CHAPTER SIX

# *cis*-Prenyltransferase and Polymer Analysis from a Natural Rubber Perspective

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## Abstract

Dolichol and natural rubber are representative *cis*-polyisoprenoids in primary and secondary metabolism, respectively. Their biosynthesis is catalyzed by *cis*-prenyltransferase (CPT) by sequential condensations of isopentenyl diphosphates (IPPs) to a priming molecule. Although prokaryotic CPTs have been well characterized, the mechanism of eukaryotic CPTs in *cis*-polyisoprene biosynthesis was only recently revealed. It was shown that eukaryotes have evolved a unique protein complex, comprised of CPT and CPT-binding protein (CBP), to synthesize *cis*-polyisoprenoids. In the context of this

*Methods in Enzymology*, Volume 576
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2016.02.026

new discovery, we found discrepancies in literature for CPT or CBP biochemical assays and in vivo CPT complementation using *rer2* (yeast *CPT*) yeast mutant. Our study here shows that *rer2* revertants occur at a frequency that cannot be disregarded and are likely accountable for the results that cannot be explained by the CPT/CBP heteroprotein complex model. To make a stable mutant, *SRT1* gene (secondary *CPT* expressed at a basal level in yeast) was additionally deleted in the *rer2Δ* mutant background. This stable *rer2Δ srt1Δ* strain was then used to individually or simultaneously express *Arabidopsis CPT1* (*AtCPT1*, *At2g17570*) and *CBP* (*AtLEW1*, *At1G11755*). We found that the simultaneous expression of *Arabidopsis CPT1* and *AtLEW1* effectively complements the *rer2Δ srt1Δ* strain, whereas the individual expression of *AtCPT1* alone or *AtLEW1* alone failed to rescue the yeast mutant. Microsomes from the dual expresser showed an efficient incorporation of IPPs into *cis*-polyisoprenoid (30% in 2 h). These results showed that the *CPT/CBP* heteroprotein complex model is valid in *Arabidopsis thaliana*. Experimental details of these results are described in this methodology paper.

## 1. INTRODUCTION

Isoprenoids (or terpenoids) are a diverse and large class of natural products with more than 70,000 known structures (Dictionary of Natural Products; http://dnp.chemnetbase.com). Some isoprenoids are indispensable in prokaryotic and eukaryotic organisms, playing essential physiological roles such as hormones (eg, testosterone, brassinolides, and gibberellin), membrane components (eg, cholesterols), and prenyl moieties of ubiquinone, plastoquinone, and prenylated proteins. Isoprenoid metabolism, therefore, forms an essential branch of primary metabolism that controls the growth and development of living organisms. On the other hand, an enormous structural diversity of isoprenoids has served as a treasured chemical reservoir, from which a number of pharmaceutical, nutraceutical, aroma, flavor, and industrial chemicals have been found or developed to benefit mankind (Gershenzon & Dudareva, 2007). Understanding the key principles governing the biosynthesis of isoprenoids has been a major research theme in the scientific community.

The two central precursors of all isoprenoids are five-carbon molecules, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Tholl, 2006). IPP and DMAPP are synthesized through either mevalonate or methylerythritol phosphate pathway, and one to three IPP molecules are sequentially condensed onto DMAPP to generate geranyl (GPP), farnesyl (FPP), and geranyl geranyl diphosphate (GGPP). Terpene synthase, a large and diverse enzyme family conserved across all the

kingdoms, utilizes the prenyl diphosphates as substrates (ie, GPP, FPP, and GGPP) to produce structurally diverse terpenes by carbocation cascade reactions (Chappell, 2002). The GPP, FPP, and GGPP synthases condense the isoprene moiety of IPP onto the DMAPP priming molecule in *trans*-configuration, and these enzymes are named *trans*-prenyltransferase (TPT). A number of biochemical studies have been carried out on TPTs, leading to the consolidated model to explain the structure and reaction mechanism of TPTs (Koksal, Jin, Coates, Croteau, & Christianson, 2011; Lesburg, Zhai, Cane, & Christianson, 1997; Starks, Back, Chappell, & Noel, 1997).

In contrast to the *trans*-configured condensation by TPT, IPP can be condensed to allylic diphosphates in the *cis*-configuration, and such reaction is catalyzed by *cis*-prenyltransferase (CPT). The most studied *cis*-isoprenoids are undecaprenol in prokaryote and dolichol in eukaryote (Fig. 1). Undecaprenol is polyisoprene alcohol synthesized in bacteria and functions as a sugar carrier molecule for peptidoglycan cell wall biosynthesis (Teng & Liang, 2012); dolichol is another polyisoprene alcohol used as a sugar carrier molecule for posttranslational modification (ie, glycosylation) of proteins on the endoplasmic reticulum in eukaryotes (Spiro, 2002). Undecaprenol and dolichol are structurally analogous except that the α-position of dolichol is saturated (Fig. 1). In the biosynthesis of these *cis*-polyisoprenoids, CPT catalyzes the condensations of 8–20 IPPs onto FPP to synthesize *cis*-polyisoprenes built on the *trans*-, *trans*-FPP priming molecule. Although the reaction mechanism of TPT and CPT are similar, the isolation of the

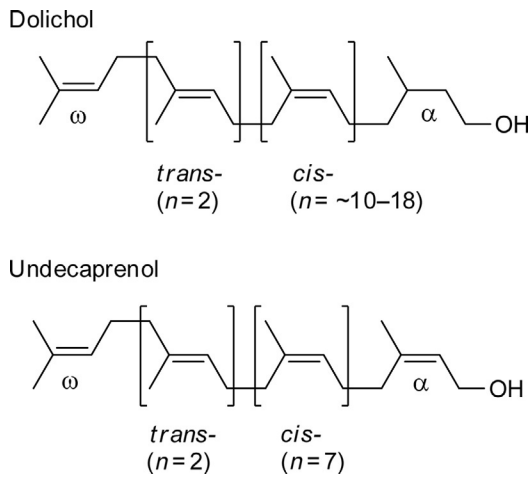


**Fig. 1** Structures of dolichol and undecaprenol.

first CPT from bacteria and its crystal structure showed that its primary and tertiary structures have no relationship to those of the TPT-family enzymes (Fujihashi et al., 2001), indicating that the TPT and CPT enzymes have evolved independently.

Another important *cis*-polyisoprenoid is natural rubber (NR), an important industrial biopolymer primarily produced from the Brazilian rubber tree (*Hevea brasiliensis*). NR possesses properties of superior resilience, heat dispersion, and abrasion resistance over synthetic rubber derived from petrochemicals, thereby being considered as an irreplaceable and strategically important industrial raw material (Cornish, 2001). Although more than 2500 plant species are known to produce NR, the Brazilian rubber tree is the only species that produces NRs in a commercially viable quantity and polymer length (van Beilen & Poirier, 2007). To be useful in industry, the average molecular weight of NR needs to be sufficiently large (>1 million Dalton). However, only a limited number of plant species, such as guayule, lettuce, and dandelion, are known to produce sufficiently lengthy NR biopolymers (Bushman et al., 2006; Mooibroek & Cornish, 2000). The simplest view of NR biosynthesis is that specialized CPTs emerged from undecaprenol and dolichol CPT catalyze the condensations of more than 10,000 IPPs without being terminated at the oligomeric length. Rubber elongation factor, small rubber particle protein, and CPT have been implicated in NR biosynthesis (Chakrabarty, Qu, & Ro, 2015; Collins-Silva et al., 2012; Hillebrand et al., 2012; Post et al., 2012), but reconstitution of NR biosynthesis in an in vitro condition or in surrogate hosts has not been achieved. The exact mechanism of NR biosynthesis remains still elusive at present.

Cumulative TPT studies have allowed consensual conclusions regarding the mechanism and metabolism of TPTs and their products, but the CPT-mediated biosynthesis of *cis*-isoprenoids including NR is not fully understood. Notably, recent four independent studies in human (Park et al., 2014), lettuce (Qu et al., 2015), dandelion (Epping et al., 2015), and tomato (Brasher et al., 2015) identified a new protein for CPT activity, conserved across the eukaryotic lineages. These works demonstrated that eukaryotes have developed a unique heteromeric CPT protein complex. Differently from the self-sufficient prokaryotic CPT, the heteroprotein complex is composed of a catalytic CPT and an unusual nonenzymatic protein, referred to as *CPTL* (CPT-like protein) in lettuce, *NgBR* (Nogo-B receptor) in human, *CPTBP* (CPT-binding protein) in tomato, and *RTA* (rubber transferase activator) in dandelion. These orthologous proteins do not

possess the conserved motifs for CPT catalysis but directly interact with catalytic CPTs. Remarkably, silencing of the respective gene markedly reduced dolichol (tomato) or NR (lettuce and dandelion). In this chapter, we will refer this nonenzymatic protein as CBP (CTP-binding protein) for simplicity.

Collectively, the data from these reports concluded that two classes of CPTs are present; self-sufficient prokaryotic CPTs in bacteria and chloroplasts of plants, and self-insufficient CPTs which must form a heteroprotein complex with CBP to acquire the CPT enzymatic activity. These eukaryotic CPT/CBP complexes are conserved from yeast to human and are involved in dolichol and NR biosynthesis. The results from different organisms are complementary to each other and also validated data from different systems. Curiously, some previously reported results are not congruent to the model of eukaryotic CPT/CBP complexes that seems nonrebuttable based on the data from the four independent groups. Some examples are: (1) *Arabidopsis* CBP ortholog, *AtLEW1* showed in vitro CPT activity by itself when expressed in *Escherichia coli* (Zhang et al., 2008); (2) dandelion eukaryotic type *CPT* alone without *CBP* could functionally complement the mutant yeast strain that has a lesion in yeast *CPT* (*rer2* temperature sensitive strain, $rer2^{ts}$) (Schmidt et al., 2010); (3) rubber tree CPT purified from *E. coli* could synthesize NR of ~1 million Dalton without *CBP* coexpression with an addition of unknown latex components (Asawatreratanakul et al., 2003). Considering the substantial influences of these works in the community of plant biochemistry, it is necessary to discuss possible technical issues and incongruent results of such previous reports in the context of recent new findings.

In this chapter, we identified the key problem of using the *rer2* yeast strain for *CPT* complementation assays, and then developed a new yeast strain suitable for *CPT* complementation. Furthermore, in the new strain background, *Arabidopsis CPT1* (*At2g17570*) and *CBP* (*AtLEW1*) were expressed to examine their biochemical activities by in vivo complementation and in vitro enzyme assays using microsomes. The experimental rationales, detailed procedures, and *Arabidopsis CPT/CBP* (*AtCPT1/AtLEW1*) functional data are described here.

## 2. RATIONALE: OBSERVATION OF REVERTANTS FROM *rer2* MUTANT

In yeast (*Saccharomyces cerevisiae*), *RER2* encodes the dehydrodolichyl diphosphate synthase that catalyzes the CPT reaction in dolichol metabolic

pathway. The null mutation of *RER2* in yeast (*rer2Δ*) is not entirely lethal, due to a weak expression of the second copy of *CPT* encoded in *SRT1*, and it grows extremely slowly (Sato, Fujisaki, Sato, Nishimura, & Nakano, 2001; Sato et al., 1999). However, such a slow growth pattern makes it cumbersome to use the *rer2Δ* for research. To overcome this, a temperature sensitive *rer2* mutant (*rer2$^{ts}$*) was identified that grows normally at a room temperature (RT) (23°C) but cannot grow at a nonpermissive temperature (37°C) (Sato et al., 1999). Since then, *rer2$^{ts}$* strain has been routinely used to evaluate in vivo activities of the *CPTs* (ie, dehydrodolichyl diphosphate synthases) from various plant species, including those of rubber tree and Russian dandelion.

We obtained the *rer2$^{ts}$* (SNH23-7D), which has *rer2* gene with a point mutation at the 209th residue (S209N), and assessed the feasibility to use this strain to measure CPT activities. To perform control experiments, the *rer2$^{ts}$* strain was first transformed with the empty yeast plasmid (p414) for a negative control or with the same vector expressing *RER2* under *GPD* promoter (p414-*RER2*) for a positive control. The two transformants were incubated on the selective solid medium at 23°C for 5–7 days. Both yeast transformants grew normally and formed a number of independent colonies on the plates at 23°C (Fig. 2A and B). These results confirmed that *rer2$^{ts}$* shows a normal growth phenotype at 23°C, as expected from other works.

In parallel, the same transformants were plated and incubated at a nonpermissive 37°C for 5–7 days. In these experiments, the *RER2*-expressing *rer2$^{ts}$* strain is expected to grow, but the empty plasmid-transformed *rer2$^{ts}$* strain is not expected to grow because the S209N point mutation makes RER2 unstable at 37°C. As predicted, the *RER2*-expressing *rer2$^{ts}$* showed a normal growth at 37°C (Fig. 2C). However, we consistently observed yeast colonies on the plates where the *rer2$^{ts}$* yeasts transformed with the empty plasmid were incubated at 37°C (Fig. 2D, arrow). The frequency of this apparent revertant was estimated to be 1 out of ~20,000 yeast cells, and this rate is not too low to ignore in complementation assays. To ensure that the revertant is not due to the problem in a specific batch of the *rer2$^{ts}$*, we received a new *rer2$^{ts}$* (SNH23-7D) strain again from Dr. Nakano laboratory (RIKEN, Japan) and repeated the experiments again with this freshly acquired strain. However, revertants still occurred in this independent experiment with a similar frequency.

To observe the growth rates of these transformants more accurately, colonies from each plate were selected and cultivated in liquid medium at a nonpermissive 37°C, and their growth rates were measured

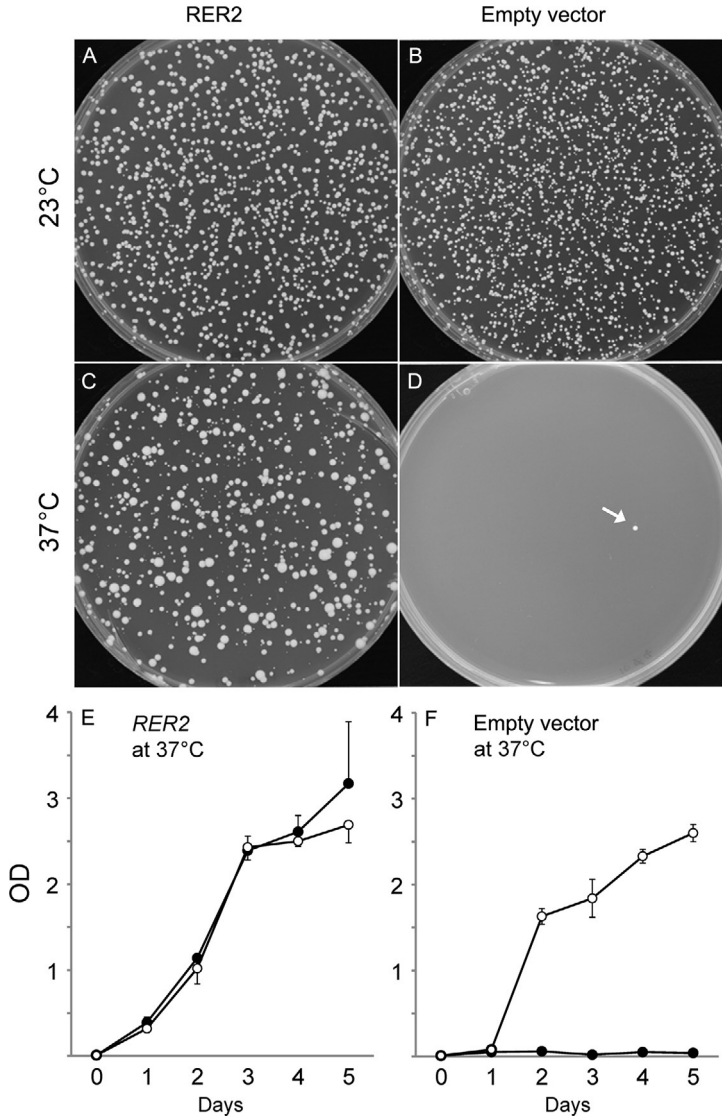


**Fig. 2** Control experiments of *rer2*$^{ts}$ mutant after transforming with the empty plasmid or expressing *RER2*. (A) and (B) Yeast *rer2*$^{ts}$ mutants were grown at a permissive temperature (23°C) after transforming with the empty plasmid or *RER2*. (C) and (D) Yeast *rer2*$^{ts}$ mutants were grown at a nonpermissive temperature (37°C) after transforming with the empty plasmid or *RER2*. The plate in D is one representative from multiple experiments, and the *arrow* in D indicates a colony from revertants. (E) and (F) Growth patterns of the yeasts at 37°C were plotted by measuring optical density (OD) at 600 nm. *Black circles* are from the yeasts preselected on solid medium at 23°C (A or B), and *white circles* are from the yeast preselected on solid medium at 37°C (C or D). Data are mean ± S.D. from three independent transformants.

spectroscopically at various time points. The *rer2*$^{ts}$ yeasts expressing *RER2* showed similar growth rates at 37°C whether they were preincubated at 23 or 37°C (Fig. 2E). This result confirmed that the overexpression of *RER2* can competently complement the *rer2*$^{ts}$ mutation at a nonpermissive temperature. On the other hand, the *rer2*$^{ts}$ yeast harboring the empty vector, preselected on the plates at 23°C, showed no growth at 37°C (Fig. 2F, black circles), displaying a predicted temperature sensitive behavior. On the contrary, the empty vector harboring *rer2*$^{ts}$ strain grown at 37°C (referred to as the revertant from here on) showed a growth rate almost identical to the *RER2*-expressing *rer2*$^{ts}$ in liquid medium (Fig. 2F, white circles). These observations demonstrate that the revertant has an equally restored growth pattern as the *RER2*-expressing *rer2*$^{ts}$.

Although the observed revertant problem in complementation assays was not explicitly stated, an article reporting the occurrence of revertants from the null *rer2* mutant (*rer2Δ*) population can be found in literature (Schenk, Rush, Waechter, & Aebi, 2001). Intriguingly, in the lipid profile analysis of the revertants, dolichols produced from the *rer2Δ* revertants were longer (19–22 isoprene units) than those synthesized from wild-type yeast with an intact RER2 (14–18 isoprene units). Because it is known that SRT1 (the second dehydrodolichyl diphosphate synthase) synthesizes isoprene polymers with 19–22 isoprene units, authors proposed that the activation of *SRT1* is the direct cause of the revertants in *rer2Δ*. Based on this and our own observation from the *rer2*$^{ts}$, we concluded that the complementation assays using *rer2*$^{ts}$ or *rer2Δ* strain must be performed with extreme cautions or is preferable to be abandoned. In addition, this implied that previous complementation results using *rer2*$^{ts}$ strain need to be interpreted with cautions. Accordingly, we pursued to generate a double knockout yeast strain (*rer2Δ srt1Δ*) for CPT and CBP complementation and biochemical assays. Detailed procedures are given below.

## 3. GENERATION OF *rer2* AND *srt1* DOUBLE KNOCKOUT YEAST STRAIN

### 3.1 Background on *rer2Δ* Strain

To generate *rer2Δ* and *srt1Δ* double knockout strain, we deleted *SRT1* on the *rer2Δ* background. The *rer2* null mutant yeast strain (*MATα rer2Δ*::*HIS3 ade2 trp1 his3 leu2 ura3 lys2* pRS316-*RER2*-*URA3*) was obtained from Dr. Nakano (RIKEN, Japan). Since the *rer2Δ* strain grows extremely slowly, this strain is maintained by expressing *RER2* in pRS3-*URA3* plasmid,

which can be removed on 5-fluoroorotic acid (5-FOA) plate where *URA3* gene product converts 5-FOA to a cytotoxic product.

## 3.2 Deletion of *SRT1* on *rer2Δ* Strain

### 3.2.1 Preparation of Homologous DNA

1. *SRT1* null mutation (*srt1Δ*) was generated in *rer2Δ* background by replacing *SRT1* open-reading frame (ORF) with a KanMX cassette. A linear piece of DNA for replacement was created by stitching together three pieces of DNA fragments—UP homologous fragment, DOWN homologous fragment, and KanMX fragment. Each of approximately 500 bp of UP and DOWN homologous regions has identical sequences at the S288C genome coordinates of 470205-469635 and 468681-468159, respectively, covering the *SRT1* ORF.
2. The three DNA fragments were PCR amplified using primer sets in Table 1 (primers are designed with $T_m$ of ~60°C). Note that the UP reverse and DOWN forward primers contain overhangs (~60–62°C, $T_m$) complementary to 5′- and 3′-end of the KanMX fragment, respectively. Each PCR fragment must be gel purified for optimal stitching. For the stitching reaction, three purified fragments were combined in an equimolar ratio to a total DNA amount of 100–200 ng in a 20 μL volume containing 0.2 units of Q5 High-Fidelity DNA Polymerase (NEB), 1 × Q5 Reaction Buffer (NEB), and 0.2 m*M* dNTP. Five cycles of PCR were performed (98°C for 20 s, 60°C for 30 s, and 72°C for 30 s).
3. Once three fragments were stitched to a single fragment by the first five cycles of PCR, this stitched fragment was further PCR amplified by UP forward and DOWN reverse primers. The entire stitching reaction is combined with additional 0.3 units of Q5 High-Fidelity DNA Polymerase in a final volume of 50 μL in 1 × Q5 Reaction Buffer, 0.2 m*M* dNTP, 0.4 μ*M* UP forward primer, and 0.4 μ*M* DOWN reverse primer in a typical PCR program for Q5 DNA Polymerase (1 cycle of 98°C/30 s, 30 cycles of 98°C/10 s, 62°C/20 s, and 72°C/1 min, 1 cycle of 72°C/2 min). The final PCR product should be gel purified as there will be undesirable spurious products (as visible on gel) that are stitched or amplified during PCR. Finally, 2–3 μg of the correct size DNA was purified in the elution volume not exceeding 60 μL.

### 3.2.2 Yeast Transformation with Linear DNA

1. Transformation was carried out using a typical yeast transformation protocol with additional steps to select for antibiotic resistant yeast.

**Table 1** Primers Used in This Study

| Primer Names | Sequences |
|---|---|
| SRT1Up_F | GAGTCGAAGCTTCAACTCG |
| SRT1Up_R | GGACGAGGCAAGCTAAACAGATCTTTGGAGTAGTTACCCTTAACAGG |
| SRT1Down_F | GATACTAACGCCGCCATCCATGCTATGGCAAGTACATGAAAATG |
| SRT1Down_R | GGGATGAAAATAGCATACCTGAG |
| KanMX_F | AGATCTGTTTAGCTTGCCTCG |
| KanMX_R | TGGATGGCGGCGTTAGTATC |
| SRT1KO_CF | GTGTTTAGAGCAGATATGCCC |
| AtCPT1_F | ATATACTAGTAACATGGCTGAACTTCCTGGTCAA |
| AtCPT1_R | CGTCATCCTTGTAATCCCCTAATTGTTTCTTCCTCTTTTCCAAGTATG |
| FLAG_R | ATATCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCCCC |
| AtLEW1_F | ATATACTAGTAACATGGATTCGAATCAATCGATGC |
| AtLEW1_R | ATATCTCGAGTTAAGTTCCATAGTTTTGGTGGACTCC |
| RER2_F | ATATACTAGTAACATGGAAACGGATAGTGGTATAC |
| RER2_R | CGTCATCCTTGTAATCCCCATTCAACTTTTTTTCTTTCAAATCGAT |

*Note*: Underlined sequences are complementary to KanMX sequence. *KO*, knockout. PCR amplification for FLAG-tagged genes (ie, AtCPT1-FLAG and RER2-FLAG) was performed twice, first with partial-FLAG reverse primer (primer pair: AtCPT1_F/AtCPT1_R, RER2_F/RER2_R) and second with complete-FLAG reverse primer (primer pair: AtCPT1_F/FLAG_R, RER2_F/FLAG_R). SRT1KO_CF (confirmation forward primer) was used with KanMX R primer for the PCR screening of yeast colonies to confirm *SRT1* deletion.

Mid-logarithmic phase yeast cells were harvested by centrifugation at $3000 \times g$, 3 min, RT. Yeast pellet was washed in sterile water once and centrifuged again as before. Approximately $10^7$ yeast cells were transformed per DNA construct. Washed yeast cells were resuspended in a small volume of sterile water and divided up to the number of transformations being performed (ie, the number of constructs) in microfuge tubes. The yeast cells were spun down again at $10{,}000 \times g$, 30 s, RT, and the water is removed.

2. A mixture of sterile Polyethylene glycol [50% PEG (w/v), average Mn 3350], sterile 1 *M* lithium acetate, and sterile denatured salmon sperm

DNA (SSD, 10 mg $mL^{-1}$ in water) was made in a ratio of 120:18:25 (v/v/v), respectively. The yeast cell pellet in the microfuge tube was combined with 2–3 μg (in <60 μL volume) of purified linear DNA and 163 μL of PEG/LiAc/SSD and mixed gently by pipetting. The yeast/DNA/PEG mixture was heat shocked in 42°C water bath for 30–40 min.

3. After incubation, yeast cells were centrifuged at 10,000 × *g*, 1 min, RT. The yeast pellet was resuspended in 1 mL of YPDA medium, transferred to a culture flask containing 10–20 mL of YPDA, and cultured for 18 h at 30°C. This recovery time in rich medium is necessary to allow for homologous integration and expression of the antibiotic resistance gene. If the recovery time is too short or too long, the transformation efficiency will suffer due to an inefficient integration or wild-type yeast override, respectively. Several 200 μL aliquots of the 18-h culture were plated on YPDA agar plates containing 300 μg $mL^{-1}$ Geneticin (G418). If selection needs to be done on a minimal medium, the nitrogen source needs to be replaced with monosodium glutamate because ammonium sulfate will significantly reduce the effectiveness of the G418.
4. Yeast colonies were screened by colony PCR method using one primer within the selection marker (ie, KanMX reverse primer) and one primer corresponding to the region outside of the homologous regions (ie, forward sequence upstream of UP forward primer in the genome, Table 1). The positive yeast strain is selected on G418 medium once more prior to glycerol stock preparation. Among the positive yeast strains, a couple of strains were verified by sequencing the PCR product of *SRT1* locus amplified from the genomic DNA to ensure the deletion of *SRT1* ORF.
5. For subsequent manipulation of this yeast strain, such as yeast transformation for complementation assay and 5-FOA selection, minimal media without G418 was used. The original *rer2Δ*::*HIS3* [pRS316-*RER2*] strain displayed a slower growth than the wild-type yeast, despite of the presence of a plasmid copy of *RER2*. This slow growth was consistently present in our newly created strain, *rer2Δ*::*HIS3 srt1Δ*::*KanMX* [pRS316-*RER2*].

## 4. COMPLEMENTATION OF *rer2Δ srt1Δ* WITH *CPT* AND *CBP*

### 4.1 Construct Generation

*Arabidopsis* CPT numbering system suggested by Kera, Takahashi, Sutoh, Koyama, and Nakayama (2012) was used for this work (Kera et al., 2012). *Arabidopsis* encodes nine *cis*-prenyltransferase genes (*AtCPT1–9*)

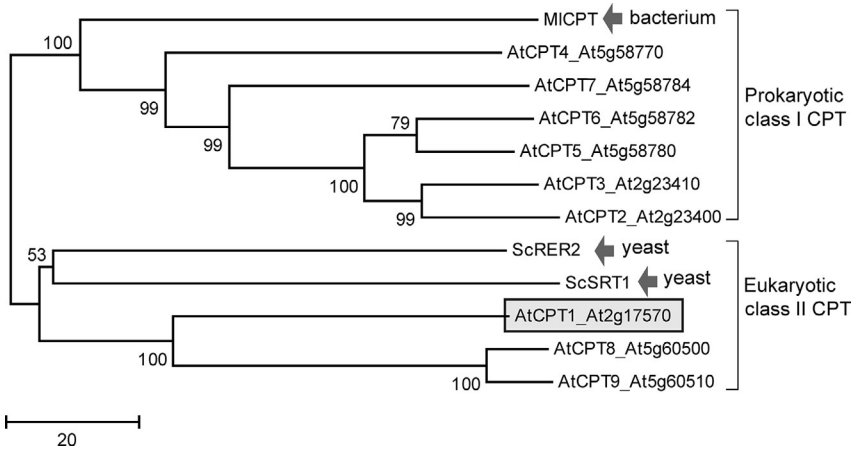


**Fig. 3** A phylogenetic analysis of the nine *Arabidopsis* CPTs. The phylogenetic tree was generated by MEGA5.2 software using a neighbor-joining algorithm (1000 replicates). Bootstrap values are shown in each node in percentage. *Arabidopsis* gene names are given beside the *CPT* numbers assigned by Kera et al. (2012). ScRER2 and ScSRT1 are yeast dehydrodolichyl diphosphate synthases; MlCPT is *Micrococcus luteus* undecaprenyl diphosphate synthase (AB004319).

and one *CBP*, *AtLEW1*. A phylogenetic analysis of the nine AtCPTs showed that AtCPT2–7 are clustered with the bacterial *Micrococcus luteus* undecaprenyl diphosphate (MlCPT), while AtCPT1/8/9 are clustered with the yeast CPTs (RER2 and SRT1, Fig. 3). The phylogenetic separation of these two groups (indicated as class I and class II in Fig. 3) is strongly supported by the bootstrap value 100 by the Neighbor-Joining algorithm. In addition, AtCPT2–7 encode a putative chloroplast-targeting motif in their N-termini, while *AtCPT1/8/9* proteins do not have such a motif. These data indicated that AtCPT2–7 are likely to be self-sufficient prokaryotic type CPTs that localize to the chloroplast, whereas AtCPT1/8/9 are not self-sufficient, eukaryotic type CPTs that require CBP, according to the CPT/CBP protein complex model. We chose *AtCPT1* (At2g17570) for functional study in this work (gray box in Fig. 3). *AtCPT1* and *AtLEW1* were amplified from *Arabidopsis thaliana* cDNA using primers listed in Table 1. *AtCPT1* was cloned into p414-GPD and *AtLEW1* was cloned into p415-GPD.

## 4.2 Yeast Transformation with Plasmids

Yeast transformation was carried out as described in Section 3.2.2 with the exception of long recovery incubation. Instead, the recovery was done by

**Table 2** Yeast Strains Before 5-FOA Selection (in *rer2Δ*::*HIS3 srt1Δ*::*KanMX* [pRS316-*RER2*] Background)

| Strain Name | Trp Marker Plasmid | Leu Marker Plasmid | Ura Marker Plasmid |
|---|---|---|---|
| Control | p414-GPD | p415-GPD | pRS316 RER2 |
| AtCPT1 | p414-GPD AtCPT1-F | p415-GPD | pRS316 RER2 |
| AtCPT1-LEW1 | p414-GPD AtCPT1-F | p415-GPD AtLEW1 | pRS316 RER2 |
| AtLEW1 | p414-GPD | p415-GPD AtLEW1 | pRS316 RER2 |
| ScRER2 | p414-GPD RER2-F | p415-GPD | pRS316 RER2 |

Grown in SC-His-Ura-Trp-Leu medium.
*Note*: F indicates FLAG tag.

incubation in YPDA medium for 1 h at 30°C in culture tubes. Recovered yeast cells were transferred to microfuge tubes and spun down at 10,000 × *g*, 1 min, RT. The yeast pellet was washed twice each with 1 mL of sterile water or selection minimal medium by spinning down as above. The entire pellet was resuspended in 200 μL of water and plated on Synthetic Complete medium (SC-His-Ura-Trp-Leu, based on Hopkins recipe). The yeast strains generated are listed in Table 2.

## 4.3 Selection in 5-FOA

1. To make 500 mL of 5-FOA medium plates, 10 g glucose, 3.35 g yeast nitrogen base without amino acid, 10 mg L-methionine, 10 mg L-arginine, 15 mg L-lysine, 10 mg adenine, 10 mg uracil, and 500 mg 5-FOA were dissolved in 250 mL water by rotating between 50°C water bath and a stir plate until completely dissolved (~30 min). This warm mixture was filter-sterilized, combined with autoclaved agar (10 g in 250 mL water), and poured to plates. Plates were dried at RT for 1–2 days before use or storage at 4°C. Minimal amount of amino acids and uracil were used in 5-FOA medium (SC-His-Trp-Leu) to maximize 5-FOA effectiveness, while regular minimal medium (SC-His-Trp-Leu-Ura) contained higher amounts of amino acids and nucleic acids (Hopkins recipe).
2. To examine the complementation of the *rer2Δ srt1Δ* strain by *AtCPT1/AtLEW1*, the *RER2* gene in URA-selection marker needs to be removed by 5-FOA selection. To achieve this, yeast colonies after a transformation were streaked on a 5-FOA-containing agar plate as well as on a regular agar plate as a control. A single colony from the

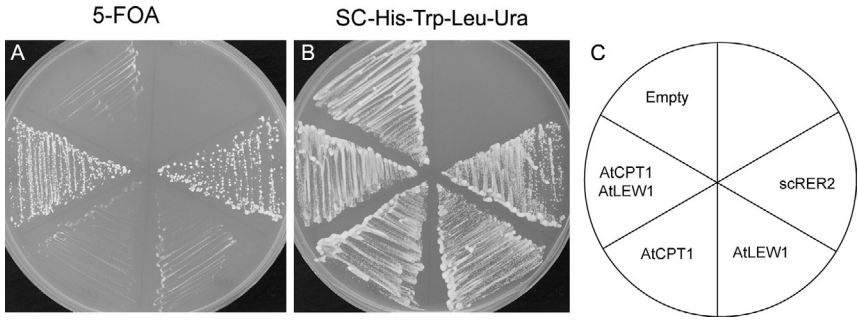


**Fig. 4** Functional complementation assays by expressing *AtCPT1* (At2g17570) and/or *AtCBP* (*AtLEW1*) in the *rer2Δ srt1Δ* double knockout yeast. (A) Yeast cultivation on 5-FOA counter-selective medium (SC-His-Trp-Leu + 5-FOA). (B) Yeast cultivation under the nutritional selective condition to keep all the plasmids listed in Table 2. (C) Experimental design. The *rer2Δ srt1Δ* double knockout is lethal and thus maintained by expressing yeast *RER2* (*ScRER2*) in pRS316-URA. The *rer2Δ* and *srt1Δ* were made by HIS and G418 marker, respectively. *CPT1* and *CBP* were expressed by TRP and LEU marker, respectively.

transformation plate was picked using a sterile flat toothpick. Approximately 5% of the picked colony was dabbed on the regular plate first (later streaked using a new toothpick), and the rest was streaked on a 5-FOA plate (Fig. 4). We recommend streaking multiple sets as 5-FOA selection requires a lengthy incubation. Growth in 5-FOA will select for yeast cells that lose the plasmid copy of *RER2* in URA3 marker and are complemented by the CPT activity from the coexpression of *AtCPT1* and *AtLEW1*. 5-FOA-containing plates were grown at 30°C for 10 days. Colonies growing on 5-FOA plates were picked and streaked on SC-His-Trp-Leu agar plates to recover from 5-FOA selection. To avoid colony variations on gene expression, multiple 5-FOA-resistant colonies were picked for generating glycerol stocks or liquid culture for microsome preparation.

## 4.4 Results

As the *rer2Δ srt1Δ* double knockout strain is lethal, this mutant was maintained by expressing *RER2* in pRS316-URA plasmid. When the *RER2* was removed on the 5-FOA counter-selective medium, no colony was detected in *rer2Δ srt1Δ* yeast strains transformed with the empty vector, *AtLEW1* alone, or *AtCPT1* alone in multiple experiments (Fig. 4). In other words, we never observed occurrences of revertants from the *rer2Δ srt1Δ* double knockout strain, and individual expression of *AtLEW1* or *AtCPT1*

cannot synthesize dolichol equivalent products to support the growth of *rer2Δ srt1Δ* yeast. These results suggested that the previously observed revertants were indeed caused by *SRT1* activation, and the *rer2Δ srt1Δ* double knockout strain is the ideal background to perform the *CPT* complementation assays. Yeast also encodes the *CBP* ortholog, *NUS1* (YDL193W), and NUS1 protein could interact with and activate eukaryotic CPTs, such as AtCPT1, to fully or partially rescue the lethal phenotype of *rer2Δ srt1Δ* strain. However, no sign of complementation was observed, when *AtCPT1* was expressed in *rer2Δ srt1Δ* strain (Fig. 4). This result indicates that yeast NUS1 cannot interact with and activate plant CPTs. The *rer2Δ srt1Δ* strain could be rescued only with the coexpression of *AtCPT1/AtLEW1* or with *RER2* on a different marker plasmid (positive control) (Fig. 4). The absence of complementation in the *rer2Δ srt1Δ* double knockout strain by *AtLEW1* alone or *AtCPT1* alone in this work is consistent with the recent reports of the CPT/CBP heteroprotein complex (Brasher et al., 2015; Epping et al., 2015; Qu et al., 2015) but is not congruent with the partial $rer2^{ts}$ complementation by *AtLEW1* and in vitro CPT activity of the *E. coli*-produced AtLEW1 (Zhang et al., 2008).

## 5. CPT BIOCHEMICAL ASSAY USING YEAST MICROSOMES

### 5.1 In Vitro CPT Assays

In order to measure eukaryotic CPT activity in vitro, a few facts from the recent studies need to be considered. First, since the *rer2* mutant ($rer2^{ts}$ or *rer2Δ*) is not stable, and the *rer2* mutant population can be overridden by revertants at any time during liquid cultivation. Therefore, *rer2* is not a suitable system to measure CPT activity. On the other hand, wild-type yeast has endogenous RER2 and SRT1 activities, making it difficult to separate the recombinant CPT activity from the endogenous ones. Second, CPT alone appears to be unstable in vivo without forming a complex with CBP. Silencing *CBP* resulted in the reduction of CPT protein without perturbing *CPT* transcripts in lettuce (Qu et al., 2015). Third, no obvious membrane-bound domain is present in CPTs, but all CBPs have clear integrating membrane domains in their N-termini. Localization studies using green fluorescent protein showed that CBPs are localized to the endoplasmic reticulum (Brasher et al., 2015; Epping et al., 2015; Qu et al., 2015). Four, CPT and CBP directly interact with each other, organizing heteroprotein complexes on the endoplasmic reticulum. Five, if CPT and CBP are separately

expressed and then mixed together in vitro, no CPT activity was observed, indicating these two must be simultaneously expressed in a single cell (Park et al., 2014). Considering all these factors together, the most appropriate approach for CPT in vitro assay is to perform assays using the microsomes isolated from the *CPT/CBP* coexpressing *rer2Δ srt1Δ* strain. Here we performed the in vitro assays of *AtCPT* and *AtLEW1* using the microsomes prepared from *RER2*-expressing (positive control) or *AtCPT1* and *AtLEW1* coexpressing *rer2Δ srt1Δ* strain.

## 5.2 Yeast Microsome Preparation

### 5.2.1 Yeast Culture

The yeast strains used to prepare microsomes are listed in Table 3. A starter culture of 1–2 mL SC-His-Trp-Leu was inoculated from freshly growing, multiple colonies on a plate and grown overnight at 30°C. A main culture of 50–100 mL SC-His-Trp-Leu was inoculated with 1 mL of the starter culture. The main culture was grown for approximately 15–20 h to a late logarithmic phase. Depending on the quality of the complementation, growth rate may be affected and may require longer culturing. The yeast culture was spun down at 3000 × *g*, 3 min, RT and washed once with 50 mL of water and once with 10 mL of TES-B buffer (0.6 *M* Sorbitol, 50 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA). The yeast pellet may be used immediately to prepare microsomes or stored at −80°C.

### 5.2.2 Bead Beating

1. The yeast pellet (from 50 mL culture) was resuspended in a final volume of 1 mL of cold TES-B and transferred to a prechilled 2-mL screw-cap vial. Prechilled glass beads (0.5 mm diameter, Biospec Products, Cat. 11079105) were added to the vial leaving approximately 15–20% void space in the vial. Bead beating was performed in a cold room by beating for 2 min at 3/4 of the intensity setting (Mini-Beadbeater-8, Biospec Products) and chilling in slurry of ice and water for 2 min. The cycle

**Table 3** 5-FOA-Selected Yeast Strains (in *rer2Δ::HIS3 srt1Δ::KanMX* Background)

| Strain Name | Trp Marker Plasmid | Leu Marker Plasmid |
|---|---|---|
| AtCPT1-LEW1 | p414-GPD AtCPT1-F | p415-GPD AtLEW1 |
| RER2 | p414-GPD RER2-F | p415-GPD |

Grown in SC-His-Trp-Leu medium.
*Note*: F indicates FLAG tag to the C-terminus of CPT.

was repeated total of three times. The broken yeast cells were examined under microscope to ensure majority (~80%) was broken. Punctured yeast cells will appear ghostly (ie, less defined cell wall than live cells) and completely broken cells will not be visible as cells, but as numerous small vesicles floating around. It is better to have less than perfect breakage since excessive bead beating will result in increased heat and increased handling time, which will jeopardize the quality of microsomal proteins.

2. To separate broken yeast cells from glass beads, various approaches can be taken. One method is pipetting out from the top of the bead-beating vial by repeated washing with a few mL of TES-B and combining the washed lysate. A better method, which is more high throughput and technically consistent, is to centrifuge yeast out of the bead-beating vial. The bead-beating vial was placed on a 1.5-mL microfuge tube with its cap completely cut off, and this structure was nested inside a 15-mL Falcon™ tube to collect the flow through yeast lysate in the microfuge tube by centrifugation at $2000 \times g$, 3 min, 4°C. All tubes were prechilled. To introduce a hole at the bottom of the bead-beating vial containing the glass beads and the yeast, 25-gauge syringe needle was flamed and used to puncture a hole. The tubes were taken out using a pair of forceps. The bead-beating vials can be washed once if desired to collect remaining yeast lysate by adding 1 mL of TES-B to the vial and centrifuging again.

### 5.2.3 Microsome Preparation

1. In order to remove large cellular debris, collected yeast lysate from above was centrifuged twice at $10{,}000 \times g$, 10 min, 4°C, once in the microfuge tube (1 mL volume) and once in a larger volume tube (10 mL volume). The supernatant from the first $10{,}000 \times g$ spin was transferred to a polycarbonate tube (the supernatant from the second wash combined) and combined with additional cold TES-B buffer to a total volume of ~10 mL, and the second $10{,}000 \times g$, 10 min, 4°C centrifugation was performed.
2. The supernatant is again transferred to a prechilled ultracentrifuge tube using serological pipet, ensuring not to disturb the pellet (to avoid contamination of microsomes with large cellular debris) and combined with additional cold TES-B buffer to a total volume of ~20 mL. The $100{,}000 \times g$, 1 h, 4°C centrifugation was performed in Beckman Coulter Ultracentrifuge with Type 70 Ti rotor. The reason for the increased

centrifugation volume especially in the ultracentrifugation step is to minimize precipitating soluble proteins with microsomes. In a highly dense solution of lysate, the soluble proteins may combine with microsomes and come down together during the ultracentrifugation. For the purpose of in vitro CPT assay, it is important to minimize the contamination by soluble proteins in the microsomal preparation, as it appears to contribute to some level of background activity.

3. The supernatant from the ultracentrifugation was discarded. The remaining microsomal pellet was washed twice gently each with 1 mL of cold TG buffer [50 m*M* Tris–HCl, pH 7.4, 20% (v/v) glycerol] to wash away soluble proteins in the tube. Note that the microsome pellet is sticky and will adhere to the pipet tip and microfuge tube. As much of the pellet as possible was picked up by a pipet tip and transferred to the microfuge tube. The remaining pellet was resuspended in cold 50 μL TG buffer in the ultracentrifuge tube and transferred to the same microfuge tube. Resuspend the microsome pellet gradually by soaking in TG buffer on ice for 10 min and pipetting up and down to resuspend. Repeat soaking/pipetting two to three times depending on how well the pellet resuspends. Ideally, no visible bits of the microsome pellet should be seen while holding the microfuge tube up against light and pipetting. Depending on the desired protein concentration, the microsome pellet can be resuspended in 50–200 μL cold TG buffer. Generally, we bring the total protein concentration to 10–15 μg $\mu L^{-1}$. Bradford assay was performed to determine the protein concentration and adequate aliquots were made for storage in –80°C and for in vitro enzyme assays.

## 5.3 *cis*-Prenyltransferase Enzyme Assays Using Yeast Microsomes and $^{14}$C-IPP

All the reaction components except for microsomes were combined into a master mix—50 m*M* HEPES, pH 7.5, 5 m*M* $MgCl_2$, 2 m*M* NaF, 2 m*M* $Na_3VO_4$, 2 m*M* DTT, 20 μ*M* FPP, and 80 μ*M* total IPP ($^{14}$C-IPP, PerkinElmer NEC773050UC, 50–60 mCi $mmol^{-1}$). The reaction was performed in organic solvent-safe 1.7 mL microfuge tubes with 50 μg of yeast microsomes in 50 μL reaction volume by incubating at 30°C for 2 h. Each reaction was set up in triplicate and extracted separately. Reaction products were extracted by adding 400 μL of 0.9% NaCl and 1 mL of chloroform: methanol (2:1), vortexing for 20 s, and centrifuging at 10,000 × *g*, 1 min, RT. The upper methanol/water phase was discarded, and the lower chloroform phase was transferred to a new microfuge tube. The chloroform phase was washed to remove unincorporated IPPs by adding 500 μL of

methanol:water:chloroform (48:47:3), vortexing and centrifuging as above. The lower chloroform phase was transferred to a new tube, and two more washing steps were performed. The chloroform phase after the final wash was transferred to a 2-mL screw-cap glass vial (gas chromatography vial). To count $^{14}C$ radioactivity, 60 μL (approximately 10% of the chloroform phase volume) was measured by scintillation counting with 4 mL scintillation cocktail. The remaining product was air-dried overnight in the fume hood.

## 5.4 Dephosphorylation

The dried product needs to be devoid of the phosphate group for size separation on reverse-phase thin layer chromatography (TLC). Dephosphorylation can be performed either enzymatically using potato acid phosphatase or chemically using acid hydrolysis.

### *5.4.1 Enzymatic Dephosphorylation*

Two to five units of potato acid phosphatase in 500 μL of buffer (50 m*M* sodium acetate, 0.1% Triton X-100, pH 4.7, 60% methanol) were added to the dried product obtained from 50 μg of microsomes. The reaction was incubated at 37°C for approximately 18 h, followed by an equal-volume extraction with benzene. The potato acid phosphatase (Sigma, Cat. P1146) is crudely purified, lyophilized powder, and when dissolving in the buffer, significant impurities will remain as particulates. The impurities are centrifuged down and only the supernatant is used for dephosphorylation.

### *5.4.2 Chemical Dephosphorylation*

Since enzymatic dephosphorylation is costly and time-consuming, we often use HCl hydrolysis. Alternative to using phosphatase, 300 μL of 1 *M* HCl was added to the dried product and incubated in 85°C water bath for 1 h. Once the reaction was cooled down to RT, the hydrolyzed product was extracted with benzene by adding 300 μL of benzene, vortexing for 30 s, and centrifuging at $4000 \times g$, 3 min, RT. Since the reactions are performed in a glass vial (ie, 2 mL screw-cap glass vials with PTFE closures) to avoid plastic contamination from benzene extraction, the glass vials are nested in 15 mL Falcon™ tubes for centrifugation. Two-third of the upper benzene phase was carefully transferred to a new glass vial without carrying over the bottom aqueous phase. To maximize recovery, additional 300 μL of benzene was added to the extraction vial, and 300 μL was transferred out to combine with the earlier transfer. The extracted product was either

air-dried overnight or dried under $N_2$ in the fume hood until an adequate volume remained (~20 μL) for spotting on a TLC plate.

## 5.5 Thin Layer Chromatography

For separating *cis*-polyisoprenoids, C18 reverse-phase silica with preadsorbent area on glass support (Analtech Cat. P90011, 20 × 20 cm) was used. Spots of 3–5 mm diameter were placed on the preadsorbent area 1 cm below the start of the reverse-phase area using microcapillary pipettes. Multiple drops were placed on the same spot until all benzene-dissolved samples was used. Too much product on the spot can result in poor separation; therefore, the optimal amount of the products for TLC separation and $^{14}C$ detection needs to be determined by loading various amounts. Ensure that the benzene is completely dried off of the TLC plate before placing in the separation solvent. Acetone:water (39:1) solvent system was equilibrated in a TLC chamber with filter papers to help maximize the amount of solvent vapor. The TLC plate should be placed in the chamber ensuring the bottom edge of the plate goes into the solvent evenly to allow for horizontal solvent front. *RER2* is known to produce dolichols of 14–18 isoprene units and was used as a positive control of the enzyme assay. An isoprenoid standard of a known size can be also spotted (C45, Solanesol). The solvent level was 5–10 mm from the bottom of the TLC plate. The run was stopped when the solvent front reached 1 cm before the end of the silica area (mark this line before you start the run). Immediately lay the TLC plate down flat to prevent solvent from pulling down the separation and to help rapid drying of the solvent. Once the plate was completely dried of the solvent, it was placed against a BAS Storage Phosphor Screen (GE Healthcare) in a film cassette, and depending on the amount of the $^{14}C$-labeled isoprenoid products, exposure was done for a few hours to a few days, followed by digitalization by scanning in a phosphorimager (Bio-Rad, Molecular Imager FX). Once adequate data are obtained, the plate can be stained in a chamber with iodine to visualize the solanesol standard. $R_f$ values may be calculated, but in our case, comparison to the products of RER2 control was adequate.

## 5.6 Results

Using $^{14}C$-IPP substrate and the microsomes from the *rer2Δ srt1Δ* yeast expressing *AtCPT1/AtLEW1* or *RER2*, we were able to measure IPP incorporation rates to *cis*-polyisoprenes as well as estimate the polymer profile on the reverse-phase TLC (Fig. 5). The microsomes containing AtCPT1

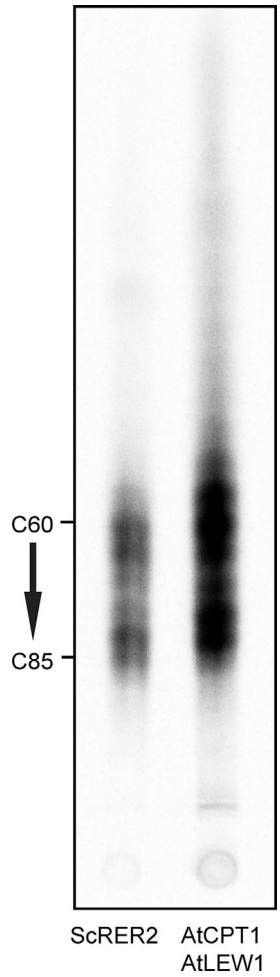


**Fig. 5** Separation of *cis*-polyisoprenols by thin layer chromatography.

and AtLEW1 showed an IPP incorporation rate of $12.0 \pm 0.3$ pmol $\mu g^{-1}$ $h^{-1}$ ($n = 3$) under the in vitro condition we used (20 $\mu M$ FPP, 80 $\mu M$ IPP, 30°C, 2 h). In 2-h reaction time, 29.9% of the total IPPs were incorporated into polyisoprene products, signifying a potent CPT activity using the microsomes having AtCPT1 and AtLEW1. In comparison, the microsomes from the yeast expressing *RER2* alone showed an IPP incorporation rate of $1.9 \pm 0.1$ pmol $\mu g^{-1}$ $h^{-1}$ ($n = 3$) with 4.8% incorporation of total IPPs. This specific activity is approximately sixfold lower than that measured from the microsomes containing AtCPT1 and AtLEW1. The yeast CBP, NUS1, has

been shown to be the partner for RER2 for the CPT activity (Park et al., 2014). Since *NUS1* was not overexpressed together with *RER2*, only the endogenous level of NUS1 is available and therefore is likely the limiting factor in CPT activity in the microsomes containing RER2. It appears that balancing the molar ratio of CPT and CBP is important for optimal CPT activity. When the radiolabeled *cis*-polyisoprene products were resolved on a reverse-phase TLC, no significant qualitative difference was observed for the polyisoprenes synthesized by RER2 or AtCPT1/AtLEW1 (Fig. 5). AtCPT1 is, therefore, a direct ortholog of yeast RER2 with respect to its biochemical property.

## 6. GENERAL DISCUSSION

Despite the long history of CPT research, it was only recently discovered that unique CPT/CBP complexes operate in the eukaryotic lineage to synthesize *cis*-polyisoprenes, including dolichol and NR. The fact that plants have two types of CPTs [the prokaryotic CPT in plastids (class I) and the eukaryotic CPT complexed with CBP on endoplasmic reticulum (class II); Fig. 3] together with the revertant occurrence in *rer2* mutants has complicated the finding of the CPT/CBP complex. However, it is now clear that the heteroprotein complexes comprised of class II CPT and CBP are required in eukaryotes to synthesize dolichol, NR, and possibly other *cis*-polyisoprenes (Brasher et al., 2015; Epping et al., 2015; Park et al., 2014; Qu et al., 2015). Since the unicellular eukaryote yeast has CPT/CBP complex, known as RER2/NUS1, the CPT/CBP complexes in higher eukaryotes are likely the direct orthologs of the RER2/NUS1, indicating the importance of such complexes in all eukaryotes. Of particular interest in plants is that lettuce has two sets of *CPT/CBP* genes—one for dehydrodolichyl diphosphate biosynthesis and the other for much longer NR biosynthesis. Thus, conservation and divergence of the CPT/CBP complexes for the biosynthesis of short to extremely long *cis*-polyisoprenes are the interesting subjects of further investigations.

Data prior to the discovery of CPT/CBP complexes for eukaryotic type CPTs (class II in Fig. 3) have puzzled us, and here we aimed to clarify the incongruent data found in literature by identifying the likely cause and providing a more reliable tool with which the activity of the eukaryotic type CPTs and CBPs can be assessed. Using the *rer2Δ srt1Δ* double knockout strain, we evaluated *Arabidopsis* CPT and CBP (AtCPT1 and AtLEW1, respectively). AtLEW1 was previously shown to partially complement *rer2*$^{ts}$

mutant, and *E. coli*-produced AtLEW1 enzyme showed dehydrodolichyl diphosphate synthase activity in vitro assays (Zhang et al., 2008). In our experiments, *rer2* revertants occur at a considerable frequency, leading to the conclusion that *rer2* mutants (*rer2*$^{ts}$ or *rer2Δ*) cannot be used for in vivo complementation or to express heterologous *CPT*s for in vitro CPT assays. On the contrary, the *rer2Δ srt1Δ* double knockout strain generated from this work is stable and cannot revert to the wild-type growth pattern, making it suitable for both in vivo CPT complementation and the microsome preparation for in vitro assays. Although revertant occurrences from *rer2* have not been explicitly stated except for one early report (Schenk et al., 2001), recent studies has started employing *rer2Δ srt1Δ* double- or *rer2Δ srt1Δ nus1Δ* triple-deletion mutant to characterize eukaryotic type CPTs possibly due to the same revertant issue found in this work (Epping et al., 2015; Grabinska et al., 2010; Park et al., 2014). The possibility of AtCPT1 activity in *rer2Δ srt1Δ* double knockout yeast through interactions with yeast NUS1 can be ruled out as AtCPT1 alone could not complement the *rer2Δ srt1Δ* strain, and therefore we concluded that the *rer2Δ srt1Δ* yeast can be used to assess plant CPT and/or CBP functions.

The methods described here can be used to assess rubber-synthesizing enzyme activities. If the rubber polymers, significantly longer than undecaprenol and dolichol, are synthesized in vitro assays, these polymer products will be immobile on the reverse-phase TLC, thereby leaving intense radiolabeled signals on or near the sample loading spots. On the other hand, in yeast complementation assays, if only high-molecular-weight rubber is synthesized (in other words, no intermediate dolichol length polymers), the lethal phenotype of *rer2Δ srt1Δ* double knockout yeast may not be rescued. The methods described here are technically reliable and do not require high-end analytical instruments. Hence, these methods can be used as the first line of experiments to examine biochemical functions of CPT and CBP in vitro and in vivo for short to long *cis*-polyisoprenes (eg, dolichol and NR) in most laboratories.

## ACKNOWLEDGMENTS

We thank Dr. Markus Aebi (Institute of Microbiology, ETH, Switzerland) for personal communication for the reported *Δrer2* revertants, Drs. Nakano and Hirata (RIKEN Advanced Science Institute, Japan) for providing us SNH23-7D and *Δrer2* strains, and Dr. Vanina Zaremberg (University of Calgary, Canada) for p414 and p415 yeast expression plasmids. We also thank Gillian MacNevin for critical reading of this manuscript. This work was supported by National Science and Engineering Research Council of Canada (NSERC) and Canada Research Chair program to D.K.R., and is also supported by the Next-Generation BioGreen 21 program (PJ00110552016) to M.K.

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