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Isolation and characterization of an *ipt* gene from the Ti plasmid Bo542

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Summary. A 1.9 kb clone of the T-DNA region of the *Agrobacterium tumefaciens* Ti plasmid Bo542 which exhibited homology to the isopentenyl transferase (*ipt*) locus of pTiA6 was identified by low stringency DNA hybridization. Introduction of this segment of pTiBo542 DNA into cells of *Nicotiana tabacum* or *N. glauca* caused tumor formation in vivo, and allowed hormone independent growth in vitro. Furthermore, this DNA segment complemented *ipt* mutant strains of *A. tumefaciens,* restoring their ability to cause tumors on *Kalanchöe* leaves and tomato stems. The complete DNA sequence of this segment has been determined, revealing an open reading frame homologous to other known *Agrobacterium ipt* genes.

Key words: *Agrobacterium tumefaciens* – Ti plasmid – T-DNA – Isopentenyl transferase – Cytokinin

Introduction

Agrobacterium tumefaciens is a free-living soil bacterium capable of transforming wounded plant tissues into tumorous growths known as crown galls (for reviews, see Nester et al. 1984; Weiler and Schröder 1987). Tumors are caused by the aberrant production of the plant growth substances auxin and cytokinin in infected tissues. This condition is brought about by the transfer of a segment of DNA, known as the T-DNA (transferred DNA), of the Ti (tumor inducing) plasmid from the bacterium into a wounded plant cell. Once in the plant cell, the T-DNA becomes integrated into the plant genome and is transcribed by endogenous plant proteins. Certain T-DNA encoded proteins catalyze the production of auxin and cytokinin. Although these compounds are normally produced by plants, their T-DNA directed biosynthesis in transformed tissues is independent of normal plant regulatory mechanisms, thus causing tumorous growth of the transformed cells.

The T-DNA regions of many Ti plasmids appear very similar by Southern blot analysis in a 'core' region which includes the *iaaH*, *iaaM*, *ipt* and *tml* loci (Chilton et al. 1978; Nester et al. 1984). However, exceptions do exist; in particular, some Ti plasmids have been shown to lack *ipt* (Yanofsky et al. 1985), a gene encoding isopentenyl transferase, an enzyme involved in cytokinin biosynthesis (Morris 1986; Weiler and Schröder 1987). In a recent study, it was observed that a probe consisting of part of the coding region of the pTiA6 *ipt* gene failed to hybridize to pTiBo542 DNA under high stringency conditions (Komari et al. 1986). However, *A. tumefaciens* strains harboring pTiBo542 are highly virulent on soybean (Hood et al. 1987), and soybean cells stringently require both auxin and cytokinin for cell proliferation (Miller 1968). Thus, based on the host range data, we reasoned that pTiBo542 is unlikely to be a member of the group of Ti plasmids which lacks genes involved in cytokinin biosynthesis, and that it would be of interest to investigate which pTiBo542 gene or genes are involved in cytokinin metabolism in transformed plant cells. We therefore searched for sequences homologous to the *ipt* gene of pTiA6 by low stringency hybridization of this gene to cloned fragments of the pTiBo542 T-DNA. This analysis revealed a 1.9 kb *Bam*HI/*Hin*dIII fragment which hybridized to the pTiA6 *ipt* probe. The 1.9 kb fragment was found to complement an *ipt* mutant strain of *A. tumefaciens*. The fragment also produced a tumorous phenotype in the absence of other T-DNA genes on certain hosts.

Nucleotide sequence analysis of the *ipt* homologous DNA segment revealed homology to both octopine- and nopaline-type Ti plasmid *ipt* genes. Analysis of the major open reading frame predicted a highly conserved amino acid sequence in the putative protein, relative to octopine and nopaline *ipt* coding sequences. Based on these data, we report here that the plasmid pTiBo542 indeed carries a gene encoding the enzyme isopentenyl transferase.

Materials and methods

Bacterial strains. A. tumefaciens strains A281, A348 and A208 contain, respectively, the wild-type Bo542, A6 and T37 Ti plasmids (Montoya et al. 1977; Nester et al. 1984). *A. tumefaciens* strains 2760 and A338 contain, respectively, the T-DNA deleted Ti plasmid, pAL4404 (Hoekema et al. 1983) and an A6 Ti plasmid containing an insertion mutation in the *ipt* gene (Lichtenstein et al. 1984). All *Agrobacterium* strains were in the A136 chromosomal background (Montoya et al. 1977) and were maintained on AB minimal agar (Chilton et al. 1974) with antibiotics added where appropriate. *Escherichia coli* strain DH5α (Hanahan 1985) was used for plasmid manipulations excluding those involv-

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ing plasmids pGA583 and pAL109, for which strain MC1000 was used. Plasmids were transferred from *E. coli* to *A. tumefaciens* via triparental mating as described by An (1987) using the helper plasmid pRK2073 (Leong et al. 1982).

Plasmid constructs. A 1.9 kb *Bam*HI-*Hin*dIII fragment of the cosmid clone pEHC65 (Hood et al. 1984) was isolated by electroelution from an agarose gel. To create pAL110A and B, the fragment ends were filled in utilizing DNA polymerase I Klenow fragment, followed by ligation of *Eco*RI linkers (GGAATTCC), which restored the *Bam*HI site of the fragment, and *Eco*RI digestion. The resulting fragments were ligated in both orientations into *Eco*RI digested, dephosphorylated pUC118 (Vieira and Messing 1987). To create pAL109, the *Bam*HI-*Hin*dIII 1.9 kb fragment was cloned directly into *Hin*dIII/*Bgl*II digested pGA583 (An 1987).

The plasmid pEND1 : tmr contains the *ipt* gene of pTiA6 in the binary vector pEND1 (Klee et al. 1985).

Restriction analysis, probe synthesis, and Southern blot hybridization. Two micrograms of total *Agrobacterium* or CsCl-purified plasmid DNA was digested with 6 units of restriction enzyme as specified by the manufacturer (New England Biolabs, Beverly, Mass or Bethesda Research Laboratories, Gaithersburg, Md). The digested DNAs were size fractionated through a 1% agarose gel in Tris-borate-EDTA running buffer (Maniatis et al. 1982) at 3 V/cm, then stained and blotted onto nylon membranes as described (Reid et al. 1988). The membrane was hybridized at low stringency to uniformly ^{32}P-labeled RNA probes at 50° C in the following hybridization buffer: 0.25 M Sodium phosphate buffer (see Amasino 1986 for preparation), 5% (w/v) SDS, 0.5% (w/v) nonfat dry milk, 10% (w/v) polyethylene glycol 8000, 2.5 mM EDTA, 100 µg/ml sheared, double-stranded salmon sperm DNA. The blot was prehybridized in this buffer for 4 h, then 10^6 cpm/ml of probe was added and hybridization was continued for 15 h. Blots were washed at 50° C in 0.25 M Sodium phosphate buffer, 1% SDS, 1 mM EDTA with 5 buffer changes at 30 min intervals. The blots were then exposed to Kodak XAR film for 15–30 h at room temperature.

The ^{32}P-labeled RNA probe complementary to the *Pst*I "fragment A" of the pTiA6 *ipt* gene was synthesized as described previously (John and Amasino 1988) from the plasmid pRA127.

Infection of plants with A. tumefaciens, *and tumor culture. Kalanchöe daigremontiana* and *Lycopersicum esculentum* (tomato variety Better Boy) plants were grown in a greenhouse in Jiffy Mix (Jiffy Products of America, West Chicago, Ill) and fertilized weekly with Hoagland's solution. Plants 10–20 cm in height were used for inoculation. *Nicotiana tabacum* and *N. glauca* plants were grown in sterile culture on Murashige-Skoog medium lacking auxin and cytokinin (MS 0/0) as described (Amasino et al. 1984). Plants were inoculated by wounding stems or leaves with a needle which had been dipped into a colony of *Agrobacterium* grown for 2 days on AB medium.

Tumors resulting from infection of *N. glauca* and *N. tabacum* were excised from the plant and cultured in the dark for 2 passages on MS 0/0 medium containing 500 µg/ml carbenicillin and 250 µg/ml cefotaxime. This treatment rendered the tissues free of bacteria, whereupon they were cultured on MS 0/0 medium without antibiotics.

Bacterial DNA isolation. Plasmid DNA from *E. coli* for subcloning, blotting or hybridization probe production was prepared by alkaline lysis and CsCl gradient ultracentrifugation (Maniatis et al. 1982). For plasmid confirmation in *Agrobacterium* the alkaline lysis protocol described by An (1987) was used. Total *Agrobacterium* DNA was isolated as follows. A 10 ml culture of cells was grown for 10–12 h in Mg/L medium (Garfinkel and Nester 1980), pelleted at $3000 \times g$ for 5 min and resuspended in 4 ml of 20 mM Tris-HCl, 20 mM EDTA, 50 mM glucose pH 8.0. Four milliliters of 100 mM Tris-HCl, 20 mM EDTA, 0.5% SDS, 0.5 M NaCl pH 8.0 and 0.1 ml of a 10 mg/ml solution of pronase (Sigma Chemical Co. St. Louis, Mo) were added to the cells and the mixture was incubated at 37° C for 1 h. The solution was extracted once with phenol:chloroform:isobutanol (25:24:1) then once with chloroform:isobutanol (24:1). The nucleic acids were then ethanol precipitated, collected by centrifugation at $3000 \times g$ for 5 min, resuspended in 4 ml of TE (TE is 10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing 100 µg/ml pre-boiled RNase A and incubated at 37° C for 30 min. Ammonium acetate was then added to a final concentration of 2 M and the DNA was ethanol precipitated, collected by centrifugation, rinsed in 70% and 100% ethanol, resuspended in TE and quantitated by UV absorbance.

DNA sequence analysis. Deletion templates of the *Bam*HI-*Hin*dIII fragment used in the sequence determination were generated as described by Henikoff (1984) except that mung bean nuclease was used to generate blunt ends. Sequencing was performed by the method of Sanger et al. (1977), with modifications as specified by Chen and Seeburg (1985). Nucleotide stock mixes, mung bean nuclease, and Klenow fragment were purchased from New England Biolabs, Beverly, Mass. Computer analysis of the sequence data (Figs. 4, 5) was performed utilizing University of Wisconsin Genetics Computer Group software as described by Devereux et al. (1984).

Results

Detection of a pTiBo542 sequence homologous to the ipt *gene of pTiA6*

Southern blot hybridizations were performed to determine if any DNA sequences carried by *Agrobacterium* strain A281, which contains the Ti plasmid Bo542, exhibited homology to the *ipt* locus of pTiA6. Total DNA from A281 was isolated and cleaved with the restriction enzymes *Hin*dIII, *Bam*HI, or *Eco*RI. After size-fractionation in agarose gels, the DNA was blotted to nylon membranes. The blots were incubated under low stringency conditions with a probe complementary to the 3′ end of the pTiA6 *ipt* mRNA (see Materials and methods). No hybridization of this probe to A281 DNA was detected under conditions which resulted in a strong hybridization signal to total DNA isolated from *Agrobacterium* strains A348 and A208 (Fig. 1). DNA from all three strains hybridized strongly to a probe corresponding to the *vir*G region of the pTiA6 plasmid (data not shown), demonstrating that the lack of hybridization of A281 DNA to the *ipt* probe was not due to A281 DNA

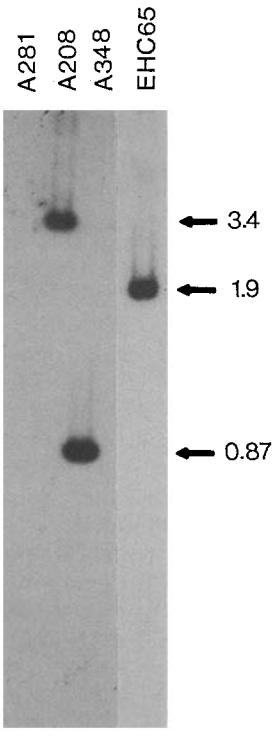

Fig. 1. Low stringency Southern blot hybridization analysis of sequence homology between the pTiA6 *ipt* gene and pTiBo542 and pTiT37 DNA. One microgram of total DNA from *Agrobacterium tumefaciens* harboring the A6 (A348), T37 (A208), or Bo542 (A281) Ti plasmids or 1 μg of purified plasmid EHC65 containing a region of the pTiBo542 T_L-DNA was digested to completion with *Hin*dIII (A208, A281), *Hin*dIII and *Bam*HI (pEHC65) or *Pst*I (A348), fractionated by electrophoresis through a 1% agarose gel, denatured, transferred to a nylon membrane and incubated at 50° C with a uniformly ^{32}P-labeled RNA probe from the pTiA6 *ipt* gene. The size in kb of the hybridizing fragments is indicated on the right

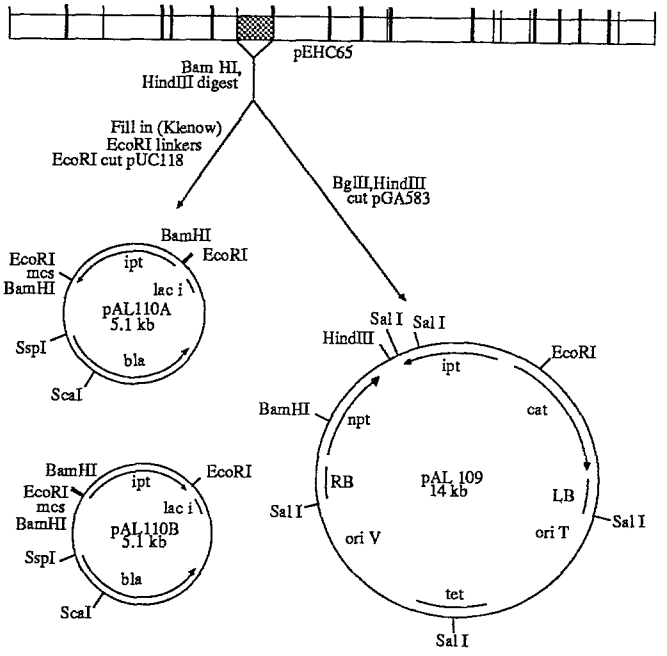

Fig. 2. Construction of plasmids containing the *ipt* cross-hybridizing 1.9 kb pTiBo542 T-DNA fragment. The 1.9 kb *Bam*HI/*Hin*dIII *ipt* homologous fragment (*shaded*) was subcloned from pEHC65. Restriction map is from Hood et al. (1986). *Bam*HI sites in the pEHC65 insert are indicated by *thin lines* and *Hin*dIII sites are indicated by *thick lines*. Abbreviations: bla, β-lactamase gene; cat, chloramphenicol acetyltransferase gene; ipt, isopentenyl transferase gene; lac i, *Escherichia coli* lactose operon gene i in pUC118; mcs, multiple cloning site; npt, neomycin phosphotransferase II gene; ori T, RK2-derived origin of conjugal transfer; ori V, RK2-derived origin of replication; RB and LB, *A. tumefaciens* T-DNA right and left border sequences respectively; tet, RK2-derived tetracycline resistance gene

degradation or inefficient transfer of DNA to the membrane. However, when purified plasmid DNA of a pTiBo542 T_L region subclone (pEHC65) was hybridized under identical conditions, a hybridization signal was observed (Fig. 1). The location of this cross-hybridizing fragment was mapped to a unique *Bam*HI/*Hin*dIII restriction fragment; the location of this region of pEHC65 is shown in Fig. 2. The same unique restriction fragment also hybridized at low stringency to a probe corresponding to the 5′ end of the pTiA6 gene (The *Bam*HI 29 fragment of pTiA6; Lichtenstein et al. 1984; data not shown). The observation of a hybridization signal in a blot of a T-DNA clone in a purified plasmid preparation but not in a blot from total *Agrobacterium* DNA is probably due to the greater quantity of DNA per restriction fragment in the plasmid blot. The hybridization signal to the cloned pTiBo542 *ipt* fragment is not observed when the hybridization is carried out under increased stringency at 65° C, although the strong hybridization signal to A348 and A208 DNA is still observed under these conditions (data not shown).

Tumorigenic properties of the ipt*-homologous pTiBo542 DNA*

The cross-hybridization of the 1.9 kb fragment of pTiBo542 to the pTiA6 *ipt* gene suggested that the fragment might contain a gene involved in cytokinin metabolism in plant cells. This hypothesis was tested by assessing the ability of the 1.9 kb fragment to complement *Agrobacterium* strain A338 which contains an inactive mutant *ipt* gene on the A6 Ti plasmid (Lichtenstein et al. 1984). Inactivation of the pTiA6 *ipt* gene has been shown to render agrobacteria harboring the mutant plasmid incapable of causing tumorous outgrowth on *K. daigremontiana* leaves and tomato stems (Garfinkel and Nester 1980; Garfinkel et al. 1981). Thus, the 1.9 kb fragment was cloned between the T-DNA borders of the binary vector pGA583 to create plasmid pAL109 (Fig. 2). The presence of pAL109 in *Agrobacterium* strain A338 restored the ability of this strain to form tumors on *Kalanchöe* leaves and tomato stems (Fig. 3A, B). Furthermore, the rate of tumor development was identical to that observed when the *ipt* mutation of A338 was complemented by the *ipt* gene of pTiA6 on the binary vector pEND1 (pEND1:tmr; Fig. 3A, B).

Also tested was the ability of the putative gene product of the 1.9 kb fragment to cause tumorous growth in the absence of other T-DNA genes in transformed *N. glauca* and *N. tabacum* cells in vivo and in vitro. The binary vectors containing the pTiBo542 fragment (pAL109) and the pTiA6 *ipt* gene (pEND1:tmr) were introduced into a strain of *Agrobacterium* which contains a Ti plasmid lacking the entire T-DNA region (pAL4404; Hoekema et al. 1983). Infec-

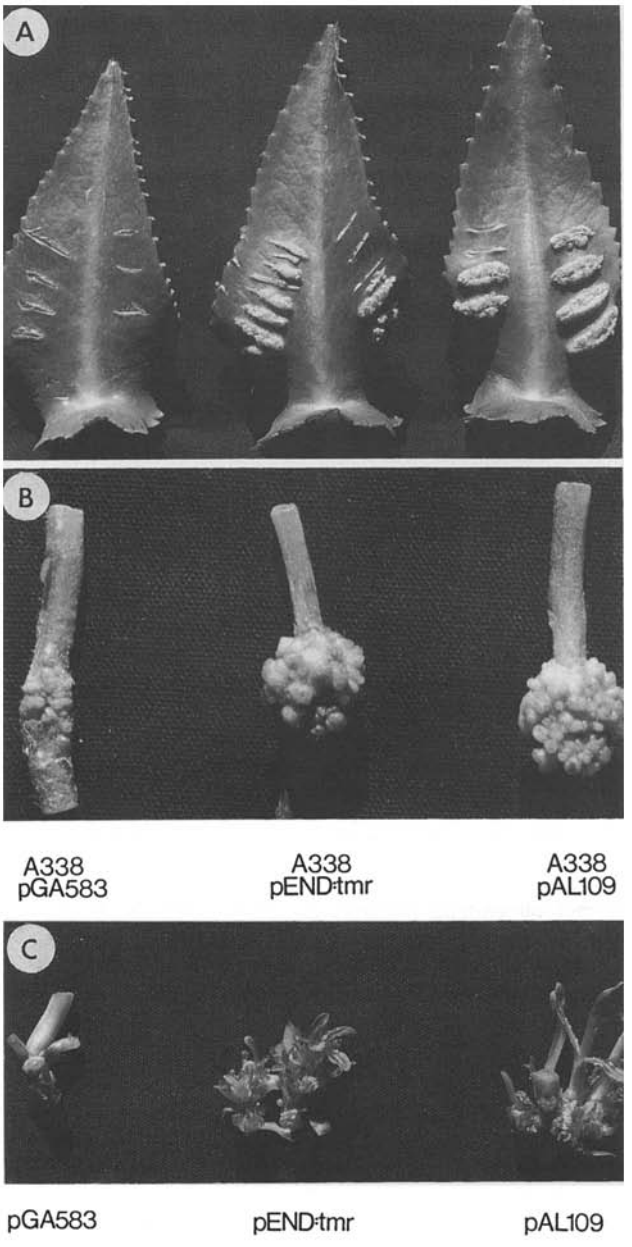

Fig. 3A–C. Oncogenic activity of the pTiBo542 T-DNA fragment is similar to that of the wild-type pTiA6 *ipt* gene. *Kalanchöe* leaves (**A**) or tomato stems (**B**) were inoculated with the *ipt* mutant strain A338 harboring either a binary vector containing the pTiA6 *ipt* gene (pEND1:tmr) or a binary vector containing the cross-hybridizing 1.9 kb fragment of pTiBo542 T-DNA (pAL109) as indicated. **C** shows stems of *Nicotiana glauca* inoculated with an *Agrobacterium* strain containing a Ti plasmid which lacks all T-DNA genes (pAL4404) and the binary vectors described above. Tumor development is shown 4 weeks after inoculation

tion with *Agrobacterium* containing either pAL109 or pEND1:tmr resulted in rapid tumor proliferation on *N. glauca* and *N. tabacum* stems; infection with *Agrobacterium* containing only the binary cloning vector pGA583 did not result in any response (Fig. 3C). The tumors developed as a mass of distorted shoots, and the phenotypes of tumors resulting from inoculation with agrobacteria containing pAL109 and pEND1:tmr were indistinguishable. When these tumors were established in axenic culture on medium without auxin or cytokinin, they continued to proliferate as shoot-forming tissues. In a soybean callus bioassay (Miller 1968), elevated levels of cytokinins characteristic of crown gall tumors (Amasino and Miller 1982) were readily detectable from extracts of these axenic tumor lines (data not shown).

Nucleotide sequence of the pTiBo542 ipt *gene*

The 1.9 kb *ipt* homologous fragment of pTiBo542 was subcloned in both orientations into the *Eco*RI site of pUC118, thus creating pAL110A and B (Fig. 2). A series of deletions of the inserts was constructed and the resulting templates were utilized to determine the entire sequence of both strands of the 1.9 kb fragment (Fig. 4). To determine if the fragment contained a diverged *ipt* gene, the sequence was compared to *ipt* sequences from pTiT37, a nopaline-type strain, and pTiAch5, an octopine-type strain (Fig. 4). Since the *ipt* genes and flanking sequences are virtually identical in all octopine-type strains analyzed to date (Barker et al. 1983; Gielen et al. 1984; Lichtenstein et al. 1984), no other octopine-type *ipt* genes were included in the comparison. The 1.9 kb fragment displays a high degree of sequence identity to the *ipt* genes and flanking sequences of pTiT37 and pTiAch5 at the level of 79% and 76%, respectively (Fig. 4). For comparison, the pTiT37 and pTiAch5 sequences are 86% identical to each other. Identity among the 3 sequences is highest toward the 5′ ends of the genes including the 5′ noncoding sequences. However, an interesting difference is observed at the 3′ noncoding ends of the sequences: whereas pTiT37 and pTiAch5 retain relatively high DNA sequence conservation with one another, at the level of 77% identity, the pTiBo542 sequence, although clearly related to the pTiAch5 and pTiT37 *ipt* genes, retains only approximately 50% identity with them.

The derived amino acid sequence of the major open reading frame in the 1.9 kb fragment is shown in Fig. 5. The predicted protein contains 239 amino acids and has an approximate molecular weight of 27 kDa, consistent with the pTiAch5 and pTiT37 isopentenyl transferase proteins, both of which contain 240 amino acids. At the amino acid level, the pTiBo542 sequence displays 87% and 81% identity to the pTiT37 and pTiAch5 sequences, respectively (Fig. 5), demonstrating that the 1.9 kb pTiBo542 fragment contains an *ipt* gene. For comparison, the pTiAch5 and pTiT37 *ipt* amino acid sequences are 87% identical to one another.

Discussion

Our initial interest in the cytokinin biosynthetic gene of pTiBo542 resulted from the observation by Komari et al. (1986) that nucleic acid hybridization between pTiBo542 DNA and a probe containing a portion of the pTiA6 *ipt* gene was not observed at high stringency. In our studies, even at reduced stringency of hybridization, we did not detect hybridization of the 3′ end of the pTiA6 *ipt* gene to DNA from an *A. tumefaciens* strain containing pTiBo542 under conditions in which DNA from octopine- and nopaline-type *Agrobacterium* strains hybridized strongly (Fig. 1). However, based on host range data, we suspected that pTiBo542 probably carried a gene or genes involved in cytokinin metabolism. Utilizing low stringency hybridization to plasmid blots, we detected a 1.9 kb fragment of pTiBo542

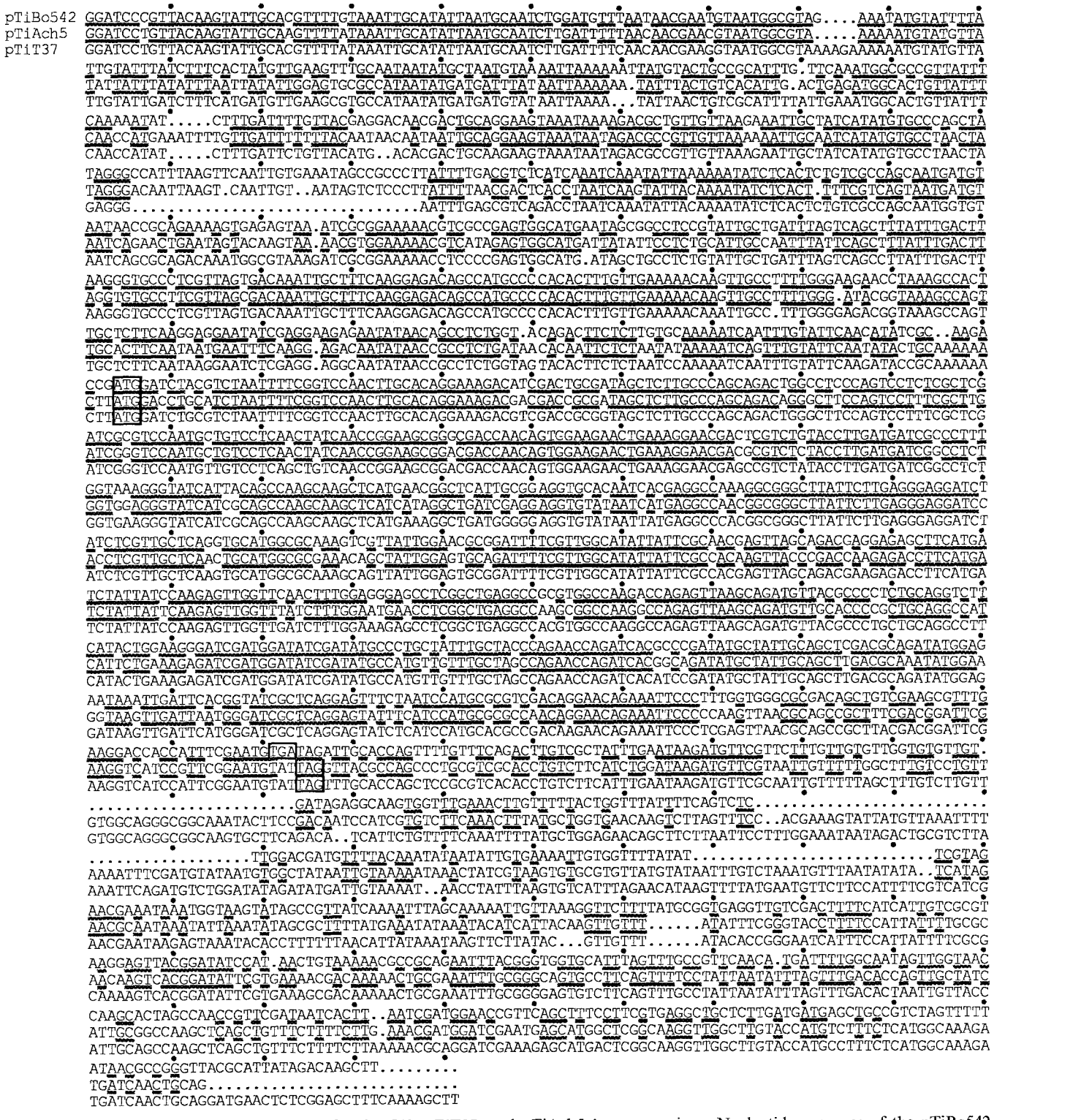

Fig. 4. Nucleic acid sequence comparisons of pTiBo542, pTiT37, and pTiAch5 *ipt* gene regions. Nucleotide sequence of the pTiBo542 1.9 kb *ipt*-homologous *Bam*HI-*Hin*dIII fragment has been aligned to *ipt* regions from pTiT37 and pTiAch5. Start and stop codons of pTiAch5 and pTiT37 genes and those of the major open reading frame of the pTiBo542 sequence are *boxed*. The identity among all three sequences is emphasized

T-DNA which weakly cross-hybridized to a pTiA6 *ipt* probe. We have isolated and characterized this 1.9 kb cross-hybridizing fragment and found it to contain a T-DNA oncogene which encodes an isopentenyl transferase enzyme of cytokinin biosynthesis. We reach this conclusion since: (1) the pTiBo542 and pTiA6 *ipt* genes are indistinguishable in their ability to complement an *ipt* mutation in the pTiA6 plasmid on *Kalanchöe* leaves and tomato stems and to cause teratomous tumors independent of other T-DNA genes on *N. glauca* and *N. tabacum* (Fig. 3); (2) comparison of the

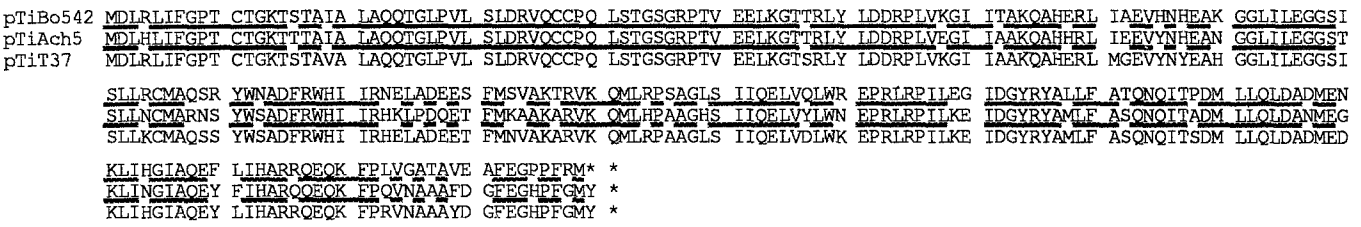

Fig. 5. Amino acid sequence comparisons of pTiBo542, pTiT37, and pTiAch5 proteins. Amino acid sequences of the pTiT37 and pTiAch5 *ipt* genes have been aligned to the pTiBo542 *Bam*HI-*Hin*dIII fragment major open reading frame. The identity among all three sequences is emphasized

nucleotide sequence of the pTiBo542 1.9 kb fragment with other *ipt* gene regions reveals substantial sequence identity (Fig. 4); and (3) conservation of the predicted pTiBo542 isopentenyl transferase amino acid sequence relative to other known isopentenyl transferase proteins is about 85% (Fig. 5).

In addition to the high level of conservation between the coding sequence of the pTiBo542 *ipt* gene and other known *Agrobacterium ipt* genes, analysis of the flanking nucleotide sequences reveals substantial regions of identity in the 5′ noncoding sequence (Fig. 4). Promoter function has been attributed to a conserved sequence in the 5′ region of the pTiA6 *ipt* gene (de Pater et al. 1987). However, the conserved sequences in the 5′ noncoding region of all the *ipt* genes characterized to date are more extensive than this particular promoter element, suggesting that other regions of sequence conservation may also be involved in transcriptional regulation of the *ipt* gene. It is unlikely that this sequence conservation is due to recent evolutionary divergence of these Ti plasmids from a common ancestor, given the striking sequence divergence of the 3′ noncoding region relative to its octopine- and nopaline-type Ti plasmid homologs (Fig. 4).

We have observed a striking decrease of sequence identity of the pTiBo542 *ipt* 3′ noncoding sequence relative to corresponding octopine- and nopaline-type sequences, beginning within a few nucleotides after the end of the coding segments of the genes. At the level of hybridization analysis, Chilton et al. (1978) demonstrated that no octopine-type T-DNA sequences extending from the 3′ half of the *ipt* coding sequence to the right border of the T-DNA were capable of cross-hybridizing with pTiBo542 DNA. Since the portion of this region which we have compared at the nucleotide sequence level is clearly related to, but quite divergent from its octopine-type analog, it will be of interest to determine if pTiBo542 contains sequences related to the remainder of the octopine-type T-DNA.

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