. 1989a, 1991).

The o/c strains constitute about half of the isolated *A. vitis* strains. Several o/c-type Ti plasmids have been mapped: pTiAg162 (Knauf et al. 1984), pTiTm4 (Otten

et al. 1992), pTiHm1, pTiAB3, pTiAg57 (van Nuenen et al. 1993) and pTi2608 (Fournier et al. 1994). These plasmids are recently evolved mosaics: some regions (e.g. the virulence region, the two T-DNAs and the octopine catabolism region) are highly conserved and found in all o/c-type Ti plasmids, whereas others are plasmid specific. We have proposed a common evolutionary origin for these plasmids and hypothetical ancestor TA and TB regions have been reconstructed (Otten et al. 1992; Otten and van Nuenen 1993). The ancestor TA-DNA region is closely related to the TL-DNA of the *Agrobacterium tumefaciens* octopine-type Ti plasmid pTiA6; the TB-DNA region is only found on o/c-type plasmids (Yanofsky et al. 1985b; Huss et al. 1989).

Vitopine-type strains are relatively rare on grapevine (L. Otten, unpublished). All contain a Ti plasmid with the same, unique restriction pattern and three T-DNAs with low homology to other T-DNAs (Gérard et al. 1992; Canaday et al. 1992).

Finally, nopaline-type *A. vitis* strains are common on grapevine (E. Szegedi, personal communication) and have been found in different parts of the world (see Table 1). They have a wide host range (Knauf et al. 1982) and their Ti plasmids display significant homology to o/c-type Ti plasmids and to the TL-DNA of pTiA6 (Thomashow et al. 1981; Knauf et al. 1983; Paulus et al. 1989a). Their Ti plasmids have not yet been investigated in detail. Here we present the map of an *A. vitis* nopaline-type Ti plasmid, pTiAB4, and an analysis of the structure and function of its T-DNA. The AB4 Ti plasmid is closely related to the o/c Ti plasmids.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table 1.

Cloning, mapping and sequencing

Cloning, mapping and dideoxy sequencing were carried out according to standard procedures (Sambrook et al. 1989). For sequence analysis of the pTiAB4, pTiTm4 and pTiAB3 T-DNA left border regions, 3.6 kb (pTiAB4), 3.4 kb (pTiTm4) and 6.6 kb (pTiAB3) *Xba*I-*Eco*RI fragments including 0.5 kb of T-DNA sequences were cloned in pUC18 and the following primers derived from the pTiTm4 sequence (Paulus and Otten 1993) were used

Table 1 Bacterial strains and plasmids. All *Agrobacterium* nopaline strains were isolated from grapevine

Name	Characteristics	Reference
Escherichia coli		
NM522	Host for pUC and pKS Bluescript vectors	N. Murray, University of Edinburgh, UK
Agrobacterium		
UBAPF2	Cured rifampicin-resistant C58 derivative	Hynes et al. (1985)
UBAPF2(pTiAB4)	UBAPF2 containing pTiAB4	Szegedi, unpublished
LBA4404	*Agrobacterium* strain with disarmed Ti plasmid	Ooms et al. (1982)
GV3101(pPM6000)	*Agrobacterium* strain with disarmed Ti plasmid	Bonnard et al. (1989b)
NI-1, AB4, AT66, IS1.1, AT1, EK2	*A. vitis* nopaline strains, Hungary	Szegedi (1985)
2179, 2609, 2643, 2673, 2674	*A. vitis* nopaline strains, France	Paulus et al. (1989a)
BS-33-6	*A. vitis* nopaline strain, China	D. Ma
NW22, NW44, NW165, NW170 NW190, NW310 NW330	*A. vitis* nopaline strains, Germany	Schulz (1992)
NCPPB1771	*A. vitis* nopaline strain, Iran	Janssens et al. (1983)
Plasmid		
pUC18	Cloning vector	Yanisch-Perron et al. (1985)
pKC7	pBR322-derived cloning vector	Rao and Rogers (1979)
pBluescript KS−/+	Sequencing vector	Stratagene
pBin19	Binary T-DNA vector	Bevan (1985)
N6, N35, N37, N62, N204, N214, N228, N231, N242	Partial *Pst*I fragments of pTiAB4 cloned in pUC18	This study
pPM1082, pPM1084, pPM1085, pPM1086, pPM1089, pPM1090	pTiAB4 T-DNA fragments cloned into pKC7	This study
pPM1100, pPM1101, pPM1102, pPM1103, pPM1104	*Sau*3AI partials of N6 cloned into pBin19	This study
pPM6000	pTiB6S3-derived disarmed Ti vector	Bonnard et al. (1989b)
pGV329	pTiC58 clone with nopaline catabolism genes	Depicker et al. (1980)
pGV342, 319, 415	pTiC58 T-DNA clones	Depicker et al. (1980)

(coordinates from EMBL DNA sequence accession number M91188): P1 (120–104), P2 (162–179), P3 (304–288), P4 (315–331) and P5 (442–426). The pTiAB4 map was constructed by comparative restriction mapping of partial *Pst*I and *Hin*dIII clones.

DNA sequence analysis

DNA sequences were analysed using the GCG sequence analysis software package version 6.0 (Devereux et al. 1987) on a MicroVax computer. Nucleotide sequences were compared using the Bestfit program.

T-DNA gene function tests

For T-DNA gene function tests, partial *Sau*3AI subfragments from pTiAB4 subclone N6 (Fig. 1a) were cloned into the binary T-DNA vector pBin19. Forty-eight randomly chosen clones were mapped with *Pst*I, *Hin*dIII, *Bam*HI and *Eco*RI. A previously established map of homology with the T-DNA of pTiC58 predicted different sets of T-DNA genes in each of the subclones. Representative clones containing various combinations of T-DNA genes were retained (pPM1100–1104). In a second set of experiments, restriction fragments from N231 (Fig. 1a), chosen on the basis of sequencing data, were cloned into the intermediate pBR322-derived vector pKC7: the 3.4 kb *Hin*dIII-*Eco*RI fragment with genes 6b and 3′ (the latter is homologous to gene 3′ from the TR-DNA of pTiAch5; Drevet et al. 1994) was ligated into pKC7, yielding pPM1082. The 1.85 kb *Pst*I-*Eco*RI fragment with gene 6b was cloned in pUC18, and recloned as a *Hin*dIII-*Eco*RI fragment in pKC7 (pPM1084). The 2.2 kb *Bam*HI fragment with gene 3′ was cloned in pUC18, and recloned as a *Hin*dIII-*Eco*RI fragment in pKC7 (pPM1085). The 5.1 kb *Nae*I-*Hin*dIII fragment with the *ipt*, 6b and 3′ genes was cloned in the *Sma*I-*Hin*dIII site of pUC18 (pPM1081), and recloned as a *Hin*dIII-(partial) *Eco*RI fragment in pKC7 (pPM1086). The 2.65 kb *Kpn*I-*Acc*I fragment with the *ipt* gene from pPM1081 was recloned in pUC18 and transferred as a *Hin*dIII-(partial) *Eco*RI fragment into pKC7 (pPM1089). The 3.5 kb *Kpn*I-(partial) *Acc*I fragment with the *ipt* and 6b genes from pPM1081 was recloned into pUC18 and transferred as a *Hin*dIII-(partial) *Eco*RI fragment into pKC7 (pPM1090). Intermediate vectors were introduced into *Agrobacterium* by triparental mating using pRK2013 for pBin19 derivatives (acceptor strain LBA4404) and GJ23 for pKC7 derivatives (acceptor strain GV3101 (pPM6000)).

Ti plasmid isolation

Ti plasmid DNA was isolated according to Currier and Nester (1976) with the following modification: before lysis, bacterial cells were washed by resuspending the pellet in one-tenth of the original volume of ice-cold washing buffer (25 mM TRIS-HCl, pH 8.0, 20 mM EDTA, 0.5 M NaCl, 0.05% sodium sarkosyl) and repelleted.

Homology studies

DNA homology studies were carried out by standard procedures, at standard stringency. For the pTiC58-pTiAB4 homology studies, pTiC58 was purified, cut with *Hin*dIII and Southern blots were prepared which were hybridized to representative pTiAB4 clones.

Virulence tests and opine assays

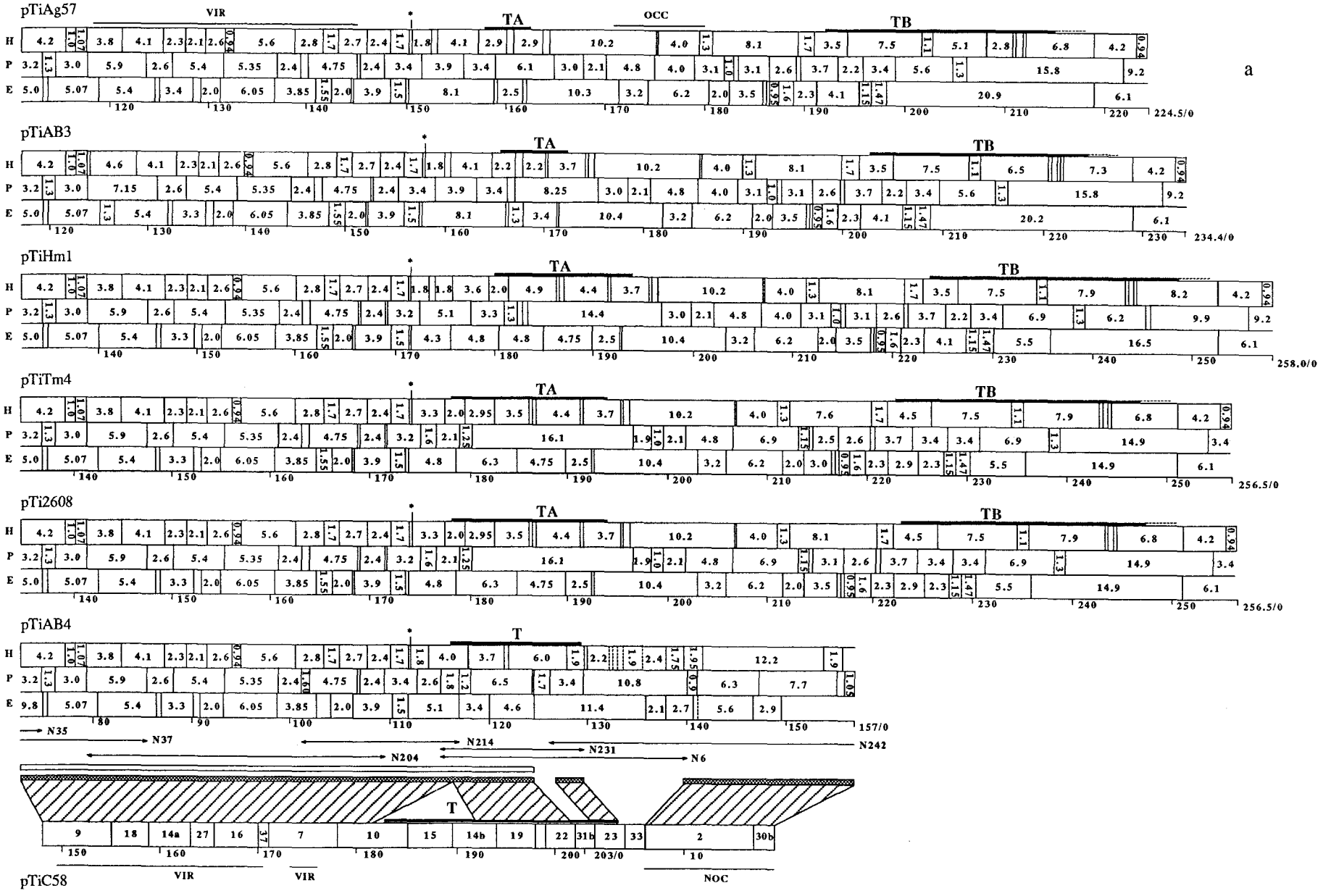
Agrobacterium strains were tested for virulence by puncturing stems of *Nicotiana rustica*, *Nicotiana tabacum* and *Kalanchoe tubiflora* and applying the bacteria grown on agar plates. Results were scored after 3–5 weeks. Assays for nopaline were done on infected *N. rustica* tissues according to Otten and Schilperoort (1978), and assays for agrocinopine according to Dahl et al. (1983).

Results

Cloning and mapping of pTiAB4

In a previous study, Southern analysis of *Hin*dIII digests of 11 *A. vitis* nopaline-type isolates with a TL-DNA probe from the *A. tumefaciens* strain Ach5 revealed a common hybridization pattern (Paulus et al. 1989a). Strain AB4 (Szegedi 1985) was chosen for subcloning and mapping of the Ti plasmid. AB4 has a wide host range: it induces tumours on *Vitis vinifera*, *Lycopersicon esculentum*, *Datura stramonium* and *K. tubiflora* (Paulus et al. 1989a). As AB4 carries two large plasmids, pTiAB4 and the tartrate utilization plasmid pTrAB4 (Szegedi et al. 1992), pTiAB4 DNA was purified from a UBAPF2 × AB4 exconjugant strain, UBAPF2(pTiAB4) (E. Szegedi, unpublished). Partially digested *Pst*I or *Hin*dIII fragments with a size between 15 and 30 kb were isolated by sucrose gradient centrifugation and cloned in pUC18. Restriction analysis of 78 *Hin*dIII clones and 112 *Pst*I clones yielded the *Hin*dIII, *Pst*I and *Eco*RI map shown in Fig. 1a, b. Eight representative clones covering pTiAB4 (Fig. 1a, b) are listed in Table 1. pTiAB4 has a size of 157 kb and is the smallest Ti plasmid found so far. As coordinate 0 we arbitrarily chose the left *Pst*I site of the 0.95 kb *Pst*I restriction fragment of clone N242. Inspection of the restriction map of pTiAB4 revealed several regions with the same restriction patterns as those of regions found in o/c-type Ti plasmids. In particular, a 45 kb region is identical to the virulence (*vir*) region of the o/c-type Ti plasmid pTiAg57 (pTiAB4 coordinates 68.5 to 113.5 correspond to pTiAg57 coordinates 107.0 to 152.5; van Nuenen et al. 1993) and is present in slightly different forms in five other o/c-type Ti plasmids for which maps have been established: pTiAg162 (Knauf et al. 1984, not shown here), pTiAB3, pTiHm1 (van Nuenen et al. 1993), pTiTm4 (Otten et al. 1993) and pTi2608 (Fournier et al. 1994). pTiAB4 has no restriction site homology to pTiC58. In Fig. 1a, an 82 kb pTiAB4 fragment containing the *vir* region and the T-DNA region is aligned with the maps of five o/c-type Ti plasmids (pTiAg57, pTiAB3, pTiHm1, pTiTm4 and pTi2608).

The remaining part of pTiAB4 (Fig. 1b) has a more complex structure. Various regions (a–e) are also found in the o/c-type Ti plasmids. Region b (b') contains the incompatibility region as defined by Knauf et al. (1984). Among the o/c-type Ti plasmids, pTiTm4 shows the highest level of restriction site homology with pTiAB4.


pTiAg57
VIR
TA
OCC
TB
H
P
E
224.5/0
a
pTiAB3
234.4/0
pTiHm1
258.0/0
pTiTm4
256.5/0
pTi2608
256.5/0
pTiAB4
T
157/0
N35
N37
N214
N231
N242
N204
N6
T
pTiC58
VIR
VIR
NOC

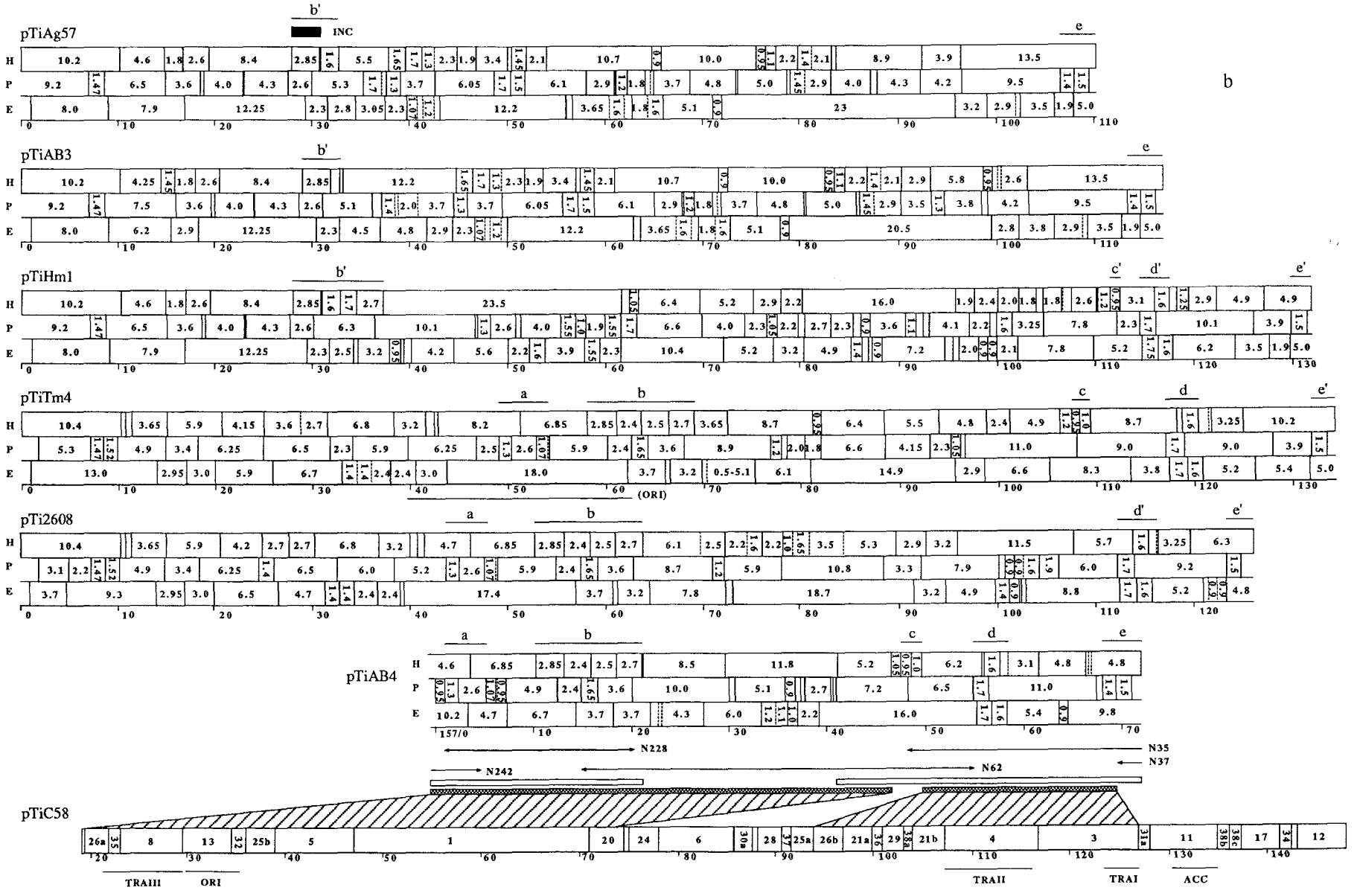


Fig. 1a, b Restriction maps of pTiAB4, pTiC58 and five related octopine/cucumopine (o/c)type Ti plasmids. Above the pTiAB4 map, are shown the maps of pTiAg57, pTiAB3, pTiHm1 and pTiTm4 (adapted from van Nuenen et al. 1993) and the pTi2608 map (Fournier et al. 1994). [*H*, *P* and *E HindIII*, *PstI* and *EcoRI*. *VIR*, *OCC* and *INC* virulence, octopine catabolism and incompatibility region (Knauf et al. 1984). *ORI* origin of replication region (Otten et al. 1992). *N-X* partial *PstI* clones of pTiAB4.] Below the pTiAB4 map the extent of the DNA homology to pTiTm4 and pTiC58 as determined by Southern hybridization is shown by *white* and *grey bars* respectively; the correlation with the *HindIII* map of pTiC58 (Depicker et al. 1980) is shown as a *hatched area*. [*TRAI*, *II* and *III* conjugational transfer regions (Engler et al. 1981), *ACC* agrocinopine catabolism region (Hayman and Farrand 1990), *NOC* nopaline catabolism region (von Lintig et al. 1991).] Coordinates in kilobases. The Ti maps are arbitrarily divided into two parts. **a** Virulence region and T-DNA region(s). The *asterisk* indicates the rightmost end of the common virulence region. **b** Remaining part of Ti plasmids with the incompatibility region and the origin of replication. Subregions *a–e* indicate regions of restriction site identity, and *b′–e′* indicate partial restriction site conservation

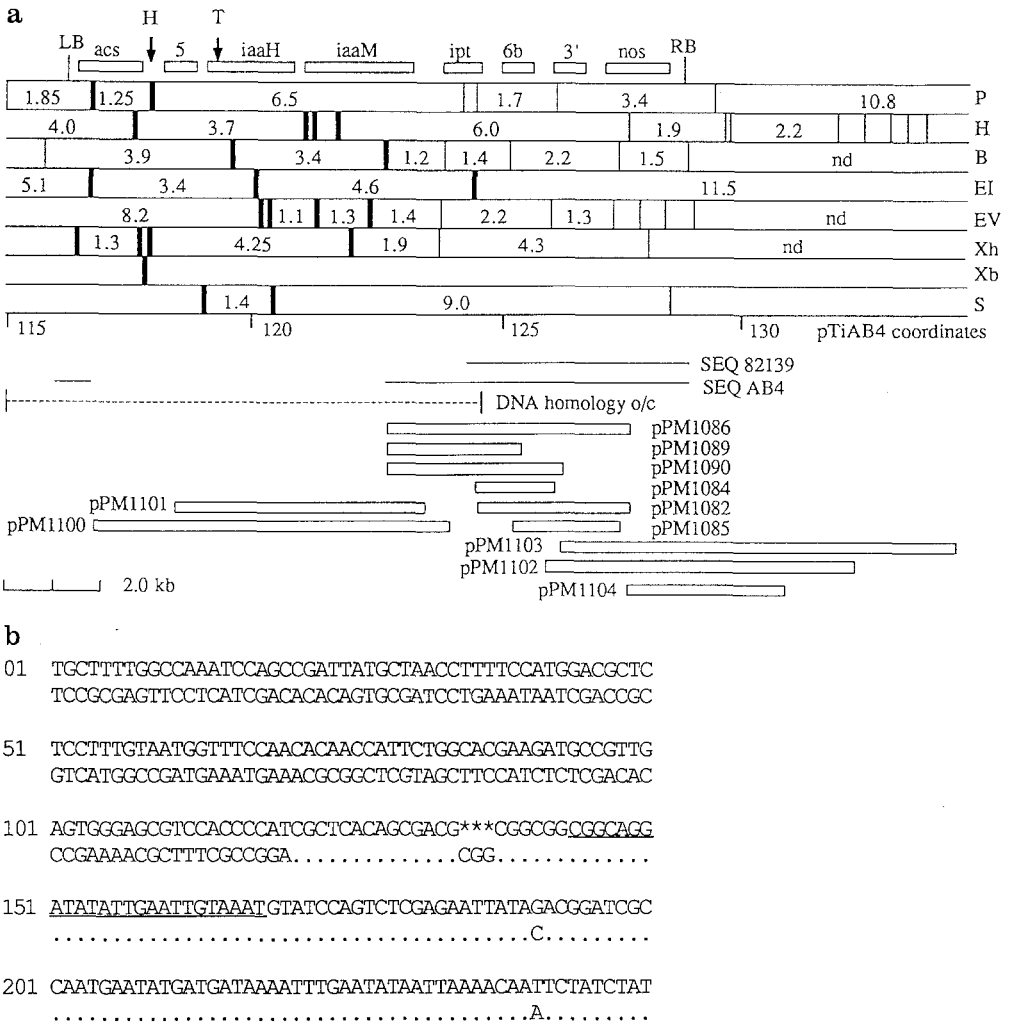


Fig. 2a, b T-DNA of pTiAB4 and surrounding sequences. **a** Map of pTiAB4 T-DNA and clones derived from this region. The pTiAB4 T-DNA is compared with the o/c ancestor TA-DNA as reconstructed from extant TA-DNAs (Otten et al. 1992). *SEQ* sequenced areas. *pPMXXXX* subclones tested on host plants. *P, H, B, EI, EV, Xh, Xb, S, Pst*I, *Hin*dIII, *Bam*HI, *Eco*RI, *Eco*RV, *Xho*I, *Xba*I, *Sma*I respectively. *nd* restriction sites not determined. *H* and *T* position of bacterial insertion elements in pTiHm1 and pTiTm4, *LB* and *RB* left and right T-DNA border.) *Heavy vertical bars* restriction sites common to pTiAB4 and pTiTm4/pTiHm1. Fragment sizes are indicated in kilobases; for pTiAB4 coordinates see Fig 1a. **b** Sequence of the left border region of the TA-DNA of pTiAB3 and pTiTm4 (*upper line*) and the T-DNA of pTiAB4 (*lower line*). *Dots* indicate identical residues within the homologous region; *asterisks* indicate gaps

DNA homology between pTiAB4 and other TI plasmids

The o/c-type plasmid pTiTm4 and the well-known *A. tumefaciens* nopaline-type Ti plasmid pTiC58 (Depicker et al. 1980) were used as probes on Southern blots with *Hin*dIII and *Pst*I digests of the eight representative pTiAB4 clones listed in Table 1. Hybridization was carried out under normal stringency. Various pTiAB4 regions without restriction site homology to pTiTm4 nevertheless hybridize strongly to this plasmid. Two large regions of pTiAB4 (140–157 and 21–41) are homologous to pTiC58, but not to pTiTm4. To establish a more precise correspondence between pTiAB4 and pTiC58, Southern blots with *Hin*dIII-digested pTiC58 DNA were hybridized to representative pTiAB4 clones. The homologous regions are clearly arranged in the same order, and confirm the homology patterns found for the pTiTm4-pTiC58 combination (Otten et al. 1992) in those regions where pTiTm4 and pTiAB4 are similar. The 140–157 region hybridizes to the nopaline catabolism (*noc*) region of pTiC58, as shown by hybridization of N242 to restriction digests of pTiC58 clone pGV329, which contains the complete *noc* region (von Lintig et al. 1991; Depicker et al. 1980). The 21–41 region hybridizes to *Hin*dIII fragments 5 and 1 of pTiC58 (no functions have been reported for this pTiC58 region). The homology data are summarized in Fig. 1a, b.

T-DNA of pTiAB4

Hybridization experiments (not shown) between pTiAB4 clones and the pTiC58 T-DNA clones pGV342, pGV319 and pGV415 (Depicker et al. 1980; Willmitzer et al. 1983) revealed strong homology between the 116–124.5 region of pTiAB4 and the central part of the pTiC58 T-DNA with the agrocinopine synthase (*acs*), 5, indoleacetamide hydrolase (*iaaH*), tryptophan monooxygenase (*iaaM*) and isopentenyladenosine phosphotransferase (*ipt*) genes (for pTiC58T-DNA map, see Willmitzer et al. 1983). No homology was found to the left part of the pTiC58 T-DNA (genes a to e), nor to genes 6a and 6b. From 127.5–129, pTiAB4 is homologous to the pTiC58 *nos* gene. A 15 kb fragment around the 116–124.5 region was mapped with eight different restriction enzymes, using different pTiAB4

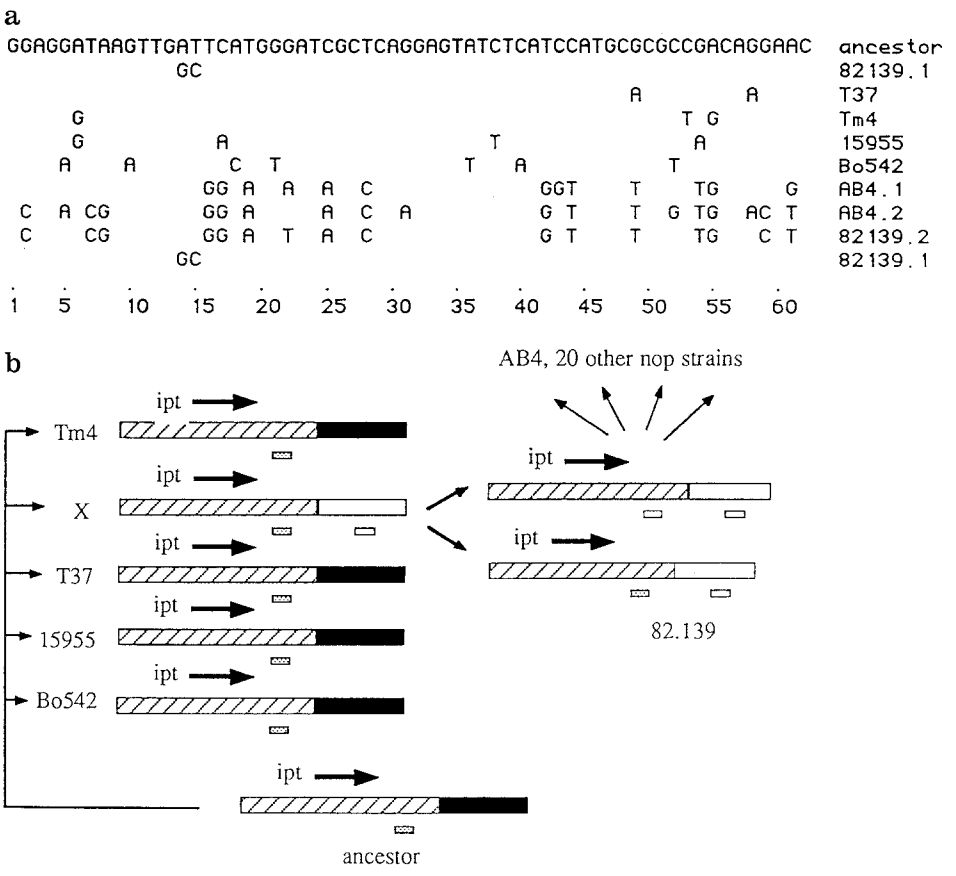


Fig. 3a, b Sequence in the 3′ non-coding region of the pTiAB4 and pTi82.139 *ipt* genes with homology to a sequence within the *ipt* coding region. **a** Sequence of the reconstructed 62 bp ancestor sequence within the *ipt* gene and the sequences of pTi82.139 (82.139.1, Drevet et al. 1994), pTiT37 (Goldberg et al. 1984), pTiTm4 (Bonnard et al. 1989b), pTi15955/pTiAch5 (Barker et al. 1983; Gielen et al. 1984), pTiBo542 (Strabala et al. 1989) and pTiAB4 (AB4.1, this study) compared with the sequence within the *ipt* 3′ non-coding region of pTiAB4 (AB4.2, this study) and pTi82.139 (82.139.2, Drevet et al. 1994). For convenience, 82.139.1 is shown twice. The AB4.1-characteristic *Pst*I site is situated at coordinates 53–58. **b** Proposed evolutionary origin of *ipt* sequences shown in **a**. We assume that all *ipt* genes are derived from the same ancestor. *Hatched area* coding sequence. In the line leading to pTi82.139 and the *A. vitis* nopaline-type Ti plasmids the 6a-6b region of the ancestor (in *black*) was replaced by another fragment (in *white*) which contained an *ipt* gene fragment of unknown origin (*small white box*; the corresponding fragment within the *ipt* coding sequence is shown in *grey*). In the Ti plasmid that generated the *A. vitis* nopaline-type Ti plasmids, the *ipt*-internal fragment came to resemble the external fragment; in the line leading to pTi82.139, the original situation persisted. Drawing not to scale

clones. The map (Fig. 2a) was compared with the T-DNA of pTiC58 (Depicker et al. 1980; Willmitzer et al. 1983) and the TA-DNAs of pTiTm4 (Paulus et al. 1989a) and pTiHm1 (Otten, unpublished). The TA-DNAs of the latter two are derived from a common ancestor T-DNA with the following gene order: *acs*-5-*iaaH*-*iaaM*-*ipt*-6b-*ocs*. In pTiHm1, an unidentified bacterial insertion element interrupts the *acs* gene (Paulus and Otten 1993); in pTiTm4 IS*866* interrupts the *iaaH* gene (Bonnard et al. 1989a). No restriction site homology was detected between the pTiAB4 and pTiC58T-DNAs. However, from the putative left T-DNA border up to the 3′ end of the *iaaM* gene, the 22 restriction sites of the pTiAB4 T-DNA (indicated by thick vertical lines in Fig. 2a) are the same as those of the left part of the reconstructed ancestor o/c TA region (Otten and van Nuenen 1993). To the left of this fragment, the maps of pTiAB4, pTiHm1, pTiAB3/pTiAg57 and pTiTm4/pTi2608 are different up to a *Hin*dIII site common to all of them and indicated by an asterisk in Fig. 1a.

To determine the left end of the common T-DNA fragment, a 650 bp fragment was sequenced (250 bp of which is shown in Fig. 2b). Starting from the *Eco*RI site at pTiAB4 coordinate 113.5 (Fig. 1a) and going left, the pTiAB4 sequence is 99.2% identical to the pTiTm4 and pTiAB3 sequences (Paulus and Otten 1993) up to 20 bp to the left end of the left border sequence. To the left of this highly conserved sequence the homology between pTiAB4 and the o/c-type Ti plasmid sequences is lost; it resumes at the *Hin*dIII site at the right end of the virulence region mentioned above.

At the right end of the T-DNA, the restriction site conservation between the o/c-type Ti plasmids and pTiAB4 ends within the *iaaM* gene. To characterize the right end of the T-DNA, a 6.5 kb fragment was sequenced. This fragment comprised 2.0 kb DNA related to pTiTm4 and 4.5 kb of DNA unrelated to pTiTm4.

```
INGVSYDFFARSGNYTEQAEEEEEEEEEEEEEVLD--REFQLGNLVSYPIIAWGSCPNSA   S4
      x          x  xxxxxxxxxxxxxx  x   x
*NNEN*GFY**G***T*EG*DDDDEMDD*G*AGGAE-P*EC*IGN*IN***I*L**CDLSA   Ach5
   x             xx xxxxxx xxx x    x   x                  x
*NGAS*GFY**R***T*EG*DDDNEIGD*G*AGGAE-I*DY*FGD*VN***V*L**SRLSA   Tm4
                 xx xxxx x  xx x    x   x    x
*NNEN*GFY**G***T*EG*DDDDEMDD*D*TGGAE-T*DS*TGN*IN***I*L**CHLSA   T37
   x             xx xxxxxx xxxxx    x   x
*RGQG*GWY**Q***V*EG*DNEGIENE*E*EE----T*EF*LSD*IH***V*L**CHLTR   AB4
                 xx xx x  x xxxxxx      x    x
*RGQG*GWY**L***V*EG*DNEGIENE*E*EEEEEET*EF*LSD*IH***V*L**CHLTR   82139
                 xx xx x  x xxxxxxxxxx  x    x
```

Fig. 4 Sequence of the last 57 amino acid residues of the pTiAB4 6b gene peptide, compared with other 6b peptides. The amino acid sequence corresponding to the C-terminal half of the deduced peptides encoded by the 6b genes of pTiS4 (Canaday et al. 1992), pTiAch5 (Gielen et al. 1984), pTiTm4 (G. Bonnard, unpublished), pTiT37 (Vanderleyden et al. 1986), pTiAB4 (this study) and pTi82.139 (Drevet et al. 1994) is shown. *Asterisks* indicate conserved residues, *dashes* lack of residues. Below each sequence, the *Xs* indicate acidic residues

Sequence of the right part of the T-DNA

The 1.2, 1.4, 2.2 and 1.5 kb *Bam*HI fragments of N231 were cloned in the sequencing vector pBluescript KS^- or KS^+ and sequenced. The 6482 bp sequence (EMBL accession number X77327) contains several open reading frames (Fig. 2a) and was compared with the available sequences of pTiTm4 (Bonnard et al. 1991), pTi15955 (Barker et al. 1983), pTiT37 (Goldberg et al. 1984), pTiBo542 (Strabala et al. 1989), pTi15955/pTiAch5 (Barker et al. 1983; Gielen et al. 1984) and pTi82.139 (Drevet et al. 1994).

Starting on the left, coordinates 1–475 correspond to the 3′ part of the *iaaM* gene, 476–1119 to the *iaaM/ipt* intergenic region and 1120–1899 to the *ipt* coding region. The pTiAB4 *iaaM* coding sequence is strongly homologous to pTi15955/pTiAch5 (93.7%) and pTiTm4 (94.7%). The *iaaM/ipt* intergenic region can be separated into two parts: a region (476–774) with strong homology to pTiT37 (86.6%), pTiBo542 (80.6%) and pTi15955/pTiAch5 (80.5%), but much less to pTiTm4 (67.5%), and a second region (775–1121) with strong homology to all sequences: 89.2%, 88.5%, 86.1% and 82.6% for pTiT37, pTiTm4, pTiBo542 and pTi15955/pTiAch5 respectively. Compared with the other *ipt* sequences, the pTiAB4 *ipt* coding sequence is 60 nucleotides longer at the 5′ end, but no obvious TATA box is found within 100 bp upstream of the new ATG codon.

On the right, the pTiAB4 sequence (1616–6285) is 97.1% homologous to the 4679 bp sequence of the right part of the T-DNA of the *A. tumefaciens* biotype II nopaline-type strain 82.139 as determined by Drevet et al. (1994). The latter starts within the *ipt* gene, 282 bp from the stop codon and ends 115 bp beyond the right border sequence. As noted by Drevet et al., part of the pTi82.139 sequence differs considerably from the T-DNA sequences of the well-known biotype I nopaline-type strains C58 and T37: a 2.5 kb fragment starting immediately after the *ipt* stop codon and ending within the 3′ non-coding end of the nopaline synthase (*nos*) gene replaces the pTiC58 6a and 6b genes. It contains a gene with 63% homology to the pTiC58 6b gene, and one with 71% homology to the 3′ gene of the TR-DNA of the biotype I octopine strain pTi15955 (Barker et al. 1983). The function of the 3′ gene is unknown. To the right of the 6b-3′ region, a *nos*-right border region with 83.9% homology to pTiT37 and pTiC58 is found (Drevet et al. 1994). Although the sequenced *ipt*-6b-3′-*nos* regions of pTi82.139 and pTiAB4 are very similar, there are two interesting differences.

1. In the 3′ non-coding region of the *ipt* gene (1981–2042 in pTiAB4) the pTiAB4 and pTi82.139 T-regions contain a 62 bp fragment with significant homology to a sequence in the coding region of the *ipt* gene (1774–1835 in pTiAB4) and in direct orientation with respect to the latter. The four sequences and their homologues from the *ipt* genes of other Ti plasmids are shown in Fig. 3a. By scoring which of the nucleotides are shared by the largest number of sequences, an ancestral *ipt* sequence can be reconstructed from which the present-day sequences with their specific mutations can be derived. The sequence within the pTi82.139 *ipt* gene (called 82.139.1) is closely related to the hypothetical ancestor sequence (60/62 bp in common), as are the sequences of pTiT37 (60/62), pTiTm4 (59/62) and pTi15955/pTiAch5 (58/62). The pTiBo542 sequence is less related to the ancestor sequence (55/62). The pTiAB4 sequence (AB4.1) has only 48 bp in common with 82.139.1 (77%) in spite of the fact that the remaining *ipt* sequence is 97% homologous to the pTi82.139 sequence (88.2%, 86.9%, 85.8% and 82.4% for pTiT37, pTiTm4, pTi15955/pTiAch5 and pTiBo542, respectively). Instead, AB4.1 is clearly related to the *ipt* fragment within the 3′ non-coding part of both pTiAB4 and pTi82.139 (called AB4.2 and 82.139.2, Fig. 3a). These observations indicate that AB4.1 results from a modification of the original ancestor sequence by a process of "sequence transfer" from the AB4.2 sequence within the 6b-3′ segment, possibly by gene conversion. This hypothesis is summarized in Fig. 3b. The modification introduces a diagnostic *Pst*I site at position 53–58 (CGACAG → CTGCAG). Total DNAs of 20 nopaline-type *A. vitis* strains isolated from grapevine in France, Hungary, Iran, Germany and China (Table 1) were digested with *Pst*I and analysed by Southern hybridization to probe N231; all strains showed the same *Pst*I pattern as AB4 (not shown). Thus, the T-DNAs of these

Table 2 Biological effects of pTiAB4 T-DNA genes. (*nt*: not tested)

Construct	T-DNA gene(s)	*Nicotiana tabacum*	*Nicotiana rustica*	*Kalanchoe tubiflora*
pPM1089	*ipt*	Tumours with shoots	Small tumours	Small tumours with shoots
pPM1084	6b	Very small tumours	Small tumours	Small tumours
pPM1085	3′	No growth reaction	No growth reaction	No growth reaction
pPM1082	6b, 3′	Very small tumours	Small tumours	Small tumours
pPM1090	*ipt*, 6b	Large tumours	Large tumours	Small tumours with shoots
pPM1086	*ipt*, 6b, 3′	Large tumours	Large tumours	Small tumours with shoots
pPM1100	5, *iaaH*, *iaaM*	nt	nt	Roots
pPM1101	*iaaH*, *iaaM*	nt	nt	Roots
pPM1102	3′, *nos*	No growth reaction	No growth reaction, nopaline in wound tissue	No growth reaction
pPM1103	*nos*	No growth reaction	No growth reaction, nopaline in wound tissue	No growth reaction
pPM1104	–	No growth reaction	No growth reaction, no nopaline in wound tissue	No growth reaction

strains have the same overall chimaeric structure as the pTiAB4 T-DNA and the same GA→TG modification.

2. Whereas the 6b gene of pTi82.139 carries four 6 bp repeats (TTCCTC) within the 3′ part of its coding sequence, the pTiAB4 gene carries only two copies of this repeat. This reduces the size of the deduced peptide by 4 glutamic acid residues. The difference is situated in a strongly acidic region present in all 6b genes sequenced to date (Fig. 4). The 6b genes of different Ti plasmids have different oncogenic properties: the o/c pTiTm4 6b gene is strongly oncogenic on *N. rustica*, whereas the nopaline pTiC58 6b gene shows only very weak tumour induction (Tinland et al. 1989). The 6b genes also affect the biological effects of cotransferred *ipt* genes to different degrees (Bonnard et al. 1989b).

Analysis of T-DNA gene function

The function of the different pTiAB4 T-DNA genes was determined by subcloning T-DNA fragments in pBin19 or pKC7 (for map positions of subclones see Fig. 2a), transferring them to *Agrobacterium* strains carrying Ti helper plasmids and testing them on different hosts chosen for their sensitivity to various oncogenes (Tinland et al. 1989). The results (Table 2) demonstrate that the *iaa*, *ipt*, 6b and *nos* genes of pTiAB4 are functional. Strains carrying the *iaa* genes induce roots on *K. tubiflora*, and a strain with the *ipt* gene induces tumours and shoots on *K. tubiflora*, small tumours with shoots on *N. tabacum*, and small tumours without shoots on *N. rustica*. Strains carrying the 6b gene induce small tumours on *N. rustica* and *N. tabacum* and those carrying the 6b gene and the *ipt* gene stimulate tumour formation on *N. tabacum* and *N. rustica*. Gene 5 does not noticeably affect the induction of roots if contransferred with the *iaa* genes. The *nos* gene encodes the synthesis of large amounts of nopaline. No agrocinopine was detected in tumours induced by AB4, suggesting that the *acs* gene is inactive. Finally, the transfer of the 3′ gene does not lead to any growth effects, nor does this gene influence the effects of the *nos* gene, the 6b gene or the *ipt* and 6b gene combination.

Discussion

The *A. vitis* nopaline-type Ti plasmid pTiAB4 has been mapped and its T-DNA characterized. pTiAB4 is relatively small (only 157 kb compared with 200–260 kb for other Ti plasmids). Large parts of pTiAB4 are very similar or identical to parts of the o/c-type Ti plasmids of *A. vitis*; the virulence region, the left part of the T-DNA and several other regions (a–d in Fig. 1b) are also found in the o/c-type Ti plasmids. Large parts of pTiAB4 hybridize to the *A. tumefaciens* nopaline-type Ti plasmid pTiC58, but they do not show any restriction site homology, showing that there is less homology between pTiAB4 and pTiC58 than between pTiAB4 and pTiTm4. The right part of the T-DNA, the 140–157 region (homologous to the pTiC58 nopaline catabolism region) and the 21–41 region (homologous to a pTiC58 region without known function) are lacking in o/c-type Ti plasmids. Thus, pTiAB4 is a composite structure which must have formed by horizontal DNA transfer between different *Agrobacterium* strains. The structure of the pTiAB4 T-DNA is itself chimaeric; the *ipt*-6b-3′-*nos* fragment of pTiAB4 is 97.1% homologous to a T-DNA fragment from the biotype II *A. tumefaciens* nopaline-type strain 82.139 isolated from wild cherry (Drevet et al. 1994), whereas the left border region is 99.2% homologous to the left border region of the o/c-type Ti plasmids (Paulus and Otten 1993). The homology at the left end of the 82.139 sequence is higher between pTiAB4 and pTi82.139 (97%) than between pTiAB4 and pTiTm4 (86.9%). We therefore assume that the junction between the pTi82.139- and o/c-like Ti plasmid sequences lies to the left of this region. The pTi82.139 T-DNA is most probably derived from a pTiC58-like T-DNA since a region 5′ to gene 5 has 98% homology with the corresponding pTiC58 region and genes a–e are present (Drevet et al. 1994).

The perfect conservation of 34 restriction sites be-

tween pTiAB4 coordinates 68.5 and 113.5 with respect to those of pTiAg57 and the 99.2% sequence homology with pTiTm4 and pTiAB3 at the left end of the T-DNA indicate that the evolutionary origin of the *A. vitis* nopaline-type plasmids is situated close to the emergence of the various o/c-type Ti plasmids. The o/c-type Ti plasmids were derived from a common structure by loss of ancestral regions and acquisition of new sequences. Point mutations played only a very minor role (sequenced areas from different o/c-type Ti plasmids are over 99.7% homologous; Paulus et al. 1991; Paulus and Otten 1993) indicating that the divergence of this group is very recent (Otten et al. 1992; van Nuenen et al. 1993). It cannot yet be decided whether the ancestor plasmid was a nopaline- or an o/c-type plasmid nor when exactly the nopaline-type Ti plasmids of *A. vitis* branched off with respect to the different o/c-type Ti plasmids. However, the ancestor plasmid encoded agrocinopine synthesis since both pTiAB4 and the o/c-type Ti plasmids carry the agrocinopine synthase (*acs*) gene, which is still active in pTiTm4. It is likely that the ancestor also carried agrocinopine utilization genes, which are still found (but not yet localized) on pTiTm4. Strain AB4 does not use agrocinopine (Paulus and Otten 1993) and agrocinopine is lacking in AB4-induced tumours. Further studies are needed to determine whether the *acs* gene of AB4 has lost its activity or functions in a different way.

Besides the virulence and T-DNA region, three other pTiAB4 regions are related to o/c Ti plasmid sequences; they are arranged in the same order and orientation. We therefore suggest that these conserved regions represent remnants of the ancestral Ti plasmid. The fact that the homology maps of the pTiAB4-pTiC58 and pTiTm4-pTiC58 couples are colinear suggests that pTiAB4 and the o/c Ti plasmids are related to pTiC58. Fragments specific for each plasmid type may have been introduced by conjugation, recombination and transposition events. It can be expected that only few insertion/deletion events could have led to viable new combinations since a number of Ti plasmid functions are interdependent: opine synthesis and utilization, various tumour functions, virulence functions and border sequences constitute an integrated system which may easily be disrupted. In this respect it is interesting to note that opine synthesis and degradation genes are closely linked, both in pTiAB4 and in o/c-type Ti plasmids.

The common occurrence of the pTiAB4-like and o/c-like plasmids shows that recombinant structures resulting in a change in opine type can be highly successful. The succinamopine-type plasmid pTiAT181 (Blundy et al. 1986) may constitute another example of such a structure: it resembles the nopaline-type Ti plasmid pTiT37 but carries a pTiAT181-specific region encoding succinamopine synthesis, replacing the *nos* gene at the right end of the T-DNA. Little is known about the remainder of this plasmid (for example, its succinamopine utilization genes) and its natural host range and relative frequency of occurrence have not been determined.

Several nopaline-type plasmids have been described: pTiT37, Ti plasmids of several Japanese isolates and pTi82.139; all of these are closely related to pTiC58 (Goldberg et al. 1984; Wabiko et al. 1989; Michel et al. 1990; Drevet et al. 1994). Our studies demonstrate that although it may be practical to group Ti plasmid types according to their opine character, such a classification clearly cannot be taken to reflect phylogenetic relatedness unless supported by molecular data.

Apart from large-scale modifications within *A. vitis* o/c- and nopaline-type Ti plasmids, the present study also revealed more subtle changes. The deduced product of the pTiAB4 *ipt* gene is 20 amino acids longer at its N-terminal end but is nevertheless biologically active; it remains to be determined whether the pTi82.139 *ipt* gene is modified in the same way. In addition, a 62 bp *ipt* gene fragment of unknown origin situated within the 3′ non-coding region of the pTiAB4 *ipt* gene apparently served to modify the original coding sequence of the pTiAB4 *ipt* gene, possibly by gene conversion. Gene conversion is generally found in higher organisms but has also been described for bacteria (see for example Yamamoto et al. 1992). The *ipt* gene change is found in all grapevine isolates studied so far; it did not occur in pTi82.139 and its functional consequences remain to be studied. We postulate that the present-day nopaline-type plasmids from *A. vitis* were derived from the plasmid that initially underwent the change; this would mean that they are of clonal origin. A clonal origin has also been postulated for the o/c-type Ti plasmids and their different subgroups (Otten and van Nuenen 1993) and for the vitopine-type Ti plasmids (Gérard et al. 1992). The frequency, mechanism and possible directionality of sequence transfers of the kind observed here remain to be studied. AB4 and 82.139 constitute natural model systems for such studies. Exchanges of Ti plasmids between agrobacteria (Petit et al. 1978) and the existence of many Ti plasmid gene homologues both within and among strains (Otten et al. 1992) may provide opportunities for sequence changes of the type described here. The T-DNAs of o/c-type Ti plasmids contain several inactivated genes: *ipt* (Yanofsky et al. 1985a), TA-*iaaH* (in pTiTm4 and related Ti plasmids; Bonnard et al. 1989a), TA-*acs* (in AB3 and Hm1) and TB-*acs* (Paulus and Otten 1993). Although inactivated, these genes may still act as donors for sequence transfers to active homologues or as sites for homologous recombination events. The direct 62 bp repeat in the pTiAB4 *ipt* region renders the *ipt* gene potentially unstable.

Whereas o/c-type Ti plasmids show at least seven different T-DNA forms (Otten and van Nuenen 1993), no variation was observed in the *Pst*I and *Hin*dIII maps of the T-DNA of 20 *A. vitis* nopaline strains. The conserved *ipt* sequences may be used to develop polymerase chain reaction (PCR) primers for the detection and identification of *A. vitis* strains in healthy and diseased grapevine material (Schulz et al. 1993). The available pTiAB4 probes will allow us to determine whether the

remainder of the nopaline-type Ti plasmids is also conserved.

The presence of a 6b-like gene with 63% homology to the pTiC58 6b gene and a gene with 71% homology to the 3′ gene on the pTi15955 TR-DNA are unusual features of 82.139 (Drevet et al. 1994) and AB4. The pTi82.139 6b and 3′ genes have not yet been tested for biological activity. The pTiAB4 6b gene, alone and in combination with the pTiAB4 *ipt* gene, is functional. The functional consequences of the four amino acid difference between the pTiAB4 and pTi82.139 6b genes located within the highly acidic stretch of the 3′ end of the coding region remain to be studied and could yield interesting information concerning the biological role of this part of the 6b coding sequence.

The 3′ gene, originally identified in the TR-DNA of pTi15955 (Barker et al. 1983) has not been reported to have any detectable function, although it is transcribed in tumour tissues (Velten et al. 1983). Its pTiAB4 equivalent does not lead to growth effects on three plant species that are highly sensitive to various tumour genes (Tinland et al. 1989) nor does it affect opine production. Its role remains to be determined.

The "patchwork" character of Ti plasmids originally noted by Engler et al. (1981), elaborated by van Nuenen et al. (1993) and confirmed in this study considerably complicates the reconstruction of the evolutionary history of these plasmids. Commonly, phylogenetic trees are constructed by comparing homology values for selected DNA fragments assuming that evolution mainly proceeds by point mutations. In the case of AB4, a comparison of available *nos* gene sequences would place strain AB4 very close to strain 82.139, and a comparison of *iaaM* gene sequences would place strain AB4 close to strain Tm4. Obviously, neither solution reflects the real situation, and it may be asked what kind of tree could reproduce the complex relationships between AB4, Tm4 and 82.139. In our opinion, only a reasonable understanding of the hypothetical steps that may have led to the AB4, Tm4 and 82.139 genomes would allow the construction of trees for these bacterial species. It should be stressed that in these cases overall levels of DNA homology [obtained for example by hybridization studies or restriction fragment length polymorphism (RFLP) analysis] cannot be used to establish phylogenetic relationships (i.e. patterns of descendance), nor can DNA homology data from single genes be used. A few recombination events (for example the loss or acquisition of a large DNA fragment) can lead to large changes in average DNA homology. In the case of Ti plasmid evolution, large-scale events are clearly much more important than point mutations. In the meantime, phylogenetic trees may be constructed for well-defined DNA fragments of several kilobases; the study on the T-DNA of pTiAB4 shows the potential complexity of such a reconstruction. By gradually extending the comparisons to adjacent sequences, trees for entire plasmids or chromosomes may eventually be obtained. However, if important evolutionary intermediates are lacking (unfortunately this will often be the case, especially if bacterial populations are rapidly dominated by certain clonal forms, as seems to be the case for the *A. vitis* strains), reconstruction of a "species tree" or "plasmid tree" may be impossible or require means to detect rare surviving intermediates. If rapid large-scale changes leading to the same degree of "scrambling" as found in *A. vitis* also occur in other bacterial species, the consequences for bacterial phylogeny may be considerable.

Acknowledgements We thank A. Hoeft for oligonucleotides, R. Wagner and P. Keltz for plants, E. Szegedi for UBAPF2(pTiAB4), C. Drevet and L. Jouanin for communicating the pTi82.139 T-DNA sequences before publication and T. Schulz for *A. vitis* strains.

References

Barker RF, Idler KB, Thompson DV, Kemp JD (1983) Nucleotide sequence of the T-region from *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol Biol 2:335–350

Bevan M (1985) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 84:8711–8721

Blundy KS, White J, Firmin JL, Hepburn AG (1986) Characterisation of the T-region of the SAP-type Ti-plasmid pTiAT181: identification of a gene involved in SAP synthesis. Mol Gen Genet 202:62–67

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

Bonnard G, Tinland B, Paulus F, Szegedi E, Otten L (1989b) Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide host range biotype III *Agrobacterium* strain. Mol Gen Genet 216:428–438

Bonnard G, Vincent F, Otten L (1991) Sequence of *Agrobacterium tumefaciens* biotype III auxin genes. Plant Mol Biol 16:733–738

Burr TJ, Katz BH (1983) Isolation of biovar 3 from grapevine galls and sap and from vineyard soil. Phytopathology 73:163–165

Burr TJ, Katz BH, Bishop AL (1987) Populations of *Agrobacterium* in vineyard and nonvineyard soils and grape roots in vineyards and nurseries. Plant Dis 71:617–620

Canaday J, Gérard J-C, Crouzet P, Otten L (1992) Organization and functional analysis of three T-DNAs from the vitopine Ti plasmid pTiS4. Mol Gen Genet 235:292–303

Currier TC, Nester EW (1976) Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal Biochem 76:431–441

Dahl GA, Gyon P, Petit A, Tempé J (1983) Silver nitrate-positive opines in Crown Gall tumors. Plant Sci Lett 32:193–203

Depicker A, de Wilde M, de Vos G, de Vos R, van Montagu M, Schell J (1980) Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. Plasmid 3:193–211

Dessaux Y, Petit A, Tempé J (1992) Opines in *Agrobacterium* biology. In: Verma DPS (ed) Molecular signals in plant-microbe communications. CRC Press, Boca Raton, Fla., p 109–136

Devereux J, Haeberli P, Marquess P (1987) The program manual for the sequence analysis software package of the genetics computer group. Nucleic Acids Res 12:387–395

Drevet C, Brasileiro ACM, Jouanin L (1994) Oncogene arrangement in a shooty strain of *Agrobacterium tumefaciens*. Plant Mol Biol 25:83–90

Engler G, Depicker A, Maenhaut R, Villarroel R, van Montagu M, Schell J (1981) Physical mapping of DNA base sequence homologies between an octopine and a nopaline Ti plasmid of *Agrobacterium tumefaciens*. J Mol Biol 152:183–208

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

Gérard J-C, Canaday J, Szegedi E, Delasalle H, Otten L (1992) Physical map of the vitopine Ti plasmid pTiS4. Plasmid 28:146–156

Gielen J, De Beuckeleer M, Seurinck J, Deboeck F, De Greve H, Lemmers M, van Montagu M, Schell J (1984) The complete nucleotide sequence of the TL DNA of the *Agrobacterium tumefaciens* plasmid pTiAch5. EMBO J 3:835–846

Goldberg SB, Flick JS, Rogers SG (1984) Nucleotide sequence of the *tmr* locus of the *Agrobacterium tumefaciens* pTiT37 T-DNA. Nucleic Acids Res 12:4665–4677

Hayman GT, Farrand SK (1990) *Agrobacterium* plasmids encode structurally and functionally different loci for catabolism of agrocinopine-type opines. Mol Gen Genet 223:465–473

Hooykaas P, Schilperoort R (1992) *Agrobacterium* and plant genetic engineering. Plant Mol Biol 19:15–38

Huss B, Bonnard G, Otten L (1989) Isolation and functional analysis of a set of auxin genes with low root-inducing activity from an *Agrobacterium tumefaciens* biotype III strain. Plant Mol Biol 12:271–283

Hynes MF, Simon R, 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

Janssens D, Kersters K, De Ley J (1983) The catabolism of 3-ketolactose in *Agrobacterium*. Sys Appl Microbiol 4:155–168

Kado CI (1991) Molecular mechanisms of Crown Gall tumorigenesis. Crit Rev Plant Sci 10:1–32

Kerr A, Panagopoulos CG (1977) Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Z Phytopathol 90:172–179

Knauf VC, Panagopoulos CG, Nester EW (1982) Genetic factors controlling the host range of *Agrobacterium tumefaciens*. Phytopathology 72:1545–1549

Knauf VC, Panagopoulos CG, Nester EW (1983) Comparison of Ti plasmids from three different biotypes of *Agrobacterium tumefaciens* isolated from grapevines. J Bacteriol 153:1535–1542

Knauf VC, Yanofsky M, Montoya A, Nester EW (1984) Physical and functional map of an *Agrobacterium tumefaciens* plasmid that confers a narrow host range. J Bacteriol 160:564–568

Michel MF, Brasileiro ACM, Depierreux C, Otten L, Delmotte F, Jouanin L (1990) Identification of different *Agrobacterium* strains isolated from the same forest nursery. Appl Environ Microbiol 56:3537–3545

Ooms G, Hooykaas PJJ, van Veen RJM, van Beelen P, Regensburg-Tuïnk AJG, Schilperoort RA (1982) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. Plasmid 7:15–29

Ophel K, Kerr A (1990) *Agrobacterium vitis* sp. nov. Strains of *Agrobacterium* biovar 3 from grapevines. Int J Syst Bacteriol 40:236–241

Otten L, Schilperoort R (1978) A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. Biochim Biophys Acta 527:497–500

Otten L, van Nuenen M (1993) Natural instability of o/c Ti plasmids of clonal origin. Microb Releases 2:91–96

Otten L, Canaday J, Gerard J-C, Crouzet P, Paulus F (1992) Evolution of *Agrobacteria* and their Ti plasmids. A review. Mol Plant-Microb Interact 5:279–287

Otten L, Gérard J-C, 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 CG, Psallidas PG (1973) Characteristics of Greek isolates of *Agrobacterium tumefaciens* (EF Smith and Townsend). Conn J Appl Bacteriol 36:233–240

Paulus F, Otten L (1993) Functional and mutated agrocinopine synthase genes on octopine T-DNAs. Mol Plant-Microb Interact 6:393–402

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

Paulus F, Ridé M, 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, 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 KL, Kado CI (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, Schell J (1978) Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature 271:570–571

Rao RN, Rogers SG (1979) Plasmid pKC7: a vector containing ten restriction endonuclease sites suitable for cloning DNA segments. Gene 7:79–82

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schulz T (1992) Untersuchungen zur Epidemiologie und Phylogenie van *Agrobacterium vitis* sp. nov., dem Erreger der Mauke an Weinreben. Ph. D. Thesis, University of Kaiserslautern, Germany

Schulz T, Lorenz D, Eichhorn KW, Otten L (1993) Amplification of different marker sequences for identification of *Agrobacterium vitis* strains. Vitis 32:179–182

Strabala TJ, Bednarek SY, Bertoni G, Amasino RM (1989) Isolation and characterization of an *ipt* gene from the Ti plasmid Bo542. Mol Gen Genet 216:388–394

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

Szegedi E, Csako M, Otten L, Koncz Cs (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, Czako M (1992) Diverse types of tartrate plasmids in *Agrobacterium tumefaciens* biotype III strains. Mol Plant-Microb Interact 5:435–438

Thomashow MF, Knauf VC, Nester EW (1981) Relationship between the limited and wide host range octopine-type Ti plasmids of *Agrobacterium tumefaciens*. J Bacteriol 146:484–493

Tinland B, Huss B, Paulus F, Bonnard G, Otten L (1989) *Agrobacterium tumefaciens* 6b genes are strain-specific and affect the activity of auxin as well as cytokinin genes. Mol Gen Genet 219:217–224

Vanderleyden J, Desair J, De Meirsman C, Michiels K, van Gool A, Jen G, Chilton M-D (1986) Nucleotide sequence of the T-DNA region encoding transcripts 6a and 6b of the pTiT37 nopaline Ti plasmid. Plant Mol Biol 7:33–41

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

Velten J, Willmitzer L, Leemans J, Ellis J, Deblaere R, van Montagu M, Schell J (1983) TR genes involved in agropine production; In: Pühler A (ed) Molecular genetics of the bacteria-plant interaction. Springer-Verlag, Berlin-Heidelberg, pp 303–312

Von Lintig J, Zanker H, Schröder J (1991) Positive regulators of the opine-inducible promoters in the nopaline and octopine catabolism regions of Ti plasmids. Mol Plant Microb Interact 4:370–378

Wabiko H, Kagaya M, Kodama I, Masuda K, Kodama Y, Yamamoto H, Shibano Y, Sano H (1989) Isolation and characterization of diverse nopaline type Ti plasmids of *Agrobacterium tumefaciens* from Japan. Arch Microbiol 152:119–124

Willmitzer L, Dhaese P, Schreier PH, Schmalenbach W, van Montagu M, Schell J (1983) Size, location and polarity of T-DNA-encoded transcripts in nopaline crown gall tumors; common

transcripts in octopine and nopaline tumors. Cell 32:1045–1056

Winans SC (1992) Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol Rev 56:12–31

Yamamoto K, Kusano K, Takahashi NK, Yoshikura H, Kobayashi I (1992) Gene conversion in the *Escherichia coli* RecF pathway: a successive half crossing-over model. Mol Gen Genet 234:1–13

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

Yanofsky M, Lowe B, Montoya A, Rubin R, Krul W, Gordon M, Nester E (1985a) Molecular and genetic factors controlling host range in *Agrobacterium tumefaciens*. Mol Gen Genet 201:237–246

Yanofsky M, Montoya A, Knauf VC, Lowe B, Gordon M, Nester EW (1985b) Limited-host-range plasmids of *Agrobacterium tumefaciens*: molecular and genetic analysis of transferred DNA. J Bacteriol 163:341–348

Zambryski P (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annu Rev Plant Mol Biol 43:465–490