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Cloning and nucleotide sequence of the *tzs* gene from *Agrobacterium tumefaciens* strain T37

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ABSTRACT

The trans-zeatin secretion locus (tzs), from the nopaline Ti plasmid of Agrobacterium tumefaciens strain T37, was cloned and the nucleotide sequence determined. This gene is located in the virulence region of pTiT37. The tzs gene is responsible for the secretion of trans-zeatin into bacterial culture medium and in addition has the cytokinin biosynthetic activity, dimethylallylpyrophosphate:AMP dimethylallyltransferase. Sequence analysis showed an open reading frame of 729 nucleotides, capable of encoding a protein of 27,545 daltons. A single new labelled protein of 27,200 daltons was detected in Escherichia coli maxicells expressing the cloned tzs gene. Significant sequence homology was observed between the tzs and the published tmr sequence from pTiT37.

INTRODUCTION

The neoplastic disease of plants known as crown gall results from the transfer and integration into the plant genome of part of a tumor-inducing (Ti) plasmid from Agrobacterium tumefaciens (1-4). The functions of several T-DNA encoded genes have been identified. One T-DNA gene (ocs or nos) is responsible for the production of unusual amino acid derivatives known as opines (5-7). Three T-DNA transcripts (tmr,tms-1,tms-2) are involved in phytohormone synthesis (8-15) and expression of these genes accounts for the observed phytohormone independent growth of transformed tissue (16). Transposon mutagenesis of these genes results in tumors with aberrant morphologies (8-10), which are associated with altered endogenous cytokinin and auxin levels (17,18).

The involvement of phytohormones in other plant-pathogen interactions has been demonstrated (19,20). Many plant-associated microorganisms, both pathogens and symbionts, produce phytohormones and some bacterially-incited plant pathologies are known to be phytohormone dependent. For example, the degree of virulence of Corynebacterium fascians, the causal agent of witches' broom, has been shown to be strongly correlated with the amount of cytokinin produced by the bacterium (21). In olive and oleander knot disease caused by

Pseudomonas syringae pv. savastanoi (22), the pathogenicity of P. savastanoi is highly dependent on the bacterial production of the auxin, 3-indoleacetic acid (IAA) by the bacterium. In this case, loss of the ability to produce IAA drastically attenuates the virulence of the bacterium.

While T-DNA phytohormone genes are expressed in crown gall tumors, other phytohormone-related loci are expressed in A. tumefaciens. The bacterium produces both auxins (23-26) and cytokinins (27-34). The production of auxins by strain C58 has been reported to involve two genetic loci, one chromosomal (iaaC) and one plasmid-borne (iaaP)(26). Cytokinin production by A. tumefaciens has been studied under several culture conditions by different investigators. Both the culture medium (27-32) and bacterial tRNA contain cytokinins (33,34). In the culture medium of both octopine and nopaline strains, the cytokinin, iso-pentenyladenine (iP), has been found (30,31). However, its hydroxylated derivative, trans-zeatin, is found in culture medium of only nopaline strains (30). The gene responsible for trans-zeatin secretion (tzs) is located on the nopaline Ti plasmid (30).

In our continuing investigation of the relationship of cytokinin production to the pathogenicity of A. tumefaciens, we report the cloning, sequencing and partial characterization of the trans-zeatin secretion gene of A. tumefaciens strain T37.

MATERIALS AND METHODS

Materials

Restriction endonucleases were purchased from BRL or New England Biolabs and used according to the supplier's instructions. DNA polymerase I Klenow fragment was purchased from Boehringer Mannheim or BRL; SI nuclease from New England Biolabs, and E. coli exonuclease III and T4 DNA ligase from BRL.

Unlabelled nucleotides, dideoxynucleotides and M13 single strand sequencing primer (17-mer) were obtained from P-L Biochemicals. [α-^{35}S]dATP was from Amersham, and [α-^{32}P]dATP and [^{35}S]cysteine were obtained from New England Nuclear.

Bacterial strains

JM83 (35) and HB101 (36) were used as the E. coli hosts for plasmid constructs and were grown in LB, YT or M9CA media (37). JM105 (38) was used as the host for M13mp19 phage clones. Agrobacterium tumefaciens strains A348 (39) and T37 (40) were grown in AB minimal (41) or MG/L medium (39).

Preparation of plasmid constructs containing the tzs locus

Plasmid DNA was isolated by the method of Birnboim and Doly (42).

Digestion, ligation and transformation of plasmid DNA were as described by Maniatis et al. (43).

From an A. tumefaciens pTiT37 BamHI cosmid library cloned into pHC79 (44), a cosmid clone, pLJ47, was obtained which contained a functional tzs locus as determined by trans-zeatin radioimmunoassay (RIA)(Figure 1). The strategy used to clone the tzs locus from pTiT37 was based on the knowledge that the tzs locus of the nopaline pTiC58 is located on HindIII fragment 9 (Regier and Morris, unpublished data). Therefore, the 38 kb insert in pLJ47 was subcloned by digestion of the plasmid with BamHI and isolation of BamHI fragment 3b (10.6 kb) by gel electrophoresis. The purified fragment was then digested with HpaI and HindIII, ligated into HincII-HindIII cut pUC18 or pUC19 (45) and transformed into JM103 (38). Recombinant clones were screened by restriction endonuclease digestion of plasmid DNA and by trans-zeatin RIA of culture medium. Only clones containing a 1.4 kb HpaI-HindIII fragment tested positive when assayed for the secretion of trans-zeatin. Two clones, pDA1-12 (in pUC18) and pDA2-18 (in pUC19), which contain the HpaI-HindIII fragment in opposite orientations, were used for further analysis.

Trans-zeatin secretion assay

Preparation of [^{3}H]-labelled trans-ribosylzeatin (tZR) dialcohol and antibodies against tZR were as previously described (46). Screening of potential plasmid and M13mp19 clones for trans-zeatin secretion was performed using a modified solution RIA (46). Briefly, to cell-free culture supernatant (500 ul), were added 50 ul each [^{3}H]tZR dialcohol (5000 cpm), 0.5 M sodium phosphate, pH 7.0 containing 1.7% ovalbumin (w/v) and tZR antibody. After 30 min incubation (25^{o}C), 100% saturated ammonium sulfate (720 ul) was added, the precipitate was collected by centrifugation after 25 min, redissolved in water (800 ul) and radioactivity was determined.

High pressure liquid chromatography (HPLC)-RIA of culture medium

Cytokinins were extracted from cell-free culture medium (15 ml) by adsorption onto a column of octadecylsilica (C_{18}-silica, 0.75 gm) and eluted with methanol as described previously (30). Samples were dried in vacuo followed by fractionation on HPLC (12). Fractions with the retention times corresponding to trans-zeatin, tZR, iP and iso-pentenyladenosine (iPA) were collected, aliquoted and the levels estimated by RIA.

Nucleotide sequencing

For nucleotide sequence analysis, the HpaI-HindIII fragment from pDA1-12 was cloned into M13mp19 (45) in both orientations. Digestion of pDA1-12 with XbaI and HindIII, followed by ligation into XbaI-HindIII digested M13mp19

resulted in the phage construct M13-8. The HpaI-HindIII fragment was cloned in the opposite orientation by digesting pDA1-12 with HindIII, filling in the ends with DNA polymerase I Klenow, ligating BamHI linkers to the ends and digesting with BamHI and XbaI. This fragment was ligated into XbaI-BamHI digested M13mp19 to generate the phage construct M13-9.

A series of subclones suitable for nucleotide sequence analysis were generated by digestion of M13-8 and M13-9 with KpnI and BamHI, followed by exonuclease III digestion as described by Guo and Wu (47). Briefly, DNA (10 ug) was treated with exonuclease III (150 units, 30°C). Aliquots were removed at 50 sec intervals, treated with SI nuclease (100 units, 0°C, 30 min), religated and transformed into JM105. Under these conditions, approximately 200 nucleotides were removed per min. Inserts in independent phage plaques were sized by electrophoresis and clones containing progressively shortened inserts were selected for sequencing.

M13mp19 single stranded template DNA was prepared by the method described in the Amersham sequencing manual. Sequencing by the dideoxy chain termination method (48) was performed using either [^{32}P]dATP (Amersham instruction manual) or [^{35}S]dATP (New England Nuclear manual). Labelled reaction mixes were electrophoresed on either 6% polyacrylamide gels or on buffer gradient gels as described by Biggin et al. (49).

Polypeptide labelling in E. coli maxicells

The maxicell host strain, CSR603, was transformed with plasmids pUC19 and pDA2-18. Carbenicillin resistant colonies were characterized by plasmid isolation, restriction endonuclease digestion and trans-zeatin secretion RIA. Labelled protein was prepared from UV irradiated cells as described (50) using [^{35}S]cysteine. Due to the increased doubling time of these strains, cells were sulfate-starved for 1.5 hours instead of 1 hour as reported (51). Cell pellets were solubilized in cracking buffer (20 ul) and electrophoresed immediately. Samples (1 ul, 100,000-200,000 cpm) were loaded onto 12.5% polyacrylamide gels using the buffer system described (52). Gels were silver stained (BioRad silver stain kit) to visualize protein molecular weight standards, dried to filter paper and autoradiographed.

Dimethylallylpyrophosphate:AMP dimethylallyltransferase (DMA transferase) assay

Preparation of bacterial cell-free extracts and assay conditions for DMA transferase activity were as previously described (12). Some of the samples were treated with alkaline phosphatase prior to HPLC (12). Samples were fractionated on HPLC using the full-gradient program previously described (12).

RESULTS

Cloning the tzs gene

In previously published work (30), nopaline Ti plasmids were shown to have a locus responsible for the secretion of trans-zeatin into the culture medium. From a BamHI library of the nopaline plasmid, pTiT37, a cosmid clone containing a functional trans-zeatin secretion locus, pLJ47, was identified by trans-zeatin RIA. This locus was first isolated on BamHI fragment 3b (10.6 kb) and subsequently recloned as a 1.4 kb HpaI-HindIII fragment (pDA1-12). Based on the restriction map of pLJ47 (G.Jen, unpublished data), this fragment maps in the pTiT37 vir region. The vir region is approximately 40 kb in size and contains genes essential for virulence but which are not maintained in the tumor.

The selection of E. coli clones containing the tzs locus was accomplished by screening for the presence of trans-zeatin in the culture medium (Table 1). Bacteria lacking the tzs locus produced no material which cross-reacted with the tZR antiserum. The identity of trans-zeatin as the

Table 1. Level of trans-zeatin secreted into the culture medium of E. coli and A. tumefaciens strains. Cell-free culture supernatants were assayed for the presence of trans-zeatin by modified solution RIA.

Strain	Trans-zeatin (ng/ml)[1]
HB101	<0.2
HB101(pUC18)[2]	0.4
HB101(pUC19)	< 0.2
HB101(pHC79)	< 0.2
HB101(pLJ47)	10
HB101(pDA1-12)[3]	>100
HB101(pDA2-18)	>100
A136	< 0.2
A348	< 0.2
T37	12

[1] Levels not corrected for recovery.

[2] Level estimated by HPLC-RIA was less than 300 pg/ml.

[3] Level estimated by HPLC-RIA was greater than 200 ng/ml.

cross-reacting material in strain HB101 (pDA1-12) was confirmed by HPLC-RIA. Based on HPLC-RIA, the amounts of trans-zeatin and tZR in the culture medium were greater than 200 ng/ml and 20 ng/ml, respectively (data not shown). The levels of iP and iPA were also measured by HPLC-RIA. In HB101 (pDA1-12), the amounts of iP and iPA in the culture medium were 40 ng/ml and 7 ng/ml, respectively. In HB101(pUC18), the iP and iPA levels were both less than 300 pg/ml (data not shown).

The culture medium of several A. tumefaciens strains was also examined for trans-zeatin (Table 1). Trans-zeatin was detected in the nopaline strain T37, but was not detected in the avirulent strain, A136 nor in the octopine strain, A348. These results parallel the results of Regier and Morris (30). In addition, no homology was observed when the tzs gene was hybridized to Southern transfers of EcoRI digested total DNA from A136 and A348 (data not shown). When the 1.4 kb HpaI-HindIII fragment containing the tzs gene was transferred into A348, the bacteria acquired the trans-zeatin secretion phenotype (data not shown).

DNA sequence determination

The M13 clones used for sequencing were generated by the exonuclease III/SI method. In both orientations, at least eight overlapping clones, progressively shortened by 100-200 bp, were selected which covered the entire HpaI-HindIII fragment. Examination of the 1.4 kb sequence revealed only one open reading frame greater than 300 nucleotides. This putative tzs open reading frame was 729 nucleotides in length and its sequence is shown in Figure 2. Based on the nucleotide sequence, the deduced protein size was 27,545 daltons. Maxicell expression of this cloned gene (pDA2-18) resulted in labelling of a single new polypeptide with an estimated molecular weight of 27,200 daltons (Figure 3). This protein was not synthesized in the control strain containing pUC19.

Trans-zeatin RIA of the M13 clones

Strains of JM105 containing the M13 clones used for sequencing were also tested for trans-zeatin secretion and results are shown in Figure 1. Of the clones derived from M13-8, only the clone containing the complete HpaI-HindIII fragment (M13-8.0) had a functional tzs locus. The next shorter clone, M13-8.1, which starts at nucleotide 152, had only partial (30-50%) activity, while activity was completely abolished in clones starting at nucleotide 340 and further downstream. In the complementary series of M13-9 clones (shortened from the HindIII end), the tzs locus was functional in clone M13-9.3, which starts at nucleotide 1037. However, it is

non-functional in clone M13-9.4, which starts at nucleotide 851. These results are in good agreement with respect to the position of the open reading frame, which extends from nucleotides 264-992.

Analysis of the 5' region

Examination of the nucleotide sequence preceding the putative coding region revealed sequences similar to consensus E. coli promoter regulatory sequences. At nucleotide position 243 (Figure 2), the sequence TTAAAT is found which is similar to the Pribnow box sequence, TATAAT (53), which is usually found 10 bp upstream from the initiation of transcription. There is also a sequence, TTGGAA, starting at nucleotide 221, which is similar to the -35 prokaryotic consensus sequence, TTGACA, necessary for RNA polymerase recognition (53). A sequence similar to the Shine-Dalgarno ribosome binding site (54) is located at nucleotide position 255.

Nucleotide and amino acid comparison of the tzs and tmr genes from pTiT37

Since the tzs locus is responsible for trans-zeatin secretion into the culture medium and, is therefore involved in cytokinin metabolism, its sequence was compared to the tmr sequence from pTiT37 (55). The tmr locus is involved in cytokinin biosynthesis (12,13) and encodes a protein of 27,025 daltons, which is close to the size (27,545 daltons) of the tzs protein.

Comparison of the tzs and tmr genes at the nucleic acid and amino acid levels showed significant overall homology, 54 and 48%, respectively (Figure 4). However, if only the N-terminal regions (amino acid residues 1-107) of the proteins are considered, homology is increased to 64% and if allowance is

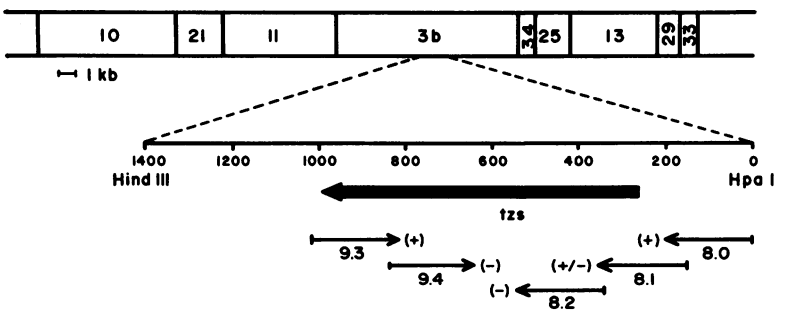

Figure 1. BamHI restriction endonuclease map of cosmid clone, pLJ47. The 1.4 kb HpaI-HindIII subfragment containing the tzs locus is shown below. The heavy arrow shows the tzs location and direction of transcription. The light arrows beneath the map represent some of the M13 clones used for trans-zeatin testing: (+) indicates trans-zeatin secretion, (-) indicates loss of trans-zeatin secretion.

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   1                                                    CTA GAGTAACGGG GTCCGCTGCA

  24   TTCACCATAA GCAGGTGTCG GGGCCAAACT CCTCAATTGT TCAGGAGGAC AACGGGATGG

  84   AAACTCTCAT GGCCGAATGA GCCGGCAACG TCAAAAATGT ACGATTACAA ATGTAGCGCG

 144   ATATTAGTTC AGCAGATTGC GTAAAGCTGG TAAAAATCAT GCAGTAAAAC ATCATAATCC

 204   GAATTGAAAA ATCTGGTTTG GAACAGATGG GATATCCCAT TAAATTTATA TGAGGCGCAC

 264   ATG ATA CTC CAT CTC ATC TAC GGA CCG ACT TGC AGC GGC AAA ACG GAC ATG
       MET Ile Leu His Leu Ile Tyr Gly Pro Thr Cys Ser Gly Lys Thr Asp Met

 315   GCG ATC CAA ATC GCA CAA GAA ACC GGG TGG CCG GTG GTT GCC CTT GAT CGT
       Ala Ile Gln Ile Ala Gln Glu Thr Gly Trp Pro Val Val Ala Leu Asp Arg

 366   GTG CAA TGC TGT CCT CAA ATC GCG ACA GGT AGC GGA AGA CCT TTG GAA TCG
       Val Gln Cys Cys Pro Gln Ile Ala Thr Gly Ser Gly Arg Pro Leu Glu Ser

 417   GAA TTG CAA TCA ACG CGG AGA ATA TAT TTG GAT TCC CGC CCC CTC ACC GAG
       Glu Leu Gln Ser Thr Arg Arg Ile Tyr Leu Asp Ser Arg Pro Leu Thr Glu

 468   GGC ATC CTT GAC GCT GAG AGT GCC CAT CGT CGA CTC ATA TTC GAA GTG GAT
       Gly Ile Leu Asp Ala Glu Ser Ala His Arg Arg Leu Ile Phe Glu Val Asp

 519   TGG CGG AAG TCC GAA GAC GGT CTT ATT CTC GAG GGC GGG TCG ATT TCG CTT
       Trp Arg Lys Ser Glu Asp Gly Leu Ile Leu Glu Gly Gly Ser Ile Ser Leu

 570   CTC AAT TGC ATG GCT AAA AGT CCG TTT TGG AGA TCG GGT TTT CAA TGG CAT
       Leu Asn Cys Met Ala Lys Ser Pro Phe Trp Arg Ser Gly Phe Gln Trp His

 621   GTC AAG CGG CTA CGT CTT GGG GAT TCG GAC GCC TTT CTC ACC CGA GCC AAG
       Val Lys Arg Leu Arg Leu Gly Asp Ser Asp Ala Phe Leu Thr Arg Ala Lys

 672   CAA CGC GTT GCG GAA ATG TTT GCC ATC CGG GAA GAT CGC CCC TCG TTG TTG
       Gln Arg Val Ala Glu Met Phe Ala Ile Arg Glu Asp Arg Pro Ser Leu Leu

 723   GAG GAG TTG GCG GAA CTC TGG AAC TAC CCT GCC GCT CGA CCG ATT TTG GAA
       Glu Glu Leu Ala Glu Leu Trp Asn Tyr Pro Ala Ala Arg Pro Ile Leu Glu

 774   GAT ATC GAC GGA TAT CGC TGC GCA ATT CGT TTT GCG CGC AAA CAC GAT CTC
       Asp Ile Asp Gly Tyr Arg Cys Ala Ile Arg Phe Ala Arg Lys His Asp Leu

 825   GCA ATC AGC CAG TTG CCA AAT ATT GAT GCA GGG CGG CAC GTA GAG CTC ATA
       Ala Ile Ser Gln Leu Pro Asn Ile Asp Ala Gly Arg His Val Glu Leu Ile

 876   GAG GCC ATA GCT AAT GAA TAT CTT GAA CAT GCG CTC TCG CAG GAG CGC GAT
       Glu Ala Ile Ala Asn Glu Tyr Leu Glu His Ala Leu Ser Gln Glu Arg Asp

 927   TTT CCT CAG TGG CCA GAA GAT GGC GCA GGA CAG CCT GTT TGC CCG GTC ACG
       Phe Pro Gln Trp Pro Glu Asp Gly Ala Gly Gln Pro Val Cys Pro Val Thr

 978   CTG ACG CGA ATT CGG TGA TTCGCCGCTT CATCATTTGA CCTGCGTGTT GGATGGCCAT
       Leu Thr Arg Ile Arg

1036   CGGCGGCGCA GCATCGGTCG GCGTCACCTC CAAGCGCAAC ATGCGGCGCG CCAAAGCTAT
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1096 TCGCACATCT TATTTGAGTA AGTGATTTCT TTCGAAGCAC CTCGGCCGGC GCTCGGTCCT

1156 CGAGGGATTT GGCCGCTTCC GAGTTCCAGC AGTTGCAGAT TTCGATTAGC CGGCGTCGGT

1216 CAGGCTTCAA AGTCGACTTC CATCAAAGTC ATTTTGGTTA CACGTCGACA TTGCATCTCA

1276 GCAAGACGAG TGGCTGTGCC GACTGATATC GACCGATTGC CATTACGTAC AGCAGGCTTA

1336 TTCGGTTACA TTTGAAACCA ATCTTTGGTC TCCTGTAATC ATTATACTGC TGATGGACTG

1396 CACATATCGT CGGTAAGC

Figure 2. Nucleotide sequence of the 1.4 kb HpaI-HindIII fragment containing the tzs locus. The 729 bp open reading frame, extending from nucleotide 264-992, and the derived amino acid sequence are shown. A sequence similar to the E. coli -10 consensus sequence is underlined and the -35 sequence is indicated by a double underline. The Shine-Dalgarno sequence is indicated by a heavy underline.

made for functionally conserved amino acid substitutions, homology is about 73%. The C-terminal region (amino acid residues 108-243) of the tzs displayed about 48% homology to the tmr gene at the nucleotide and amino acid levels. The last 20 amino acid residues of tzs showed no homology to the tmr gene. The tzs gene contained an additional proline residue at amino acid residue 150.

DMA transferase activity

Since the tzs and tmr gene products are similar in size and exhibit a high degree of homology at the nucleotide and amino acid levels, the tzs locus was tested for DMA transferase activity, which is encoded by the tmr locus (12,13). E. coli strains containing either the vector, pUC18, or the plasmid, pDA1-12, were assayed for DMA transferase activity. The radiolabelled assay products were isolated by adsorption to C_{18}-silica, fractionated by HPLC and the distribution of radioactivity was determined.

In Figure 5, the radioactivity profiles are shown for four samples. One sample set was treated with alkaline phosphatase prior to chromatography, while the other set was not. Very low levels of radioactivity were recovered in the vector control, pUC18 (Figure 5B,D) but, in the strain containing pDA1-12 (Figure 5A,C) large quantities of radiolabelled products were recovered. In the sample which was not treated with alkaline phosphatase (Figure 5A), approximately 6 x 10^5 cpm eluted at the retention time of iso-pentenyladenosine monophosphate. Labelled material also eluted in the iP/iPA region. In the companion sample treated with alkaline phosphatase prior to chromatography, no labelled material eluted in the

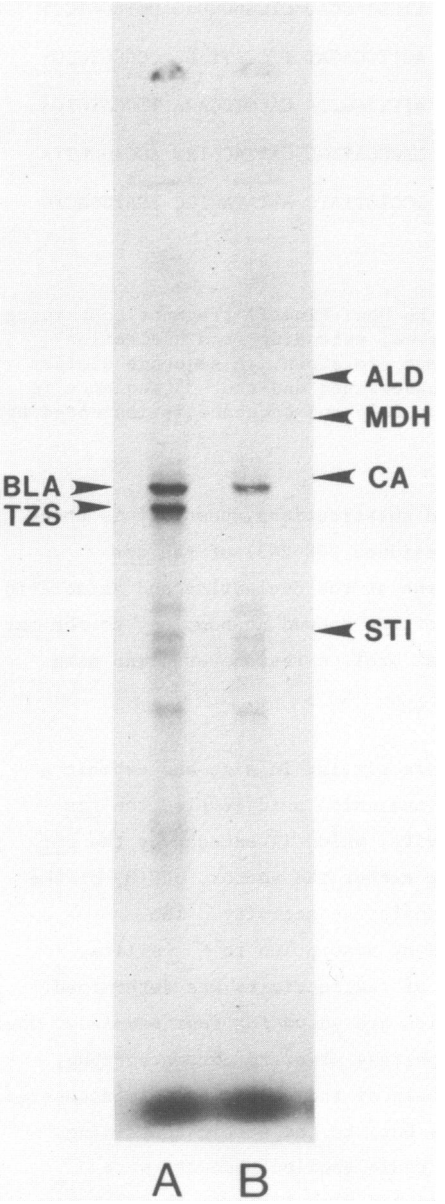

Figure 3. E. coli maxicell expression of a strain containing the tzs locus. Autoradiogram of proteins from strain CSR603 containing pDA2-18 (lane A) or pUC19 (lane B). The positions of the molecular weight markers are shown on the right side: STI, soybean trypsin inhibitor (21 kdal), CA; carbonic anhydrase (29 kdal); MDH, malate dehydrogenase (34 kdal) and ALD, aldolase (40 kdal). The positions of β-lactamase (BLA) and the tzs protein are shown on the left side.

iso-pentenyladenosine monophosphate region, but the iP/iPA region showed a corresponding increase in radioactivity (Figure 5C). This radioactivity was confirmed to be iP/iPA by immunoprecipitation of the radioactivity by

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  1  MET Ile Leu His Leu Ile Tyr Gly Pro Thr Cys Ser Gly Lys Thr Asp Met
     ... Asp ... Arg ... ... Phe ... ... ... ... Thr ... ... ... Ser Thr

 18  Ala Ile Gln Ile Ala Gln Glu Thr Gly Trp Pro Val Val Ala Leu Asp Arg
     ... Val Ala Leu ... ... Gln ... ... Leu ... ... Leu Ser ... ... ...

 35  Val Gln Cys Cys Pro Gln Ile Ala Thr Gly Ser Gly Arg Pro Leu Glu Ser
     ... ... ... ... ... ... Leu Ser ... ... ... ... ... ... Thr Val Glu

 52  Glu Leu Gln Ser Thr Arg Arg Ile Tyr Leu Asp Ser Arg Pro Leu Thr Glu
     ... ... Lys Gly ... Ser ... Leu ... ... ... Asp ... ... ... Val Lys

 69  Gly Ile Leu Asp Ala Glu Ser Ala His Arg Arg Leu Ile Phe Glu Val Asp
     ... ... Ile Ala ... Lys Gln ... ... Glu ... ... Met Gly ... ... Tyr

 86  Trp Arg Lys Ser Glu Asp Gly Leu Ile Leu Glu Gly Gly Ser Ile Ser Leu
     Asn Tyr Glu Ala His Gly ... ... ... ... ... ... ... ... ... ... ...

103  Leu Asn Cys Met Ala Lys Ser Pro Phe Trp Arg Ser Gly Phe Gln Trp His
     ... Lys ... ... ... Gln ... Ser Tyr ... Ser Ala Asp ... Arg ... ...

120  Val Lys Arg Leu Arg Leu Gly Asp Ser Asp Ala Phe Leu Thr Arg Ala Lys
     Ile Ile ... His Glu ... Ala ... Glu Glu Thr ... Met Asn Val ... ...

137  Gln Arg Val Ala Glu Met Phe Ala Ile Arg Glu Asp Arg Pro Ser Leu Leu
     Ala ... ... Lys Gln ... Leu Arg Pro Ala Ala Gly Leu xxx ... Ile Ile

154  Glu Glu Leu Ala Glu Leu Trp Asn Tyr Pro Ala Ala Arg Pro Ile Leu Glu
     Gln ... ... Val Asp ... ... Lys Glu ... Arg Leu ... ... ... ... Lys

171  Asp Ile Asp Gly Tyr Arg Cys Ala Ile Arg Phe Ala Arg Lys His Asp Leu
     Glu ... ... ... ... ... Tyr ... Met Leu ... ... Ser Gln Asn Gln Ile

188  Ala Ile Ser Gln Leu Pro Asn Ile Asp Ala Gly Arg His Val Glu Leu Ile
     Thr Ser Asp Met ... Leu Gln Leu ... ... Asp Met Glu Asp Lys ... ...

205  Glu Ala Ile Ala Asn Glu Tyr Leu Glu His Ala Leu Ser Gln Glu Arg Asp
     His Gly ... ... Gln ... ... ... Ile ... ... Arg Arg ... ... Gln Lys

222  Phe Pro Gln Trp Pro Glu Asp Gly Ala Gly Gln Pro Val Cys Pro Val Thr
     ... ... Arg Val Asn Ala Ala Ala Tyr Asp Gly Phe Glu Gly His Pro Phe

239  Leu Thr Arg Ile Arg
     Gly Met Tyr
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Figure 4. Comparison of the deduced amino acid sequences of the tzs (upper line) and the tmr (lower line) loci from pTiT37. Identical amino acid residues are indicated (...). Functionally conserved amino acid changes are underscored. The amino acid residues are numbered from the methionine starting at nucleotide position 224 in the tzs sequence.

iPA-specific antiserum (data not shown). Conversion of AMP to iP derivatives was estimated to be greater than 20% under our assay conditions. This was significantly greater than the incorporation previously reported for the tmr gene (12) under identical assay conditions.

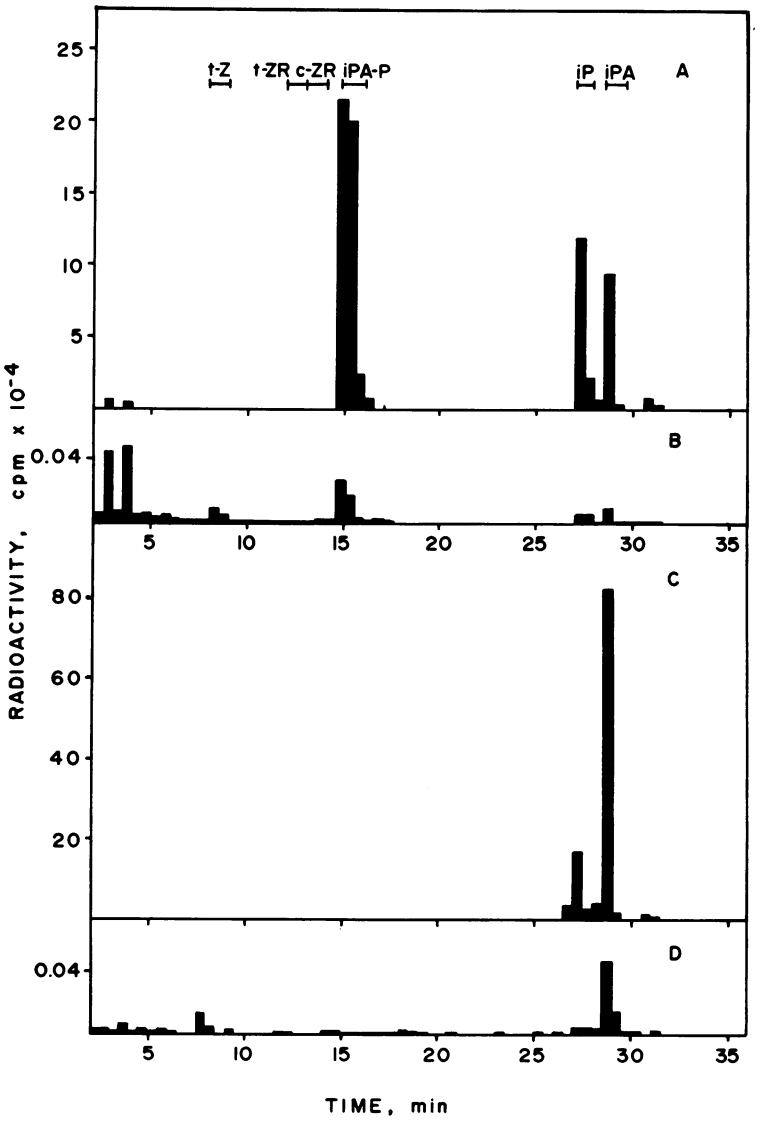

Figure 5. Radioactivity profile of DMA transferase assay products on HPLC. To E. coli cell-free extracts (from 0.5 gm cells), DMAPP (1 mg) and [^{3}H]AMP (3 uCi; specific activity 15 Ci/mmole) were added and incubated (30 min, 27°C). Cytokinins were adsorbed onto C_{18}-silica, eluted and fractionated by HPLC: (B and D) HB101 containing the plasmid vector, pUC18; (A and C) HB101 containing pDA1-12. One sample set (C and D) was treated with alkaline phosphatase prior to HPLC. Retention times of cytokinin standards are shown by horizontal bars (⊢⊣) in panel A: t-Z, trans-zeatin; t-ZR, trans-ribosylzeatin; c-ZR, cis-ribosylzeatin: iPA-P, iso-pentenyladenosine monophosphate; iP, iso-pentenyladenine; iPA, iso-pentenyladenosine.

DISCUSSION

The *tmr* gene encodes DMA transferase, which catalyzes addition of the isoprenoid side chain to AMP during the initial step in cytokinin biosynthesis (12,13). The nucleotide and amino acid sequences of the *tmr* gene from *A. tumefaciens* strains T37 (55), 15955 (56), A6S2 (57) and ACH5 (58) have been determined and show greater than 90% homology to each other.

The degree of homology between *tzs* and *tmr* both at the nucleic acid and amino acid levels is striking. The greatest homology is in the N-terminal portion of the protein sequence but extends through to near the C-terminus, with only the last 20 amino acid residues showing no homology. The homology does not extend outside the coding region. The *tzs* has sequences typical of prokaryotic promoters, while the *tmr* has sequences typical of eukaryotic promoters and also has a polyadenylation site (55).

The similarity between *tzs* and *tmr* sequences is consistant with the known biochemical activity of the two proteins, cytokinin biosynthesis. Both gene products have DMA transferase activity *in vitro*. Additionally, bacterial hosts containing *tzs* and plant cells containing *tmr* (17) show phenotypic overproduction of *trans*-zeatin. As expected, the expression of *tmr* is poor in prokaryotic hosts, which have undetectable levels of *trans*-zeatin in the culture medium. However, when the *tmr* is fused to an inducible phage promoter, bacterial hosts show high levels of *trans*-zeatin secretion characteristic of *tzs* expression (data not shown). Therefore, in addition to DMA transferase activity, both *tzs* and *tmr* may specify another catalytic activity involved in cytokinin biosynthesis, such as a *trans*-zeatin specific hydroxylase, a *cis*-*trans* zeatin isomerase or a cytokinin permease. However, none of these activities have yet been demonstrated by *in vitro* assay and further biochemical analyses of *tzs* and *tmr* must await development of such assays.

The presence of two genes involved in cytokinin biosynthesis on pTiT37 is intriguing. The importance of *tmr* in tumorigenesis is supported by the observation that *tmr* is capable of transforming plant cells in the absence of other T-DNA genes (15). On the other hand, the role of *tzs*, which is located in the *vir* region, is not known. The *tzs* gene may possibly function in the initial stages of infection by possibly conditioning the plant cells for more efficient transformation, extending host range of the bacterium, retarding senesence or mobilizing nutrients to the wound site. The role of a cytokinin gene in expanding host range has been demonstrated in *Agrobacterium*. Strains containing the limited host range plasmid, pTiAg57, normally incite tumors on

grapevine and a few other plant hosts. Introduction of the octopine tmr gene into these strains expand their host range to include tomatoes, carrots and N. rustica (59,60). Determination of which, if any, of these mechanisms is functioning during tumor initiation by A. tumefaciens will come with construction of mutants lacking the trans-zeatin secretion gene and assessment of their virulence and host range.

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