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Tumour genes in plants: T-DNA encoded cytokinin biosynthesis

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Gene 4 from the T-region of Ti plasmids is responsible for cytokinin effects in crown gall cells; we investigated whether it codes for an enzyme of hormone biosynthesis. In a first set of experiments, gene 4 from octopine plasmid pTiAch5 and nopaline plasmid pTiC58 was expressed in *Escherichia coli*, and the gene products were identified by reaction with antiserum raised against a decapeptide derived from the DNA sequence of the gene. Extracts from cells expressing the gene contained high isopentenyl-transferase activity catalyzing the formation of N^6-(Δ^2-isopentenyl)adenosine from 5'-AMP and Δ^2-isopentenylpyrophosphate. The cytokinin was identified by sequential h.p.l.c. chromatography and mass spectrometry. In a second set of experiments it was shown that crown gall cells contained isopentenyltransferase activity and a protein of mol. wt. 27 000 which was identified as the product of gene 4 by reaction with the antiserum. Isopentenyltransferase activity was specifically inhibited by the antiserum. No comparable enzyme activity or immunoreactive protein was detected in cytokinin-autotrophic, T-DNA free tobacco cells. The results establish that gene 4 from the T-region of octopine and nopaline Ti plasmids codes for an enzyme of cytokinin biosynthesis.

Key words: cytokinin biosynthesis/isopentenyltransferase/peptide antiserum/crown gall/Ti plasmids

Introduction

In contrast to most plant tissues, cells transformed with Ti plasmids of *Agrobacterium tumefaciens* do not require supplementation with auxins and cytokinins for continued cell growth and division. Hormone independence is the result of gene expression from the T-DNA, the part of Ti plasmids which is transferred from Agrobacteria to plant cells during tumorgenesis (for recent reviews, see Schell *et al.*, 1984; Nester *et al.*, 1984; Hille *et al.*, 1984).

Genetic experiments indicate that the T-DNAs of octopine and nopaline plasmids contain a conserved set of three genes which are responsible for hormone independence and other hormone-controlled reactions; two of the genes cooperate to achieve auxin effects, while the third causes cytokinin effects (Garfinkel *et al.*, 1981; Leemans *et al.*, 1982; Joos *et al.*, 1983; Ream *et al.*, 1983; Van Slogteren *et al.*, 1983; Inzé *et al.*, 1984). One of the possible explanations for the function of these genes is that they code for enzymes of hormone biosynthesis. Since in crown galls the contribution of plant-coded enzymes to hormone concentrations cannot easily be determined, it is difficult to test this hypothesis in plant cells. Recent results indicate, however, that the three genes also express defined proteins in procaryotic cells, including *Escherichia coli* (Schröder and Schröder, 1983; Schröder *et al.*, 1983). If one assumes that these proteins are functional, *E. coli* should provide a suitable system to investigate the hypothesis. This approach led to the demonstration that one of the two auxin genes produces an enzyme synthesizing the auxin indole-3-acetic acid (Schröder *et al.*, 1984; Kemper *et al.*, 1984; Thomashow *et al.*, 1984).

If the same rationale is applied to the single T-DNA gene responsible for cytokinin effects, one would expect that it codes for an enzyme synthesizing an active cytokinin from precursors present in all plant cells. A possible candidate would be prenyltransferase catalyzing the formation of N^6-(Δ^2-isopentenyl)-5'-AMP (IPAR-monophosphate) from 5'-AMP and Δ^2-isopentenylpyrophosphate (Δ^2-IPP) (see Figure 1), since this has been defined as the first step in cytokinin biosynthesis (Taya *et al.*, 1978; Chen and Melitz, 1979), and since such enzyme activity has been described in crown gall cells (Morris *et al.*, 1982). Here we describe the subcloning and expression in *E. coli* of cytokinin gene from the octopine and nopaline T-region (gene 4, see Figure 2), the identification of the proteins with antibodies raised against a decapeptide derived from the DNA sequence, and we present evidence that the protein is an isopentenyltransferase active in cytokinin biosynthesis. We also show that this protein is responsible for the same enzyme reaction in T-DNA containing plant cells.

Results

Antiserum against a peptide derived from the DNA sequence

The DNA sequences of gene 4 in octopine plasmids pTiAch5 (Gielen *et al.*, 1984; Heidekamp *et al.*, 1983), pTiA6NC (Lichtenstein *et al.*, 1984), and pTi15955 (Barker *et al.*, 1983) predict the amino acid sequence Phe-Asp-Gly-Phe-Glu-Gly-His-Pro-Phe-Gly close to the C-terminal end of the protein. The corresponding sequence in nopaline plasmids pTiT37 (Goldberg *et al.*, 1984) and pTiC58 (J. Schell, personal communication) is

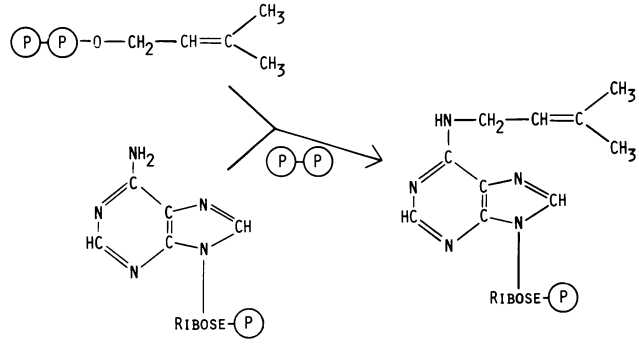

Fig. 1. Prenyltransferase reaction in biosynthesis of cytokinins. The enzyme combines Δ^2-isopentenylpyrophosphate (Δ^2-IPP) with 5'-AMP to N^6-(Δ^2-isopentenyl)-5'-AMP (IPAR-monophosphate), and pyrophosphate is released in the reaction (Taya *et al.*, 1978; Chen and Melitz, 1979).

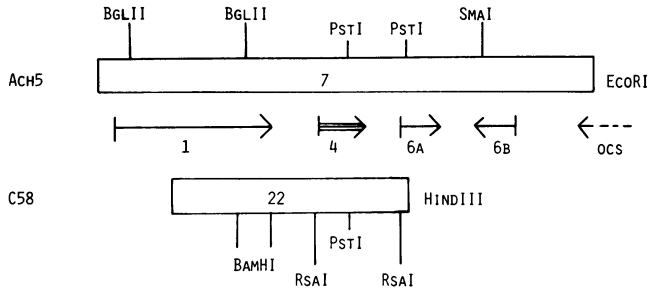

Fig. 2. Position of gene 4 in *Eco*RI 7 (octopine plasmid pTiAch5) and in *Hind*III 22 (nopaline plasmid pTiC58). Lines with arrows represent the coding regions derived from the DNA sequence of *Eco*RI 7, and the numbering of the genes is according to Gielen *et al.* (1984). Fragment *Hind*III 22 was aligned by the position of gene 4 (Goldberg *et al.*, 1984), and by common restriction sites with *Eco*RI (Engler *et al.*, 1981). Only restriction sites used in subcloning are indicated. ocs: octopine synthase.

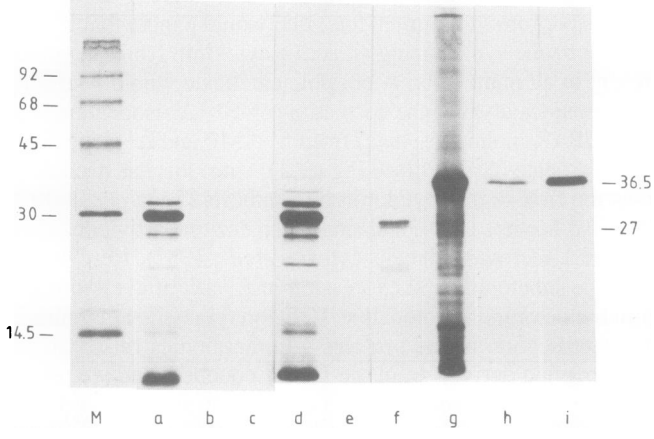

Fig. 3. Gel electrophoretic analysis of radioactive proteins synthesized in *E. coli* minicells. **Lanes a–c:** control (vector pINIIA without insert); **d–f:** pGS207.2 (gene 4 from octopine T-region in pINIIA); **g–i:** pGS208.2 (gene 4 from nopaline T-region, as gene fusion in pACYC184). **Lanes a,d,g:** total incorporation; **b,e,h:** immunoprecipitates with CAT-antiserum; **c,f,i:** immunoprecipitates with decapeptide antiserum. M: marker proteins; the numbers at the left indicate the size in mol. wt. x 10^{-3}.

identical except that the first Phe is replaced by Tyr. Since this amino acid is convenient for covalent attachment to proteins (Walter *et al.*, 1980), the latter peptide was coupled to bovine serum albumin, and the conjugate was used to raise antibodies. The antiserum specifically recognized the gene 4 encoded proteins from octopine and nopaline plasmids (see below).

Subcloning and expression of gene 4

Fragment *Eco*RI 7 from octopine plasmid pTiAch5 expressed gene 4 into protein in *E. coli* with promoters from vector pINIIA reading into the fragment (Schröder and Schröder, 1983). Since the distance between promoter and gene was too large for high expression, the *Bgl*II fragment (see Figure 2) was removed by restriction and religation. This left the presumed transcription stop signals of gene 1 intact, and therefore the plasmid was opened with *Bgl*II, digested with *Bal*31 to remove ~0.5 kb at both ends, and then it was religated. One of the plasmids was analyzed in detail by restriction mapping, and the results indicated that the left *Eco*RI site of the original fragment was retained, that gene 1 including its transcription stop signals was deleted, and that the complete coding region of gene 4 was retained. The plasmid (named pGS207.2) could be tested in the minicell system without further modification since the orientation of the gene in

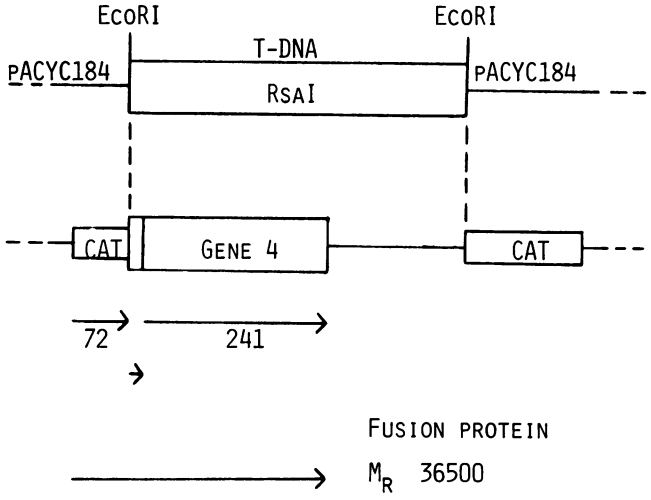

Fig. 4. Structure of the gene fusion in pGS208.2. The open reading frame for synthesis of the mol. wt. 36 500 protein contains the first part of the CAT-gene (72 amino acids) connected in-frame with the complete coding region of gene 4 (241 amino acids) by the peptide encoded in the DNA sequence between the left *Rsa*I site and the presumed start methionine of gene 4. M_r, mol. wt.

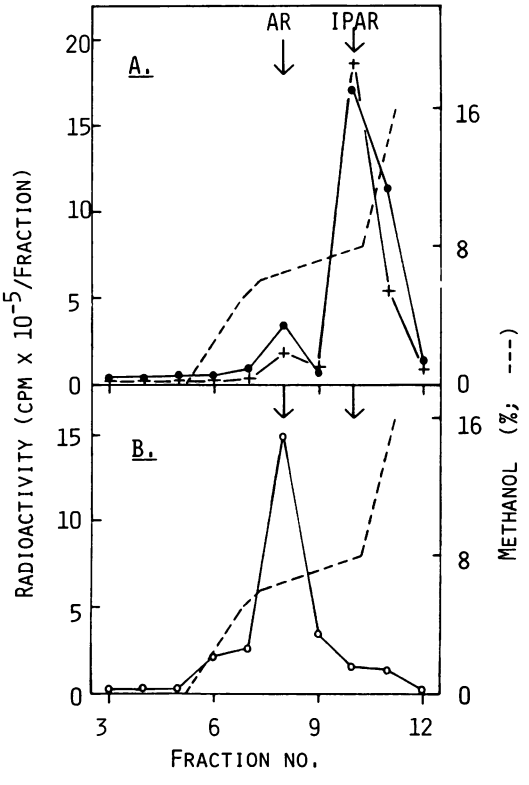

Fig. 5. H.p.l.c. analysis of prenyltransferase incubations with *E. coli* extracts. The figure shows only part of the elution pattern; the other fractions contained <0.1 x 10^5 c.p.m. Arrows indicate the position of authentic adenosine (AR) and N^6-(Δ^2-isopentenyl)adenosine (IPAR). **Panel A:** pGS207.2 (•–•; gene 4 from octopine T-region), pGS208.2 (+–+; gene 4 fusion, nopaline T-region). **Panel B:** pGS200.2 (control without gene 4; the plasmid is described in Materials and methods). Dotted lines indicate the methanol gradient of the elution system.

the vector plasmid was not changed in the cloning procedure. Figure 3 shows that pGS207.2 expressed gene 4 into protein. This is difficult to see with total minicell extracts (lane d), since a vector-encoded protein from the ampicillin resistance gene has a similar mobility (see control in lane a), but immunoprecipita-

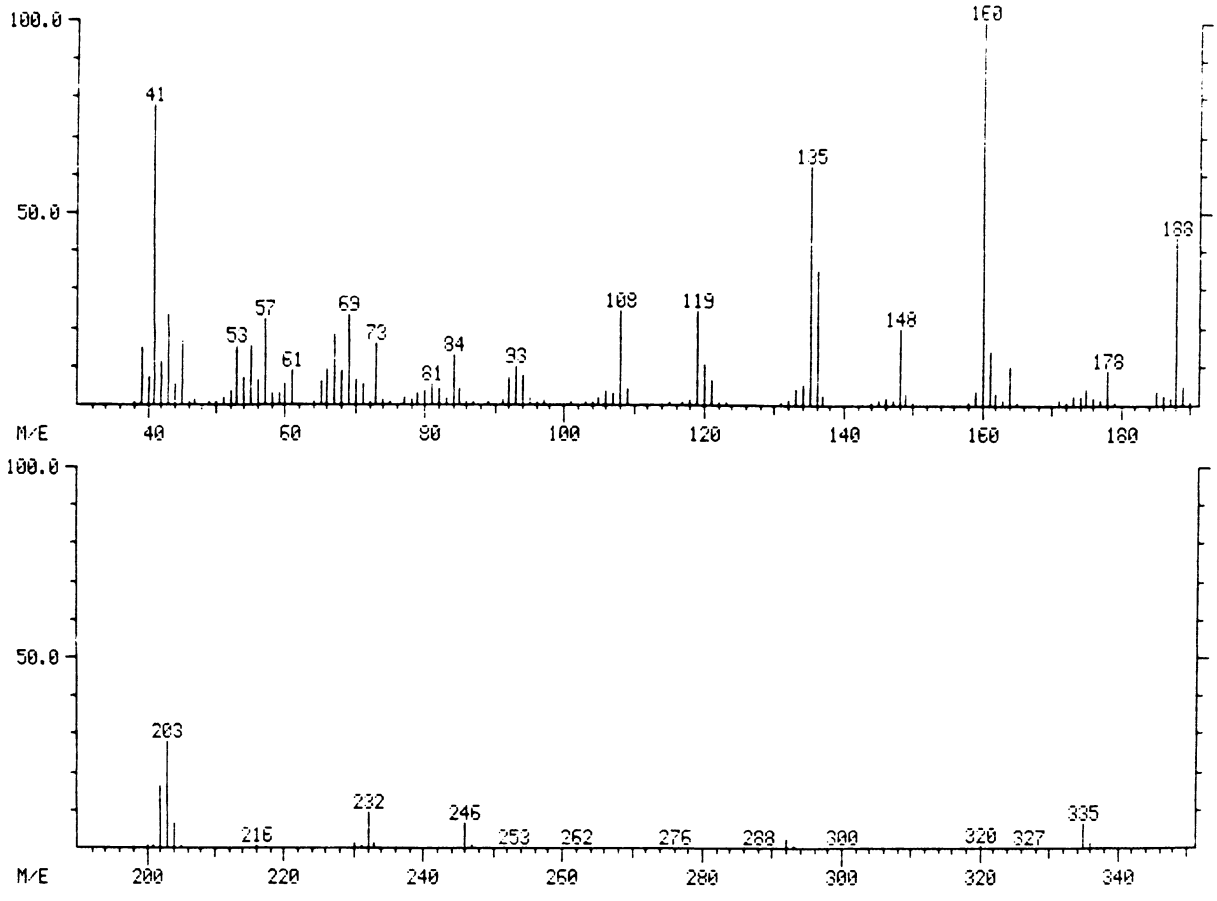

Fig. 6. EI-mass spectrum (70 eV) of the cytokinin IPAR synthesized in extracts from *E. coli* cells containing pGS208.2 (gene 4 from nopaline plasmid pTiC58 fused to CAT-gene of vector pACYC184).

tions with the antiserum demonstrated the presence of immunoreactive material. The gene 4 product appeared as a doublet at mol. wt. 27 000 rather than as a single band (lane f), and in most experiments a much smaller protein was also detected (see Discussion). None of these proteins were synthesized with vector pINIIA without insert (lane c). In addition to gene 4, pGS207.2 contains genes 6a and 6b (Figure 2), but these are not expressed into protein in *E. coli* without further subcloning (Schröder *et al.*, 1983; and unpublished).

Nopaline plasmid pTiC58 contains gene 4 on fragment *Hind*III 22 (Figure 2), and the fragment shows a weak expression of the gene in *E. coli* (Schröder *et al.*, 1983). In a first step, the *Bam*HI/*Hind*III subfragment containing the complete coding region on a *Rsa*I fragment was recloned into pBR322. Inspection of the sequence (Goldberg *et al.*, 1984; J. Schell, personal communication) showed that the right *Rsa*I site is ~540 bp downstream of the end of the coding region, that the left *Rsa*I site is 52 bp upstream of the presumed start methionine, and that the DNA sequence from the left *Rsa*I site to the first methionine contains an open reading frame in-phase with that of gene 4. The *Rsa*I fragment was isolated, cloned with *Eco*RI linkers into vector pACYC184, and the asymmetric *Pst*I site was used to screen for recombinants containing the gene in the same direction of transcription as the gene for chloramphenicol acetyltransferase (CAT) in the vector. This plasmid was named pGS208.2, and when it was assayed in the minicell system, it produced a mol. wt. 36 500 protein (Figure 3, lane g). The sequences indicated that this protein represented a fusion of the first part of the CAT-protein from vector pACYC184 (Alton and Vapnek, 1979; Shaw *et al.*, 1979) connected to the complete gene 4 protein by the amino acids encoded between *Rsa*I site and first methionine of gene 4 (Figure 4). The size of the protein was consistent with the prediction from the DNA sequence, and the structure was confirmed by the finding that the fusion protein was recognized by antisera against the CAT-protein (Figure 3, lane h) and against the decapeptide encoded in gene 4 (lane i). Controls showed that the CAT antiserum did not react with the protein from the T-DNA gene when this was expressed by itself (lane e).

When the *Rsa*I fragment was cloned into vector pINIIA in a different phase which precluded a fusion protein, the recombinant plasmid produced a low level of a doublet protein at mol. wt. 27 000 and very strongly two polypeptides of mol. wt. 15 000 and 12 000 (not shown). The doublet and the larger of the two polypeptides were specifically recognized by the antiserum against the DNA sequence-derived decapeptide. This indicated that gene 4 from the nopaline T-region, like the gene from the octopine T-region, expressed a doublet protein in *E. coli*, and furthermore that the protein was unstable when expressed at high levels. Since this was not observed with the fusion protein obtained with pGS208.2, the latter was used for investigating the enzyme activity.

Enzyme activity in E. coli

The product of the prenyltransferase reaction shown in Figure 1 is the monophosphate of IPAR (Taya *et al.*, 1978; Chen and Melitz, 1979). However, since crude enzyme extracts contain

Table I. Isopentenyltransferase acivities in extracts from *E. coli* and plant cells. Results were calculated from radioactivity peaks at the position of IPAR in h.p.l.c. chromatography.

Gene 4 from	Enzyme source		Enzyme activity (pmol/h/mg)
pTiAch5 (octopine)	*E. coli*/pGS207.2		2470
pTiC58 (nopaline)	*E. coli*/pGS208.2		2080
–	*E. coli*/pGS200.2		$<10^{a}$
pTiA6 (octopine)	tobacco/A6-S1		0.6
–	tobacco/NW-S1		<0.001
		Pre-treatment	
pTiA6 (octopine)	tobacco/A6-S1	decapeptide antiserum	0.05
pTiA6 (octopine)	tobacco/A6-S1	control antiserum	1.0

[a]Calculated from radioactivity at the shoulder of AR; no distinct peak discernible at position of IPAR (see text).

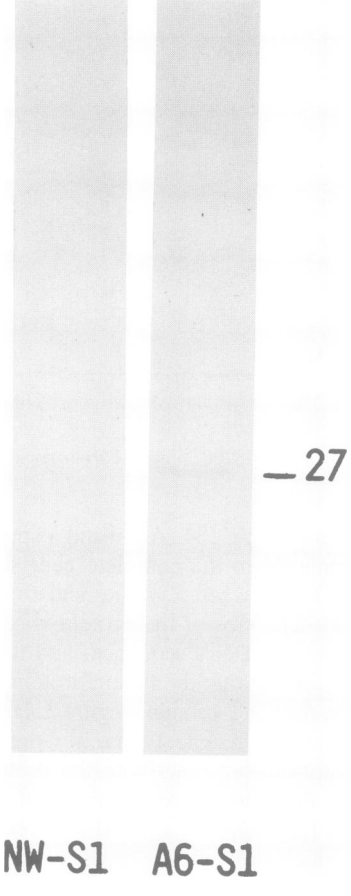

Fig. 7. Immunological detection of the gene 4 encoded protein in crown gall cells. Proteins (200 μg) of partially purified extracts from tumor cells (A6-S1) or habituated cells (NW-S1) were separated by gel electrophoresis, transferred to nitrocellulose, and incubated sequentially with decapeptide antiserum and with phosphatase-conjugated anti-antibodies. 27: Size of the immunoreactive protein (mol. wt. x 10^{-3}).

active phosphatases, at least part of the product and of the substrate are converted into IPAR and AR in the course of the incubation. To facilitate the analysis we found it useful to follow the initial prenyltransferase reaction with a second incubation in presence of excess phosphatase, so that the radioactivity in substrate and product could be quantitated as AR and IPAR, and a h.p.l.c. system separating these two components was employed for analysis. Figure 5A shows that extracts from *E. coli* cells expressing gene 4 synthesized from radioactive 5′-AMP and Δ^2-IPP a substance co-migrating with the cytokinin IPAR, and little radioactivity was found at the position of AR. The fractions containing the presumed IPAR from four standard incubations were pooled, concentrated and further analyzed by mass spectrometry. Figure 6 shows the mass fragmentation pattern of the substance synthesized in extracts containing the fusion protein encoded in pGS208.2; the pattern was identical with that of authentic IPAR, and the same result was obtained with gene 4 from the octopine T-region expressed in pGS207.2. In contrast to this, control incubations with extracts not containing the product of gene 4 revealed most of the radioactivity co-migrating with AR, and only a small shoulder was detected at the position of IPAR (Figure 5B). Attempts to analyze this material by mass spectrometry failed, since no identifiable fragmentation pattern could be obtained. This does not exclude, however, that the shoulder contained a small amount of IPAR mixed with other non-identified substances. A conservative estimate calculated from the quantitative data indicated that extracts containing the T-DNA gene product formed at least 20 times more IPAR than control extracts (Table I).

Protein and enzyme activity in plant cells

Since a specific antiserum was available, we first investigated whether immunoreactive material was present in plant cells. Figure 7 shows that a partially purified enzyme preparation from octopine tumor line A6-S1 contained a protein of the expected size (mol. wt. 27 000) which reacted with the antibodies, and a protein with the same properties was also detected in a tobacco cell line containing the T-DNA from pTiC58 (not shown). The corresponding preparation from the habituated cell line NW-S1 revealed no significant reaction (Figure 7). Measurements of isopentenyltransferase activity were negative with extracts from the habituated strain, while the protein fraction from the tumor cell line A6-S1 synthesized IPAR (Table I), as identified by h.p.l.c. analysis. Enzyme activity was comparable with previous determinations with other crown gall cells (Morris *et al.*, 1982; Akiyoshi *et al.*, 1984), but low when compared with activity in *E. coli* cells. This was to be expected, however, since measurements were performed after expression-cloning of the gene in multicopy plasmids. The correlation between protein and enzyme activity in crown gall cells suggested that the T-DNA encoded protein was responsible for the activity, and this conclusion was confirmed by the following experiment: Extracts from the tumor cell line were incubated either with decapeptide antiserum or with control antiserum, and enzyme was measured after removal of antibodies and immunocomplexes with Protein A-Sepharose. The results showed that the antiserum against the

T-DNA sequence derived decapeptide led to disappearance of most of the isopentenyltransferase activity, while the control antiserum had no such effect (Table I).

Discussion

The amino acid sequences encoded in gene 4 of octopine and nopaline plasmids are very similar, and in both cases they predict a protein of mol. wt. ~27 000 (Gielen *et al.*, 1984; Heidekamp *et al.*, 1983; Lichtenstein *et al.*, 1984; Barker *et al.*, 1983; Goldberg *et al.*, 1984), but our results show that *E. coli* minicells contained a doublet rather than a single protein. The same doublet was observed in previous experiments analyzing gene expression in *E. coli* with larger fragments of the T-region. At that time it seemed possible that independent, overlapping coding regions were involved (Schröder *et al.*, 1983). The present results indicate that both proteins are products of the gene 4 coding region. This is based on the findings that both proteins are specifically recognized by the antibodies, and that in case of nopaline gene 4, the *Rsa*I fragment cloned in pINIIA also expressed a doublet although there was no second open reading frame which could account for a protein of mol. wt. 27 000. Although this has not been further analyzed, it seems likely that the smaller protein of the doublet and a polypeptide also detected with antibodies in minicells containing gene 4 from pTiAch5 (Figure 3, lane f) are the result of protein instability in the foreign background *E. coli*. Such instability was most pronounced with the gene product from nopaline plasmid pTiC58 when not expressed as fusion protein. Two polypeptides appeared as main products, and since one of them (mol. wt. 15 000) reacted with the antibodies (which are directed against the C-terminal part of the protein), it was possible to localize the region of instability in the amino acid sequence. This region (amino acids no. 100 – 109) differs in pTiAch5 and pTiC58 by four closely spaced amino acid exchanges, and one or several of them may render the protein more susceptible to proteolytic attack in *E. coli*. Although one might speculate that different protein conformations are involved, the precise reason for the stability of the fusion protein remains to be established.

Extracts from *E. coli* cells expressing gene 4, either as doublet (pGS207.2, octopine T-region) or as fusion protein (pGS208.2, nopaline T-region) contained an isopentenyltransferase very active in the first step of cytokinin biosynthesis, and the product of the reaction was unambiguously identified by sequential h.p.l.c. chromatography and mass spectrometry. In control extracts the presence of the activity would not be clearly demonstrated, since the small amount of radioactive material at the position of the cytokinin gave no identifiable mass fragmentation pattern. It seems possible that the radioactivity represented trailing of the AR peak, or alternatively, that the only known prenyltransferase in *E. coli*, which utilizes tRNA as normal substrate (Rosenbaum and Gefter, 1972; Schaeffer *et al.*, 1973), also accepted 5′-AMP as unspecific substrate in a slow reaction under our assay conditions. Interestingly, the stable fusion protein possessed enzyme activity, and so far we have not observed significant differences in enzyme properties with extracts from pGS207.2 and pGS208.2, except that enzyme activity appeared to be somewhat lower with the fusion protein expressed from pGS208.2 (Table I).

While this work was in progress, two independent publications also reported experiments suggesting that gene 4 expressed a prenyltransferase in *E. coli*. Akiyoshi *et al.* (1984), investigating the gene from pTiA6NC (an octopine plasmid), did not analyze the proteins, and enzyme activity was very low when compared with our results. The product of the reaction was analyzed by h.p.l.c. chromatography, and the identification of the cytokinin was supported by binding studies with antibodies specific for IPAR. Interestingly, they also detected enzyme activity with a gene fusion containing the first 10 codons of β-galactosidase fused to the 5′ end of the T-DNA gene. This is comparable with our construction with the gene from the nopaline T-region which led to an enzymatically active fusion protein. Barry *et al.* (1984) used expression cloning of the gene from pTiT37 (a nopaline plasmid), but enzyme activity in crude extracts of *E. coli* (5 pmol/h/mg) was ~400-fold lower than in our experiments. The reasons are not clear; one of them may be an undetected protein instability as we describe for the gene product from nopaline plasmid pTiC58. They also used 5′-AMP as radioactive substrate, and co-migration of the expected radioactive product with authentic IPAR in h.p.l.c. chromatography was the sole means of identification. AR, the dephosphorylation product of 5′-AMP, was not discussed in the product analysis, and its position was not indicated in the h.p.l.c. chromatograms. Since the radioactive material co-migrating with IPAR was not further analyzed by other techniques, the product identification appears insufficient.

The data discussed above and our results, which in addition present the immunological identification of the proteins and the unambiguous identification of the product of the enzyme reaction, indicate that gene 4 from the T-region of Ti plasmids codes in *E. coli* for an enzyme active in cytokinin biosynthesis. It was equally important, however, to test whether the gene has the same function in transformed plant cells. Our results show that partially purified extracts from a crown gall cell line contained a protein which specifically reacted with the decapeptide antiserum and which had the size predicted by the DNA sequence. The same protein fraction contained isopentenyltransferase activity; this activity was much reduced after immunoprecipitation with the decapeptide antiserum, but not with control antiserum. This indicates that the T-DNA encoded protein was responsible for the enzyme activity. This is to our knowledge the first direct demonstration of the protein in plant cells, and also the first direct evidence that the isopentenyltransferase activity in crown gall cells is encoded in gene 4. The name *ipt* (for *iso*pentenyl*t*ransferase) was suggested for the gene (Barry *et al.*, 1984), and since our results substantiate this suggestions, we follow this proposal.

The role proposed for gene 4 is consistent with results from genetic experiments that the gene is responsible for cytokinin effects, including cytokinin independence, in tumor cells (Garfinkel *et al.*, 1981; Leemans *et al.*, 1982; Joos *et al.*, 1983; Van Slogteren *et al.*, 1983; Inzé *et al.*, 1984). It is also consistent with findings that cytokinin levels are often unusually high in transformed plants cells (Weiler and Spanier, 1981; and other references in Letham and Palni, 1983), and that the presence of gene 4 is correlated with high cytokinin contents (Akiyoshi *et al.*, 1983) and prenyltransferase activity (Morris *et al.*, 1982; Akiyoshi *et al.*, 1984). In view of the debate on pathways to cytokinins in normal plant tissues (isopentenylation of free 5′-AMP *versus* degradation of isopentenylated tRNA; see review by Letham and Palni, 1983), it is of interest whether the enzyme reaction encoded in the T-DNA gene is tumor-specific or also present in non-transformed cells. Our experiments with the habituated tobacco line NW-S1 did not detect measurable enzyme activity, although these cells produce cytokinins (Weiler, Schröder *et al.*, unpublished). However, our protein fractionation procedure was primarily designed to detect the T-DNA en-

coded protein, and it is quite possible that other proteins with isopentenyltransferase activity were lost in other fractions of the cell extracts. The few enzymatic data available in higher plants suggest that isopentenylation of free 5'-AMP also occurs in normal tissues (Chen and Melitz, 1979; Nishinari and Syono, 1980; Chen, 1982). However, the most convincing results were reported for cytokinin-independent cell cultures (Chen, 1982), and since crown gall cells share this property, it would seem necessary to establish that no T-DNA gene was present before a definite conclusion can be reached.

Materials and methods

Cloning and expression

E. coli plasmids with fragment *Eco*RI 7 from octopine plasmid pTiAch5 and fragment *Hind*III 22 from nopaline plasmid pTiC58 have been described (Schröder and Schröder, 1983; Schröder *et al.*, 1983). Procedures used in subcloning, the *E. coli* minicell system, and the analysis of radioactive proteins are published (Schröder *et al.*, 1983). Digestions with *Bal*31 were performed as recommended by the enzyme manufacturer (Boehringer).

Antisera

The decapeptide derived from the DNA sequence was synthesized by UCB-Bioproducts (Brussels) and coupled without further purification to bovine serum albumin (Walter *et al.*, 1980). The conjugate (0.7 mg) was injected with complete Freund's adjuvant intradermally at 10 different sites into 4-month-old rabbits. Animals were boostered monthly, and antisera were taken 7 days later. Four boosters were necessary to obtain an antiserum with high activity. Antiserum against CAT-protein (chloramphenicol acetyltransferase) was raised with highly purified enzyme (a gift from W.V.Shaw) with essentially the same schedule.

Immunoprecipitation

Minicells labelled with radioactive methionine were lysed in 25 mM Tris-HCl (pH 6.8) containing 0.6% SDS for 10 min at 95°C. Lysates were mixed with six volumes of wash buffer (50 mM Tris-HCl, pH 8, 5 mM EDTA, 0.15 M NaCl, 2% Triton X-100) and incubated for 3 h at 4°C with 5 μl antiserum. Immunocomplexes were isolated with Protein A-Sepharose (Pharmacia), washed three times with wash buffer, and then they were solubilized in sample buffer for gel electrophoresis.

Enzyme extracts from E. coli

Pellets from a 30 ml culture grown to $A_{600}=0.8$ were resuspended in 1.9 ml buffer A (10 mM Tris-HCl, pH 8, 1.3 mM EDTA, 10 mM 2-mercaptoethanol, 2.7 mM Mg-acetate, 25 mM KCl, 1 mg/ml lysozyme) and incubated on ice with repeated vigorous mixing. After 10 min the cells were lysed, and the extracts were dialyzed for 2 h against four changes of buffer B (10 mM Tris-HCl, pH 8, 1 mM EDTA, 20 mM $MgCl_2$, 10% glycerol, 10 mM 2-mercaptoethanol). They were then mixed with 0.5 ml 0.25 M Hepes-KOH (pH 7) and used immediately for enzyme assays.

Enzyme assay in E. coli extracts

Standard incubations were performed in a final volume of 0.4 ml. They contained 0.35 ml dialyzed enzyme extract (0.63 mg protein), additional $MgCl_2$ to a final concentration of 28 mM, 16 μM unlabelled 5'-AMP, 10 μCi [2-^{3}H]5'-AMP (Amersham Buchler, 13 Ci/mmol), and 0.25 mM Δ^2-isopentenlypyrophosphate (a gift from D. Akiyoshi, Seattle). After 2 h at 25°C, assays were mixed with 50 μl 0.2 M Tris-HCl (pH 8) containing 20 units alkaline phosphatase (Boehringer), and incubations were continued for 2 h at 37°C to dephosphorylate the nucleotides. After freeze-drying, material extracted into 0.5 ml water (pH 6.5) was subjected to further analysis (see below). Enzyme activity was expressed as pmol IPAR/h/mg protein; it was calculated from the amount of radioactivity in the peak at the position of IPAR in h.p.l.c. chromatography.

Analytical techniques

H.p.l.c. analysis. The phosphatase treated, lyophilized enzyme incubations were dissolved in 0.5 ml water and for a first purification adsorbed on a small preparative column filled with RP18 (30–40 μm). After washing the column with 10 ml water the less polar material was eluted with 10 ml methanol. The methanol was evaporated under reduced pressure; the residue was dissolved in 50 μl water (pH 6.5) and applied to an analytical h.p.l.c. column (RP18, 5 μm). Elution was performed at a flow-rate of 1.5 ml/min with a non-linear gradient from 0 to 40% methanol in water (5 min from 0 to 5%, 2 min from 5 to 6%, 10 min from 6 to 8%, 10 min from 8 to 40%). Authentic AR and IPAR were eluted at 6.5% and 7.9% methanol, respectively.

Mass spectrometry. Radioactive material co-migrating with IPAR in h.p.l.c. chromatography was pooled from four standard incubations, to obtain enough substance. Samples were lyophilized, redissolved in 5 μl methanol, and portions of 2 μl were used to record the mass spectrum of the substance (Finnigan MAT4510 mass-spectrometer, solid probe inlet with ballistic heating from 50 to 450°C, 70 eV, electron impact ionization).

Controls in E. coli experiments

E. coli cells containing vector pINIIA without insert were used as controls in most experiments. In some of the enzyme assays, the vector contained pTiAch5 *Eco*RI 7 with a deletion covering genes 4, 6a, and 6b. This plasmid (named pGS200.2) was constructed in two steps: (i) removal of the *Pst*I fragment by restriction and religation, (ii) restriction with *Sma*I and subsequent digestion with *Bal*31 to remove ~1.6 kb at both ends.

Experiments with plant cells

Suspension cultures of tobacco crown gall A6-S1 (T-DNA from octopine plasmid pTiA6) and the normal tobacco line NW-S1 (no T-DNA, but habituated for hormone-independent growth) have been described (Schröder and Schröder, 1982). Extracts were prepared by homogenization of 20 g frozen tissue with 4 g Dowex (1 x 2) in 20 ml buffer (0.15 M Tris-HCl, pH 7, 15 mM Mg-acetate, 5 mM EDTA, 10 mM 2-mercaptoethanol, 25% glycerol). After removal of insoluble material by centrifugation (30 min at 21 000 *g*), proteins in the supernatants were fractionated by ammonium sulfate precipitation. The fraction precipitating between 35% and 65% was redissolved in 50 mM Tris-HCl, pH 7, containing 15 mM Mg-acetate and 10 mM 2-mercaptoethanol, passed through Sephadex G-25 equilibrated with the same buffer, and portions of 0.35 ml (corresponding to 1.5–2.8 mg protein) were immediately assayed for isopentenyltransferase as described for *E. coli* extracts. When inactivation by antibodies was tested, extracts (0.35 ml) received NaCl to a final concentration of 0.15 M and 20 μl decapeptide antiserum or control antiserum. After 60 min at 4°C, immunocomplexes and antibodies were removed with Protein A-Sepharose (see *E. coli* experiments), and the supernatant fluids were assayed for isopentenyltransferase activity. For immunological detection of the protein, portions of the extracts were concentrated by protein precipitation with seven volumes of acetone, and 0.2 mg of the proteins redissolved in sample buffer were applied to gel electrophoresis in the presence of SDS (Schröder *et al.*, 1983). After completion of the run, proteins were electrophoretically transferred to nitrocellulose sheets (Towbin *et al.*, 1979). Immunoreactions with the decapeptide antiserum and alkaline phosphatase-conjugated anti-antibodies were performed as described (Knecht and Dimond, 1984; Blake *et al.*, 1984).

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