

# Molecular Cloning, Expression, and Functional Analysis of a *cis*-Prenyltransferase from *Arabidopsis thaliana*

IMPLICATIONS IN RUBBER BIOSYNTHESIS\*

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Soo Kyung Oh, Kyung Hwan Han‡, Stephen B. Ryu, and Hunseung Kang§

From the Kumho Life and Environmental Science Laboratory, 1 Oryong-Dong, Puk-Gu, Kwangju, 500-712, Korea

*cis*-Prenyltransferase catalyzes the sequential condensation of isopentenyl diphosphate with allylic diphosphate to synthesize polyprenyl diphosphates that play vital roles in cellular activity. Despite potential significance of *cis*-prenyltransferase in plant growth and development, no gene of the enzyme has been cloned from higher plants. Using sequence information of the conserved region of *cis*-prenyltransferase cloned recently from *Escherichia coli*, *Micrococcus luteus*, and yeast, we have isolated and characterized the first plant *cis*-prenyltransferase from *Arabidopsis thaliana*. Sequence analysis revealed that the protein is highly homologous in several conserved regions to *cis*-prenyltransferases from *M. luteus*, *E. coli*, and yeast. *In vitro* analyses using the recombinant protein overexpressed in *E. coli* revealed that the enzyme catalyzed the formation of polyprenyl diphosphates ranging in carbon number from 100 to 130 with a predominance of C<sub>120</sub>. The enzyme exhibited a higher affinity for farnesyl diphosphate than for geranylgeranyl diphosphate, with the *K<sub>m</sub>* values being 0.13 and 3.62 μM, respectively, but a lower affinity for isopentenyl diphosphate, with a *K<sub>m</sub>* value of 23 μM. *In vitro* rubber biosynthesis analysis indicated that the *Arabidopsis cis*-prenyltransferase itself could not catalyze the formation of higher molecular weight polyprenyl diphosphates similar to natural rubber. A reverse transcriptase-polymerase chain reaction analysis showed that the gene was expressed at low levels in *Arabidopsis* plant, in which expression of the *cis*-prenyltransferase in leaf and root was higher than that in stem, flower, and silique. These results indicate the tissue-specific expression of *cis*-prenyltransferase and suggest a potential role and significance of the enzyme in the polyisoprenoid biosynthesis in plants.

terpenes, quinones, glycosyl carrier lipids, prenyl proteins, and natural rubber. Based on the configuration of isoprene units in the final reaction products, prenyltransferases are classified into two classes: *trans*- and *cis*-prenyltransferase. In both prokaryotes and eukaryotes, *trans*-prenyltransferases catalyze the formation of isoprenoid compounds, such as geranyl diphosphate (C<sub>10</sub>), farnesyl diphosphate (FPP<sup>1</sup>; C<sub>15</sub>), and geranylgeranyl diphosphate (GGPP; C<sub>20</sub>), which serve as initiating molecules to produce many other longer chain length isoprenoid compounds necessary for cellular growth and survival. The structural genes for FPP synthase (1–6) and GGPP synthase (7–13) have been cloned and characterized from various organisms. In addition, the genes for hexaprenyl diphosphate synthase (14), heptaprenyl diphosphate synthase (15), octaprenyl diphosphate synthase (16), solanesyl diphosphate synthase (17), and decaprenyl diphosphate synthase (18) have been cloned. Mutational analyses and x-ray crystallographic investigations of the structure of *trans*-prenyltransferase revealed the importance of several amino acid residues in the conserved domains for the mechanism of chain length determination (19–23).

In contrast, only three *cis*-prenyltransferase genes have recently been cloned. Two genes are the structural genes for undecaprenyl diphosphate synthase, which catalyzes the formation of undecaprenyl diphosphate (C<sub>55</sub>) that serves as a glycosyl carrier lipid during the biosynthesis of cell wall polysaccharide components in *Escherichia coli* and *Micrococcus luteus* (24–26). The other is the gene encoding a *cis*-prenyltransferase involved in the biosynthesis of dolichols used for the glycosylation of proteins in yeast (27). In addition to these genes whose functions have been verified by *in vitro* and *in vivo* analyses, several other hypothetical proteins from diverse sources have been suggested to be *cis*-prenyltransferases based on the sequence alignments (Refs. 25 and 27 and GenBank™ accession numbers therein).

Although the *cis*- and *trans*-prenyltransferases catalyze similar reactions of the sequential condensation between isopentenyl diphosphate (IPP) and allylic diphosphates in the presence of Mg<sup>2+</sup> ions, no similarity in the sequence was found between *cis*- and *trans*-prenyltransferases. The *cis*-prenyltransferases cloned from *E. coli*, *M. luteus*, and yeast and other hypothetical proteins share a low level of sequence homology (~30% identity) among them. Several regions with highly conserved amino acid sequences were, however, identified. In order to understand the genomic structure of *cis*-prenyltrans-

Prenyltransferase is an enzyme that catalyzes the synthesis of linear prenyl diphosphates involved in the biosynthesis of various isoprenoid compounds, including sterols, carotenoids,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF162441.

‡ Present address: Dept. of Forestry, Michigan State University, East Lansing, MI 48824-1222.

§ To whom correspondence should be addressed: Kumho Life and Environmental Science Laboratory, 1 Oryong-Dong, Puk-Gu, Kwangju, 500-712, Korea. Tel.: 82-62-970-2648; Fax: 82-62-972-5085; E-mail: hskang@ksc.kumho.co.kr.

<sup>1</sup> The abbreviations used are: FPP, farnesyl diphosphate; ACPT, *Arabidopsis cis*-prenyltransferase; GGPP, geranylgeranyl diphosphate; GPC, gel permeation chromatography; IPP, isopentenyl diphosphate; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; IPTG, isopropyl-1-thio-β-D-galactopyranoside; bp, base pair(s).

ferase and to verify the role of these conserved amino acid sequences for the catalytic activity of the enzyme, it is necessary to identify and clone more genes for *cis*-prenyltransferase from different organisms including animals and plants. In addition, it is of critical importance to test whether *cis*-prenyltransferase could catalyze the formation of higher molecular weight polymers similar to natural rubber (*cis*-1,4-polyisoprene), which is synthesized by the action of a rubber polymerase or rubber transferase that catalyzes the sequential condensation of IPP with allylic diphosphates similar to *cis*-prenyltransferase.

In this study, using the sequence information of the conserved regions of *cis*-prenyltransferases isolated from microorganisms, we isolated and characterized a full-length cDNA encoding *cis*-prenyltransferase from *Arabidopsis thaliana*, thus designated ACPT. The deduced amino acid sequence is highly homologous in several conserved regions to that of *cis*-prenyltransferases from *M. luteus*, *E. coli*, and yeast. *In vitro* analysis of the recombinant protein revealed that the enzyme catalyzed the formation of polyprenyl diphosphates with predominant carbon number C<sub>120</sub>. *In vitro* rubber biosynthesis analysis indicated that the *Arabidopsis cis*-prenyltransferase itself could not catalyze the formation of high molecular weight polyprenyl diphosphate such as natural rubber.

#### MATERIALS AND METHODS

**Plant Material and RNA Isolation**—*A. thaliana* samples were obtained from mature plants grown under controlled greenhouse conditions. Total RNA was extracted by using a plant RNA isolation kit, and mRNA was isolated by using the Oligotex-dT<sup>TM</sup> mRNA kit (Qiagen Inc., Chatsworth, CA).

**Reverse Transcription-Polymerase Chain Reaction Amplification of ACPT**—First-strand cDNA was synthesized by reverse transcription with 0.5  $\mu$ g of total RNA. Two primers, AC1 (5'-TGGATGGGAACCGGAGATGGGCC-3') and AC2 (5'-TTCTCCACTTGCTCTAATCATTA-3'), were designed according to the sequence information of *cis*-prenyltransferase from *E. coli*, *M. luteus*, yeast, and *Arabidopsis* genomic sequences (accession no. AC003040). PCR was performed using 10  $\mu$ l of the first-strand cDNA and two primers. PCR was performed for 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, with a 5-min preheat and a 7-min final extension at 72 °C. The PCR product was used to screen the *Arabidopsis* cDNA library.

**Screening of cDNA Library**—The pGAD 424 library containing *Arabidopsis* cDNA inserts was a gift from G. T. Choi. The PCR product was used to screen 4  $\times$  10<sup>6</sup> colonies of the cDNA library. Colony hybridization was performed at 60 °C in 2 $\times$  SSC hybridization solution (32). The cDNA clones hybridized to the probe were sequenced. One clone carrying a full-length cDNA insert was chosen and designated pACPT.

**Sequencing of cDNA Clones**—Plasmid DNA for sequencing reactions was prepared by the alkaline lysis method (28) by using the Wizard Plus SV Minipreps DNA Purification System kit (Promega). The sequence was determined by the dideoxy chain termination method using the dye Terminator cycle sequencing kit and ABI prism<sup>TM</sup> 310 DNA sequencer (Perkin-Elmer).

**Analysis of ACPT Gene Expression by Northern Blot and Quantitative RT-PCR**—Total RNAs of various tissues were prepared using RNA isolation kit (Quiagen) and were treated with DNase I to remove contaminating genomic DNA. Tissues were collected from 4-week-old *Arabidopsis* plants except young leaf (5 days) and old leaf (8 weeks). For Northern blot, total RNA (10  $\mu$ g) was subjected to electrophoresis on a 0.8% agarose-formaldehyde gel and blotted as for Southern blot. A <sup>32</sup>P-labeled full-length ACPT cDNA was used as a probe. For quantitative RT-PCR, the first-strand cDNA was synthesized from total RNA (3  $\mu$ g) by using reverse transcriptase and oligo(dT). A 200-unit reverse transcriptase (RT), 1  $\times$  RT buffer, 0.5 mM dNTP, and 0.5  $\mu$ g of oligo(dT) primer (Promega) were added to the heat-denatured total RNA. After reverse transcription for 1 h at 37 °C, the first-strand cDNA was used for PCR performed in standard conditions: 5 min at 95 °C; 35 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s); 7 min at 72 °C. Two primers, 5'-GCGAGGATGGGTTACAAAGC-3' (208 region) and 5'-TTC-CCCAATTCTCTGTGGAG-3' (414 region), were used for RT-PCR. As a control, 18 S ribosomal RNA standard primers (Ambion) were used. An equal volume of each PCR sample was separated by 1.2% agarose gel electrophoresis.

**Heterologous Expression and Purification of ACPT Protein in *E. coli***—The ACPT gene was cloned in the *Bam*HI-*Eco*RI site of pET-22b(+) (Novagen) to construct pET-ACPT. The *E. coli* BL21 (DE3) transformed with pET-ACPT was grown to midstationary phase in TB medium (1.2% bactotryptone, 2.4% yeast extract, 0.4% glucose, and 10% potassium phosphate solution) containing 500  $\mu$ g/ml carbenicillin at 30 °C with vigorous aeration. The cultures were induced by adding IPTG to a concentration of 1 mM and then incubating for another 2 h. All subsequent steps were carried out at 4 °C. The cells were harvested, washed with 0.1 M potassium phosphate (pH 7.4) by centrifugation (5000  $\times$  g, 10 min), and then disrupted by sonication. The expressed proteins were purified using Ni<sup>2+</sup>-nitrilotriacetic acid-agarose (Quiagen). The lysate was incubated with Ni<sup>2+</sup>-nitrilotriacetic acid slurry at 4 °C for 60 min, and the mixture was loaded to a column. The column was washed four times with washing buffer, and the proteins were then eluted with 0.5 ml of elution buffer. The lysate was subjected to SDS-polyacrylamide gel electrophoresis according to the standard method of Laemmli (30).

**Western Blot Analysis**—The purified protein was separated by SDS-10% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane by electroblotting, and the membrane was blocked with 5% skim milk in phosphate-buffered saline with 0.1% Tween 20 for 1 h at room temperature. After washing, the membrane was incubated with Penta Anti-His antibody (Quiagen) for 1 h, and the proteins were detected using anti-mouse IgG/horseradish peroxidase and enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

**Assay of ACPT Activity**—Enzyme activity was measured in a 50- $\mu$ l reaction mixture containing 100 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 20  $\mu$ M FPP, and 80  $\mu$ M [<sup>14</sup>C]IPP (55 mCi mmol<sup>-1</sup>, Amersham Pharmacia Biotech). The reaction was initiated by the addition of indicated amounts of the recombinant enzyme. The mixture was incubated for 1 h at 30 °C and then treated with 1-butanol saturated with water to extract the products of the prenyltransferase reaction. The butanol phase was collected and washed with water saturated with NaCl. The extract was mixed with Ready Solv HP scintillation mixture (Beckman), and the radioactivity was determined by a liquid scintillation counter (Beckman).

**Analysis of ACPT Reaction Products**—The washed organic extract was dried under N<sub>2</sub>, and the residues were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to Fujii *et al.* (31). The alcohols were extracted with hexane and analyzed by TLC on a reverse phase RP-18 plate (Merck) with a solvent system of acetone/H<sub>2</sub>O (19:1). Normal phase TLC of the reaction products was also carried out on a Silica Gel-60 plate (Merck) with a solvent system of 1-propanol/ aqueous NH<sub>3</sub>/H<sub>2</sub>O (6:3:1). The distribution of <sup>14</sup>C-labeled reaction products on the TLC plate was analyzed by a Bio-image analyzer BAS 1500 (Fuji). The positions of authentic standards were visualized with *p*-anisaldehyde spray reagent (Sigma).

**In Vitro Rubber Assay and Product Analysis**—Rubber biosynthetic activity of the enzyme was measured according to the procedure as described (29), which is similar to the method used for the *cis*-prenyltransferase assay described above. After incubation for 5 h at 25 °C, the reaction products were extracted with benzene and dried under N<sub>2</sub>. The extract was dissolved in tetrahydrofuran, filtered through a membrane of 0.4- $\mu$ m porosity, and analyzed using a gel permeation chromatography (GPC). Gel permeation chromatography was carried out in a Waters high performance liquid chromatograph using three columns in series, a mixed bed polydivinylbenzene column with molecular weight cut-off from 100 to 10,000 (Jordi) and two polystyrene-divinylbenzene copolymer gels having an exclusion limit of 4  $\times$  10<sup>7</sup> and 6  $\times$  10<sup>4</sup> (Supelco). Measurements were made at 35 °C using tetrahydrofuran as eluent at a flow rate of 0.5 ml min<sup>-1</sup>, and the reaction product was monitored by an evaporative light scattering detector (Alltech). The effluents were collected at 0.5-min intervals and assayed for radioactivity. The molecular weight of the reaction product was estimated by comparing the elution profile of the sample to that of standard *cis*-polyisoprene or polystyrene.

#### RESULTS

**Isolation and Characterization of ACPT Gene**—PCR and cDNA library screening were employed to clone the gene. The fragment of the ACPT gene (0.5 kilobase pairs) was obtained by RT-PCR and used to screen 4  $\times$  10<sup>6</sup> colonies of the cDNA library. Seven positive colonies were selected in the first screening, and of these only one colony was hybridized to the ACPT probe in the second screening. Sequence analysis showed

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1  caaagccaaattaagttgtcacaacaatggtgtctctctcttcttctgattcttctctat
      M L S L L S S D S S L 11
61  taccactcctcttctcttctctcctctgtctctttatcacaagctatataggcttcc
      L S L L F L F L I P C L F I T S Y I G F 31
121 ctgtgttcttgctaaaactcatcgactcatcaaaatcaaagctgctcgagacaacgaga
      P V F L L K L I G L I K I K A A R D N E 51
181 aacgtgatgagggaaacttatggttccgcgaggatgggttacaaagagaactaatgccga
      K R D E G T Y V V R E D G L Q R E L M P 71
241 gacacgtggcattcactactggatgggaaccggagatgggccaacggcgccgattgacga
      R H V A F I L D G N R R W A K R A G L T 91
301 cgtcacaaggccacgagcgggagctaaacggccttatagatatcgccgagctctgcttcg
      T S Q G H E A G A K R L I D I A E L C F 111
361 agttgggggttcatacagtcctcagcttttctctccacagagaattggggaagagaca
      E L G V H T V S A F A F S T E N W G R D 131
421 agattgagattgataacttgatgtctttgatccaacactaccgcaataagtccaacatca
      K I E I D N L M S L I Q H Y R N K S N I 151
481 aattcttccacagatcggaagttcgcgtttctggtatcggaataaaacgaagatccctg
      K F F H R S E V R V S V I G N K T K I P 171
541 aatctctcctcaaagaaatccatgagatagaggaagctacaaaggctacaagaataaac
      E S L L K E I H E I E E A T K G Y K N K 191
601 atctcatcatggcagtagattacagcgggaaatgatcatgcatgcttgcaagagtc
      H L I M A V D Y S G K F D I M H A C K S 211
661 ttgtgaagaaatcagaaaaagggttgatccgagaggaagatgtagacgagggcattgatcg
      L V K K S E K G L I R E E D V D E A L I 231
721 agagagagcttttgacaaattgtagtgacttccaagtcctgatttaagattaggacaa
      E R E L L T N C S D F P S P D L M I R T 251
781 gtggagaacagaggattagtaacttcttctgtggcaacttgcttattccgagcttttct
      S G E Q R I S N F F L W Q L A Y S E L F 271
841 tctcgcgggtatcttgccctgatttcgataaggataagcttctagaggctcttgcttcgt
      F S P V F W P D F D K D K L L E A L A S 291
901 atcagcgtagggaaagacgatttgggtgtcgggttgatccaccttctcctctctatt
      Y Q R R E R R F G C R V * 303
961 atgttttgttcatctgtaatttctaccaaacttcttgcgtatcattgtaatttgtgtcgg
1021 taaaaatcgaataaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

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FIG. 1. Nucleotide (accession no. AF162441) and deduced amino acid sequences of ACPT. Numbers of nucleotide sequence and amino acid sequence are indicated on the left and right, respectively. The underlined regions are the sequences from which the primers were designed for RT-PCR amplification of the ACPT gene.

that the cDNA insert was 1062 bp long and contained a 909-bp open reading frame. The ACPT contains a 26-bp-long 5'-untranslational region and a 127-bp-long 3'-untranslational region including a poly(A) tail of 33 bp. The ORF encodes for a 303-amino acid polypeptide with a predicted molecular mass of 33 kDa (Fig. 1). The deduced protein is basic with an isoelectric point of 8.05, which is similar to that of undecaprenyl pyrophosphate synthase in *M. luteus* ( $pI = 8.58$ ) and *RER2* (for return to the endoplasmic reticulum) gene in yeast ( $pI = 7.75$ ). Hydropathy and transmembrane motif analyses of the deduced amino acid sequence show that ACPT is hydrophilic and has N terminus transmembrane helices large enough to span the lipid bilayers (data not shown), in contrast to other *cis*-prenyltransferases from yeast, *M. luteus*, and *E. coli* that do not have transmembrane helices.

The predicted amino acid sequence of the ACPT has low similarity to those of *cis*-prenyltransferases from *E. coli* (SWISS-PROT Q47675; 30% identity), *M. luteus* (accession no. AB004319; 30% identity), and yeast *S. cerevisiae* (accession no. AB013497; 28% identity) (Fig. 2). Several regions with identi-

cal or highly conserved amino acids are, however, identified throughout the sequence. These conserved domains are likely to be functionally important for the catalytic activity and mechanism of chain elongation by *cis*-prenyltransferase.

**Expression of the ACPT Gene**—Our initial attempt to check the expression pattern of ACPT gene by Northern blot analyses failed due to the extremely low level of gene expression. Therefore, we performed a RT-PCR using total RNAs obtained from different tissues. The cycle number for RT-PCR was chosen in the linear range of PCR for the products. As shown in Fig. 3, the predicted size of 207 bp for ACPT was detected in all of the tissues used. Varied expression levels were observed among the different tissues. The expression of ACPT in root and leaf was higher than that in stem, flower, and silique. When the intensity of RT-PCR product in young leaf (5 days old) was set at 100 by Eagle-eye software (Stratagene), the relative intensities of RT-PCR products in other tissues were quantified as follows: mature leaf, 75; old leaf, 76; root, 51; stem, 28; flower, 33; and silique, 18. In order to modulate the amplification efficiency of PCR template and to compare the expression levels of the

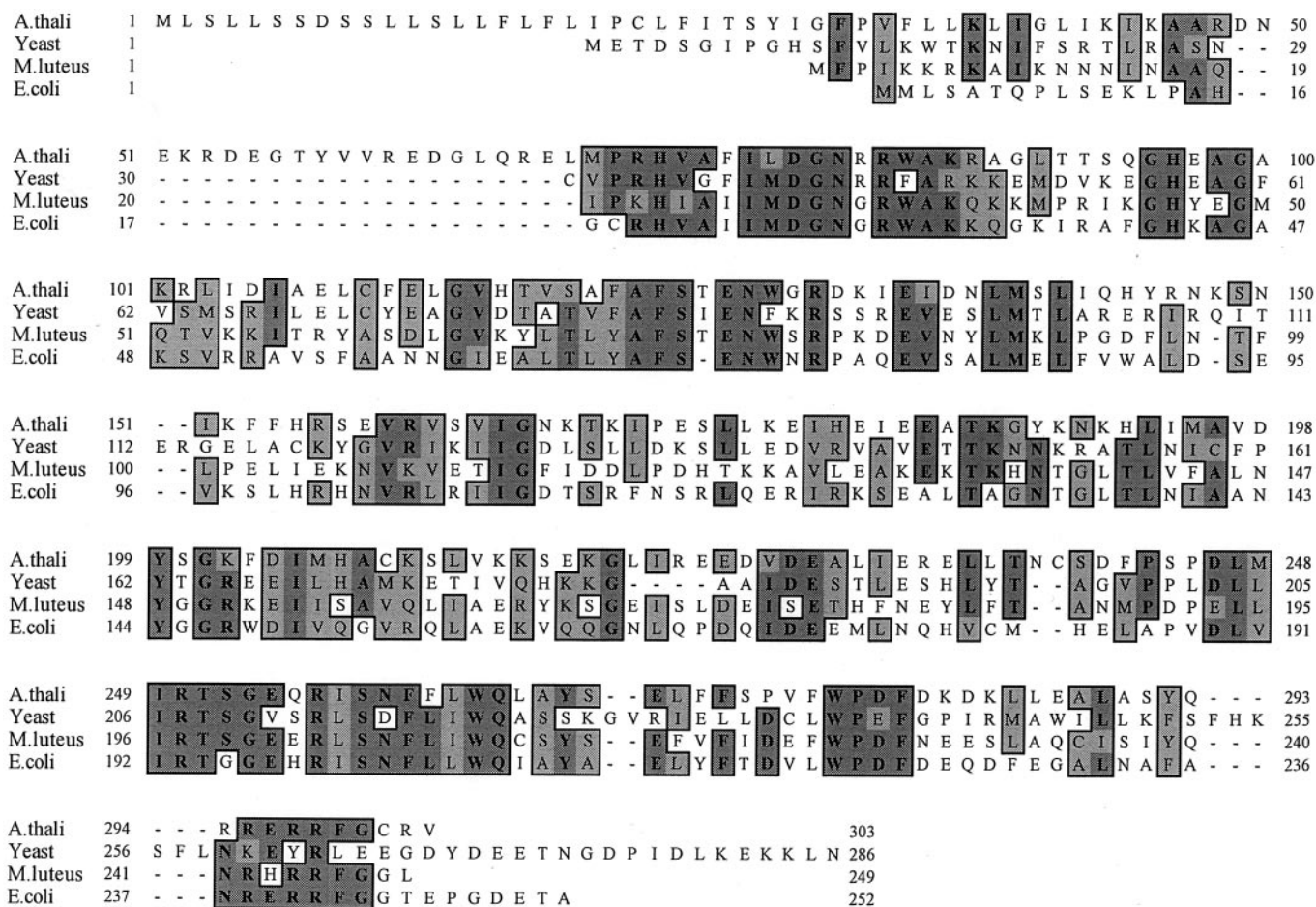


FIG. 2. Comparison of the deduced amino acid sequence of ACPT with *cis*-prenyltransferase from *E. coli* (accession no. P15252), *M. luteus* (accession no. AB004319), and yeast (accession no. AB013497). The alignment was made using the ClustalW program. Gaps in the sequences are indicated by dashes. Numbers of amino acids are indicated on the right.

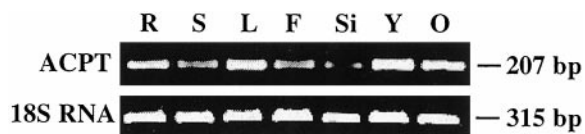


FIG. 3. RT-PCR analysis of RNA transcripts extracted from various tissues. The RT-PCR products for ACPT and control 18 S rRNA were separated on 1.2% agarose gel. The sizes of amplified bands are indicated at the right. R, root; S, stem; L, leaf; F, flower; Si, siliqua; Y, young leaf; O, old leaf.

ACPT gene, a control reaction using the 18 S primers and Competimer<sup>®</sup> provided by the manufacturer (Ambion) were performed. The ratio between 18 S primer and Competimer was 2:8, which is designed to detect the rare transcripts (Ambion). The intensities of the PCR products of ACPT in different tissues were weaker than that of control 18 S ribosomal RNA (Fig. 3), indicating that ACPT is expressed in an extremely small amount in *Arabidopsis* plant.

**Overexpression of the Recombinant ACPT in *E. coli***—In order to characterize the *cis*-prenyltransferase from *A. thaliana*, the enzyme was overexpressed in *E. coli* by using a pET-22b(+) expression system. Our first attempt to overexpress the recombinant ACPT based on the pGEX expression system failed, possibly due to a toxic effect of the gene that is involved in cell wall biosynthesis of host cells. Therefore, we used the pET-22b(+) expression system containing the *pelB* signal sequence to the N terminus of the ACPT gene so that the translated protein could be secreted to the periplasmic region.

After inducing the *E. coli* with IPTG, the cells were har-

vested and disrupted by sonication. Since the *cis*-prenyltransferase is a membrane-bound protein, it was necessary to treat Triton X-100 at a 1% concentration to solubilize the protein from the membranes. The proteins were purified by using a His tag affinity column from the supernatant and analyzed by SDS-12% polyacrylamide gel electrophoresis (Fig. 4). The major band at an expected size of 36 kDa (ACPT plus His tag) was clearly observed in the extract from the IPTG-induced cells. In contrast, no band was observed in the extract from the same *E. coli* cells but without IPTG induction. Since we do not have the antibody for ACPT, it was not feasible to directly detect the ACPT protein by Western blot. However, Western blot analysis of the ACPT-His tag fusion protein using the His tag antibody revealed that the major protein of 36 kDa in size is the translated product of the pET-ACPT construct (Fig. 4).

**Substrate Specificity and Characterization of ACPT**—To determine whether the gene we have cloned is really the *cis*-prenyltransferase, a standard activity assay of *cis*-prenyltransferase was performed with IPP and FPP as substrates. In a time course experiment using the recombinant enzyme, it was clearly noted that [<sup>14</sup>C]IPP incorporation increased linearly with incubation time up to 3 h (data not shown). It was also observed that [<sup>14</sup>C]IPP incorporation increased with the amount of enzyme added in the reaction mixture (data not shown). Since Mg<sup>2+</sup> ion is known to be required for the activity of *cis*-prenyltransferase from *E. coli* (25), the effect of Mg<sup>2+</sup> ion on catalytic activity of ACPT was investigated. No activity was observed in the absence of Mg<sup>2+</sup> ion, and a rapid increase of enzyme activity was detected with the addition of Mg<sup>2+</sup> ion up

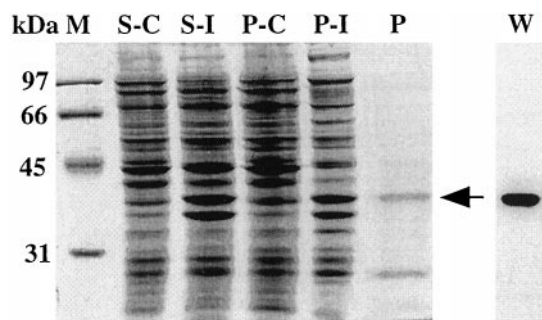


FIG. 4. **Overexpression of ACPT in *E. coli*.** The pET-22b(+) expression vector was constructed, in which the ACPT gene is fused to *pelB* signal sequence in order to localize the recombinant protein into periplasmic region and transformed into *E. coli* BL21. The cell was induced by the addition of 0.1 mM IPTG, and ACPT proteins were purified by a His tag column. *S-C*, supernatant control without IPTG induction; *S-I*, supernatant after IPTG induction; *P-C*, pellet control without IPTG induction; *P-I*, pellet after IPTG induction; *P*, purified after IPTG induction; *W*, Western with His tag antibody; *M*, molecular weight markers. An arrow indicates the migration of ACPT.

to 2 mM. A further addition of  $Mg^{2+}$  ion inhibited the activity (Fig. 5). Triton X-100 was absolutely required for the activity of bacterial undecaprenyl pyrophosphate synthase (25). During the purification of recombinant ACPT, Triton X-100 was necessary to solubilize the protein from membrane. To test whether Triton X-100 is necessary for the activity of the enzyme, we investigated several times the ACPT activity with varying concentration of Triton X-100. Maximum activity was observed at 0.01% Triton X-100. However, the purified enzyme showed about 80% activity in the absence of Triton X-100 (Fig. 5), indicating that ACPT is not strictly dependent on Triton X-100 for its activity.

The substrate specificities of ACPT were investigated with different amounts of allylic diphosphate substrates (Fig. 6). The substrate concentrations were plotted against the total extractable radioactivities. Between the two initiating molecules tested (*trans,trans*-FPP and *trans,trans,trans*-GGPP), *trans,trans*-FPP was a better substrate than *trans,trans,trans*-GGPP, with enzyme saturation being achieved at 2 and 10  $\mu M$ , respectively (Fig. 6). The condensing substrate, IPP, was a poor substrate, since the enzyme saturation was achieved at 200  $\mu M$ . The data from Fig. 6 were used to calculate  $K_m$  and  $V_{max}$  values for the different substrates, according to Lineweaver-Burk analysis. The enzyme exhibits a higher affinity for *trans,trans*-FPP than for all-*trans*-GGPP, with the  $K_m$  values being 0.13 and 3.62  $\mu M$ , respectively. The enzyme shows a lower affinity for IPP, with a  $K_m$  of 23  $\mu M$ .

**Products of the ACPT Reaction**—The purified enzymes were incubated with FPP and [ $^{14}C$ ]IPP, and the reaction products were analyzed on TLC and GPC. The products formed were subsequently extracted, dephosphorylated enzymatically, and separated using reverse phase TLC (Fig. 7). The dephosphorylated reaction products migrated more slowly than the polyprenol standard with carbon number of 90, indicating that ACPT synthesized polyisoprenes with carbon number higher than 90. In order to determine the size distribution of polyisoprenes, the reaction products extracted with butanol were analyzed by GPC. The effluents were collected at 0.5-ml intervals, and their radioactivities were measured by liquid scintillation counter. The peak activity was observed at the point that corresponded to the polyisoprene with carbon number  $C_{120}$ .

In order to test whether ACPT can catalyze the formation of higher molecular weight polyisoprenes such as natural rubber, an *in vitro* rubber biosynthesis assay was conducted. Gel permeation chromatography analysis of the benzene extract of the reaction products indicated that the predominant reaction

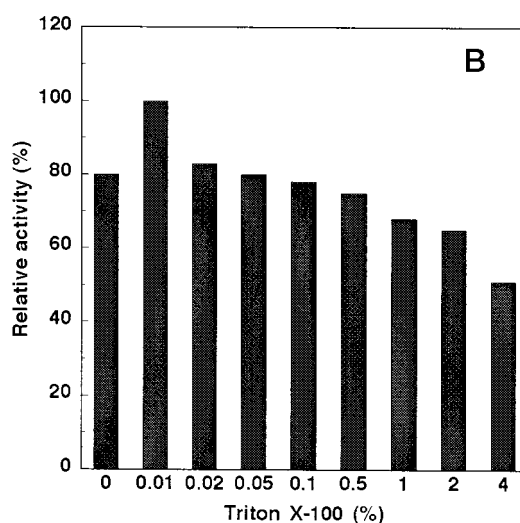
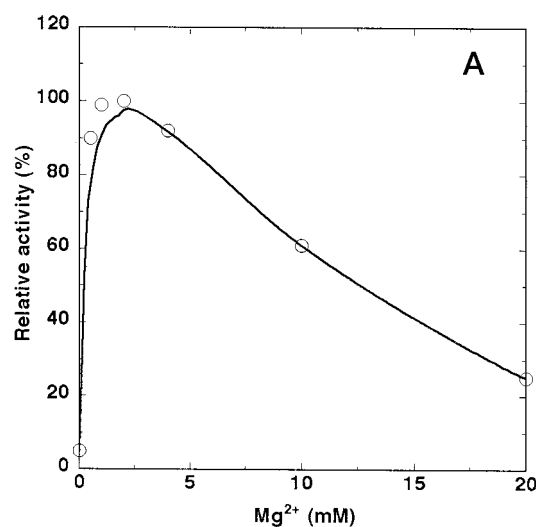


FIG. 5. **Effects of  $Mg^{2+}$  and Triton X-100 on the enzymatic activity.** *cis*-Prenyltransferase assay was performed in 50  $\mu l$  of standard reaction mixture containing 100 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 10  $\mu M$  FPP, 60  $\mu M$  [ $^{14}C$ ]IPP, 20 mM KF, 5  $\mu l$  of enzyme solution and the indicated amount of  $MgCl_2$  (A) or 1 mM  $MgCl_2$  and the indicated amount of Triton X-100 (B). After incubation at 30  $^{\circ}C$  for 1 h, the reaction products were extracted with butanol, mixed with scintillation mixture, and subjected to liquid scintillation counting. Each value is the mean of three separate experiments.

product of ACPT was prenyl diphosphate with carbon number 120, and no polymers with higher molecular size was produced. This result suggests that ACPT itself could not catalyze the formation of higher molecular weight rubber-like polymers.

#### DISCUSSION

The plant *cis*-prenyltransferase from *A. thaliana* shares a common feature in its primary structure to *cis*-prenyltransferases from *E. coli*, *M. luteus*, and yeast. A number of conserved domains are identified throughout the sequence. However, the potential significance of these domains in substrate binding and catalytic activity remains to be investigated. The DDXXD motif conserved for the allylic substrate and IPP bindings in *trans*-prenyltransferase is not present in *cis*-prenyltransferase. For *trans*-prenyltransferase, aspartate residues are involved for the liganding of multiple  $Mg^{2+}$  ions required for the substrate binding (19–23). Absence of the conserved DDXXD motif in *cis*-prenyltransferase suggests that the catalytic mechanism of the enzyme is different from that of *trans*-prenyltransferase.

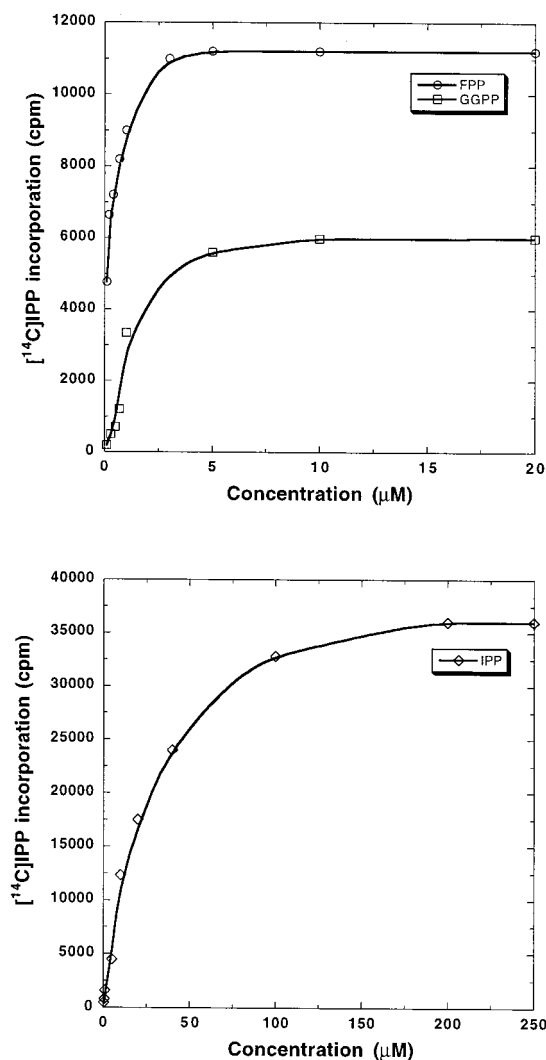


FIG. 6. **Substrate specificity of ACPT.** Purified ACPT was incubated with increasing amounts of FPP and GGPP and saturated amounts of IPP (*top*) and increasing amounts of IPP and saturated amounts of FPP (*bottom*). The amount of product formed was plotted against substrate concentration.

The gene related to ACPT we have cloned by PCR and cDNA library screening has been reported as a hypothetical protein based on an *Arabidopsis* genomic sequence analysis. The amino acid sequence of ACPT with 303 amino acids is identical to the hypothetical protein in chromosome II BAC F26B6 (accession no. AC003040) with 290 amino acids, except 13 extra amino acids in ACPT and a few mismatch in N terminus. The ACPT also shares a high sequence homology (30% identity) with the hypothetical protein in chromosome II P1 MJB20 (accession no. AC007584), which is relatively short with 260 amino acids and lacks the corresponding amino acids in its N terminus. In order to investigate the copy number of *cis*-prenyltransferase in *Arabidopsis*, high molecular weight total DNA was prepared by using a plant genomic isolation kit (Omega Biotek), digested with appropriate restriction enzymes, blotted on a nylon membrane by capillary method, and was subjected to Southern blot analysis. The presence of these two related genes with high sequence homology was supported by two to three signals in Southern blot analysis of the genomic DNA of *A. thaliana* (data not shown).

*Arabidopsis cis*-prenyltransferase contains additional 30 amino acids in N terminus compared with the enzymes from *E. coli*, *M. luteus*, and yeast. Hydropathy and PSORT analyses

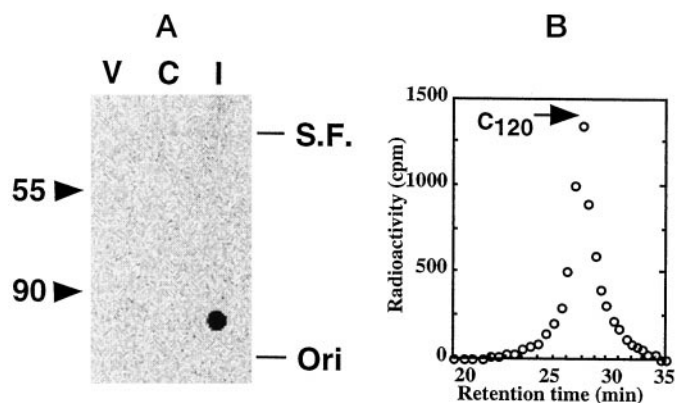


FIG. 7. **Analysis of the ACPT reaction products *in vitro*.** A, after extraction and dephosphorylation, the polyprenols were separated on reverse phase-TLC with a solvent system of acetone/water (19:1). The plate was exposed on image plate and analyzed by a Bio-image analyzer BAS 1500 (Fuji). V, vector only after IPTG induction; C, pET-ACPT construct without induction; I, pET-ACPT construct after induction; S.F., solvent front; Ori., origin. The numbers on the left indicate the positions of authentic polyprenol standards. B, the reaction extract was subjected to GPC, the fractions were collected at 0.5-ml intervals, and the radioactivity of each fraction was counted.

revealed that this extra N-terminal amino acid sequence of ACPT is hydrophobic and has transmembrane helix motif, which contrasts with other *cis*-prenyltransferases from *E. coli*, *M. luteus*, and yeast that do not contain the motif. During the purification of ACPT, we were not able to detect the enzyme in the soluble fraction of the cell lysate in the buffer not containing Triton X-100. The addition of Triton X-100 to the cell lysis buffer was required to solubilize the enzyme from the membranes and to purify the recombinant protein. These results suggest that ACPT does exist in association with membrane in plants. The enzyme displayed higher affinity for *trans,trans*-FPP than for all-*trans*-GGPP (Fig. 6). This characteristic was also observed in *cis*-prenyltransferase from rat liver microsomes (33).

We do not have direct evidence for the biological function of *cis*-prenyltransferase in plants. The prokaryotic *cis*-prenyltransferase, undecaprenyl diphosphate synthase, catalyzes the formation of undecaprenyl diphosphate ( $\text{C}_{55}$ ) that serves as a glycosyl carrier lipid during the biosynthesis of cell wall polysaccharide components (24–26). The eukaryotic *cis*-prenyltransferase is involved in the biosynthesis of dolichols that are used for the posttranslational glycosylation of proteins (27). In the analysis of ACPT transcripts in *Arabidopsis*, we were not able to detect the mRNA by Northern blot analysis. Our RT-PCR analysis of the transcripts indicated that ACPT is expressed in low levels in various tissues including leaf, stem, flower, and silique, but at slightly higher levels in roots and leaves. The tissue-specific expressions suggest a functional role of the enzyme in *Arabidopsis* growth and development.

Rubber polymerase or rubber transferase belongs to a family of *cis*-prenyltransferases that catalyze the formation of poly-prenyl diphosphates by sequential condensation of IPP to allylic diphosphate. Our primary interest is to identify *cis*-prenyltransferase involved in rubber biosynthesis. The present data revealed that *Arabidopsis cis*-prenyltransferase itself could not catalyze the formation of rubber. It is possible that in addition to *cis*-prenyltransferase other factors such as rubber elongation factor and termination factor are required for the synthesis of rubber. It is also possible that a rubber polymerase is an entirely different protein from *cis*-prenyltransferase, as suggested by Oh *et al.* (29). Identification and isolation of more *cis*-prenyltransferases from different rubber producing plant species are necessary to further determine whether *cis*-prenyl-

transferase is related to rubber polymerase. We are currently searching for the related genes of *cis*-prenyltransferase from various rubber producing plant species.

In conclusion, we have isolated and characterized the first plant *cis*-prenyltransferase from *Arabidopsis thaliana*. The deduced amino acid sequence is highly homologous in several conserved regions to those of *cis*-prenyltransferases from *M. luteus*, *E. coli*, and yeast. *In vitro* analysis of the recombinant protein overexpressed in *E. coli* revealed that the enzyme catalyzes the formation of polyprenyl diphosphates with predominant carbon number C<sub>120</sub>. *In vitro* rubber biosynthesis analysis indicated that *cis*-prenyltransferase could not catalyze the formation of higher molecular weight polyprenyl diphosphates such as natural rubber. The *cis*-prenyltransferase was expressed in low levels in all tissues of *Arabidopsis* tested, but at slightly higher levels in roots and leaves, and only barely in siliques. The results described here represent an important step in understanding the primary structure of *cis*-prenyltransferase in higher plants and provide a basis for the further investigation of the catalytic mechanism of chain elongation in polyprenyl diphosphates biosynthesis.

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