Mol Gen Genet (1992) 235:292–303

MGG

Organization and functional analysis of three T-DNAs from the vitopine Ti plasmid pTiS4

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Received April 15, 1992 / Accepted May 26, 1992

Summary. The vitopine Ti plasmid pTiS4 of *Agrobacterium vitis* has an unusual T-DNA organization. The pTiS4 oncogenes, localized by screening selected pTiS4 clones for growth-inducing activity, are localized on three T-DNAs, whereas in all other characterized Ti plasmids one or two T-DNAs are found. The nucleotide sequences and predicted amino acid sequences of the pTiS4 oncogenes set them apart from the corresponding genes from other Ti or Ri plasmids. The oncogenes induce the same type of reaction on various test plants as the well-known pTiAch5 oncogenes but the pTiS4 *ipt* gene induces considerably more shoots than its Ach5 homologue. We have also identified the gene coding for vitopine synthase as well as a vitopine synthase pseudogene. Both sequences show homology to the octopine synthase gene. In terms of both nucleotide sequence and overall organization, the pTiS4 T-DNAs appear to be only distantly related to previously characterized T-DNAs.

Key words: *Agrobacterium* – Ti plasmid – T-DNA – Vitopine

Introduction

Agrobacteria are well-known for their capacity to induce crown gall tumors and hairy root disease by stable transformation of their plant host. Well-defined fragments of the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid or the *A. rhizogenes* root-inducing (Ri) plasmid are transferred during infection. The expression of the transferred DNA (T-DNA) leads to tumor or root formation and the production of opines which the *Agrobacterium* uses as a source of nutrition. The T-DNAs are defined by specific border sequences and are transferred by Ti-borne functions encoded by the virulence genes (for a review, see Zambryski et al. 1989).

Numerous *Agrobacterium* strains have been isolated. Chromosomally encoded metabolic and physiological characteristics of the bacteria have been used to classify strains into three biotypes or biovars (Kerr and Panagopoulos 1977). The most widely studied Ti plasmids originate from strains belonging to the biotype I group: Ach5 (very similar to 15955 and A6; de Vos et al. 1981) and C58 (very similar to T37; Willmitzer et al. 1983). The biotype II group contains *A. rhizogenes* strains which cause hairy root disease. The biotype III strains induce crown gall tumors on grapevine. It has been proposed recently (Ophel and Kerr 1990) that the latter strains be referred to as *Agrobacterium vitis* since they are isolated from grapevine (*Vitis vinifera*) and show characteristics which distinguish them from both *A. tumefaciens*, *A. rhizogenes*, *A. rubi* and *A. radiobacter*. Within the different biotypes, opine utilization is used to distinguish the different Ti plasmids. The *A. vitis* Ti plasmids can be divided into octopine/cucumopine (o/c), nopaline (nop) and vitopine (vit) plasmids (Szegedi et al. 1988; Paulus et al. 1989a). While octopine and nopaline Ti plasmids are also found in *A. tumefaciens*, and cucumopine plasmids are found in *A. rhizogenes*, plasmids encoding vitopine synthesis have only been found in *A. vitis*.

Tumorigenic *Agrobacterium* strains have been found to contain different oncogenes whose structure and function have been studied in detail. The *ipt* gene encodes isopentenyl transferase which synthesizes isopentenyladenosine 5′-monophosphate, a cytokinin that induces shoot formation on suitable host plants (Akiyoshi et al. 1984; Buchmann et al. 1985). The *iaa* genes, also found in mannopine/agropine (m/a) strains of *A. rhizogenes* (Offringa et al. 1986), include *iaaM*, encoding tryptophan monooxygenase, and *iaaH*, encoding indole acetamide hydrolase (Thomashow et al. 1984, 1986;

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Abbreviations: Ap, ampicillin; IS, insertion sequence; *iaaH*, indole acetamide hydrolase; *iaaM*, tryptophan monooxygenase; *ipt*, isopentenyl transferase; Km, kanamycin; LB, Luria broth; m/a, mannopine/agropine; o/c, octopine/cucumopine; *ocs*, octopine synthase

Schröder et al. 1984). The combined activity of *iaaM* and *iaaH* leads to indole acetic acid (IAA) synthesis which induces roots on certain host plants. The 6b gene, whose gene product has not yet been characterized, is qualified as an oncogene (Hooykaas et al. 1988; Tinland et al. 1989) since it leads to the formation of tumors on certain hosts.

Both the octopine/cucumopine and nopaline Ti plasmids of *A. vitis* show homology to the oncogenes of the *A. tumefaciens* model strain Ach5 in hybridization experiments. Oncogenes of an o/c model strain Tm4 have been studied in detail (Huss et al. 1989b). The vitopine plasmids did not show hybridization homology (Paulus et al. 1989a, b), suggesting that the vitopine T-DNAs have distinctive oncogenes. To investigate the oncogene content and T-DNA organization of vitopine Ti plasmids, we began a detailed study of the Ti plasmid from the strain S4 (Szegedi 1985). The S4 Ti plasmid has been cloned and mapped (Gérard et al. 1992) and here we report the analysis of the pTiS4 T-DNAs.

Materials and methods

Bacterial strains and media. Standard *Escherichia coli* strains, plasmids and media can be found in Sambrook et al. (1990). *Agrobacterium* strains listed in Table 1 were grown as described (Leemans et al. 1983).

Construction and screening of the S4 plasmid library. S4 carries four plasmids (Gérard et al. 1992), which were extracted and purified (Currier and Nester 1976) and used to construct a library in the pBR322-derived vector pKC7 (Ap^r, Km^r; Rao and Rogers 1979). This vector was chosen since pKC7 clones can be mobilized to *Agrobacterium* and selected by growth on kanamycin or neomycin. Plasmid DNA was partially digested with *Sau*3AI, size-fractionated on a sucrose gradient and ligated into *Bam*HI-digested, dephosphorylated vector DNA. Transformation of *E. coli* strain NM522 yielded a library of 1100 recombinant clones. The clones referred to in this study are listed in Table 1.

Colonies were grown and stored in microtitre plates as described by Gergen et al. (1979). For screening, colonies were seeded onto LB agar (Ap, Km) plates using a Multipoint Inoculator, transferred to Whatman 540 paper and hybridized as described (Gergen et al. 1979) with minor modifications. The probe for IS*867* was the *Sma*I-*Eco*RI fragment from clone T-9 of the Tm4 TA region (Otten, unpublished). The IS*868* probe was the 0.95 kb *Hin*cII fragment used previously (Paulus et al. 1991b).

Table 1. Strains and plasmids

Designation	Characteristics	Reference
Agrobacterium strains:		
GV3101	C58 pTi cured, rif resistant	Depicker et al. (1980)
S4	vitopine	Szegedi (1985)
Agrobacterium plasmids:		
pGV3850	disarmed Ti vector	Zambryski et al. (1983)
pPM6000	disarmed Ti vector	Bonnard et al. (1989)
pGV2260	disarmed, borderless Ti vector	Deblaere et al. (1985)
pPM6000::pPM1023	pTiAch5 *iaa* genes	Huss et al. (1989a)
pPM6000::pPM1023–32	pTiAch5 *iaaM* gene	Huss et al. (1989a)
pPM6000::pPM1023–39	pTiAch5 *iaaH* gene	Huss et al. (1989a)
pPM6000::pPM1010	pTiAch5 6b gene	Tinland et al. (1989)
pPM6000::pPM33	pTiAch5 *ipt* gene	Bonnard et al. (1989)
Escherichia coli plasmids:		

Clones	Fragment	Vector	Reference
pPM601-pPM812	partial *Sau*3AI	pKC7	This work
pPM605 subclones			
pPM605.1	1.85 kb *Eco*RI	pKC7	This work
pPM605.2	1.6 kb *Eco*RI	pKC7	This work
pPM605.3	3.1 kb *Eco*RI	pKC7	This work
pPM605.4	2 kb partial *Sau*3AI	pKC7	This work
pPM674 subclones			
pPM674.1	5.8 kb *Eco*RI	pBluescript KS	This work
pPM674.2	0.7 kb *Eco*RI	pBluescript KS	This work
pPM674.3	4.5 kb *Eco*RI	pBluescript KS	This work
pPM674.4	6.0 kb *Eco*RI	pBluescript KS	This work
pPM674.4.1	3.0 kb *Eco*RI/*Hin*dIII	pBluescript KS	This work
pPM674.4.2	3.1 kb *Hin*dIII/*Eco*RI	pBluescript KS	This work
pPM674.5	partial *Hin*dIII	pKC7	This work
pPM674.6	2.4 kb *Cla*I/*Eco*RI	pKC7	This work
pPM674.7	3.1 kb *Bam*HI/*Eco*RI	pKC7	This work
pPM674.8	1.7 kb *Hin*dIII/*Dra*I	pKC7	This work

Subcloning of pTiS4 DNA fragments. DNA fragments were isolated from clones from the S4 library and subcloned using standard techniques (Sambrook et al. 1990). The subclones are listed in Table 1. pPM605.4 was obtained by partial *Sau*3AI digestion of pPM605. DNA fragments of 2 kb were selected after sucrose gradient fractionation and cloned in the *Bam*HI site of pKC7. Twenty recombinant clones were mapped by hybridization and restriction analysis. pPM674.5 was obtained by partial *Hin*dIII digestion of pPM674 and religation. In addition to the region shown in Fig. 2c, this clone contains 400 bp from the left extremity of pPM674, extending from the *Sau*3AI cloning site to the first *Hin*dIII site. The 3.2 kb *Bam*HI-*Eco*RI fragment in pPM674.7 was subcloned from pPM674.3 as a 3.1 kb *Bam*HI fragment using the pBluescript KS polylinker *Bam*HI site. pPM674.8 was obtained by digesting pPM674.4.2 containing the 1.7 kb *Hin*dIII-*Dra*I fragment with *Bam*HI (which cuts in the pBluescript KS polylinker) and *Dra*I and cloning this fragment into *Bam*HI + *Sma*I-digested pKC7.

Sequence analysis. Clones used for sequencing were derived from pPM602, pPM605 and pPM674 by subcloning appropriate fragments in pBluescript KS− and KS+ vectors (Stratagene, La Jolla, Calif.). Nested deletions of the subclones were obtained with exonuclease III (Henikoff 1987). Single-stranded DNA template was used for sequencing by the dideoxy chain termination technique (Sanger et al. 1977). Additional information was obtained by sequencing restriction fragments cloned in M13 vectors mp18 and mp19 (Sambrook et al. 1990). When necessary, specific oligonucleotide primers were used. To resolve band compression and sequence ambiguities, clones were sequenced using dITP instead of dGTP (United States Biochemicals). All sequences were determined on both strands and the data were analyzed using the GCG sequence analysis software package Version 6.0 (Devereux et al. 1987) on a MicroVax computer. Nucleotide sequences and deduced amino acid sequences were compared using the Bestfit program.

Introduction of clones into Agrobacterium *plasmids.* DNA fragments cloned in pKC7 were recombined into the disarmed *Agrobacterium* vectors pPM6000, pGV3850 or pGV2260 by conjugation using the mobilization functions of GJ23 (van Haute et al. 1983). The structure of each construct was verified by hybridization analysis of *Agrobacterium* DNA prepared according to Dhaese et al. (1979).

Plant infections. Kalanchoe daigremontiana, Nicotiana tabacum cv Samsun and *Nicotiana rustica* plants grown in a greenhouse were infected by puncturing the stems and inoculating the wounds with a concentrated bacterial suspension. The *Agrobacterium* strains used were grown overnight, washed and resuspended in 10 mM $MgSO_4$ to give a tenfold concentrated suspension. Comparisons were made between batches of plants infected at the same time.

Vitopine detection. Opines were extracted from tumors obtained by infection of *N. rustica* stems with pPM6000::pPM674. To induce growth of transformed tissue, non-oncogenic clones were coinfected with pPM6000::pPM1023 containing the *iaa* genes from pTiAch5. Vitopine was detected by high-voltage paper electrophoresis according to Szegedi et al. (1988).

Analysis of tumor DNA. Tumor tissue was obtained by infection of sterile *N. rustica* leaf discs (Huss et al. 1989b) with an S4 bacterial suspension. Tumors were propagated on LS (Linsmaier and Skoog 1965) agar with 500 mg/ml Claforan (Hoechst). The tissue was frozen in liquid nitrogen and stored at −80° C. DNA was extracted from tumor or from normal leaf tissue according to Chaubet et al. (1989). Southern blots of DNAs digested with *Bam*HI, *Eco*RI and *Hin*dIII were probed with insert DNA from clones pPM605.1, pPM605.2, pPM605.3, pPM674.3 and pPM674.4.2 (Table 1 and Fig. 2).

Results

Localisation of pTiS4 oncogenes

Among the 68 grapevine plasmid isolates in our collection, nine belong to the vitopine group. Since hybridization results (Paulus et al. 1989a, b; our unpublished results) indicated that the vitopine plasmids are very simi-

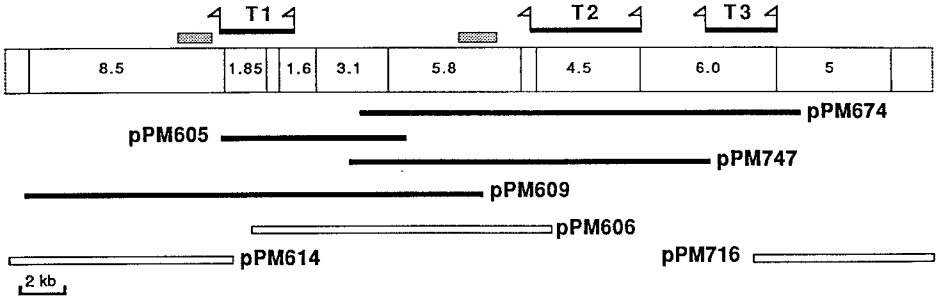

Fig. 1. T-DNA containing region of pTiS4. The map positions of the three pTiS4 T-DNAs are shown *above* the *Eco*RI restriction map. T-DNA borders are indicated by *flags*. *Dotted boxes* show the regions homologous to the IS*868* probe. *Below* the restriction map, are shown selected clones from the S4 plasmid bank: *solid bars*, clones that induce a growth response on *Nicotiana rustica*; *empty bars*, clones that do not induce a growth response on *N. rustica*

lar, the strain S4 (Szegedi 1985) was arbitrarily chosen for the construction of a plasmid library. Subsequently, analysis of the plasmid content showed that S4 contains four different plasmids, whereas the other vitopine strains (Sz1, Sz2, NW11 and NW221) each contain only one.

Oncogene probes from the octopine plasmid pTiAch5 (Paulus et al. 1989a, b) and the nopaline plasmid pTiC58 or pRiHRI (*A. rhizogenes*) do not hybridize with S4 DNA (Gérard et al. 1992). However, plasmid DNA from S4 did hybridize to probes from insertion sequences IS*867* and IS*868*. Since both of these elements are associated with T-DNA regions in the o/c Ti plasmids (Paulus et al. 1991b), it was possible that the same would be true in pTiS4. Therefore, the S4 clones from the plasmid library which hybridized to IS*867* and IS*868* probes were tested for oncogene activity. Twenty-four clones with IS*867*- or IS*868*-like sequences were identified, mapped and linked by restriction and hybridization analysis, and were found to form a contiguous 60 kb fragment which was subsequently shown to be part of pTiS4 (Gérard et al. 1992). The two regions of pTiS4 which hybridize to the IS*867* probe are situated in the left part of this 60 kb region (not shown). The right-hand 40 kb region is shown in Fig. 1 and contains two regions homologous to IS*868*.

To test for oncogene activity, selected clones covering the 60 kb fragment were transferred to the disarmed *Agrobacterium* Ti integration vector pGV3850 (see the Materials and methods). The resulting strains were used to infect *N. rustica* stems. The map positions of four oncogenic clones (pPM605, pPM609, pPM747 and pPM674) and three negative clones (pPM614, pPM606 and pPM716) are shown in Fig. 1. Three different tumor phenotypes were observed. The overlapping clones pPM605 and pPM609 induced small tumors on *N. rustica* stems (identical to the tumors described below for pPM605.1 and shown in Fig. 3a). From the map position of the overlapping negative clones pPM614 and pPM606, we predicted that at least part of the oncogene(s) responsible for the phenotype observed was located in the 1.85 kb *Eco*RI fragment. Of the three *Eco*RI fragments subcloned from pPM605 (pPM605.1, pPM605.2 and pPM605.3 in Table 1), only the 1.85 kb *Eco*RI fragment (pPM605.1) induced tumor formation upon infection (Fig. 3a). To identify the oncogene(s), the 1.85 kb *Eco*RI fragment was sequenced (see below).

Clone pPM674 led to the formation of large tumors on *N. rustica* stems. On Kalanchoe stems, it induced tumors and roots below the tumor (not shown). The neighboring clone, pPM747, however, showed only root production on Kalanchoe stems (Fig. 7b), indicating that the oncogenes responsible for root formation are located in the region common to both pPM747 and pPM674, whereas the additional activity leading to tumor formation is localized in the right part of pPM674. Since pPM606 covers part of the region common to pPM747 and pPM674 and gives a negative reaction, it seemed likely that the 4.5 kb *Eco*RI and part of the 6 kb *Eco*RI fragment absent from pPM606 contained the root-inducing oncogenes.

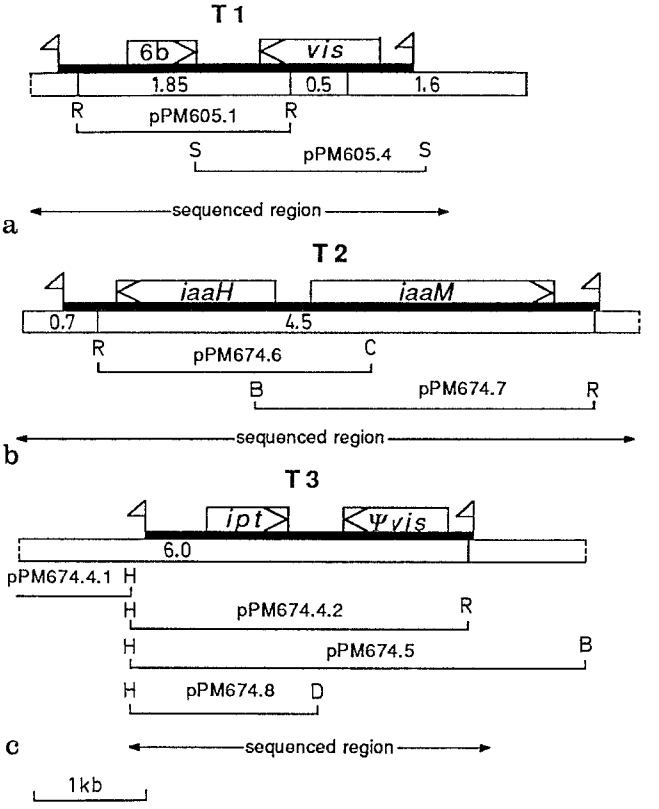

Fig. 2a–c. pTiS4 T-DNA regions. The map positions of the pTiS4 oncogenes are shown *above* the *Eco*RI restriction map. The direction of transcription is indicated by *arrowheads*. T-DNA borders are indicated by *flags*. The extent of the clones (listed in Table 1) is shown *below* the restriction map. Restriction site abbreviations: B, *Bam*HI; C, *Cla*I; D, *Dra*I; H, *Hin*dIII; R, *Eco*RI; and S, *Sau*3AI. **a** T1 region; **b** T2 region; **c** T3 region. Clone pPM674.4.1 extends to the *Eco*RI site (not shown here, see Fig. 1). Clone pPM674.5 contains an additional 400 bp *Sau*3A-*Hin*dIII fragment not shown in this figure (see the Materials and methods)

The *Eco*RI fragments from pPM674 were subcloned as pPM674.1–4 (Table 1). The 6 kb *Eco*RI fragment from pPM674.4 was subcloned as pPM674.4.1 (3.0 kb *Eco*RI-*Hin*dIII) and pPM674.4.2 (3.1 kb *Hin*dIII-*Eco*RI) (Fig. 2c). The extremities of the subcloned *Eco*RI and *Eco*RI-*Hin*dIII fragments were sequenced. The 4.5 kb *Eco*RI fragment (pPM674.3) was shown to contain *iaaM*- and *iaaH*-like sequences. Homology to the *ipt* gene was found in the 3.1 kb *Hin*dIII-*Eco*RI fragment (pPM674.4.2). The complete sequences of the 4.5 kb *Eco*RI fragment and the 3.1 kb *Hin*dIII-*Eco*RI fragment were therefore determined (see below). Subsequently, 25 additional pTiS4 clones, situated outside the 60 kb fragment described above, were tested for oncogene activity. These clones, covering 90% of the pTiS4 map (Gérard et al. 1992), did not show oncogenic activity. We cannot, however, exclude the possibility that oncogenes that have no effect on *N. rustica* remained undetected in our screening procedure.

Sequences of the pTiS4 oncogenes

The complete sequences of the 1.85 kb *Eco*RI (pPM605.1), the 4.5 kb *Eco*RI (pPM674.3) and the

3.1 kb *Hin*dIII-*Eco*RI (pPM674.4.2) fragments were determined (available in the EMBL data base under accession numbers M91608, M91609 and M91610). Analysis of these sequences allowed us to identify four pTiS4 oncogenes: 6b, *iaaM*, *iaaH* and *ipt*, termed S-6b, S-*iaaM*, S-*iaaH* and S-*ipt* (the Ach5 genes are preceded by the prefix A- and the Tm4 genes by the prefix T-). The 1.85 kb *Eco*RI fragment (Fig. 2a) contains an open reading frame (ORF) showing 60–62% nucleotide homology to the coding sequence of 6b genes from Ach5, T37 and Tm4. As predicted from preliminary sequencing results, the 4.5 kb *Eco*RI fragment (Fig. 2b) contains two ORFs corresponding to *iaaH* and *iaaM* genes. The two genes are in opposite orientation and show 58–61% (for *iaaH*) and 59–60% (for *iaaM*) nucleotide homology with their counterparts from pTiAch5, pTiTm4 and pRiA4. The 3.1 kb *Hin*dIII-*Eco*RI fragment (Fig. 2c) contains a complete coding sequence for an *ipt* gene which shows 60–62% nucleotide homology to the pTiAch5, pTiT37 and pTiTm4 *ipt* genes. For all four pTiS4 oncogenes, the nucleotide homology to known oncogenes ranges from 58 to 62% and is limited to the coding sequences. No significant nucleotide homology was found on comparing the 3′ or 5′ non-coding regions of pTiS4 oncogenes with their counterparts from other Ti or Ri plasmids.

The amino acid sequences deduced from the various nucleotide sequences are compared in Table 2. All four pTiS4 peptides are only distantly related to their counterparts from other Ti or Ri plasmids since for each type of peptide, the pTiS4 peptide presents the lowest degree of amino acid identity (47–58%). In addition, comparable values are obtained when a given pTiS4 peptide is compared to its counterpart from a Ti plasmid from *A. tumefaciens*, *A. vitis* or from *A. rhizogenes* in the case of the *iaa* peptides. When similar amino acids are taken into consideration, higher percentages of homology are obtained but the same conclusions hold.

We have also compared the amino acid sequences of oncogenes of the o/c *A. vitis* strain Tm4, with those of pTiAch5 and pTiT37. In comparison to pTiS4 peptides, T-6b and T-*ipt* peptides show significantly higher homology (72–89% amino acid identity) with their counterparts from pTiAch5 and pTiT37. Judging from the nucleotide and amino acid comparisons, the oncogenes from pTiAch5, pTiT37 and pTiTm4 form a closely related group. The pTiS4 oncogenes do not belong to this group. Additional comparisons (not shown) indicate that the S-*iaa* genes are not closely related to the *Pseudomonas syringae* (pv. *savastanoi*) *iaa* genes (Yamada et al. 1985) nor is the S-*ipt* gene closely related to the pTiT37 *tzs* gene (Beaty et al. 1986). A detailed analysis of the sequences of pTiS4 oncogenes will be presented elsewhere.

Table 2. Comparison of the deduced amino acid sequences of *Agrobacterium* oncogenes

a. 6b polypeptides

Strain	Tm4[1]	Ach5[2]	T37[3]
S4	55% (74%)[a]	55% (70%)	55% (70%)
Tm4		72% (84%)	72% (84%)
Ach5			90% (94%)

b. *iaaM* polypeptides

Strain	Tm4[4]	Ach5[2]	A4[5]
S4	47% (65%)	48% (66%)	49% (68%)
Tm4		87% (92%)	58% (75%)
Ach5			58% (74%)

c. *iaaH* polypeptides

S4	58% (73%)	58% (73%)	58% (72%)
Tm4		88% (95%)	70% (84%)
Ach5			71% (83%)

d. *ipt* polypeptides

Strain	Tm4[6]	Ach5[2]	T37[7]
S4	52% (69%)	49% (67%)	51% (68%)
Tm4		87% (92%)	89% (93%)
Ach5			87% (93%)

[a] The values shown indicate percentage identity and in parentheses, percentage homology

[1] G. Bonnard, unpublished, accession number X56185; [2] Gielen et al. 1984; [3] Vanderleyden et al. 1986; [4] Bonnard et al. 1991; [5] Camilleri and Jouanin 1991; [6] Bonnard et al. 1989; [7] Goldberg et al. 1984

Functional analysis of pTiS4 oncogenes

To test the activity of the pTiS4 oncogenes, individual genes were cloned in pKC7 and introduced into the disarmed pTi vector pPM6000. (Details of the constructions are given in the Materials and methods and the positions of the clones are indicated on the maps in Fig. 2). The resulting *Agrobacterium* strains were used to infect different plant species, since no single host reacted strongly towards all types of oncogene. The pTiS4 oncogenes were compared with their well-characterized counterparts from pTiAch5 (*iaa*, Schröder et al. 1984; *ipt*, Buchmann et al. 1985; and 6b, Hooykaas et al. 1988), carried on pPM6000.

The S-6b gene was tested in three different ways. It induces small tumors on *N. rustica* stems (Fig. 3a) and on *N. tabacum* stems (Fig. 3i). It has been shown that the A-6b gene and T-6b genes increase the size of A-*iaa*-induced tumors on *N. rustica* stems (Tinland et al. 1989). A similar coinfection phenotype is found for S-6b and the A-*iaa* genes (Fig. 3b). In coinfections with the A-*ipt* gene, 6b genes from different Ti plasmids lead to different phenotypes on *N. tabacum* (Tinland et al. 1989). The S-6b/A-*ipt* gene combination (Fig. 3j) induces white tumors with traces of necrosis whereas coinfection of A-6b and A-*ipt* induces green tumors (Tinland et al. 1989).

The S-*iaa* genes induce roots on Kalanchoe, like the A-*iaaM* and A-*iaaH* genes. The S-*iaa* genes induce slightly more roots than do the A-*iaa* genes (cf. Fig. 3d

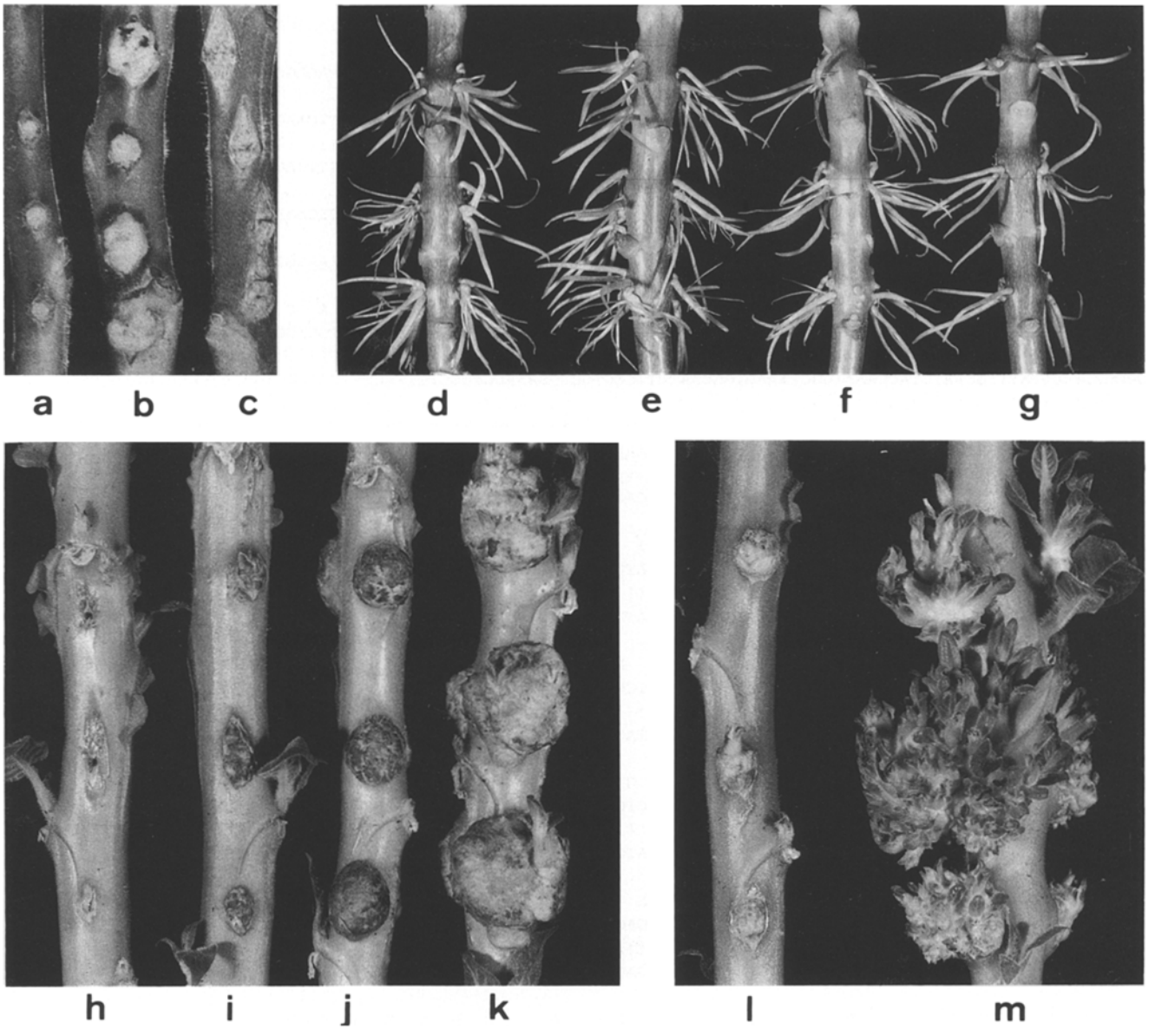

Fig. 3a–m. Oncogene activity on test plants *N. rustics* (**a–c**), Kalanchoe (**d–g**) 4 weeks after infection and *N. tabacum* (**h–m**) 6 weeks after infection. **a** S-6b; **b** S-6b + A-*iaaM* + A-*iaaH*; **c** A-*iaaM* + A-*iaaH*; **d** S-*iaaM* + S-*iaaH*; **e** A-*iaaM* + S-*iaaH*; **f** S-*iaaM* + A-*iaaH*; **g** A-*iaaM* + A-*iaaH*; **h** pPM6000, disarmed vector; **i** S-6b; **j** S-6b + A-*ipt*; **k** S-6b + S-*ipt*; **l** A-*ipt*; **m** S-*ipt*

and g). This difference could be due to the S-*iaaH* gene since A-*iaaM*/S-*iaaH* (Fig. 3e) produces more roots than does S-*iaaM*/A-*iaaH* (Fig. 3f) or A-*iaaM*/A-*iaaH* (Fig. 3g). Infection of *N. tabacum* stems with the S-*ipt* gene construct leads to much more abundant shoot formation (Fig. 3m) than with the A-*ipt* gene (Fig. 3l). In coinfection experiments with the S-6b gene, the S-*ipt* gene induces larger tumors (Fig. 3k) than the A-*ipt* gene (Fig. 3j). In addition, the tumor morphologies differ, since shoot formation is observed only on S-6b/S-*ipt* tumors. In summary, while the S-*iaa* genes differ only slightly from the A-*iaa* genes, both the S-*ipt* and S-6b genes induce a stronger growth response than the corresponding genes from Ach5.

Identification of the pTiS4 vitopine synthase gene

In the course of sequence analysis of the pTiS4 oncogenes, two ORFs showing homology to the pTiAch5 octopine synthase (*ocs*) gene were found. The first, situated 3′ to the 6b gene, extends into the adjacent 0.50 kb and 1.6 kb *Eco*RI fragments (Fig. 2a) and is part of pPM606 (Fig. 1). The 6b gene and this *ocs* homolog are oriented in opposite directions whereas in all octopine Ti plasmids so far characterized, the 6b and *ocs* genes are in the same orientation. The second region of pTiS4 showing sequence homology to the *ocs* gene lies on pPM674 (Fig. 1) and is found in the 3.1 kb *Hin*dIII-*Eco*RI fragment 3′ to the *ipt* gene (Fig. 2c).

Since S4-induced tumors do not contain octopine but vitopine, it was surprising to find two sequences homolo-

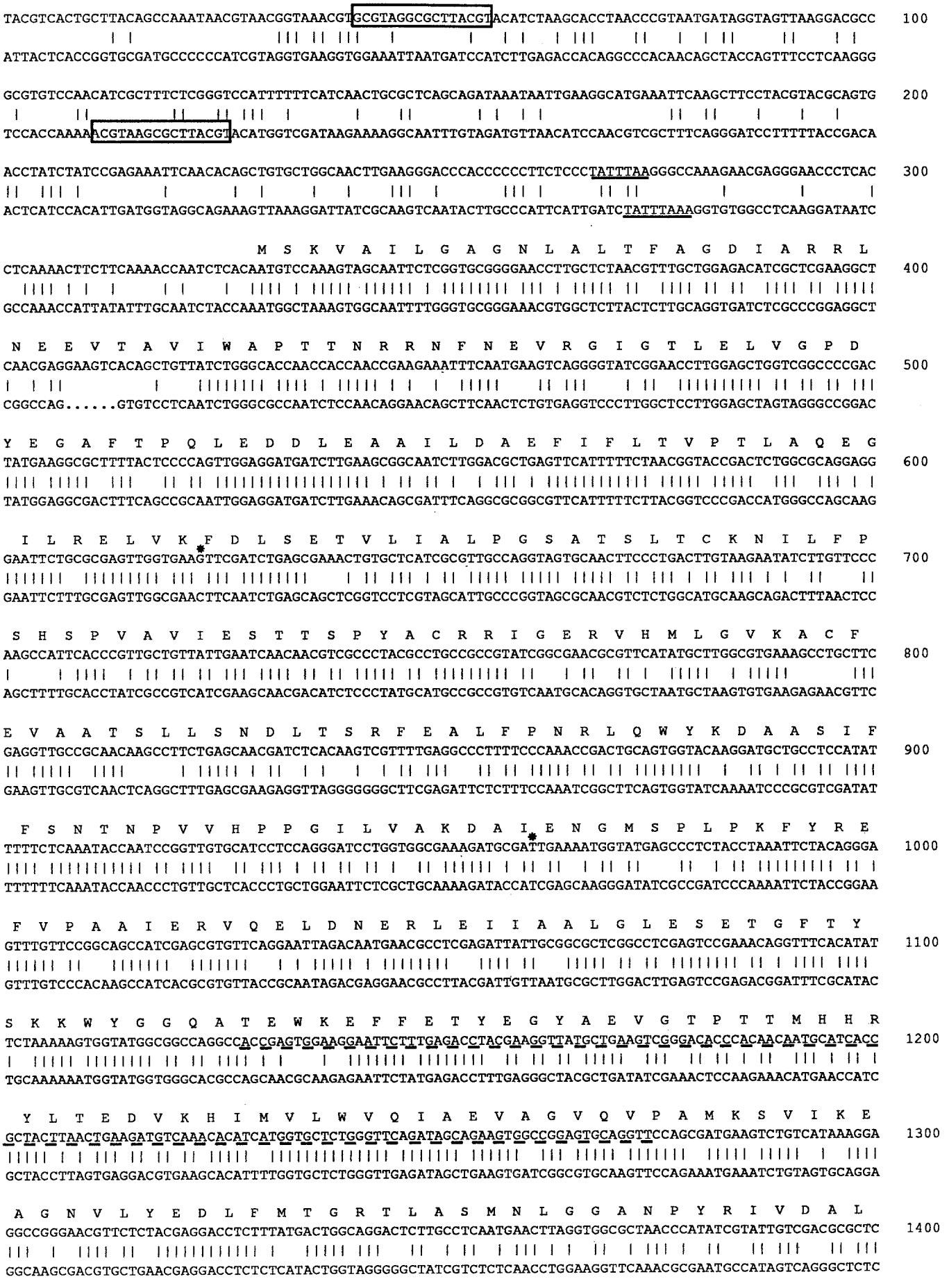

Fig. 4

Fig. 4. Alignment of *vis* and *ocs* sequences. The *numbered line* corresponds to the *vis* sequence (reverse complement of the sequence from positions 1556 to 3455 in accession number M91608). The deduced amino acid sequence is shown above and the *ocs* sequence (reverse complement from positions 11466 to 13359; Gielen et al. 1984) is given below. *Boxes* indicate *ocs* enhancer and *vis* homologue, *underlined* sequences indicate TATA boxes, *overlined* sequences indicate polyadenylation signals, and *asterisks* and *dotted lines* indicate deletions in *ψvis* (see text)

Fig. 5. a Schematic diagram of the T1, T3 and pTiAch5 sequence comparisons, part of which (indicated by *vertical arrows*) is shown in **b**. Homologous sequences are shown by *gray boxes* (*ipt*) or *slashed boxes* (*vis* and *ψvis*). **b** Alignment of sequences from T1, S-6b/*vis* (positions 1439–1574 in accession number M91608) from T3 S-*ipt* and *ψvis* (positions 1261–1399 in accession number M91610) and from pTiAch5 (positions 8773–8920; Gielen et al. 1984). The S-6b, S-*ipt* and A-*ipt* stop codons are *boxed* and the Chi sequence in S-*ipt* is *underlined*. The predicted A-*ipt* amino acid sequence is shown below the A-*ipt* sequence; amino acids common to S-*ipt* are *underlined*

gous to the *ocs* gene. To test whether these *ocs* homologs might be involved in vitopine synthesis, opine assays were done on tumor tissue containing the corresponding regions (as described in the Materials and methods). Vitopine was present in tumor tissue containing pPM6000::pPM606 (map in Fig. 1) but not in tumors induced by pPM6000::pPM674 (map in Fig. 1). Since pPM606 covers 13 kb, a pPM605 subclone, pPM605.4 (Table 1 and Fig. 2a), containing only the *ocs* homolog (as shown by sequencing, see below) was also tested for vitopine production and found to be positive. This confirmed the identification of the *ocs* homolog in this region as the vitopine synthase gene (*vis*). The second *ocs* homolog is indicated as *ψ*vis (see below).

The complete sequences of both *vis* and *ψvis* were determined. The *vis* coding sequence shows 68% nucleic acid homology to the pTiAch5 *ocs* coding sequence. The alignment of the two sequences (Fig. 4) shows that the stretches of perfect homology do not exceed 18 nucleotides. Within the coding sequence, 56% of the differences between *vis* and *ocs* are found in the third codon positions. Comparison of the deduced amino acid sequences shows 67% identity. Both the nucleic acid homology and peptide homology indicate that *ocs* and *vis* are more closely related than are the pTiS4 oncogenes to their counterparts. While no significant overall homology is found in the 5′ and 3′ non-coding regions of *vis* and *ocs*, the *vis* sequence contains a motif similar to the *ocs* enhancer element at positions 41–56. The 16 bp *ocs* palindrome binds an *ocs* transcription factor (Singh et al. 1989) and has been shown to be essential for enhancer activity (Ellis et al. 1987). Functional homologs of this element are found upstream from other T-DNA genes that are involved in opine synthesis and show between 8 and 13 matches with the 16 bp *ocs* palindrome (Bouchez et al. 1989). The *ocs*-like element of the *vis* gene,

situated farther upstream (289 bp from the ATG) than the *ocs* element (119 bp upsteam), has not been tested for enhancer activity but shows 14 matches with the *ocs* palindrome.

Comparison of the *vis* sequence with the *ψvis* coding sequence (not shown) indicates that single nucleotide deletions are found in positions corresponding to 623 and 961 in the *vis* sequence (Fig. 4). These deletions interrupt the *ψvis* reading frame. In addition, a 146 bp deletion extends from positions 1128 to 1274 (Fig. 4). Since the *ψvis* nucleotide sequence is more similar to *vis* (77%) than to *ocs* (68%), it can be called a *vis* pseudogene or *ψvis*. The 5′ and 3′ non-coding regions of the *vis* and *ψvis* genes can be aligned. *ψvis* shows 68% nucleic acid homology with the upstream region of the *vis* gene from position 131 to the ATG initiation codon in position 330 (data not shown). The region from the end of *ψvis* to *ipt* can be aligned with the intergenic region between *vis* and 6b (Fig. 5b).

The similarity between *vis* and *ψvis* and their surrounding sequences suggests that they result from the duplication of an ancestral *vis* gene. One copy, the *vis* gene, is now found to be linked to the 6b gene and the second, *ψvis*, is linked to the *ipt* gene. The homology between the two diverged copies ends at the 6b stop codon but extends 39 bp into the *ipt* gene. The last 39 bp of the *ipt* gene encode 12 amino acids and the stop codon. The S-*ipt* predicted amino acid sequence is homologous to other *ipt* amino acid sequences, and includes this region. It would therefore appear that the duplicated region includes part of the S-*ipt* gene as well as the *vis* gene and its adjacent sequences. While it is not clear how such a duplication event could have occurred or whether other recombination events were involved, we note the presence in the S-*ipt* gene of a Chi sequence (Fig. 5b), which is implicated in bacterial RecBCD-mediated recombination events (Smith 1988).

Identification of pTiS4 border sequences

A T-DNA border consensus sequence has been established (van Haaren et al. 1988) by comparison of known left border (LB) and right border (RB) sequences, and consists of two conserved regions of 13 and 5 nucleotides separated by 5 variable nucleotides. The regions surrounding the pTiS4 oncogenes were sequenced (Fig. 2) to identify potential T-DNA borders. Such sequences (Fig. 6) were found at the positions indicated by flags in Fig. 2 and define three putative T-DNAs, called T1, T2 and T3. In each case, the putative borders were close (within 550 bp) to the sequenced pTiS4 genes (or pseudogene). No additional ORFs were found between the identified pTiS4 genes and the border sequences.

Each pTiS4 left border sequence has one mismatch (shown in small letters, Fig. 6a) with the consensus sequence. The mismatch found in the T1 left border is also present in the TL (oct) and TA (o/c) left borders. The T1 and T2 left borders are preceded by GG as are other left border sequences. The pTiS4 right borders each show three differences from the consensus sequence

a Left Border Sequences

```
                    1                       24
T1 (vit)    ggcGGCAGGATATATttagtTGTAAAa
T2 (vit)    ggTGGCAGGATgTATtgtcaTGTAAAc
T3 (vit)    gaTGGCAGGATATATcaaagTGTAAgt

consensus   NNTGGCAGGATATATNNNNNTGTAA(A/T)N

TL (oct)    ggcGGCAGGATATATtcaatTGTAAAc
TR (oct)    ggTGGCAGGATATATcgaggTGTAAAa
T  (nop)    ggTGGCAGGATATATtgtggTGTAAAc
TL Ri(m/a)  ggTGGCAGGATATATtgtgaTGTAAAc
TA (o/c)    ggcGGCAGGATATATtgaatTGTAAAt
```

b Right Border Sequences

```
                    1                       24
T1 (vit)    taTGaCAGGATtTATcgttaTGTcATg
T2 (vit)    ctTGaCAGGATATATggtgaTGTcAcg
T3 (vit)    gtTGGCAGGATtTATtgctaaGTcATc

consensus   NNTGGCAGGATATATNNNNNTGTAA(A/T)N

TL (oct)    acTGGCAGGATATATaccgtTGTAATt
TR (oct)    gaTGGCAGGATATATgccgtTGTAATt
T  (nop)    ttTGaCAGGATATATtggcggGTAAAc
TL Ri(m/a)  acTGaCAGGATATATgttccTGTcATg
TA (o/c)    gcTGaCAGGATATATaccgtTGTAATt
```

Fig. 6a, b. Comparison of border sequences. **a** Left border sequences; **b** right border sequences: TL, TR (oct) from pTi15955 (Barker et al. 1983), T(nop) from pTiT37 (Yadav et al. 1982), TL Ri from pRiA4 (Slightom et al. 1986), TA (o/c) from pTiAB3 (Paulus et al. 1991a). *Lower case* letters indicate mismatches from the consensus

(Fig. 6b). The A in position 3 is found in both T1 and T2 borders as well as in the right border from T-DNA (nop), TL Ri (m/a) and TA (o/c). In position 10, T is found in T1 and T3 but not in other borders (where it is A). The C found in position 22 in all three pTiS4 right borders is also found in the left TL of pRiA4.

A conserved overdrive sequence necessary for efficient T-DNA transmission is found immediately to the right of the right border in Ti and Ri plasmids (Peralta et al. 1986). The consensus overdrive sequence of 24 nucleotides contains an 8-nucleotide conserved core sequence (TGTTTGTT). Neither the overdrive sequence nor the core sequence are found in the sequenced regions to the right of the three pTiS4 right borders covering 241 bp (T1), 311 bp (T2) and 170 bp (T3). Comparison of the sequences to the right of the pTiS4 right borders does not reveal any conserved motifs. However, to the right of the T2 right border a distinctive sequence containing nine GTGCATCC repeats is found. The first repeated motif starts in position 25 (Fig. 6b) of the T2 right border sequence.

To determine whether the pTiS4 borders are functional, clones containing the T1 region (pPM609, Fig. 1), T2 region (pPM747, Fig. 1) and T3 region (pPM674.5, Fig. 2c) were recombined into pGV2260, a disarmed borderless pTiAch5 derivative. T-DNA transfer in such a system uses the *A. tumefaciens* virulence genes and requires at least one functional border in the inserted DNA. The pGV2260 transconjugants were used to infect appropriate test plants. The phenotype obtained was compared to that obtained for the same clone recom-

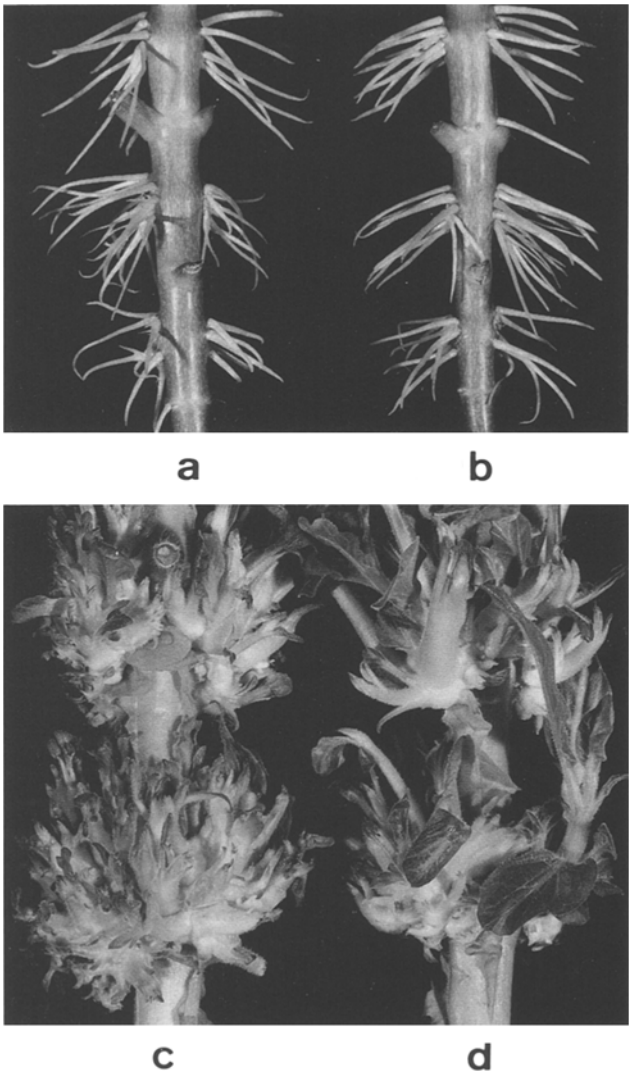

Fig. 7a–d. Functional analysis of pTiS4 borders: Kalanchoe (**a**, **b**) 4 weeks after infection, *N. tabacum* (**c**, **d**) 6 weeks after infection. **a** pGV2260::pPM747; **b** pPM6000::pPM747; **c** pGV2260::pPM674.5; **d** pPM6000::pPM674.5

bined between the borders of the TL DNA of the disarmed pTiAch5 derivative pPM6000.

The T1-containing clone (pGV2260::pPM609) induced only a very small growth reaction on *N. rustica* stems compared to pPM6000::pPM609 (not shown), indicating that the T1 region is not efficiently transferred from pGV2260. The T2 region (pPM747) is transferred, since both the pGV2260 construct (Fig. 7a) and the pPM6000 construct (Fig. 7b) induce roots on Kalanchoe. Similarly, the T3 clone (pPM674.5) induces shoots on *N. tabacum* when integrated in pGV2260 (Fig. 7c) or in pPM6000 (Fig. 7d).

While the T1 borders do not function efficiently in the pGV2260 vector, we expected that this region would be transferred by S4 since it contains the *vis* gene responsible for vitopine synthesis by the tumor tissue. Hybridization of DNA from S4-induced tumors (results not shown) to both the 1.85 kb *Eco*RI and 1.6 kb *Eco*RI fragments from the T1 region demonstrate that the T1 region is indeed transferred. The adjacent *Eco*RI fragment (pPM605.3) situated outside the T1 region did not hybridize to S4 tumor DNA. Probes from the T2 and T3 regions (see the Materials and methods) also hybridized to tumor DNA from S4-induced tumors, confirming that all three T-DNAs are transferred.

Discussion

Three T-DNAs from the Ti plasmid of the representative *A. vitis* vitopine strain S4 have been identified, characterized and sequenced. This is the first example of a Ti plasmid containing three T-DNAs. Since pTiS4 did not show homology with the T-DNA probes used (Paulus et al. 1989a, b), the pTiS4 T-DNAs were localized by testing cloned pTi fragments for oncogene activity. Based on these results, the sequences of the oncogene-containing fragments and adjacent regions were determined. Three oncogene-containing regions, delimited by T-DNA border sequences, were found and designated T1, T2 and T3.

The unusual tripartite T-DNA organization in pTiS4 raises the question of the origin of these T-DNAs. We have noted that the oncogenes are related, albeit distantly, to those of the pTiAch5/pTiT37/pTiTm4 group; homology is also found to pRiA4 *iaa* genes. It is conceivable that pTiS4 shares a common ancestor with these Ti plasmids but so far we have not found other Ti plasmids of the pTiS4 lineage. In terms of organization, the pTiS4 T1 region containing 6b and *vis* genes can be compared to the TA region of *A. vitis* o/c plasmids like pTiAB3 which contain only a 6b and an *ocs* gene. This TA region originated from a TL-like ancestor by deletion of the *iaa* and *ipt* genes (Paulus et al. 1991b). The pTiS4 T1 region could also derive from a TL-like ancestor by a series of rearrangements different from those leading to the pTiAB3 TA region. One of the rearrangement steps would be the inversion of the 6b gene ancestor, since S-6b is now found in the opposite orientation to that of 6b genes in all other T-DNAs in which 6b is found. We have shown that the 6b/*vis* intergenic region and *vis* gene in T1 are homologous to the *ipt*/ψ*vis* region of T3. This homology suggests that a duplication event was involved in generating the present T1 and T3 structures. The T2 region, like the TB region of o/c *A. vitis* Ti plasmids and the TR region of *A. rhizogenes* m/a strains, contains a set of *iaa* genes. However T2 is much smaller and, unlike TB and TR, does not contain an opine-synthesizing gene. This situation could result from fragmentation of a large T-DNA which led to the separation of the *iaa* genes, *ipt*, 6b and *vis* genes. While other origin schemes, including the integration of independent T-DNAs into pTiS4, can be proposed, the fragmentation of a large T-DNA ancestor is consistent with the present pTiS4 organization.

The border sequences which delimit the T-DNAs in pTiS4 differ from those previously described, showing one to three mismatches with the consensus border sequence. While all three T-DNAs are present in tumor tissue, only the T2 and T3 borders function in a disarmed borderless *A. tumefaciens* vector. The transfer of

T2 and T3 appears to be as efficient using its own borders (in pGV2260 constructs) as with Ach5 borders (pPM6000 constructs). This efficient transfer is surprising since, in all three pTiS4 T-DNAs, the overdrive sequence that is found immediately adjacent to the right border in *A. tumefaciens* Ti plasmids, is absent. However, since the overdrive sequence can promote efficient T-DNA transfer even when situated at 2 kb from the border (van Haaren et al. 1987), we cannot exclude the possibility that in pTiS4, overdrive sequences might occur at a distance from the right borders in the unsequenced regions of pPM747 and pPM674.5. In *A. tumefaciens* Ti plasmids, the overdrive region interacts with the *virC1* gene product (Toro et al. 1989). While pTiS4 hybridizes to heterologous virulence regions (Gérard et al. 1992) including the *virC* region, no information on the pTiS4 virulence functions is yet available.

A distinctive repeated motif GTGCATCC was found adjacent to the T2 right border. It would be interesting to determine whether this sequence plays a role in T-DNA transfer since repeated motifs found adjacent to the right border in *A. rhizogenes* pRi8196 have been shown to be necessary for efficient T-DNA transfer (Hansen et al. 1992). To determine how T-DNA transfer is mediated in S4, detailed functional analysis of the pTiS4 *vir* genes and the interaction of their gene products with the pTiS4 border regions will be necessary. Since in pTiS4 the 6b, *ipt* and *iaa* oncogenes are located on different T-DNAs, the efficiency of transfer of each T-DNA could be a factor in tumor formation by pTiS4. In addition to the oncogenes, we have identified the vitopine synthase gene in the T1 region. While the *vis* gene shows more homology (67%) to the *ocs* gene than do the pTiS4 oncogenes to their homologues (58–62%), it codes for a different enzyme and is responsible for vitopine synthesis in S4 tumor tissue.

All four pTiS4 oncogenes were shown to be active: S-6b induces tumors and enhances auxin and cytokinin effects, the S-*iaa* genes and S-*ipt* gene lead to root and shoot formation respectively. Whereas the root formation by the pTiS4 *iaa* genes is comparable to that of A-*iaa* genes, both S-6b and S-*ipt* show significantly more growth induction in infection and coinfection experiments. These differences could be due to higher activity of the S-6b and/or S-*ipt* promoters or to differences in the gene products. This question can be addressed by testing the activity of heterologous promoter/gene constructs.

The vitopine strains, like the o/c strains, are found on grapevine. Functional studies of the o/c pTiTm4 oncogenes showed that the T-*ipt* gene (Bonnard et al. 1989) as well as the T-*iaa* genes from the TB region (Huss et al. 1989a) induce less growth than their pTiAch5 homologues. Mutation of the T-*ipt* gene in pTiTm4 showed that this gene was not essential for tumor formation on grapevine (Huss et al. 1989b). The activity of both the S-*iaa* genes and S-*ipt* genes distinguishes them from those of pTiTm4, since the S-*iaa* genes are as active as A-*iaa* genes and S-*ipt* shows even higher activity on test plants. It would therefore be particularly interesting to determine the role of the pTiS4 oncogenes in grapevine tumor formation by mutating the different oncogenes in the pTiS4 context.

Acknowledgements. We thank Y. Dessaux and A. Petit (ISV, Gif-sur-Yvette) for vitopine assays. We thank F. Paulus for helpful discussions, J. Brevet for communicating the border sequence of pRi8196 and G. Bonnard for the unpublished T-6b sequence. Oligonucleotide primers were synthesized by Mrs. A. Hoeft (IBMC, Strasbourg). P. Crouzet and J.-C. Gérard received grants from the French Ministry of Research and Technology.

References

Akiyoshi D, Klee H, Amasino R, Nester EW, Gordon MP (1984) T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc Natl Acad Sci USA 81:5994–5998

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

Beaty JS, Powell GK, Lica L, Regier DA, MacDonald EMS, Hommes NG, Morris RO (1986) *Tzs*, a nopaline Ti plasmid gene from *Agrobacterium tumefaciens* associated with trans-zeatin biosynthesis. Mol Gen Genet 203:274–280

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

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

Bouchez D, Tokuhisa JG, Llewellyn DJ, Dennis ES, Ellis JG (1989) The ocs-element is a component of the promotors of several T-DNA and plant viral genes. EMBO J 8:4197–4204

Buchmann I, Marner FJ, Schröder G, Waffenschmidt S, Schröder J (1985) Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. EMBO J 4:853–859

Camilleri C, Jouanin L (1991) The TR-DNA region carrying the auxin synthesis genes of the *Agrobacterium rhizogenes* agropine-type plasmid pRiA4: nucleotide sequence analysis and introduction into tobacco plants. Mol Plant-Microbe Interact 4:155–162

Chaubet N, Philipps G, Gigot C (1989) Organization of the histone H3 and H4 multigenic families in maize and in related genomes. Mol Gen Genet 219:404–412

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

Deblaere R, Bytebier B, De Greve H, Deboeck F, Schell J, van Montagu M, Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer in plants. Nucleic Acids Res 13:4777–4785

Depicker A, de Wilde M, 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

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

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

Dhaese P, De Greve H, Decraemer H, Schell J, van Montagu M (1979) Rapid mapping of transposon insertion and deletion mutants in the large Ti-plasmids of *Agrobacterium tumefaciens*. Nucleic Acids Res 7:1837–1849

Ellis JG, Llewellyn DJ, Walker JC, Dennis ES, Peacock WJ (1987) The ocs element: a 16 base pair palindrome essential for activity of the octopine synthase enhancer. EMBO J 6:3203–3208

Gérard JC, Canaday J, Szegedi E, Delasalle H, Otten L (1992) Physical map of the vitopine Ti plasmid pTiS4. Plasmid (in press)

Gergen JP, Stern RH, Wensink PC (1979) Filter replicas and permanent collections of recombinant DNA plasmids. Nucleic Acids Res 7:2115–2136

Gielen J, De Beuckeleer M, Seurinck J, Deboeck F, De Greve H, Lemmers H, 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 *Agrobacterium tumefaciens* pTiT37 T-DNA. Nucleic Acids Res 12:4665–4677

Hansen G, Tempé J, Brevet J (1992) A T-DNA transfer stimulator sequence in the vicinity of the right border of pRi8196. Plant Mol Biol (in press)

Henikoff (1987) Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol 155:156–165

Hooykaas PJJ, Den Dulk-Ras H, Schilperoort RA (1988) The *Agrobacterium tumefaciens* T-DNA gene *6b* is an onc gene. Plant Mol Biol 11:791–794

Huss B, Bonnard G, Otten L (1989a) 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

Huss B, Tinland B, Paulus F, Walter B, Otten L (1989b) Functional analysis of a complex oncogene arrangement in biotype III *Agrobacterium tumefaciens* strains. Plant Mol Biol 14:173–186

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

Leemans J, Hernalsteens JP, Deblaere R, De Greve H, Thia-Toong L, van Montagu M, Schell J (1983) Genetic analysis of T-DNA and regeneration of transformed plants. In: Pühler A (ed) Molecular genetics of the bacteria-plant interaction. Springer, Berlin, pp 322–330

Linsmaier EM, Skoog F (1965) Organic growth requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Offringa I, Melchers L, Regensburg-Tuink A, Costantino P, Schilperoort R, Hooykaas P (1986) Complementation of *Agrobacterium tumefaciens* tumor-inducing aux mutants by genes from the TR-region of the Ri plasmid of *Agrobacterium rhizogenes*. Proc Natl Acad Sci USA 83:6935–6939

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

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-Microbe 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, Huss B, Tinland B, Herrmann A, Canaday J, Otten L (1991a) Role of T-region borders in *Agrobacterium* host range. Mol Plant-Microbe Interact 4:163–172

Paulus F, Canaday J, Otten L (1991b) Limited host range Ti plasmids: recent origin from wide host range Ti plasmids and involvement of a novel IS element, IS*868*. Mol Plant-Microbe Interact 4:190–197

Peralta EG, Hellmiss R, Ream LW (1986) Overdrive, a T-DNA transmission enhancer on the *Agrobacterium tumefaciens* tumour-inducing plasmid. EMBO J 6:1137–1142

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 (1990) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schröder G, Waffenschmidt S, Weiler EW, Schröder J (1984) The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. Eur J Biochem 138:387–391

Singh K, Tokuhisa JG, Dennis ES, Peacock WJ (1989) Saturation mutagenesis of the octopine synthase enhancer: correlation of mutant phenotypes with binding of a nuclear protein factor. Proc Natl Acad Sci USA 86:3733–3737

Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D (1986) Nucleotide sequence analysis of the TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J Biol Chem 261:108–121

Smith GR (1988) Homologous recombination in Procaryotes. Microbiol Rev 52:1–28

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

Szegedi E, Czako 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

Thomashow LS, Reeves S, Thomashow MF (1984) Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. Proc Natl Acad Sci USA 81:5071–5075

Thomashow MF, Hugly S, Buchholz WG, Thomashow LS (1986) Molecular basis for the auxin-independent phenotype of crown gall tumor tissues. Science 231:616–618

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

Toro N, Datta A, Carmi OA, Young C, Prusti RK, Nester EW (1989) The *Agrobacterium tumefaciens virC1* gene product binds to overdrive, a T-DNA transfer enhancer. J Bacteriol 171:6845–6849

Vanderleyden J, Desair J, De Meirsman C, Michiels K, van Gool A, Jen G, Chilton MD (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 Haaren MJJ, Sedee NJA, Schilperoort RA, Hooykaas PJJ (1987) Overdrive is a T-region transfer enhancer which stimulates T-strand production in *Agrobacterium tumefaciens*. Nucleic Acids Res 15:8983–8997

van Haaren MJJ, Sedee NJA, Krul M, Schilperoort RA, Hooykaas PJJ (1988) Function of heterologous and pseudoborder repeats in T region transfer via the octopine virulence system of *Agrobacterium tumefaciens*. Plant Mol Biol 11:773–781

van Haute E, Joos H, Maes M, Warren G, van Montagu M, Schell J (1983) Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens*. EMBO J 2:411–417

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: evidence for common transcripts present in both octopine and nopaline tumors. Cell 32:1045–1056

Yadav NS, Vanderleyden J, Bennet DR, Barnes WM, Chilton MD (1982) Short direct repeats flank the T-DNA on a nopaline Ti plasmid. Proc Natl Acad Sci USA 79:6322–6326

Yamada T, Palm CJ, Brooks B, Kosuge T (1985) Nucleotide sequences of the *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. Proc Natl Acad Sci USA 82:6522–6526

Zambryski P, Joos H, Genetello C, Leemans J, van Montagu M, Schell J (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J 2:2143–2150

Zambryski P, Tempé J, Schell J (1989) Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. Cell 56:193–201

Communicated by A. Kondorosi