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Biochemistry

Identification of a cloned cytokinin biosynthetic gene

(*Agrobacterium tumefaciens*/Ti plasmid/*tmr* locus/isopentenyltransferase/phytohormone biosynthesis)

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ABSTRACT **A small region of the Ti plasmid (the *tmr* locus), thought to be involved in phytohormone metabolism in *Agrobacterium tumefaciens*-transformed plant tissue, was cloned and expressed in *Escherichia coli*. By enzyme assay, the *tmr* locus was shown to encode isopentenyltransferase, an enzyme that catalyzes the first step in cytokinin biosynthesis.**

Crown gall disease, characterized by the development of neoplastic growth on the infected plant, affects many dicotyledonous plants and is caused by the soil bacterium *Agrobacterium tumefaciens* (1). The induction and maintenance of the tumor is dependent upon the integration into the plant genome and expression of a DNA fragment (T-DNA) from the large virulence plasmid (Ti) harbored by the bacterium (2–7). In tissue culture the *Agrobacterium*-induced tumors have been shown to be autotrophic for phytohormones (8), specifically for auxins and cytokinins. That the transforming T-DNA was involved in the alteration in the endogenous hormone synthesis was suggested by the identification of *A. tumefaciens* isolates that induced tumors with abnormal morphologies, characteristic of hormonal imbalance, such as the overproduction of shoots or roots (9–13). The loci responsible for such altered morphologies have been mapped to the T-DNA and have been shown to correlate with alterations in the phytohormone levels in the tumors (14, 15). Specifically, mutants in the *tms* ("shooty") and *tml* ("large") loci result in over production of cytokinins, while those in the *tmr* ("rooty") locus result in a very large decrease in endogenous cytokinin levels.

The enzyme central to all cytokinin biosynthesis is Δ^2-isopentenyl pyrophosphate:5′-AMP Δ^2-isopentenyltransferase (EC 2.5.1.-) commonly referred to as isopentenyl transferase (16). The product of this reaction is isopentenyladenosine monophosphate (iPeAMP) (Fig. 1), which by dephosphorylation is converted to the cytokinin isopentenyladenosine (iPeA) and which is the precursor to all other cytokinins (17). The *tms* and *tml* defects result in a large increase in isopentenyltransferase activity in the tumors, while the *tmr* defects result in a decrease in activity to a level lower than that found even in normal tissue (14). While the *tms*, *tml*, and *tmr* results are consistent with the regulatory role of these loci on the expression or activity of endogenous cytokinin biosynthesis, the results are also consistent with the *tmr* locus directly encoding a biosynthetic enzyme.

Transcriptional and translation maps of the T-DNA regions have been determined recently for both the octopine and nopaline Ti plasmids, and the *tmr* gene transcript has been identified in tumors induced by both plasmid types (11, 18–22). The *tmr* region from the nopaline plasmid pTi T37 was cloned and the nucleotide sequence was determined (23). An open reading frame encoding a protein of 27 kilodaltons (kDa) was identified in this sequence, as has also been found for the same region of the octopine Ti plasmid (24). A protein of this size has been shown to be expressed from the octopine *tmr* region both in *Escherichia coli* minicells and in cell-free extracts of *E. coli* and *A. tumefaciens* (22).

In this study, we engineered, *in vitro*, the nopaline *tmr* locus open reading frame to maximize expression from a strong *E. coli* promoter and, by assay for isopentenyltransferase, we provide definitive evidence that the *tmr* locus encodes this enzyme. The steps in the isolation and identification of the cytokinin biosynthetic gene are described in the following.

MATERIALS AND METHODS

Δ^2-Isopentenyl pyrophosphate (iPePP) was synthesized by the method of Cornforth and Popjak (25). 5′-[2-^{3}H]AMP was purchased from Amersham. Synthetic DNA oligonucleotides were synthesized by a modification of the phosphite method (26).

Construction of pMON230. All DNA manipulations were carried out by standard procedures (27). Plasmid pMON99, containing the 1983-base-pair (bp) *Bam*HI/*Hin*dIII segment of *Hin*dIII-22 of pTi T37 (23), served as source of the *tmr* sequences. The construction of pMON230 (outlined in Fig. 2) was accomplished as follows: pMON99 was digested with *Sal* I and *Hin*dIII and the 1279-bp *Sal* I/*Hin*dIII fragment was isolated; the 41-bp *Mbo* I/*Sal* I fragment was purified from *dam*$^-$ pMON99 DNA. The isolated fragments were ligated with a synthetic DNA fragment (sequence in Fig. 2) that regenerated the putative 5′ end and inserted into the *Bam*HI/*Hin*dIII sites of pKC7 (28). The reconstituted open reading frame was then cloned downstream from a *trp* promoter (29), using the *Xba* I site within the synthetic fragment. This plasmid was used in the *E. coli* expression studies of the *tmr* locus described below.

Expression of the *tmr* Open Reading Frame. Expression of the *tmr* coding region was studied in *E. coli* CSR603 maxicells and the ^{35}S-labeled proteins were examined by NaDodSO$_4$/PAGE (30). Cultures for enzyme assay were grown in minimal M9 Casamino acids medium (31) to derepress tryptophan biosynthesis and expression from the P_{trp}–*tmr* recombinant plasmid pMON230.

Preparation of Cell Extracts for Enzyme Assay. *E. coli* W3110 (32) and W3110/pMON230 were grown in M9 minimal Casamino acid medium and harvested by centrifugation, and the cells were washed with M9 salts and stored at −70°C. Frozen cell pellets were resuspended in buffer A [10 mM Hepes, pH 7.0/10 mM magnesium acetate/6 mM KCl/1 mM EDTA, 6 mM 2-mercaptoethanol/25% (vol/vol) glycerol (33)] and disrupted by mixing with glass beads on a Vortex mixer. After removal of the cell debris by centrifugation, a partially purified enzyme extract was prepared from the cell supernatant as described (33), except that DEAE-cellulose (Whatman DE52) was used in the chromatography step. This enzyme preparation was stored for no longer than 16 hr at

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Abbreviations: iPeA, N^6-(Δ^2-isopentenyl)adenosine; iPeAMP, N^6-(Δ^2-isopentenyl)adenosine monophosphate; iPePP, Δ^2-isopentenyl pyrophosphate; bp, base pair(s).

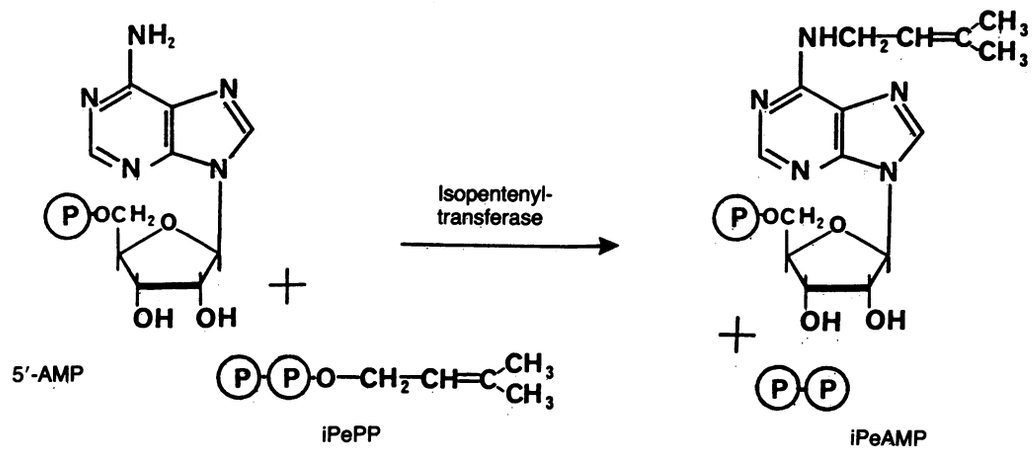

FIG. 1. Reaction catalyzed by isopentenyltransferase (16): Formation of N^6-(Δ^2-isopentenyl)adenosine monophosphate (iPeAMP) from Δ^2-isopentenyl pyrophosphate (iPePP) and adenosine 5'-phosphate (5'-AMP).

−70°C before being assayed for isopentenyltransferase. Crude enzyme extracts were prepared by disruption of the cell pellet with glass beads and the supernatant was dialyzed twice (500 vol of buffer A for 2 hr each at 4°C) and assayed immediately. The protein concentrations of the enzyme preparations and crude extracts were determined by using the Bio-Rad reagent with a bovine serum albumin standard and following the manufacturer's directions.

Isopentenyltransferase Assay. Isopentenyltransferase was assayed in a final volume of 6 ml containing Hepes (pH 7.0) at 20 mM, magnesium acetate at 30 mM, iPePP at 0.15 mM, 2-mercaptoethanol at 6 mM, 5–10 μCi of 5'-[^{3}H]AMP (11.7 Ci/mmol; 1 Ci = 37 GBq) (16), and 1 ml of partially purified enzyme or cell extract. The reaction was incubated, with gentle agitation, for 1 hr at room temperature. The reaction was stopped by passage through a C_{18} cartridge (SEP-PAK, Waters Associates) and washing twice with 5 ml of water (pH 6.5). The retained material was then eluted with two 5-ml portions of methanol, dried under reduced pressure, and stored dry at 4°C. Samples for phosphatase treatment were resuspended in 20 mM Tris·HCl, pH 9.0/10 mM $MgCl_2$, calf alkaline phosphatase (Boehringer Mannheim) was added to 26 units/ml, and the mixture was incubated for 30 min at 37°C. Phosphatase substrate (Sigma 104, disodium *p*-nitrophenyl phosphate) was included at 0.5 mg/ml to monitor the reaction. The reaction was stopped by passage through a C_{18} cartridge and processed as before.

HPLC Analysis of Isopentenyltransferase Reaction Products. Samples for HPLC were dissolved in water (titrated to pH 3.0 with phosphoric acid) immediately before analysis by reverse-phase chromatography on a C_{18} μBondapak analytical column (Waters Associates) with a 50-min linear gradient of 0–40% acetonitrile. Samples (1.5 ml) were collected at 1-min intervals and mixed with 5 ml of Insta-Gel (Packard) and the ^{3}H radioactivity was measured. Standards, consisting of iPeA and iPeAMP (P-L Biochemicals) were chromatographed and the elution was monitored by absorbance at 269 nm. The identity of the labeled reaction products was determined by comparison of the elution patterns. One unit of activity is defined as the incorporation of 1 pmol of 5'-

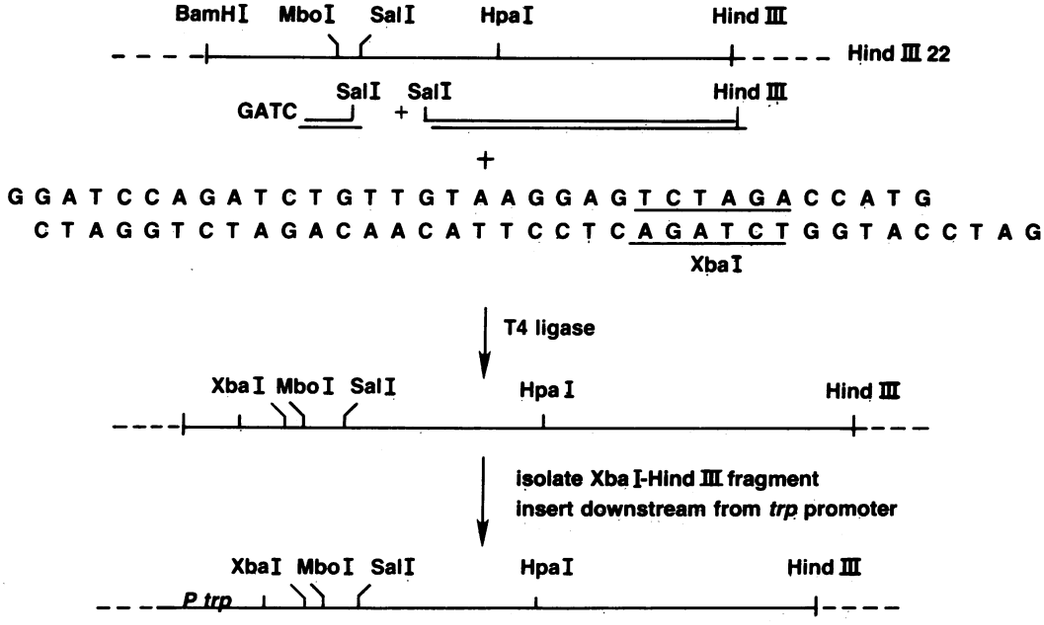

FIG. 2. Construction of pMON230. The cloning scheme, with the restriction sites used and the sequence of the synthetic linker, is shown. The *Hpa* I site that was known to occur in the *tmr* coding sequence is shown as a reference (21, 23). The diagram is not to scale. Restriction sites shown are for clarity and are not necessarily unique.

[^{3}H]AMP into iPeA or iPeAMP in 1 hr at 25°C (1 pmol = 6250 cpm) (14).

RESULTS

Engineered *tmr* Locus Open Reading Frame Encodes a 27-kDa Protein in *E. coli*. After nucleotide sequencing of the *tmr* region from the nopaline plasmid pTi T37 and computer-aided examination of the sequence, an open reading frame of 720 nucleotides, sufficient to encode a protein of molecular weight 27,025, was found (23). This open reading frame was engineered *in vitro* to determine if this protein could be expressed in *E. coli* and to provide enough material to test for function. An *Mbo* I site was conveniently located at position 663 (23) (the junction of amino acids 2 and 3 of the putative protein), which allowed a synthetic DNA linker to be easily attached to the 5′ terminus of the open reading frame sequence. The procedure employed and the sequence of the synthetic fragment are shown in Fig. 2. Attachment of this linker to the *Mbo* I site regenerates the 5′ terminus of the open reading frame and introduces additional cloning sites upstream from the putative ATG start codon. This hybrid molecule was then joined to the *trp* promoter (29), using the *Xba* I site contained in the linker sequences.

The expression of proteins from the recombinant plasmid was studied in *E. coli* maxicells and the proteins synthesized were examined by NaDodSO$_4$/PAGE (Fig. 3). CSR603 transformants of the promoter-plus and promoter-minus constructs show different proteins expressed; specifically, a new protein of 27 kDa is seen in the promoter-plus construct. The *bla* gene product (34) is visible in both lanes. The expression of a protein of 27 kDa has previously been observed in *A. tumefaciens* lysates, in *E. coli* minicells harboring recombinant plasmids containing the octopine *tmr* region (22), and in the *in vitro* translation products of RNA from Ti plasmid transformed plant cells selected by hybridization to *tmr* region DNA fragments (20).

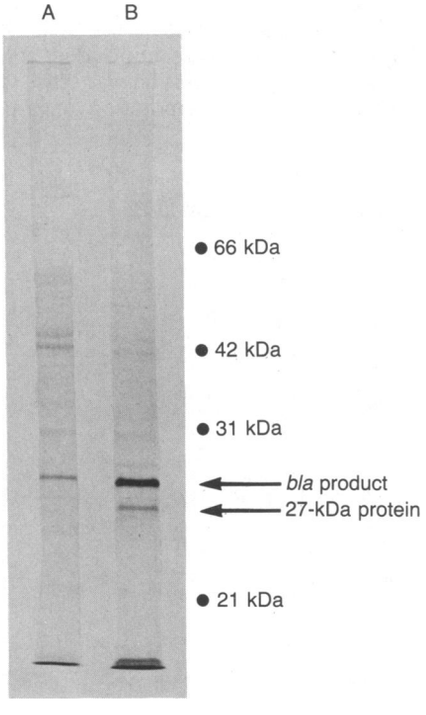

FIG. 3. Proteins expressed in *E. coli* maxicells from plasmids containing the *tmr* coding region. The autoradiogram shows a comparison between constructs containing a promoter (lane B) or lacking one (lane A). The positions of the molecular weight marker proteins are indicated, as are the *bla* gene product (34) and the new 27-kDa protein.

The 27-kDa Protein Is Isopentenyltransferase. After expression of the putative *tmr* protein in *E. coli* had been observed an attempt was made to identify the function of this protein. This locus was thought to be involved in cytokinin synthesis, and one explanation of the observed T-DNA mutant phenotypes was that the region encoded a cytokinin biosynthetic enzyme.

Isopentenyltransferase has previously been found in a number of plant tissues and in the plant pathogenic bacterium *Corynebacterium fascians* (14–16, 33, 35). The enzymes from all of these sources appear to be very similar in specificity and requirements for activity. Furthermore, the same purification procedures result in the purification of the enzyme from tobacco crown gall tissue and from *C. fascians* (35). The assay protocol for isopentenyltransferase involves collecting the products of the reaction on a C_{18} SEP-PAK, followed by chromatography on an analytical C_{18} μBondapak column. The primary product of the reaction is iPeAMP which, on the subsequent addition of alkaline phosphatase, can be converted to the less polar iPeA.

The product of the engineered *tmr* gene construct was assayed in *E. coli* extracts after growth of the transformants under conditions to maximize expression. The elution profile for the reaction products from the assay of the *E. coli* crude extracts shows the typical peaks of radioactivity observed (Fig. 4). The first peak (I), which has not been identified, does not elute with any known cytokinin, does not correlate with the presence of the *tmr* gene, and is formed in enzyme reactions lacking iPePP. Peaks II and III, on the other hand, are found only with extracts of transformants expressing the *tmr* open reading frame. The peak (II) eluting at ≈19 min has been identified as iPeAMP on the basis of its coelution with the iPeAMP standard and its conversion to peak III by treatment with alkaline phosphatase (data not shown). Peak III has been identified as iPeA on the basis of its coelution with the iPeA standard both on this chromatography system and on a C_8 Biophase column (Bioanalytical Systems, West Lafayette, IN) under isocratic conditions (data not shown). No activity was detected in the nontransformed strain [<0.005 unit (Table 1)]. The relative amounts of radioactivity in peak II and peak III varied somewhat in different extracts; this presumably is due to the level of contaminating dephosphorylating activities, but these activities were not actually measured. The strains were assayed both in the crude system and after partial purification of the enzyme (Table 1). The activity in the crude extract was quite high, and purification resulted in approximately a 10-fold concentration of the enzyme. The reaction is absolutely dependent on both iPePP and 5′-AMP and adenosine cannot be substituted for the latter in the reaction (16). A reaction time of 1 hr was chosen, but the reaction products are stable in the reaction mix for at least 8 hr.

DISCUSSION

The nucleotide sequence of the *tmr* region from the nopaline Ti plasmid pTi T37 revealed an open reading frame capable of encoding a protein of 27 kDa. This sequence was engineered *in vitro* to express this coding sequence in *E. coli*. The cloning and expression of genes in *E. coli* has frequently been used both to identify the proteins encoded by the genes and to provide enough material to assign functions to the gene products (36, 37). The expression of a 27-kDa protein was observed in maxicells from the engineered pTi T37 *tmr* construct, roughly the same size as found from the homologous region of an octopine Ti plasmid in *A. tumefaciens* cells and *in vitro* from RNA extracted from transformed plant cells (20, 22). That the same gene is being expressed in all these cases is suggested but not proven by these data.

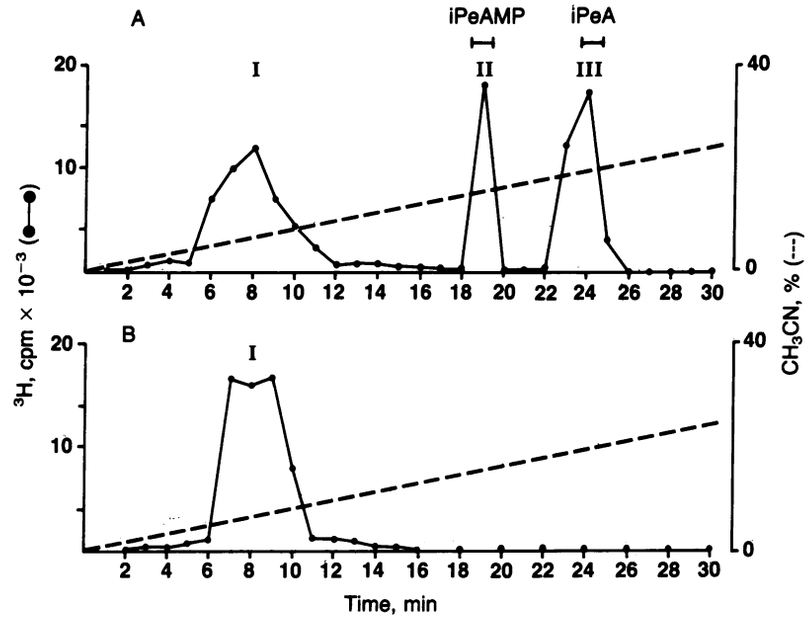

FIG. 4. Examination of the products of the isopentenyltransferase assay. (*A*) Elution profile of an assay of a crude lysate of tryptophan-derepressed W3110/pMON230. The positions of the cytokinin standards are indicated by the short bars. For an explanation of I, II, and III, see text. (*B*) Profile for W3110 lacking plasmid.

Strains harboring the recombinant *tmr* plasmid were assayed for the cytokinin biosynthetic enzyme isopentenyltransferase, an enzyme not normally found in *E. coli*; *E. coli* does contain an enzyme that transfers the isopentenyl moiety of iPePP to AMP residues in tRNA, but this enzyme has an absolute specificity for unmodified or demodified tRNA (38, 39). An isopentenyltransferase activity was detected that correlated with the expression of the nopaline *tmr* gene. This activity satisfied the biochemical criteria for similar enzymes previously assayed from other sources (16). In addition, this activity could be purified by using methods similar to those used for the enzyme in Ti plasmid-transformed plant cells (16, 35).

The sequence of the octopine *tmr* gene has also been determined (24), and the coding sequences of the octopine and nopaline genes are very similar (for a detailed comparison, see ref. 23). On the basis of identical phenotypes, the expression of proteins of apparently identical molecular weight, and the high degree of homology between the genes, it is probable that the octopine *tmr* locus also encodes isopentenyltransferase.

Phytohormone production has been observed in both Ti plasmid-transformed plant cells and the inciting bacteria (40–43, 15). It is possible that the same genes are responsible for this synthesis in the plant tissue and in *A. tumefaciens*, but further work will be necessary to establish this point.

The *tmr* locus was named originally for the rooty phenotype; mutants at this locus developed tumors with root-like growths, and later this phenotype was shown to correlate with the low levels of cytokinin and isopentenyltransferase (14, 15). By isolating and expressing this *tmr* coding region in *E. coli* we have provided definitive evidence that this locus encodes isopentenyltransferase. We propose that the *tmr* locus be renamed *ipt* (for isopentenyltransferase) now that this specific function has been assigned to the gene product.

Table 1. Isopentenyltransferase activities in crude and partially purified extracts of *E. coli* W3110 with and without pMON230

	Specific activity, pmol 5′-AMP incorporated per hr per mg of protein	
E. coli source	Crude lysate	Purified extract
W3110	<0.005	<0.005
W3110/pMON230	5.0	48.0

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