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

IMPLICATIONS IN RUBBER BIOSYNTHESIS*

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 polypropenyl 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 polypropenyl diphosphates ranging in carbon number from 100 to 130 with a predominance of C120. The enzyme exhibited a higher affinity for farnesyl diphosphate than for geranylgeranyl diphosphate, with a K_m values being 0.13 and 3.62 μM, respectively, but a lower affinity for isopentenyl diphosphate, with a K_m 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 polypropenyl 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.

Prenyltransferase is an enzyme that catalyzes the synthesis of linear prenyl diphosphates involved in the biosynthesis of various isoprenoid compounds, including sterols, carotenoids, 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_{10}), farnesyl diphosphate (FPP; C_{15}), and geranylgeranyl diphosphate (GGPP; C_{20}), 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), selenyl 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-prenyltransferases genes have recently been cloned. Two genes are the structural genes for undecaprenyl diphosphate synthase, which catalyzes the formation of undecaprenyl diphosphate (C_{15}), 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^{2+} 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-
cis-Prenyltransferase from A. thaliana

Heterologous Expression and Purification of ACPT Protein in E. coli—The ACPT gene was cloned in the BamHI-EcoRI 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% bactotrypton, 2.4% yeast extract, 0.4% glycerol, and 0.5% magnesium phosphate solution) containing 100 μg/ml ampicillin 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 × g, 10 min), and then disrupted by sonication. The expressed proteins were purified using Ni2+-nitrilotriacetic acid-agarose (Qiagen). The lysate was incubated with Ni2+-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 (50).

Western Blot Analysis—The purified protein was separated by SDS-10% polyacrylamide gel electrophoresis and transferred to polyvinyl-dene 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 (Qiagen) 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-μl reaction mixture containing 100 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM dithiothreitol, 20 μM FPP, and 80 μM [3^14C]IPP (55 mCi mmol−1, 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 5800-μl Teflon). The specific activity is expressed as dpm/mg of protein.

Analysis of ACPT Reaction Products—The washed organic extract was dried under N2, and the residues were hydrolyzed to the corresponding alcohols with hydrochloric acid. The residues were then dissolved in methanol and analyzed by gas chromatography/mass spectrometry (Varian 3400) with an HP-5 column. The retention times of authentic standards were compared with those of the reaction products. The products were quantified by a liquid scintillation spectrometer (Beckman 5800-μl Teflon).

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 × 10^6 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...
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 \textit{M. luteus} (pI 5.85) and \textit{RER2} (for return to the endoplasmic reticulum) gene in yeast (pI 5.75).

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 \textit{cis}-prenyltransferases from yeast, \textit{M. luteus}, and \textit{E. coli} that do not have transmembrane helices.

The predicted amino acid sequence of the ACPT has low similarity to those of \textit{cis}-prenyltransferases from \textit{E. coli} (SWISS-PROT Q47675; 30% identity), \textit{M. luteus} (accession no. AB004319; 30% identity), and yeast \textit{S. cerevisiae} (accession no. AB013497; 28% identity) (Fig. 2). Several regions with identical 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 \textit{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...
ACPT gene, a control reaction using the 18 S primers and Competimer 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 harvested 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 [14C]IPP incorporation increased linearly with incubation time up to 3 h (data not shown). It was also observed that [14C]IPP incorporation increased with the amount of enzyme added in the reaction mixture (data not shown). Since Mg2+ ion is known to be required for the activity of cis-prenyltransferase from E. coli (25), the effect of Mg2+ ion on catalytic activity of ACPT was investigated. No activity was observed in the absence of Mg2+ ion, and a rapid increase of enzyme activity was detected with the addition of Mg2+ 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 petB 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-trans,trans,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 μM, respectively (Fig. 6). The condensing substrate, IPP, was a poor substrate, since the enzyme saturation was achieved at 200 μM. 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 $K_m$ values being 0.13 and 3.62 μM, respectively. The enzyme shows a lower affinity for IPP, with a $K_m$ of 23 μM.

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 polypropenol 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 eluents 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 polyisoprenes 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 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.
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 its 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 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 faction 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 microsome (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 (C_{10}) 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-

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**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.

**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.
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 polypropenyl diphosphates with predominant carbon number C120. In vitro rubber biosynthesis analysis indicated that cis-prenyltransferase could not catalyze the formation of higher molecular weight polypropenyl 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 polypropenyl diphosphates biosynthesis.

**Acknowledgments**—We are grateful to G. T. Choi for providing the *Arabidopsis* cDNA library and to M. H. Lee for help in growing *Arabidopsis*. We thank Pill-Soon Song for critical reading of the manuscript.

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Molecular Cloning, Expression, and Functional Analysis of a cis-Prenyltransferase from Arabidopsis thaliana: IMPLICATIONS IN RUBBER BIOSYNTHESIS
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doi: 10.1074/jbc.M002000200 originally published online April 7, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002000200

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