

cis-Prenyltransferase AtCPT6 produces a family of very short-chain polyisoprenoids in planta

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ARTICLE INFO

Article history:

Received 22 August 2013

Received in revised form 13 November 2013

Accepted 22 November 2013

Available online 1 December 2013

Keywords:

Polyisoprenoid alcohol

Dolichol

cis-Prenyltransferase

Protein N-glycosylation

Arabidopsis

rer2Δ Saccharomyces cerevisiae mutant

ABSTRACT

cis-Prenyltransferases (CPTs) comprise numerous enzymes synthesizing isoprenoid hydrocarbon skeleton with isoprenoid units in the *cis* (Z) configuration. The chain-length specificity of a particular plant CPT is in most cases unknown despite the composition of the accumulated isoprenoids in the tissue of interest being well established. In this report AtCPT6, one of the nine *Arabidopsis thaliana* CPTs, is shown to catalyze the synthesis of a family of very short-chain polyisoprenoid alcohols of six, seven, and eight isoprenoid units, those of seven units dominating. The product specificity of AtCPT6 was established *in vivo* following its expression in the heterologous system of the yeast *Saccharomyces cerevisiae* and was confirmed by the absence of specific products in AtCPT6 T-DNA insertion mutants and their overaccumulation in AtCPT6-overexpressing plants. These observations are additionally validated *in silico* using an AtCPT6 model obtained by homology modeling. AtCPT6 only partially complements the function of the yeast homologue of CPT-Rer2 since it restores the growth but not protein glycosylation in *rer2Δ* yeast. This is the first *in planta* characterization of specific products of a plant CPT producing polyisoprenoids. Their distribution suggests that a joint activity of several CPTs is required to produce the complex mixture of polyisoprenoid alcohols found in Arabidopsis roots.

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1. Introduction

Polyisoprenoid alcohols are accumulated in all organisms, from bacteria to higher eukaryotes, and exhibit unique structures and features. They are classified in two groups: polyprenols – allylic alcohols with a single double bond in each isoprenoid unit, and dolichols with no double bond in the OH-terminal isoprenoid unit (Fig. 1). Dolichols are mainly found in animal and yeast cells and in plant roots whereas polyprenols in bacteria and plant photosynthetic tissues (summarized in [1]). Polyisoprenoids are accumulated as free alcohols and/or esters of carboxylic acids; with a small fraction of mono- and diphosphates. Polyisoprenoids modulate properties of biological membranes, while polyisoprenoid phosphates serve as sugar carriers for the biosynthesis of glycosyl-phosphatidylinositol membrane (GPI) anchor, protein C- and O-mannosylation, and protein N- and O-glycosylation; they are also utilized as donors of isoprenoid groups during protein prenylation [2–4]. The content of dolichols and polyprenols increases during the life-course of a tissue/organism and upon environmental stress (summarized in [1,5]).

Abbreviations: AtCPT, *Arabidopsis thaliana cis*-prenyltransferase; HPLC, High Performance Liquid Chromatography; IPP, isopentenyl diphosphate; i.u., isoprenoid unit; Pren/Dol-n, prenol/dolichol composed of n i.u.

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The key enzyme of polyisoprenoid synthesis is *cis*-prenyltransferase (CPT, also called dehydrololichyl diphosphate synthase, polyprenyl diphosphate synthase or undecaprenyl diphosphate synthase – UPPS) responsible for construction of their long hydrocarbon skeleton. Most of typical CPTs elongate a short all-*trans* (E) precursor, oligoprenyl diphosphate, by sequential additions of a desired number of isopentenyl diphosphate (IPP) molecules, which results in the formation of a chain of *cis* (Z) isoprenoid units in the CPT end-product – polyisoprenoid diphosphate (polyprenyl-PP). Subsequent dephosphorylation of polyprenyl-PP gives the corresponding polyprenol which may undergo hydrogenation to form dolichol [6]. Koyama and coworkers has classified *cis*-prenyltransferases into three subgroups according to their product chain length: short- (composed of three isoprenoid units), medium- (from ten to eleven i.u.) and long-chain (from fourteen to twenty four i.u.) CPTs [7] although recent studies on plant CPTs summarized below indicate that this classification should be redefined. An interesting feature of all eukaryotic CPTs is the formation of mixtures of homologous polyisoprenoid diphosphates called ‘families’. In contrast, a single polyprenyl-PP (most often composed of eleven i.u., Pren-11PP) is synthesized by prokaryotic CPTs [8,9].

Several genes encoding CPT have been cloned from bacteria, yeast, plants and animals. Two *Saccharomyces cerevisiae cis*-prenyltransferases (Rer2 and Srt1) catalyzing the synthesis of two dolichol families have been described. The products of Srt1 (Dol-21 dominating) are longer by several isoprenoid units than those of Rer2 (Dol-15 dominating). These enzymes have different localization and physiological roles during the

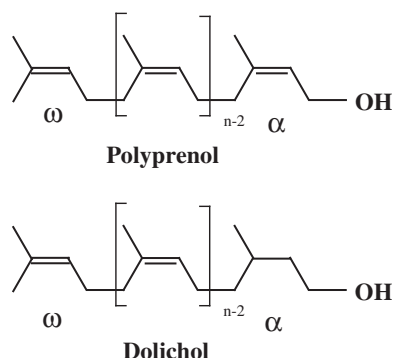


Fig. 1. Structure of polyprenol and dolichol composed of n isoprenoid units. α and ω represent terminal isoprenoid units.

life cycle of the yeast [10,11]. Only one gene encoding CPT has been identified in humans [12,13], the fish [14] and the parasite [15]. In contrast, plants usually express several CPTs. Thus, seven genes encoding CPT producing almost exclusively short-chain polyisoprenoids have been identified in tomato (SlCPT1–7) [16]. Interestingly, only one CPT (LAA66) has been described in *Lilium logiflorum* [17] which in fact does not preclude the existence of a family of CPT encoding genes in this plant. Additionally, CPTs catalyzing the formation of long-chain polyisoprenoids of natural rubber have been partially characterized, two from *Hevea brasiliensis* (HRT1 and HRT2) [18] and three from *Taraxacum koksaghyz* (TkCPT1–3) [19].

The model plant *Arabidopsis thaliana* has a family of nine genes homologues to yeast CPTs – AtCPT1 to AtCPT9 [5]; however only two of which have been partially characterized at the molecular level. *In vitro*, AtCPT1 (ACPT/DPS, At2g23410) catalyzes the synthesis of a single dolichol composed of 18 i.u. [20,21] while AtCPT6 (AtHEPS, At5g55780) a mixture of polyprenols with dominating prenol composed of seven i.u. [22]. The *in vivo* activity of any of the AtCPTs has not been studied yet. A recently discovered gene, *LEW1* (leaf wilting 1), encoding an atypical *cis*-prenyltransferase with low sequence similarity to the AtCPTs was reported in 2008. Its product plays a critical role in protein glycosylation and has been suggested to be involved in formation of dolichols of 15 and 16 i.u. [23].

This study reports the *in vivo* activity of AtCPT6 (At5g55780). Upon heterologous expression in yeast, AtCPT6 catalyzed the synthesis of a family of very short-chain polyisoprenoid diphosphates which are after dephosphorylation/reduction accumulated in the cells as mixture of polyprenols/dolichols composed of 6 to 8 i.u., with Pren/Dol-7 dominating. That specificity was confirmed in the native system of *A. thaliana*. The content of Pren/Dol-7 in roots was considerably lower in insertion mutants of AtCPT6 than in wild type plants, while overexpression of the gene increased it. Analysis of a structural model of AtCPT6 obtained by homology modeling further supported its capacity to synthesize short-chain products. Taken together, we conclude that AtCPT6 catalyzes the formation of very short-chain polyprenyl products. To our knowledge this is the first report showing the *in vivo* activity of a plant polyisoprenoid producing CPT.

2. Materials and methods

2.1. Yeast material

S. cerevisiae strains used in this study, SS328 (wild type, MAT α ade2-101 ura3-52his3 Δ 200 lys2-801) and YG932 (*rer2* Δ mutant, MAT α *rer2* Δ ::kanMX4 ade2-101 ura3-52his3 Δ 200 lys-801) were kind gifts of Dr. C.J. Waechter (University of Kentucky College of Medicine, Lexington, KY, USA).

2.2. Plant material

A. thaliana ecotype Columbia 0 (Col-0) and At5g55780 insertion mutants *cpt6-1* (SALK_071255) and *cpt6-2* (SALK_064499) were obtained from the Nottingham Arabidopsis Stock Center.

2.3. Generation of Arabidopsis plants overexpressing AtCPT6

The S35::AtCPT6 construct was introduced into *Agrobacterium tumefaciens* GV3101 strain and then used for transformation of *A. thaliana* (Col-0) wild-type plants by the floral dip method [24]. F1 seeds were screened on Murashige and Skoog (MS) agar plates supplemented with 50 mg/l kanamycin (Sigma). The putative F1 transgenic lines were transferred into soil and the leaves used for genotyping by PCR, using the forward primer of the S35 promoter (5'-caatcccatatcc ttcgc) and the reverse primer of AtCPT6 (5'-ctcttgactgttcggagga). The F3 of the CPT6-OE line was selected and used for the expression analysis and lipid content characterization.

2.4. Plant growth conditions

Seeds were sterilized with calcium hypochlorite and stratified at 4 °C for 2 days. Seedlings were grown with half MS medium supplemented with 1% sucrose and kanamycin on agar (0.8%) plates or in hydroponic culture (modified Gibeaut medium) [25] under long-day (16 h light/8 h dark) conditions at 22°/18 °C in a Percival chamber.

2.5. Preparation of pYES-DEST52-AtCPT6 construct

Total RNA was isolated from wild type *A. thaliana* ecotype Col-0 roots using RNeasy Plant Mini Kit (Qiagen) and reverse transcribed to cDNA using SuperScript® First-Strand Synthesis System (Invitrogen). The cDNA was used as a template for PCR with gene-specific primers designed according to the cDNA sequences of the putative Arabidopsis *cis*-prenyltransferase At5g58780 (NM_125265) from the GenBank, AtCPT6-F (5'-caccatgtgtctattctctctctc-3') and AtCPT6-R (5'-aacccgacagccaaatcg-3'). PCR was performed in a final volume of 20 μ l containing 20 pmol of amplification primer pair for 50 cycles at 30 s at 98 °C, 40 s at 62 °C and 1 min at 72 °C, with a 3-min preheat at 98 °C and a 10-min final extension at 72 °C. The PCR product was purified by agarose gel electrophoresis, subcloned into the pENTR vector (Invitrogen) and transformed into chemically competent TOP10 *Escherichia coli*.

The AtCPT6 cDNA was recombined from the pENTR vector into the destination vector pYES-DEST52 Gateway Vector using the Gateway LR Clonase enzyme mix (Invitrogen).

2.6. Heterologous expression and purification of His-tagged AtCPT6 protein

pYES-DEST52-AtCPT6 construct was transformed into *S. cerevisiae* INVSc1 strain according to the protocol of S.c.EasyComp Kit (Invitrogen) and expressed using 2% galactose induction for 32 h at 28 °C. The His-tagged AtCPT6 fusion protein was purified using Ni-NTA agarose (Qiagen), resolved by 10% SDS-PAGE and stained by Coomassie Blue or transferred onto nitrocellulose membrane for Western blot analysis.

2.7. In vivo complementation of *rer2* Δ mutation in *S. cerevisiae*

The YG932 *rer2* Δ mutant yeast strain was transformed with empty vector pYES-DEST52 or pYES-DEST52-AtCPT6 as above. The transformants were grown for 5 days at 30 °C in SC-ura medium containing 2% glucose. The cells were pelleted, washed and resuspended in SC-ura medium containing 2% glycerol and grown for 4 h at 30 °C to permit derepression of the galactose-inducible promoter in the expression plasmid. Next, serially diluted yeast cultures were spotted on solid YP-galactose medium and incubated at 23 or 37 °C for 5 days. The wild type strain, which grew normally at 37 °C, and nontransformed *rer2* Δ mutant were used as controls.

2.8. Preparation of yeast microsomes

Yeast cultures were grown at 28 °C in adequate medium: wild type in YPD, nontransformed *rer2Δ* mutant in YPD supplemented with G418 (200 mg/l), and *rer2Δ* transformed with empty vector pYES-DEST52 or pYES-DEST52-*AtCPT6* in Ura-selective medium (SC Minimal Medium) containing yeast nitrogen base (6.7 g/l) and required amino acids without uracil supplemented with G418 (200 mg/l). Yeast cells were harvested at logarithmic phase, $OD_{600} = 1.1$, resuspended in 3 volumes of lysis buffer [50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 10 mM MgCl₂, 0.1% Igepal, 1 mM PMSF, 1 tablet/10 ml Complete ULTRA Mini, EDTA-free (Roche)] plus an equal volume of 0.4–0.6 mm glass beads and vortexed vigorously 7 times at 4 °C for 1 min each with 1-min intervals of cooling on ice. The homogenates were centrifuged at 2500 ×g for 5 min at 4 °C to remove cellular debris and supernatant was subsequently centrifuged at 50,000 ×g for 80 min at 4 °C (Beckman) to sediment crude microsomes. The microsomal pellet was resuspended in the lysis buffer and stored at –20 °C before resolution by SDS-PAGE.

2.9. Preparation of plant microsomal fractions

Roots and leaves collected from 6-week-old WT and *cpt6-1* mutant plants grown in hydroponic culture were homogenized on ice using a mortar and pestle with 1 volume (v/w) of homogenization buffer (50 mM Tris, pH 7.5, 5 mM MgCl₂, 10 μM ZnCl₂, 2 mM DTT, 100 mM NaCl, 250 mM saccharose) containing protease (Complete Mini, Roche) and phosphatase (PhosSTOP, Roche) inhibitor cocktails. The homogenates were centrifuged at 2500 ×g for 10 min at 4 °C to remove cell debris and subsequently the supernatant was centrifuged at 12,000 ×g for 30 min (Sorvall RC-5B) to separate pellet (P1) containing mitochondria and chloroplast and supernatant (S1). The S1 was next spun at 200,000 ×g for 1.5 h (Sorvall Discovery 90SE) to yield microsomal (pellet P2) and cytosolic (supernatant S2) fractions. Protein concentrations were determined by the Lowry method using bovine albumin (fraction V, Sigma-Aldrich) as a standard.

2.10. SDS-PAGE and Western blotting

Protein samples were resolved on 10% or 12% SDS polyacrylamide gel and transferred to ECL nitrocellulose membrane by wet transfer (Mini Trans Blot, Biorad Laboratories, Hercules, CA). The membrane was blocked for 45 min with 4% non-fat milk in PBS-T (0.1% Tween-20 in 1 × PBS). Immunodetection was performed using appropriate primary antibody – rabbit anti-peptide *AtCPT6* antibody (1:100, custom service Agrisera), rabbit anti-peptide *AtCPT7* antibody (1:1000, Agrisera), mouse anti-CPY antibody (1:3000, Invitrogen) or anti-His(C-term)-HRP antibody (1:5000, Invitrogen) overnight at 4 °C. Blots were washed three times in PBS-T and incubated for 1 h with HRP-conjugated anti-rabbit IgG (1:5000, Agrisera) or HRP-conjugated anti-mouse IgG (1:1000, Sigma-Aldrich), respectively. After washing (three times, PBS-T) the signal was detected using SuperSignal West Pico (Pierce).

2.11. Extraction of polyisoprenoids from yeast

Stationary-phase yeast cells (1- to 4-day culture depending on the strain) grown at 28 °C were harvested by centrifugation at 3600 rpm (Allegra; Beckman) for 5 min at 4 °C and washed once with sterile water. The pellets were dissolved in 10 ml of hydrolytic solution (25 g of KOH, 35 ml of sterile distilled water, brought to 100 ml with 99.8% ethanol) [26], vortexed for 1 min and incubated at 95 °C for 1 h. Nonsaponifiable lipids were then extracted three times with hexane, pooled extracts were evaporated and purified on a silica gel 60 column using isocratic elution with 10% diethyl ether in hexane. Purified polyisoprenoids were analyzed by HPLC/UV using external and internal standards.

2.12. Extraction of polyisoprenoids from plants

Liquid-nitrogen frozen roots were homogenized using a mortar and pestle. Lipids were extracted with a mixture of chloroform:methanol:water (C/M/W, 1:1:0.3 by vol.) for 48 h at room temperature. After supplementation with chloroform and water (to reach the final solvent ratio of C/M/W 3:2:1 by vol.) the chloroform phase was collected and evaporated under a stream of nitrogen. Residual lipids were dissolved in 5 ml of a mixture containing toluene/7.5% KOH/95% ethanol (20:17:3 by vol.) and hydrolyzed for 1 h at 95 °C [27], then extracted with hexane, purified and analyzed as above.

2.13. HPLC/UV analysis of polyisoprenoids

Lipids were analyzed as described earlier with modifications [28]. A Waters dual-pump HPLC device with a ZORBAX XDB-C18 (4.6 × 75 mm, 3.5 μm) reversed-phase column (Agilent, USA), a Waters Photodiode Array Detector (spectrum range: 210–400 nm) with the solvent system: A—methanol/water, 9:1 (by vol.); B—methanol/propane-2-ol/hexane, 2:1:1 (by vol.) and a flow rate of 1.5 ml/min controlled by a Waters gradient programmer were used. The chain length and identity of lipids were confirmed by comparison with external standards of a polyprenol mixture (Pren-9, 11–23, 25). Pren-28 and Pren-29 were used as internal standards in plant lipid analysis. Integration of the HPLC/UV chromatograms was performed with the Empower (Waters) software.

2.14. LC/MS analysis of polyisoprenoids

LC/MS analysis of polyisoprenoids was performed on an Ultra-Performance Liquid Chromatograph ACQUITY UPLC I-Class (Waters Inc.) coupled with a MALDI Synapt G2-S HDMS (Waters Inc.) mass spectrometer equipped with an electrospray ion source and q-TOF type mass analyzer. The separation of polyisoprenoid alcohols was carried out using a BEH C18 column (Waters Inc.). The following solvents were used: solvent A—methanol/water, 9:1 (by vol.), solvent B—methanol/propane-2-ol/hexane, 2:1:1 (by vol.) and a gradient from 0% B to 75% B in 10 min, from 75% B to 90% B in the next 5 min, from 90% B to 100% B in 1 min, isocratic 100% B for 4 min, then from 100% B to 0% B in 1 min. The polyisoprenoid fractions were injected in a mixture of methanol/chloroform (1:1, by vol.). The LC/MS analysis was carried out at a flow rate 0.4 ml/min.

High resolution mass spectra of polyisoprenoid alcohols were recorded in the positive ion mode. Nitrogen was used as the desolvation gas (flow 448 l/h), cone gas (flow 76 l/h) and nebulizer gas (4.6 bar). The capillary voltage was set to 3800 V, the sampling cone—30 V and the desolvation temperature—250 °C. The instrument was controlled and recorded data were processed using MassLynx V4.1 software package (Waters Inc.).

2.15. Real time PCR analysis of *AtCPTs* expression level

Total RNA was isolated from 6-week-old roots and leaves of Col-0, *cpt6-1* and *CPT6-OE* Arabidopsis plants using the RNeasy Plant Mini Kit (Qiagen) and transcribed to cDNA using the SuperScript™ II First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's procedures. Real-time PCR analysis using *AtCPTs* and *Actin2* gene-specific primers [35] was performed in a real-time thermal cycler PikoReal 96 (Thermo Scientific) according to the manufacturer's instructions. The reaction mixture contained 6 μl of cDNA (30-fold diluted), 2 μl (5 μM) of both forward and reverse primers in a total volume of 20 μl of Maxima™ SYBR Green qPCR Master Mix (Fermentas). Cycling conditions were: 50 °C for 2 min; 95 °C for 10 min; then 50 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate along with a no-template control.

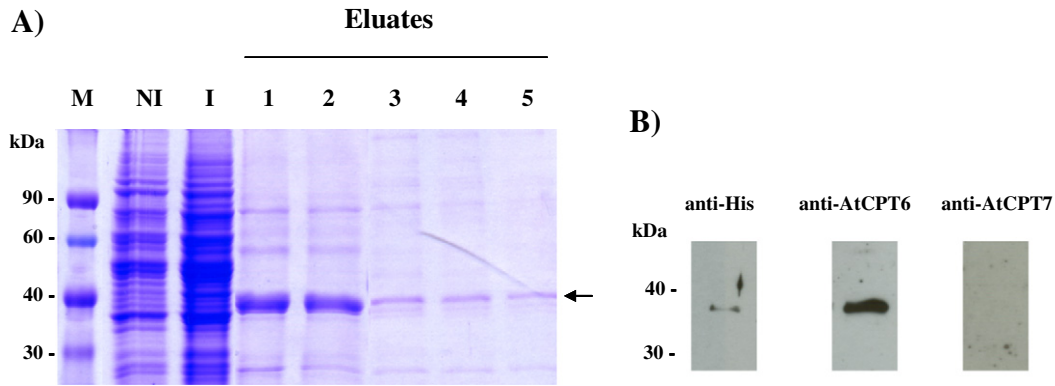


Fig. 2. Expression of recombinant His-tagged AtCPT6 protein in *S. cerevisiae*. (A) The expression of AtCPT6 in yeast was induced by addition of 2% galactose to culture medium. His-tagged AtCPT6 protein was purified on Ni-NTA gel under native conditions. Fractions from each purification step were resolved by 8% SDS-PAGE and stained with Coomassie Blue. M—molecular weight standard; NI—noninduced cells; I—cells after induction with 2% galactose; 1–5 sequential eluates. Arrow indicates purified His-tagged AtCPT6 protein. (B) Western blot analysis of purified recombinant AtCPT6 using specific antibodies directed against: His-tag, AtCPT6, and AtCPT7 proteins, respectively.

The expression level of AtCPTs was determined using Real Time PCR Absolut Quantification using standard curve obtained by serial dilutions of template DNA stock (4×10^5 copies/ μ l). The cycle threshold (Ct) values were calculated under default settings for the absolute quantification using PikoReal Software 2.0 (Thermo

Scientific). The relative expression level of each gene was analyzed after normalization with Actin2 (*ACT2*) gene used as the internal reference. The cycle threshold (Ct) was used to determine the relative expression level of a given gene using the $2^{-\Delta\Delta C_t}$ method.

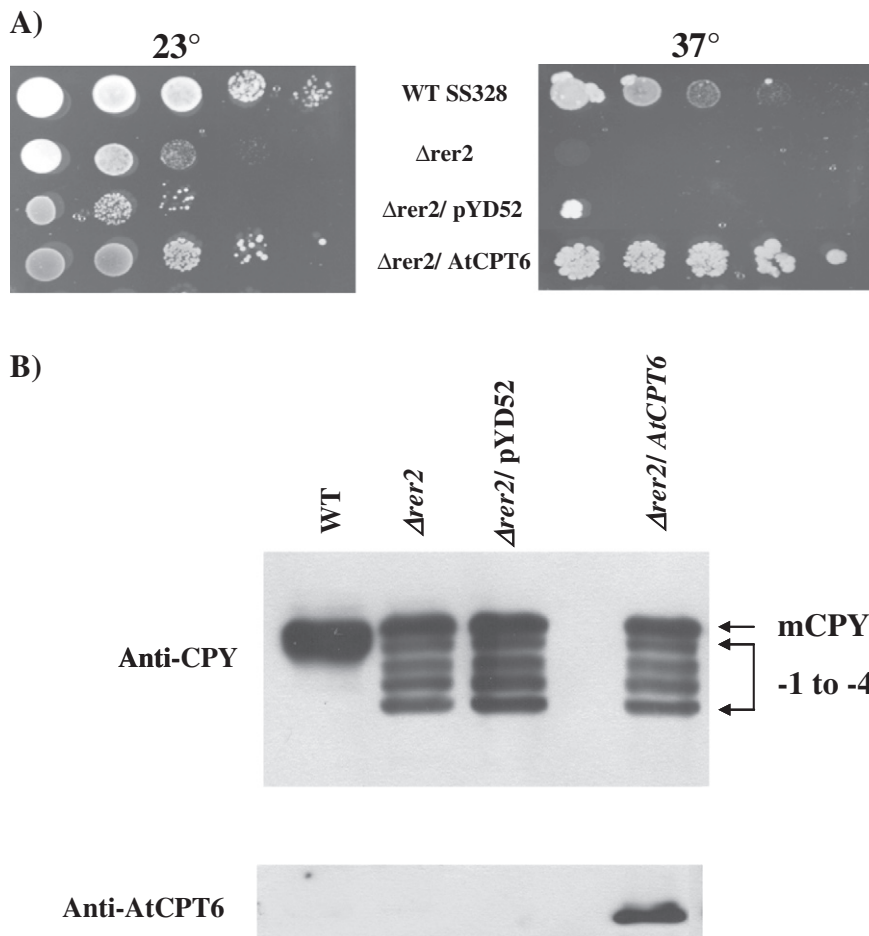


Fig. 3. Analysis of cell growth and CPY processing in yeast *rer2Δ* mutant cells expressing AtCPT6. (A) *rer2Δ* yeast mutant was transformed with pYES-DEST52-AtCPT6 expression construct or empty vector pYES-DEST52. WT strain SS328, which grew normally at 37 °C, and nontransformed *rer2Δ* mutant were used as controls. Serially diluted yeast cultures were plated on solid YP-2% galactose medium and incubated at 23 or 37 °C for 5 days. (B) proteins from microsomal fractions from WT, *rer2Δ*, *rer2Δ* transformed with empty vector, and *rer2Δ* transformed with pYES-DEST52-AtCPT6 were separated by 8% SDS-PAGE and analyzed by Western blotting with anti-CPY antibody. The positions of mature CPY (mCPY) and hypoglycosylated glycoforms lacking between one and four N-linked oligosaccharide chains are indicated. Western blotting with anti-AtCPT6 antibody was performed to confirm AtCPT6 expression in *rer2Δ* transformant.

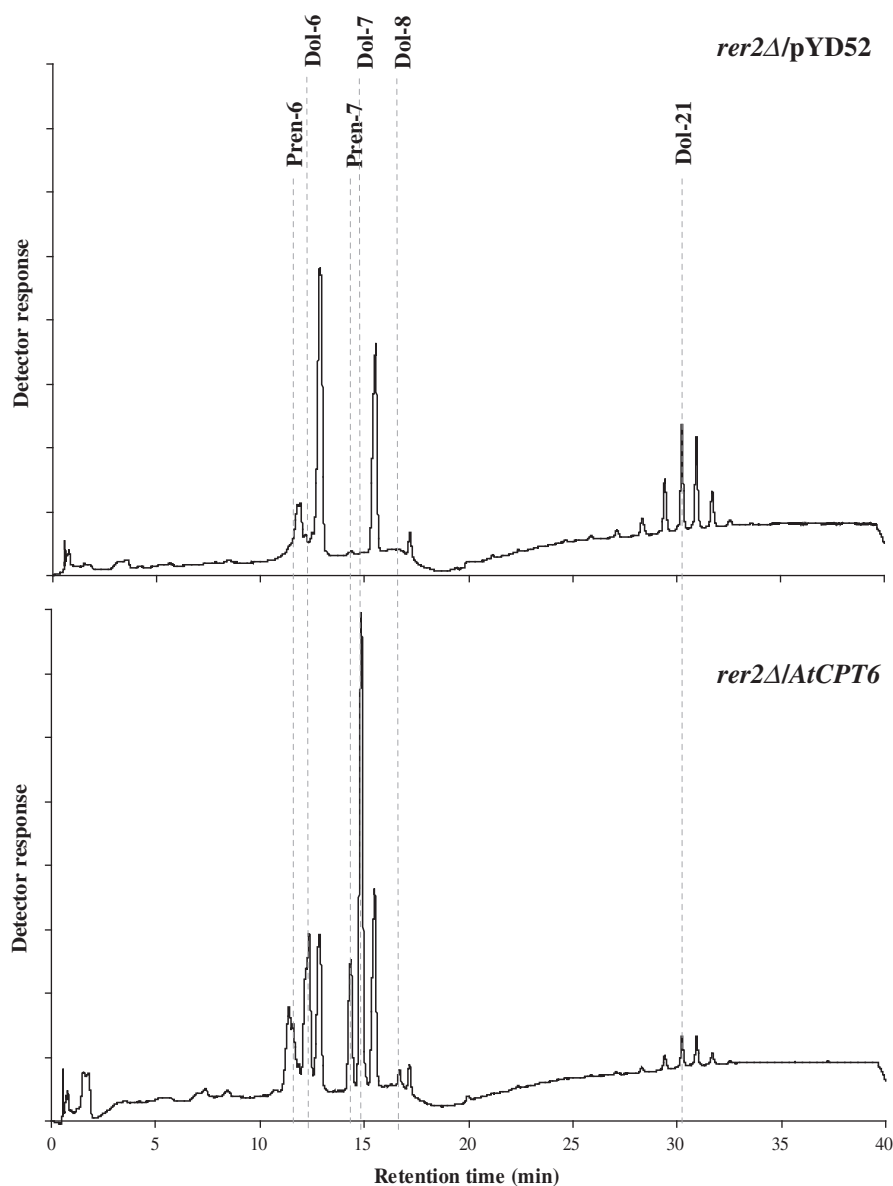


Fig. 4. Composition of polyisoprenoids isolated from yeast *rer2Δ* mutant transformed with empty pYES-DEST52 vector or pYES-DEST52-*AtCPT6* construct. Polyisoprenoids were isolated and their profile analyzed by HPLC/UV as described in [Materials and methods](#).

2.16. Homology modeling

HHpred [29] and BLAST [30] servers indicated that among solved structures the closest structural homologue of AtCPT6 was CPT from *Staphylococcus aureus* called undecaprenyl diphosphate synthase, UPPS [31]. Coordinates of UPPS deposited in the Protein Data Bank [32] as 4H8E were used as a template to model a fragment of AtCPT6 comprising amino acids L65–V302 using SYBYLx2.0, TRIPOS Inc.

Table 1

Mass spectrometry analysis of polyisoprenoid alcohols isolated from yeast *rer2Δ* strain expressing AtCPT6. *m/z* values of monoisotopic pseudomolecular ions $[M + Na]^+$ were recorded with HPLC/HR-ESI-MS.

Polyisoprenoid alcohol	Molecular formula	Molecular mass	
		Calculated	Measured
Pren-6	C ₃₀ H ₅₀ ONa	449.3759	449.3745
Dol-6	C ₃₀ H ₅₂ ONa	451.3916	451.3904
Pren-7	C ₃₅ H ₅₈ ONa	517.4385	517.4385
Dol-7	C ₃₅ H ₆₀ ONa	519.4542	519.4529
Dol-8	C ₄₀ H ₆₈ ONa	587.5168	587.5176

2.17. Chemicals

All dolichol and polyprenol standards were from the Collection of Polyrenols (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw). Silica gel plates and silica gel 60 for column chromatography were from Merck (Darmstadt, Germany), organic solvents (HPLC or p.a. grade) were from POCh (Gliwice, Poland). Murashige and Skoog Basal Salt Mixture and other chemicals were purchased from Sigma-Aldrich and were of analytical grade. RNeasy Plant Mini Kit was obtained from Qiagen (Hilden, Germany). DNase I, RNase-free, Taq DNA Polymerase, GeneRuler™ DNA Ladder and DNA Loading Dye were from Fermentas (Vilnius, Lithuania). SuperScript™ II First-Strand Synthesis System for RT-PCR was from Invitrogen (Carlsbad, CA).

3. Results and discussion

3.1. Expression of recombinant AtCPT6 protein in *S. cerevisiae*

Based on a putative Arabidopsis *cis*-prenyltransferase sequence available from GenBank (ID At5g58780) we amplified full-length coding

cDNA for AtCPT6 by RT-PCR using RNA isolated from roots and specific primers. To obtain the AtCPT6 protein, the cDNA was cloned using Gateway System (Invitrogen) into pYES-DEST52 vector and expressed in *S. cerevisiae* INVS c1 strain using galactose induction. The His-tagged AtCPT6 fusion protein was purified under native conditions using Ni-NTA gel and sequential eluates were analyzed by SDS-PAGE. The major band of ~37 kDa mainly recovered in eluates 1–5 corresponded well to the predicted mass of the His-tagged AtCPT6 (1 kDa + 35 kDa) (Fig. 2A). The identity of the purified fusion protein was analyzed by Western blotting. Anti-His-tag and specific anti-AtCPT6-peptide antibodies showed a positive signal only with the ~37 kDa product, whereas an antibody directed against another Arabidopsis CPT (AtCPT7) did not show cross reactivity with it (Fig. 2B). This observation further supported the identification of the obtained polypeptide as AtCPT6. Finally, mass spectrometry analysis (Mass Spectrometry Lab, IBB PAS) clearly confirmed the sequence of AtCPT6 expressed in *S. cerevisiae*.

3.2. Functional characterization of AtCPT6 activity in yeast *rer2Δ* mutant

To determine whether AtCPT6 is a functional enzyme with a *cis*-prenyltransferase activity it was expressed in a yeast mutant defective in *cis*-prenyltransferase activity. We tested the ability of the putative plant CPT to complement the activity of Rer2 in the *rer2Δ* yeast mutant which shows temperature-sensitive growth, defects in *N*-glycosylation and abnormal membrane morphology. Transformation with pYES-DEST52-AtCPT6, in contrast to the empty vector, restored growth of the *rer2Δ* mutant at 37 °C (Fig. 3A). This observation is in agreement with a previous report [22]. Since the putative involvement of AtCPT6 in protein glycosylation has not been studied so far we followed the effect of AtCPT6 expression in yeast on glycosylation of carboxypeptidase Y (CPY). In WT yeast cells only mature, fully glycosylated form of CPY

(mCPY) was detected by specific anti-CPY antibody, whereas in the *rer2Δ* mutant both the mature and four hypoglycosylated forms of CPY were observed (Fig. 3B). A similar profile of CPY was also noted for the *rer2Δ* mutant transformed with the empty vector. Transformation with AtCPT6 cDNA did not restore normal *N*-glycosylation of CPY, since both mature and hypoglycosylated forms of CPY were observed despite the fact that the AtCPT6 protein was present in the yeast cells as confirmed by Western blotting with a specific anti-AtCPT6 antibody (Fig. 3B).

HPLC/UV analysis of polyisoprenoids synthesized by *rer2Δ* yeast expressing AtCPT6 revealed a family of very short polyisoprenoid alcohols composed of 6 to 8 i.u., with the homologue composed of 7 i.u. dominating (Fig. 4). Accumulation of these polyisoprenoids was obviously dependent on the presence of AtCPT6 since in the *rer2Δ* mutant transformed with the empty vector no such products were observed (Fig. 4). These data prove that the family of very short-chain polyisoprenoids is a product of AtCPT6 in the heterologous system.

Mass spectrometry analysis of these products revealed the presence of dolichols accompanied by traces of respective polyprenols and their identity was conclusively confirmed by the accurate mass measurements (HPLC/HR-ESI-MS) summarized in Table 1; the MS analysis additionally revealed the presence of minute amounts of Pren-5 (data not shown).

Studies on the product specificity of typical plant CPTs producing polyisoprenoids described so far have been performed *in vitro*. The only exception is partial *in vivo* characterization of a unique CPT-SICPT1 synthesizing a single oligoprenyl chain composed of two isoprene units – it has been shown that RNAi-mediated suppression of SICPT1 led to a large decrease in β -phellandrene (metabolite produced from neryl diphosphate) [16]. Chemotype of SICPT1-OE plants has not been described yet. Studies of the typical plant CPTs include the analysis

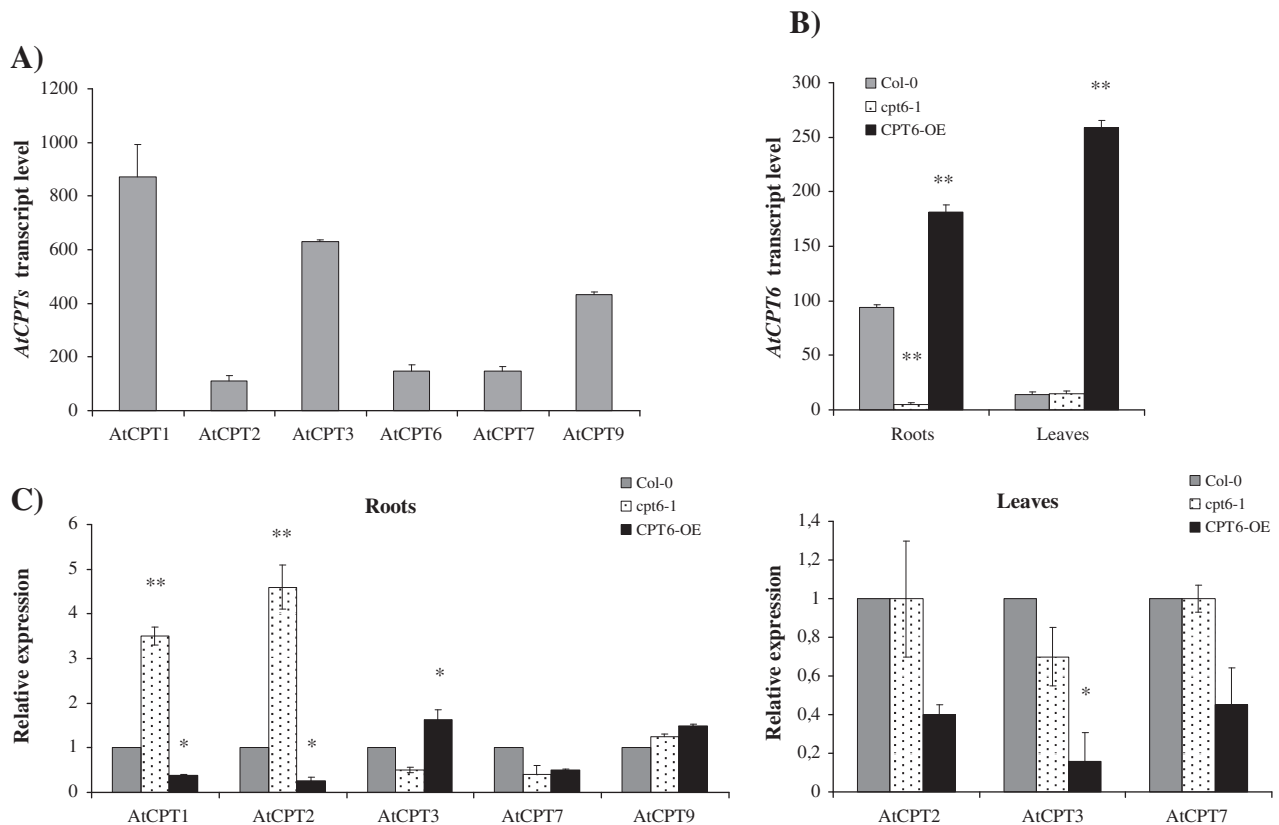


Fig. 5. Expression of AtCPT encoding genes in *A. thaliana*. A) Expression of *AtCPTs* in roots of WT (Col-0) plants. B) *AtCPT6* mRNA in roots and leaves of Arabidopsis plants: WT (Col-0), *AtCPT6* insertion mutant (*cpt6-1*) and *AtCPT6* overexpressing mutant (CPT6-OE). C) Effect of inhibition or overexpression of *AtCPT6* on the expression of other genes encoding *cis*-prenyltransferase isoenzymes in roots and leaves of Arabidopsis WT and mutants (*cpt6-1* and CPT6-OE). *AtCPTs* mRNA level was determined by qRT-PCR relative to Actin2. Data are mean $\pm$ SD of three independent measurements, error bars are indicated. *P* value was determined by Student's *t*-test. * indicates $p \leq 0.05$ and ** – $p \leq 0.01$.

of AtCPT1 and AtCPT6 for which Dol-18 and a mixture of polyprenols with dominating prenol composed of 7 i.u., respectively [21,22], have been identified as products. Similarly, various products were ascribed to the seven tomato SICPTs (a single prenol in a range Pren-2–Pren-4 to SICPT1, SICPT2 and SICPT6, a single Pren-13 to SICPT3, and medium-chain length products Pren-5–Pren-13 with no clear distribution profile to SICPT4, SICPT5 and SICPT7) [16]. Interestingly, since only one of the tomato CPTs, SICPT3, was shown to complement the growth defect of the *rer2Δ* mutant only this enzyme has been postulated to be involved in dolichol biosynthesis although its role in protein glycosylation has not been elucidated [16].

As mentioned above some of the plant CPTs produce *in vitro* a single product [16,21], in some cases mixtures of products are observed [16,22]. This calls into question the relevance of some results since accumulation of mixtures of mainly *cis*-polyisoprenoid alcohols has been reported *in vivo* in all eukaryotic tissues studied so far [1,33]. Moreover, yeast and human CPTs have been shown to synthesize *in vitro* mixtures of polyisoprenoid diphosphates which are then converted to dolichols [10,13]. A plausible explanation of the disturbed activity of plant CPT *in vitro* is a lack of regulatory/stabilizing effects exerted on the CPT activity by cellular regulatory/accessory proteins. Putative orthologs of a hCTP-interacting protein, NogoB receptor, identified recently in human cells [34], seems a good candidate for such a regulatory activity,

although its existence in plant cells is yet to be confirmed. Thus, results of *in vitro* assays of CPT activity should be viewed with caution and verified *in vivo*.

3.3. Expression of AtCPT6 and other AtCPTs in WT and mutant Arabidopsis plants

Previously, using semi-quantitative RT-PCR we have found that six AtCPTs, including AtCPT6 are expressed in Arabidopsis roots [5]. Real Time PCR (qRT-PCR) analysis performed here confirmed this and indicated that AtCPT1, -3 and -9 mRNA were most abundant in Arabidopsis WT roots (Fig. 5A). As expected, in the homozygous T-DNA insertion mutant line *cpt6-1*^{-/-} AtCPT6 mRNA was undetectable both in roots or leaves (Fig. 5B). In turn, the AtCPT6 transcript level was significantly increased in both studied organs of transgenic plants overexpressing AtCPT6 (CPT6-OE) relative to the WT control (Fig. 5B).

Analysis of the relative transcript level of other AtCPT encoding genes revealed variable changes. In the roots of the *cpt6-1*^{-/-} mutant noticeably increased expression of AtCPT1 and -2 relative to WT was observed while expression of the remaining CPTs was either slightly decreased (AtCPT3 and AtCPT7) or remained unchanged (AtCPT9) (Fig. 5C). As expected, AtCPT6 deficiency did not affect the expression of the other AtCPTs in the leaves of *cpt6-1* plants (Fig. 5C).

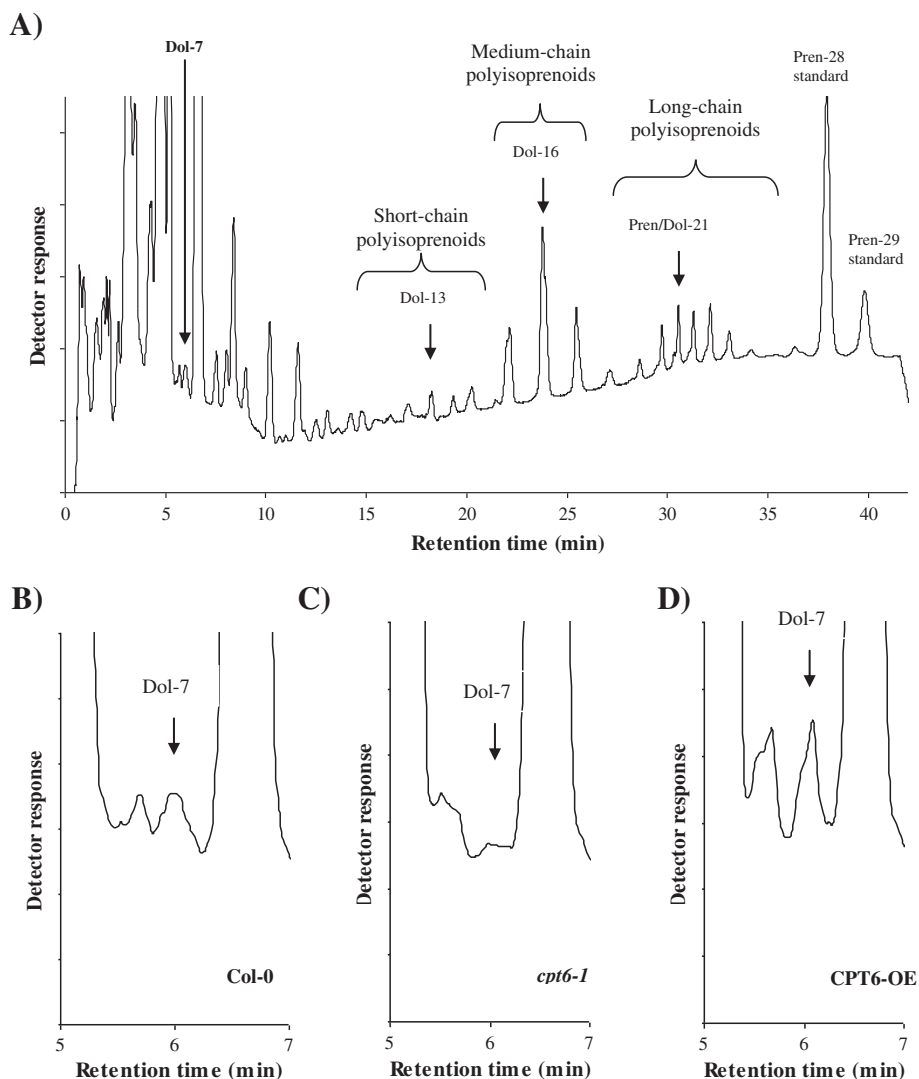


Fig. 6. Composition of polyisoprenoids isolated from *A. thaliana* roots. Shown are an HPLC/UV chromatogram of a total polyisoprenoid mixture from a 6-week-old Col-0 plant (A) and expanded regions of HPLC/UV tracks corresponding to very short-chain polyisoprenoids from the roots of WT (Col-0) (B), insertion mutant *cpt6-1* (C) and CPT6-OE plants overexpressing AtCPT6 (D). Short-, medium- and long-chain polyisoprenoid families and Dol-7 are indicated.

In the roots of AtCPT6 overexpressing plants (*CPT6-OE*) transcript levels of AtCPT1, -2 and -7 were decreased in contrast to the modest increase of the mRNA of the two remaining AtCPTs (AtCPT3, -9). In the leaves of *CPT6-OE* plants expression of all the AtCPTs was decreased.

Observed changes of the level of the AtCPT6 transcript in *cpt6-1* and *CPT6-OE* plants are in line with that expected for both types of mutants. Interestingly, increased levels of AtCPT1 and -2 mRNA in the roots of the *cpt6-1* mutant might suggest possible compensation of the AtCPT6 deficiency by these two enzymes and simultaneously it might indicate the existence of a mechanism responsible for regulation of these CPTs at the transcriptional level. Additionally, decreased expression of AtCPT1 and -2 in the roots of the *CPT6-OE* mutant confirms this supposition. Modulation of the level of AtCPT transcripts expressed in leaves of the *CPT6-OE* mutant points in the same direction although AtCPT6 is a root-specific enzyme and effects observed in the leaves have to be carefully considered.

AtCPT6 is postulated to play a role in the root cell response to abiotic stress [22]. Accordingly, AtCPT6 expression is induced by increased concentrations of sugars in the medium (3% glucose or sucrose), a form of stress [35]. As long as the cellular function of AtCPT6, as well as other AtCPTs, remains not fully established the concept of their cellular compensation remains elusive. Observations described above prompted us to analyze the profile of polyisoprenoid lipids accumulated in the roots of mutant plants.

3.4. AtCPT6 biosynthetic activity in planta

To identify the products of AtCPT6 activity in its native setting we compared the profiles of polyisoprenoids extracted from three types of plants: Col-0, the *cpt6-1* and *cpt6-2* insertion mutants, and plants overexpressing AtCPT6 (*CPT6-OE*). Recently, in wild type Arabidopsis hairy root culture three families of dolichols were detected: short-chain Dol-12 to Dol-14, with Dol-13 dominating, medium-chain Dol-15 to Dol-18, with Dol-16 dominating and long-chain Dol-19 to Dol-30, with Dol-21 or Dol-23 dominating [35]. A similar composition of the dolichol mixture was found in the roots of the wild type plants grown in hydroponic medium (Fig. 6A). Additionally, a careful inspection of the HPLC chromatograms revealed a small amount of Dol-7, a representative of very short-chain polyisoprenoids (Fig. 6B). Notably, the content of Dol-7 was considerably decreased in the roots of the *cpt6-1* and *cpt6-2* plants (Fig. 6C) – to approximately 23% and 42% of the WT control level for *cpt6-1* and *cpt6-2*, respectively (Table 2). Additionally, the content of short-chain dolichols (Dol-13 dominating) was also slightly decreased (to respectively, 73% and 89% of the control) whereas the content of the medium- and long-chain dolichols was not significantly different between *cpt6-1* and *cpt6-2*, and WT plants (Table 2). These observations strongly suggest that AtCPT6 inactivation suppresses the biosynthesis of the family of very short-chain polyisoprenoids (Dol-7 dominating) in planta.

To further confirm that AtCPT6 is responsible for the biosynthesis of the very short-chain polyisoprenoids we analyzed the composition of

polyisoprenoids extracted from roots of *CPT6-OE* plants (Fig. 6C). The amount of the very short-chain product (Dol-7) was increased to 271% in the level of WT plants. Also, the content of the short-chain dolichol (Dol-13) was increased (147% of the control). The content of the medium-chain polyisoprenoids (Dol-16) was increased only slightly (113% of the control), and of the long-chain ones (Dol-23) decreased (86% of the control) (Table 2).

Taken together these results show that the family of very short-chain polyisoprenoids (Dol-7 dominating) is the major product of AtCPT6 in the roots of Arabidopsis, confirming the results obtained in the heterologous yeast system in this study as well as in the *in vitro* AtCPT6 activity assay [22]. The observed fluctuations of the content of the family of short-chain polyisoprenoids (Dol-13 dominating) accompanying the affected Dol-7 levels suggest a possible involvement of AtCPT6 in their biosynthesis as well. Further detailed studies are needed to confirm this supposition.

3.5. Subcellular localization of AtCPT6

The majority of eukaryotic CPTs of non-plant origin are postulated to be associated with the ER membrane. *cis*-Prenyltransferase activity has been detected in the microsomal fraction from pig, calf and rat brain [36,37], and rat liver [38]. Subcellular localization of CPT of plant origin is far from being clear. ER localization of GFP-AtCPT6 in *A. thaliana* cells has been shown by Kera et al. [22]; however, only one of the seven tomato CPTs has been found in cytoplasm/ER (SICPT3) while six remaining SICPTs have been localized in plastids [16]. Moreover, CPT activity has been detected in ER of spinach leaves [39]. To follow the subcellular distribution of AtCPT6 we analyzed cytosolic (supernatant, S) and microsomal (pellet, P) fractions isolated from leaves and roots of Col-0 and *cpt6-1* plants using Western blotting. A specific antibody raised against AtCPT6 revealed an immunoreactive polypeptide with the expected molecular mass ~37 kDa in the microsomal fraction from Col-0 roots only (Fig. 7). As expected, no immunoreactive bands were detected in the subcellular fractions prepared from leaves or roots of an AtCPT6 T-DNA insertion mutant. The endoplasmic reticulum membrane marker BiP2, used as a positive control, was present predominantly in the microsomal fractions from both leaves and roots of WT and mutant plants. These observations are in agreement with the ER localization of GFP-AtCPT6 fusion protein in *A. thaliana* cells [22]. Accordingly, an *in silico*

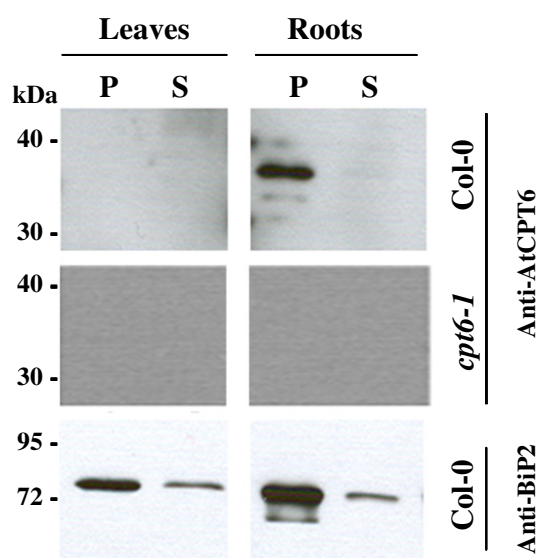


Fig. 7. Subcellular localization of AtCPT6. Proteins from cytosolic (supernatant, S) and microsomal (pellet, P) fractions isolated from leaves and roots of Col-0 and *cpt6-1* Arabidopsis plants were resolved by 12% SDS-PAGE, transferred onto nitrocellulose membrane and probed with specific antibodies directed against AtCPT6 and BiP2 (ER marker).

Table 2

Effect of AtCPT6 gene disruption or overexpression on accumulation of polyisoprenoid alcohols in Arabidopsis roots. Content of dominating dolichols of very short-, short-, medium- and long-chain polyisoprenoid families was determined in *cpt6-1* and *cpt6-2* T-DNA insertion mutants, and plants overexpressing AtCPT6 (*CPT6-OE*) and expressed as percentage of the corresponding values in WT (Col-0) plants. Values are means $\pm$ SD of four independent measurements.

	Dominating polyisoprenoid alcohol			
	Dol-7 (very short chain)	Dol-13 (short chain)	Dol-16 (medium chain)	Dol-23 (long chain)
WT Col 0	100%	100%	100%	100%
<i>Cpt6-1</i>	23% (± 9)	89% (± 9)	105% (± 15)	97% (± 10)
<i>Cpt6-2</i>	42% (± 4)	73% (± 17)	106% (± 19)	78% (± 23)
<i>CPT6-OE</i>	271 (± 83)	147 (± 42)	113 (± 3)	86 (± 10)

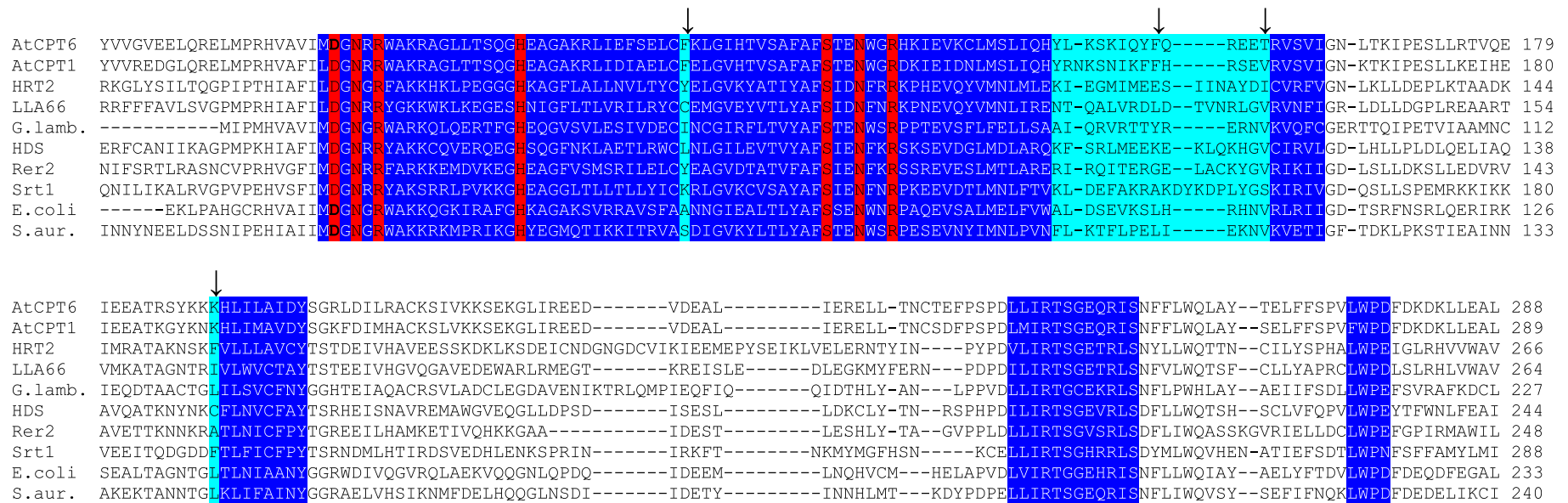


Fig. 8. Amino acid sequence alignment of selected *cis*-prenyltransferases. CPTs from *Saccharomyces cerevisiae*: Rer2 and Srt1 (P35196 and Q03175), *Giardia lamblia*: G.lamb. (A8B1Z2), *Arabidopsis thaliana*: AtCPT1 and AtCPT6 (O80458 and Q8RX73), *Hevea brasiliensis*: HRT2 (Q8W3U4), *Lilium longiflorum*: LLA66 (B2BA86), *Homo sapiens*: HDS (Q865Q9), *Escherichia coli*: E.coli (P60472), *Staphylococcus aureus*: S.aur. (P60477). Residues surrounding the active center and hydrophobic channel are shown in dark blue, those critical in determining polyprenyl chain length are indicated in light blue and residues forming the active center are shown in red. Arrows indicate residues postulated to limit the chain length of AtCPT6 products.

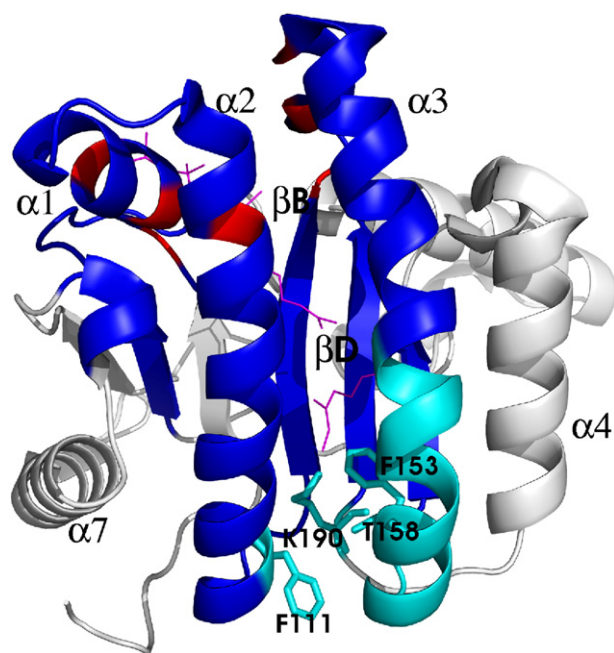


Fig. 9. Homology-based model of AtCPT6. Dark blue ribbon is adjacent to the hydrophobic channel (substrate/product binding pocket) with amino acid residues determining product chain length in light blue. Active center is in red, and substrate/product polyisoprenoid skeleton in magenta. Residues essential for product chain length are shown as sticks.

analysis (TMHMM server) suggested the presence of a transmembrane motif in the AtCPT6 sequence. Additionally, a search in the databases (e.g. BaCelLo, TargetP) also indicates ER/Golgi localization of AtCPT6, similar to AtCPT1 and AtCPT8, while plastidial or nuclear localization is predicted for remaining AtCPTs.

3.6. Homology modeling of AtCPT6

Based on the approximately 40% identity between the L65–V302 fragment of AtCPT6 and *S. aureus* UPPS revealed by the sequence alignment (Fig. 8), we expected a high similarity of the structures of these enzymes. The only difference was the flexible region between α -helix 6 and β -sheet E (L235–P242), which presumably is not important for the enzyme catalytic function. A model of the structure of AtCPT6 monomer is shown in Fig. 9. All the residues suggested to be involved in the catalysis for *E. coli* UPPS [40], namely D79, N81, R83, H96, S124, N127, and R130 (numbering according to AtCPT6) were conserved between these remote organisms (eukaryotic and prokaryotic), indicating the location of the AtCPT6 active center (Figs. 8, 9). On the other hand, residues comprising fragment of helix α 3 and the loop between α 3 and β C defined earlier as important for determination of the polyprenol chain length [7,41], highlighted by the blue box in Fig. 8 and indicated as a blue ribbon in Fig. 9, were highly variable. The substrate/product binding pocket – a long hydrophobic channel (Fig. 9, adjacent to the blue ribbon) surrounded by helices α 2 and α 3- and β -sheets A, B, C and D – was mainly composed of hydrophobic amino acid residues. The substrate/product binding pocket of *E. coli* UPPS is blocked by the side chain of L137 [42]. In AtCPT6 the corresponding is K190 residue, which is in fact bulkier. Interestingly, in other aligned CPTs (Fig. 8) the residues corresponding to L137 of *E. coli* were highly diverse. In contrast, V105 of *E. coli* also predicted to be of importance in determining the product chain length [42] was rather conserved among the CPTs compared, although in AtCPT6 it was replaced by a smaller residue of T158. The proximal location of L137 and V105 at the bottom of the hydrophobic channel of the *E. coli* UPPS, and correspondingly of K190 and T158 for AtCPT6 justified the volume sum of these pairs of amino acids to be compared; they turned out to be almost identical (228 Å³ and 229 Å³

for AtCPT6 and EcUPPS, respectively; approximate amino acid volumes according to [43]). This observation suggests an involvement of some other amino acids in the determination of the chain-length of the polyisoprenoids produced by the respective enzyme. Two non-conserved phenylalanine residues, F111 and F153 (Fig. 9), located at the very end of the hydrophobic channel of AtCPT6, corresponding to A58 and L100 in EcUPPS, might be possible candidates for this role.

Additionally, several small amino acids located in EcUPPS in the middle of the amino acid sequence and creating the hydrophobic channel are replaced by larger ones in AtCPT6 (e.g. S49, A53 and S99 EcUPPS are replaced by R102, F106 and Y152 AtCPT6, respectively). According to our model, however, these amino acids are not located in the interior of the channel and it precludes their role in determination of the chain length of AtCPT6 products (data not shown). Finally, Y145 AtCPT6 replacing A92 EcUPPS does not seem a good candidate to act as polyisoprenoid chain length determinant since other bacterial UPPSs, e.g. *Micrococcus luteus*, *Helicobacter pylori*, and *S. aureus*, contain larger cyclic amino acids at the equivalent positions (as shown in Fig. 8 for *S. aureus*). This supposition, however, requires further experimental verification.

Taken together, a comparison of the amino acids surrounding the hydrophobic channel described above suggested a stronger steric hindrance faced by the polyisoprenoid chain penetrating the substrate/product binding pocket of AtCPT6 than in the case of EcUPPS. Thus, keeping in mind that EcUPPS produces Pren-11 AtCPT6 should be expected to synthesize even shorter-chain polyisoprenoids, in accordance with the experimental characteristics of the AtCPT6 products presented above.

4. Conclusions

Taken together, both the model of AtCPT6 and the *in vivo* characteristics of the AtCPT6 products in the homologous and heterologous systems presented in this study indicate that a family of very short-chain polyisoprenoid diphosphates which are subsequently converted to polyisoprenoid alcohols composed of 6–8 i.u., with the dominating homologue composed of 7 i.u., is the main product of this enzyme. This is in accordance with AtCPT6 products recorded *in vitro* for partially purified enzyme [22]. A possible involvement of AtCPT6 in the formation of short-chain polyisoprenoid family with Dol-13 dominating seems intriguing yet so far speculative.

The complex mixtures of mainly *cis*-isoprenoids produced by CPTs in plant cells comprise species-specific metabolic fingerprints. The biological functions of these metabolites remain in most cases elusive. Some of them are utilized as substrates to generate, e.g., monoterpenes [44], some have been postulated to be involved in the plant adaptive response to adverse environmental stimuli [45]. A similar role has been suggested for polyisoprenoids [22,46].

The reason for the presence of as many as nine highly similar genes encoding putative AtCPTs in the Arabidopsis genome is still not clear. Dissection of the products of particular AtCPTs will provide arguments to resolve this problem. Although redundant role of AtCPTs cannot be excluded, a tissue-specific expression pattern and product specificity could, in contrast, suggest specific functions of these enzymes in the plant cell metabolism.

Acknowledgements

The *S. cerevisiae* strains SS328 and YG932 used in this study were kind gifts of Dr. C.J. Waechter from the University of Kentucky College of Medicine, Lexington, KY, USA.

This investigation was supported by a grant from the National Science Centre [DEC-2011/03/B/NZ1/00568] and a grant funded by the Polish National Cohesion Strategy Innovative Economy [UDA-POIG 01.03.01-14-036/09].

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