



Molecular Studies of the Protein Complexes Involving *Cis*-Prenyltransferase in Guayule (*Parthenium argentatum*), an Alternative Rubber-Producing Plant

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Guayule (*Parthenium argentatum*) is a perennial shrub in the Asteraceae family and synthesizes a high quality, hypoallergenic *cis*-1,4-polyisoprene (or natural rubber; NR). Despite its potential to be an alternative NR supplier, the enzymes for *cis*-polyisoprene biosynthesis have not been comprehensively studied in guayule. Recently, implications of the protein complex involving *cis*-prenyltransferases (CPTs) and CPT-Binding Proteins (CBPs) in NR biosynthesis were shown in lettuce and dandelion, but such protein complexes have yet to be examined in guayule. Here, we identified four guayule genes – three *PaCPTs* (*PaCPT1-3*) and one *PaCBP*, whose protein products organize *PaCPT/PaCBP* complexes. Co-expression of both *PaCBP* and each of the *PaCPTs* could complemented the dolichol (a short *cis*-polyisoprene)-deficient yeast, whereas the individual expressions could not. Microsomes from the *PaCPT/PaCBP*-expressing yeast efficiently incorporated ¹⁴C-isopentenyl diphosphate into dehydrodolichyl diphosphates; however, NR with high molecular weight could not be synthesized in *in vitro* assays. Furthermore, co-immunoprecipitation and split-ubiquitin yeast 2-hybrid assays using *PaCPTs* and *PaCBP* confirmed the formation of protein complexes. Of the three *PaCPTs*, guayule transcriptomics analysis indicated that the *PaCPT3* is predominantly expressed in stem and induced by cold-stress, suggesting its involvement in NR biosynthesis. The comprehensive analyses of these *PaCPTs* and *PaCBP* here provide the foundational knowledge to generate a high NR-yielding guayule.

Keywords: terpenoid, *cis*-prenyltransferase, guayule (*Parthenium argentatum*), polyisoprene, protein complex

INTRODUCTION

Natural rubber (NR) is an isoprene biopolymer, chemically defined as *cis*-1,4-polyisoprene, and it is used to manufacture a number of medical and industrial products (Cornish, 2001; van Beilen and Poirier, 2007). NR is best known for its prized physical properties, such as elasticity, abrasion resistance, heat dispersion, and hysteresis, which have special importance for the products

used in medical and heavy-duty industries. Despite significant advances in polymer science, no material can so far fully replicate the qualities of NR (Mooibroek and Cornish, 2000). The key feature that distinguishes the NR from other synthetic polymers is that NR is a stereo-regular polymer. The isoprene monomeric units of NR are condensed entirely in *cis*-1,4-configuration in average Mw of ~1 million Da. Such uniform stereo-structures are theorized to permit NR to form an extremely lengthy spring-like biomolecule, whereas synthetic rubbers are not exclusively stereo-regular (Morton, 2013). These properties, therefore, make NR a strategically important, irreplaceable commodity in modern manufacturing industries.

Natural rubber is synthesized in >7,000 plant species (Rivano et al., 2013). Nonetheless, the rubber tree (Para rubber tree or the Brazilian rubber tree; *Hevea brasiliensis*) from the Euphorbiaceae family is an almost exclusive NR-producing plant species. The rubber tree synthesizes NR on rubber particles in latex, the cytoplasmic content of the specialized laticifer cells (Cornish et al., 1999). The supply of NR solely depended on the rubber trees, mostly (>90%) cultivated in Southeast Asian countries (van Beilen and Poirier, 2007). During World War II, the supply route of the NR from Southeast Asia was blocked by the Japanese Empire, and in search of alternative NR-producing plants, American and Russian researchers identified guayule (*Parthenium argentatum*) and the Russian dandelion (*Taraxacum kok-saghyz*), respectively, as these plants can also synthesize high quality NR (Mooibroek and Cornish, 2000; van Beilen and Poirier, 2007). However, the research activities to develop alternative rubber plants ceased at the end of World War II.

Guayule is a perennial desert shrub native to the northern states of Mexico, but it also grows in the southern states of Texas, New Mexico, and Arizona in the United States. Guayule is unique in its ability to grow in some of North America's most arid lands, receiving as little as 25–38 cm of rainfall annually (Rasutis et al., 2015). In addition to having little need of water, guayule is known to have dry weights ranging from 4 to 12% in NR (Cantú et al., 1997). Unlike rubber tree, guayule does not have laticifers, but instead, synthesizes NR in the parenchyma cells in stem (Kajiura et al., 2018).

The NR biosynthetic mechanism has not been fully elucidated yet, and researchers have focused on the *cis*-prenyltransferase (CPT) class of enzymes, as these enzymes are able to catalyze the condensation of isopentenyl diphosphate (IPP) onto a priming molecule in an exclusively *cis*-configuration (Teng and Liang, 2012). The prokaryotic CPT family enzyme, undecaprenyl diphosphate synthase (UPPS), catalyzes the condensations of IPP onto farnesyl diphosphate (FPP) to form primarily C55 undecaprenyl diphosphate (eight IPP molecules condensed to one FPP) (Shimizu et al., 1998). Its monophosphate form, undecaprenyl phosphate, is a vital lipid molecule that mediates sugar transfer for peptidoglycan cell-wall biosynthesis in prokaryotes. The crystal structure study demonstrated UPPS to be a soluble, homo-dimeric enzyme localized in the prokaryotic cytosol (Fujihashi et al., 2001). Similarly, a homologous eukaryotic CPT enzyme, dehydrololichyl diphosphate synthase (DDS), catalyzes the analogous *cis*-condensation reaction to form C80–C120 *cis*-polyprenyl diphosphate, which is converted

to dolichyl phosphate by a phosphatase, polyprenol reductase, and dolichol kinase (Denecke and Kranz, 2009). The resulting dolichyl phosphate serves as a sugar carrier molecule for the post-translational modification of proteins in all eukaryotes. Therefore, both UPPS and DDS are essential metabolic enzymes, and lesions on these genes are lethal (Sato et al., 1999; Zhang et al., 2008). Interestingly, the primary and tertiary structures of CPTs display no similarities to those of *trans*-prenyltransferases, indicating that *cis*- and *trans*-prenyltransferases do not share a common origin and have independently evolved for distinct stereo-selective catalytic reactions (Fujihashi et al., 2001; Lu et al., 2009).

Although comprehensive biochemical data for the prokaryotic CPTs have been documented in the literature, *in vitro* biochemical characterizations of eukaryotic CPTs has been relatively scarce in the literature until recently. This discrepancy was first resolved by the discovery of the human CPT binding partner, NogoB receptor. Human CPT forms a hetero-protein complex with the NogoB receptor and becomes active in dolichol biosynthesis (Harrison et al., 2011). Similar complexes comprised of CPT and its binding protein homolog were also found in lettuce, tomato, and Arabidopsis for dehydrololichyl diphosphate biosynthesis (Brasher et al., 2015; Qu et al., 2015; Kwon et al., 2016). The CPT binding proteins, including the NogoB receptor, were named differently in each species. In this work, they are referred to as CBP (CPT-binding protein). CBP is only found in the eukaryotic lineage from yeast to humans, but interestingly, not in prokaryotes (Brasher et al., 2015; Qu et al., 2015). CBP displays low sequence homology to CPTs, forms a complex with CPT on the endoplasmic reticulum (ER), and lacks all conserved motifs required for CPT activity. Therefore, it is believed that CBP interacts with, activates, and tethers CPT onto the eukaryotic ER, but does not function directly in polymerization.

Intriguingly, RNA interference studies showed that analogous CPT–CBP complexes are also involved in NR biosynthesis in lettuce and dandelion (Epping et al., 2015; Qu et al., 2015). However, *in vitro* reconstitution solely using microsomal CPT and CBP recombinant proteins failed to produce *cis*-polyisoprenes longer than the short polymer, dolichol (Qu et al., 2015). It is worth noting that lettuce encodes two pairs of CPT–CBP genes – one for the dehydrololichyl diphosphate biosynthesis within primary metabolism, and the other for NR biosynthesis for the specialized (or secondary) metabolism in latex (Qu et al., 2015). Therefore, at least in lettuce and related species, such as dandelion, it is evident that the NR biosynthesis has functionally diverged from the primary dolichol metabolic pathway.

Aside from CPT and CBP, rubber elongation factor (REF) and its homolog, small rubber particle protein (SRPP), have been suggested to play roles in NR biosynthesis (Dennis and Light, 1989; Oh et al., 1999). However, silencing *REF/SRPP* homologs showed no decrease in lettuce and no substantial reduction (40–50%) in dandelion (Hillebrand et al., 2012; Chakrabarty et al., 2015). These results are in contrast to the results from CPT- or CBP-silenced dandelion and lettuce where almost complete disappearance of NR was observed (Post et al., 2012;

Qu et al., 2015). Thus, the REF and SRPP are not likely to be critical catalytic components in NR biosynthesis. It should be also noted that *REF/SRPP* homologs are also found as the lipid-droplet associated proteins in non-NR producing plants (Arabidopsis, tobacco, and avocado; Horn et al., 2013). The REF and SRPP are thus not restricted to the NR-producing plants but are likely to be common structural proteins of lipid-droplets or related monolayer structures in all plants. Recently, three new proteins were identified by using photo-affinity substrate and were proposed to be the components of NR-biosynthesizing protein complexes (Cornish et al., 2018). However, their *in vivo* roles and protein sequences remain unknown.

While the evidence to support the model for CPT–CBP complex in the NR biosynthesis exists in lettuce and dandelion, both species are closely related to each other. Each of these species belongs to the same Cichorieae tribe of the Cichorioideae subfamily within the Asteraceae family. Outside lettuce and dandelion (Cichorieae tribe), the rubber tree and guayule are the only plants that are commercially cultivated for NR production. Of these two, guayule (*P. argentatum*) is from the same Asteraceae family as lettuce and dandelion, but it comes from a different subfamily and tribe (Asteroideae and Heliantheae, respectively). On the other hand, the rubber tree (*H. brasiliensis*) belongs to an entirely different family, Euphorbiaceae. Recently, studies of CPT and CBP from the rubber tree suggested that its CPTs interact with its CBP (Yamashita et al., 2016).

The Heliantheae (for guayule) and the Cichorieae (for lettuce and dandelion) tribes are distantly related within the Asteraceae family as they share a common ancestor from ~39 million years ago¹. Considering such a divergence between guayule and lettuce/dandelion, along with guayule's commercial potential to produce NR in arid regions, we conjecture that in-depth molecular studies of guayule CPTs and CBP can provide the knowledge necessary to help develop guayule as an alternative NR-producer. Guayule is transformable (Ponciano et al., 2018), and utilization of its biomass via bio-refinery process has been studied (Orts and McMahan, 2016). Isolated rubber particles from guayule have been used for biochemical studies (Cornish and Siler, 1995), but guayule CPT and CBP have not been investigated at the molecular level.

In this work, CPT and CBP homologs were identified from guayule, followed by the investigation of their biochemical activities, interactions, and expression patterns. In addition, the findings from the guayule CPT and CBP studies are discussed in the context of the current knowledge of NR biosynthesis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Guayule plant seeds (PI478663) were obtained from the United States Department of Agriculture (USDA). Seeds were soaked in fresh water for 4 days before germination, and potted to a mixture of 35% autoclaved sands and 65% potting soil.

The germinated plants were grown in a growth chamber under conditions of 16 h light, 8 h dark, at 25°C.

RNA Isolation, Gene Isolation, and Phylogenetic Analysis

Two months old guayule plants were ground in liquid nitrogen using mortar and pestle, and 100 mg of powder was mixed with 1 mL of Trizol (Invitrogen), and total RNA was extracted according to the manufacturer's protocol. First strand synthesis of cDNA was performed using 1 µg of the extracted RNA (leaf and stem) as template, combined with the M-MuLV reverse transcriptase (NEB) and anchored oligo dT₂₂ (IDT). The synthesis of cDNA was performed according to NEB's provided protocol. *PaCPT1* was amplified from guayule cDNA by primers 1/2 and cloned into the *pGEMT-easy* vector (Promega) using TA/blunt ligase mix (NEB). *PaCPT2/3* and *PaCBP* were synthesized after yeast codon optimization (*SynPaCBP*, MF688939; *SynPaCPT2*, MF688937; *SynPaCPT3*, MF688939). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* ver. 6 (Tamura et al., 2013). All sequences were retrieved from TAIR or NCBI database. The CPT sequences are from AtCPT1 (At2g23410), AtCPT2 (At2g23400), AtCPT3 (At2g17570), AtCPT4 (At5g60510), AtCPT5 (At5g60500), AtCPT6 (At5g58780), AtCPT7 (At5g58770), AtCPT8 (At5g58782), AtCPT9 (At5g58784), CeCPT (NP_001023351), EcCPT (WP_077899378), HbCPT1 (BAB92023), HbCPT2 (BAB83522), HsCPT (NP_995583), LsCPT1 (XP_023749459), LsCPT2 (XP_023743551), LsCPT3 (XP_023762616), PaCPT1 (ATD87115), PaCPT2 (ATD87118), PaCPT3 (ATD87116), RER2 (NP_009556), SaCPT (CEH25875), SICPT1 (NP_001234633), SICPT2 (AFW98426), SICPT3 (AFW98427), SICPT4 (AFW98428), SICPT5 (AFW98429), SICPT6 (AFW98430), SICPT7 (AFW98431), SRT1 (NP_013819), TbcPT1 (AGE89403), TbcPT2 (AGE89404), TbcPT3 (AGE89405). The CBP sequences are from AtLew1 (At1g11755), CeCBP (NP_495928), HaCBP (OTG12218), HbCBP (XP_021659156), HtCBP (GHBN01046996), LsCBP1 (AIQ81186), LsCBP2 (AIQ81187), NogoBR (NP_612468), NUS1 (NP_010088), PaCBP (ATD87120), PtCBP (XP_002305421), SICBP (NP_001333078), ToCBP1 (DY820019), ToCBP2 (DY832694), VvCBP (XP_003631635). The guayule sequences were deposited at NCBI: *PaCBP* (MF688938), *PaCPT1* (MF688933), *PaCPT2* (MF688936), *PaCPT3* (MF688934).

Yeast Complementation Assay and *in vitro* Biochemical Assay

Details of yeast complementation, *in vitro* enzyme assay, and reverse-phase thin layer chromatography were previously described (Kwon et al., 2016). All primer sequences are given in **Supplementary Table S1**. *PaCPT1-3* were amplified using primers 3/4, 5/6 or 7/8, respectively, and the amplicons were cloned into p414-GPD using *SpeI/SalI*, *SpeI/XhoI* or *BamHI/ClaI* sites, respectively. *PaCBP* was cloned into p415-GPD using *SpeI/XhoI* sites. The cloned vectors were introduced to a yeast strain deficient in both

¹<http://www.timetree.org>

copies of CPTs, *rer2Δ::HIS3 srt1Δ::KanMX* [pRS316-RER2], described in Kwon et al. (2016). This strain is referred to as *rer2Δ srt1Δ* hereafter, and Ro lab yeast stock number is ROY13. The constructed PaCBP/CPT pairs were transformed in this yeast strain. To serve as a positive control of complementation, p414-GPD ScRER2-TRP1 (previously cloned in Kwon et al., 2016) with empty p415-GPD was also transformed in the *rer2Δ srt1Δ* strain. The transformants were streaked on both SC-Leu-Trp-Ura-His plates (positive control) and SC-Leu-Trp-His plates containing 5FOA (5-fluoroorotic acid) and uracil to allow for counter-selection of pRS316 ScRER2-URA3. Growth on 5FOA/Ura-containing media indicates functional complementation of PaCBP/CPTs in place of yeast CPTs. Growth on both media was observed after a 7-day incubation period at 30°C.

The yeast strains, rescued from the 5FOA selection in the complementation assay, were cultured overnight and inoculated into 100 mL of SC-Leu-Trp-His media. The cells were harvested at late logarithmic phase by centrifugation at $3,000 \times g$ for 3 min. The pelleted cells were then resuspended in 1 mL of TES-B buffer (0.6 M sorbitol, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4) and homogenized by bead-beating. The yeast lysate (1 mL) was centrifuged at $10,000 \times g$ for 10 min to remove large cellular debris. The supernatant was diluted with 10 mL of TES-B and centrifuged again similarly. The final supernatant was centrifuged at $100,000 \times g$ for 1 h, 4°C. The pellet was re-suspended in TG buffer [50 mM Tris-HCl and 20% (v/v) glycerol, pH 7.4] to a concentration appropriate for *in vitro* biochemical assays.

The *in vitro* biochemical assays of CPB/CPT were performed in 50 μ L reaction mixtures containing 50 μ g of prepared microsomal proteins, 20 μ M FPP, 80 μ M of IPP (14 C-IPP PerkinElmer, 50–60 mCi mmol^{-1}), 50 mM HEPES, 5 mM MgCl_2 , 2 mM NaF, 2 mM Na_3VO_4 , and 2 mM DTT, pH 7.5. The reaction mixture was incubated at 30°C for 2 h. For each reaction, the extraction of polyisoprenoid products was performed in 1 mL of chloroform/methanol (2:1, v/v) and 0.4 mL of 0.9% NaCl, by vortexing for 20 s followed by centrifugation at $10,000 \times g$ for 1 min at room temperature. The chloroform phase was transferred to a new tube and washed twice with 0.5 mL of methanol/water/chloroform mixture (48:47:3, v/v/v) by vortexing and centrifuging as above to remove unincorporated IPP. The lower chloroform phase was transferred to a glass vial and allowed to evaporate. Dephosphorylation of the product to polyprenol was carried out by incubating the dried product in 300 μ L of 1 M HCl at 85°C for 1 h, followed by benzene extraction. The concentrated products in benzene were used for thin layer chromatography (TLC). The concentrated products were spotted on a C18 reverse-phase silica plate (Analtech cat. P90011, 20 \times 20 cm) and separated by acetone/water (39:1, v/v) mobile phase. The TLC plate was then exposed to a BAS Storage Phosphor Screen (GE Healthcare) and the screen was scanned by a phosphorimager (Bio-Rad, Molecular Imager FX). The R_f value of known RER2 products (Brasher et al., 2015) was confirmed in our TLC system, and the R_f values were used to determine the carbon numbers.

Split Ubiquitin Membrane Yeast Two-Hybrid (MYTH) and β -Galactosidase Assay

MYTH assays were performed according to the published methods (Gisler et al., 2008; Snider et al., 2010). The bait plasmid (*PaCBP-Cub-LexA-VP16*) was generated by ligating the *PaCBP* amplified with primers 9/10 into pAMBV α using StuI/NcoI sites. For the prey plasmids, *PaCPT1-3* were amplified with primers 11/12, 13/14 or 15/16, and cloned into pPR3C-NubG in SpeI/BamHI (*PaCPT1*) or BamHI/ClaI (*PaCPT2 and 3*) to yield *PaCPT-HA-NubG* constructs. *PaCPT1-3* were also amplified with primers 17/18, 19/20 or 21/22 and cloned into pPR3N-NubG in BamHI/SalI (*PaCPT1*) or BamHI/ClaI (*PaCPT2 and 3*) to generate *NubG-HA-PaCPT* constructs. The bait and prey plasmid pair was co-transformed into the Y2H reporter yeast strain, THY.AP4. The control prey vectors pOST1-NubG and pOST1-NubI were also co-transformed with the bait vector. Individual transformed-yeast colonies were cultured in 30°C until OD₆₀₀ reached to 3.0–3.5. The cell densities were then normalized to 0.05 (OD₆₀₀ value) by dilution. The normalized cells were diluted to produce dilution of 1/10 and 1/100. Ten μ L of cells was spotted on SC-Leu-Trp (SC-LT) and on SC-Leu-Trp-Ade-His (SC-LTAH) containing 3-amino-1,2,4-triazole (3-AT) at concentrations of 5, 10, or 25 mM. Spotted plates were incubated for 3 days at 30°C to observe cell growth.

The strength of protein interactions was quantified using β -galactosidase assay with *O*-nitrophenyl- β -D-galactoside (ONPG) as the substrate. From SC-LTAH selection plates, the positive colonies were cultured overnight in SC-LTAH medium without 3-AT. Cells from the overnight culture were inoculated to 4 mL of SC-LTAH medium and cultured for 5 h. The cells were harvested at $9,000 \times g$ for 3 min at 4°C and resuspended in 1 mL of Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , and 50 mM β -mercaptoethanol, pH 7.0), 20 μ L of chloroform and 20 μ L of 0.1% SDS. After vortexing, the mixture was pre-incubated for 5 min at 28°C. The reaction was initiated by adding 0.2 mL of ONPG (4 mg mL^{-1} in Z buffer) and terminated by the addition of 1 M of Na_2CO_3 . The mixture was centrifuged at $20,000 \times g$ for 10 min, and the absorbance at 420 nm and 550 nm were measured to determine the Miller units (Miller, 1972).

Cloning and *in vitro* Translation of *PaCPT1-3* and *PaCBP*

DNA constructs for *in vitro* translation were cloned into pT7CFE1-3xFLAG (constructed using pT7CFE1-NHa) for *PaCPT1-3* and pT7CFE1-NHa (Thermo Fisher Scientific) for *PaCBP*. pT7CFE1-3xFLAG was generated by replacing HA tag in pT7CFE1-NHa with 3xFLAG. The 3xFLAG (atggactacaagaccatgacgggtattataaagatcatgatatcgattacaaggatgacgatgacaag) was amplified using primer sets 23/24, 23/25, and 23/26 sequentially using the IRES region in pT7CFE1-NHa as a template. The final PCR product containing partial IRES sequence and 3xFLAG was digested by KpnI and BamHI and ligated to corresponding restriction sites in pT7CFE1-NHa. Synthetic PaCPT1 and PaCPT2 (GenScript, Piscataway, NJ,

United States) were ligated to BamHI and XhoI sites in pT7CFE1-3xFLAG. PaCPT3 was amplified from *P. argentatum* leaf cDNA using a primer set, 27/28. The PaCPT3 PCR product was cloned into pT7CFE1-3xFLAG via BamHI and XhoI restriction sites. Synthetic PaCBP (GenScript, Piscataway, NJ, United States) was amplified using a primer set 29/30 and digested with BamHI and XhoI, followed by ligation to corresponding sites in pT7CFE1-NHa. A control construct, pT7CFE1-NHa-GFP for co-immunoprecipitation described below, was generated by cloning GFP sequence into pT7CFE1-NHa. GFP was amplified from pCFE-GFP (Thermo Fisher Scientific) using a primer set 31/32, containing BamHI and XhoI, respectively, followed by ligation to corresponding sites in pT7CFE1-NHa. The purified plasmid preparations of all IVT constructs were cleaned up further by ethanol precipitation, followed by resuspension in nuclease-free water to a concentration of 500 ng μL^{-1} prior to IVT. IVT was carried out using 1-Step Human Coupled IVT Kit (Thermo Fisher Scientific), following the manufacturer's procedure except for reducing the reaction to half of the recommended amounts. Immediately following incubation of the IVT reaction mixture at 30°C for 4 h, 1 μL of the 12.5 μL IVT mixture was saved as input for Western blotting by adding SDS-PAGE loading buffer and storage in -20°C until use. The remaining IVT mixture was used immediately for immunoprecipitation as described below.

Co-immunoprecipitation of PaCPT1-3 and PaCBP

For pre-binding of protein G magnetic beads (NEB, ON, Canada) to monoclonal mouse anti-FLAG M2 antibody (Sigma, ON, Canada) or monoclonal rat anti-HA 3F10 antibody (Roche, Laval, QC, Canada), for each co-immunoprecipitation reaction, 10 μL of the bead suspension was washed (invert five times/vortex 5 s) in 100 μL of room temperature PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4) four times and incubated with 0.5 μL of the antibody in 100 μL of cold PBST (PBS, pH 7.4, 0.1% Tween 20) at 4°C for 16 h on a Labquake™ Tube Rotator. The separation of magnetic beads from solution was achieved by applying magnetic field for 30 s on a magnetic stand. To remove unbound antibody, the antibody-immobilized beads were washed (invert five times/vortex 5 s) three times, each in 100 μL of cold IP buffer (150 mM NaCl, 100 mM Tris-HCl pH 7.4, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% NP-40, 1 mM PMSF). The immobilized beads were resuspended in 500 μL of cold IP buffer, followed by addition of the 11.5 μL of the completed IVT reaction mixture. Co-immunoprecipitation reaction was incubated at 4°C for 1 h on the rotator. To remove unbound proteins, the mixture was washed (invert five times/vortex 5 s) five times, each in 700 μL of cold IP buffer. Immunoprecipitated proteins were eluted from the beads by resuspending in 20 μL of 2X SDS-PAGE loading buffer [100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% glycerol, 0.01% (w/v) bromophenol blue, 200 mM β -mercaptoethanol] and incubating at 95°C for 5 min with intermittent gentle flicks. This elution also liberates anti-FLAG and anti-HA antibodies from the magnetic beads as the heavy (50 kDa) and light

(25 kDa) chains of the IgG antibodies. After bringing the eluted mixture to room temperature, the mixture was spun down by a brief centrifugation, followed by transfer of the eluate while on a magnetic stand. Half of the eluate was used to run 12% SDS-PAGE followed by Western blotting with mouse anti-FLAG M2 antibody and the other half with rat anti-HA 3F10 antibody. The IVT reaction mixture (1 μL of 12.5 μL total IVT reaction) saved as input was also divided in half to run in SDS-PAGE and Western blotting with anti-FLAG and anti-HA antibodies. SDS-PAGE gel was transferred to PVDF membrane using a Tris-glycine-methanol buffer system, and the antibody hybridization was performed in 5% skim milk TBST (5% skim milk, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) at 1:2000 for both anti-FLAG and anti-HA antibodies by overnight incubation at 4°C. HRP-conjugated anti-mouse and anti-rat secondary antibodies were also hybridized in 5% skim milk TBST at 1:10,000 at room temperature for 1 h.

RNA-Seq Analysis

For *de novo* transcriptome assembly and differential gene expression analysis, 4-month-old greenhouse-grown guayule plants (AZ-2 cultivar) were transferred to a simulated summer condition in a growth chamber (16 h daylight, 25°C daytime and 15°C night time temperatures). At 6 months of age, plants were transferred to a simulated winter condition (13 h daylight, 25°C daytime and 5°C nighttime temperatures) for induction of rubber production. Plants were harvested for analysis at 8 months of age. RNA was extracted from three biological replicates each for leaf and stem tissues from induced and control plants using Trizol (Invitrogen) following the manufacturer's instructions. Following Trizol extraction, RNA samples were treated with DNAase using the Turbo DNA-free kit (Ambion) and further purified using RNeasy column purification (Qiagen). Directional, bar-coded Illumina sequencing libraries were prepared using NEBNext® Ultra™ Directional RNA Library Prep Kit (NEB) with the suggestions for size selection of an average insert size of 300–450 bp. Libraries were then pooled and sequenced on Illumina Miseq and HiSeq2500 platforms producing 28 million 300 bp paired end reads and 155 million 150 bp paired end reads, respectively, with an average of 2.4 million MiSeq and 12.9 million HiSeq reads per sample. RNA-seq data was quality-controlled using Trim Galore (Babraham Bioinformatics group) to eliminate adapter sequence contamination and to trim data with a quality score below Q30. Miseq and HiSeq reads were pooled for transcriptome assembly and differential gene expression analysis. The transcriptome was assembled using Trinity assembler version 2.04 set to minimum Kmer coverage of 2 (Haas et al., 2013). The initial transcriptome assembly was filtered using a utility included within the Trinity package to eliminate transcript isoforms with low abundance and low coverage. Differential gene expression analysis was performed using EdgeR (Robinson et al., 2010). The assembly data are available in the NCBI short read archive under submission number SRP107961.

RESULTS

Identification of Guayule *PaCPT* and *PaCBP*

Commercially cultivated guayule (*Parthenium argentatum*) plants for NR production are polyploids, making the analysis of gene family difficult due to gene redundancy. However, the guayule transcriptome assembly from the diploid accession PI478640 is accessible in the public NCBI database (Hodgins et al., 2014), and we used the data set from diploid guayule in this work. In this transcriptome data, 51,947 unigenes were assembled from 983,076 reads (454 GS FLX Titanium) from four different guayule tissues (root, leaf, flower, and stem). We screened this assembled transcriptome using lettuce *CPT* and *CPT-Like* (*CPTL*) in our previous study in lettuce (Qu et al., 2015). *CPTL* was renamed as *CBP* (*CPT-Binding Protein*) in the previous work (Kwon et al., 2016) and will be referred to as *CBP* in this work. From this analysis, one guayule *CBP* and three *CPT* cDNAs were identified, and accordingly these cDNAs were named as *PaCBP* and *PaCPT1-3*. Unlike lettuce that has two *CBP* isoforms, one of which displays a laticifer-specific gene expression, guayule does not have laticifer cells and has only a single copy of *CBP*.

Phylogenetic Analysis of *PaCPT1-3* and *PaCBP*

To understand phylogenetic relatedness of *PaCPT1-3* to other known eukaryotic and prokaryotic *CPTs*, a phylogenetic tree was reconstructed using the deduced amino acid sequences from *PaCPT1-3* cDNAs. It has been established that two clades of *CPTs* (clade I and clade II) are present in plants (Figure 1A). Clade I is comprised of chloroplastic *CPTs* that likely originated from prokaryotic homo-dimeric *CPTs*, whereas clade II consists of cytosolic/ER *CPTs* that interact with *CBPs* to form hetero-protein complexes, which are then recruited to ER. The *CPT* clade II is further divided into two sub-clades – one type of *CPT* for dolichol biosynthesis in the primary metabolism and the other type for NR biosynthesis in the specialized secondary metabolism.

In the phylogenetic analysis, all three *PaCPTs* belong to the cytosolic/ER clade II *CPT*. At the sub-clade level, with strong statistical supports, *PaCPT1* is clustered with *LsCPT1* (lettuce), *SICPT3* (tomato), and *AtCPT1* (Arabidopsis), all of which are known to synthesize dehydrololichyl diphosphate (Brasher et al., 2015; Qu et al., 2015; Kwon et al., 2016). On the other hand, *PaCPT2* and *PaCPT3* form a close cluster with *LsCPT3* and *TbCPT1-3* (dandelion), known to have implications in NR biosynthesis (Post et al., 2012; Qu et al., 2015). This phylogenetic tree suggested that guayule possesses two sub-clades of diverged *CPTs* within the *CPT* clade II. It can be inferred from these results that *PaCPT1* is likely to be involved in the dolichol metabolism, while *PaCPT2* and *PaCPT3* are involved in NR metabolism.

The phylogenetic tree for *CBPs* was also constructed. *CBP* is exclusively found in eukaryotes, and thus the phylogenetic structure of *CBP* shows a monophyletic group (Figure 1B). The duplications of *CBP* (i.e., two *CBP* isoforms) are restricted to lettuce and dandelion in the Cichorieae tribe (within

Cichorioideae sub-family under Asteraceae family), and other plants with high quality genome sequences encode a single copy of *CBP*. Interestingly, *PaCBP* and other single copy *CBPs* from the Asteroideae sub-family (sunflower *HaCBP* and Jerusalem artichoke *HtCBP*) are tightly clustered with the laticifer-specific *CBPs* (lettuce *LsCBP2* and common dandelion *ToCBP2*), even though there is no developed laticifer in sunflower and artichoke. Thus, the *CBP* gene duplication event likely occurred prior to the divergence of the two sub-families and the advent of laticifer in Cichorioideae subfamily.

Complementation in *rer2Δ srt1Δ* Yeast

In eukaryotes, dolichol is an indispensable carrier molecule of sugar moieties for post-translational protein modifications. Therefore, all eukaryotes have at least one set of *CPT* and *CBP* to synthesize dehydrololichyl diphosphate (i.e., dolichol precursor), and the absence of either *CPT* or *CBP* is lethal. Yeast (*Saccharomyces cerevisiae*) has one homolog of *CBP* (*NUS1*) and two homologs of *CPTs* (*RER2* and *SRT1*). We previously developed a yeast strain that has double deletions in *RER2* and *SRT1*, in which its lethality is rescued by expressing a plasmid copy of *RER2* (Kwon et al., 2016). To examine the ability of *PaCPT* and/or *PaCBP* to rescue the dolichol-deficiency of *rer2Δ srt1Δ*, these guayule genes were first transformed to this strain individually or in *PaCPT/PaCBP* pairs, and then the URA-selective plasmid containing *RER2* was removed by 5-fluoroorotic acid (5FOA) selection (Figure 2). This enabled us to assess the *PaCPT* activity in the absence of both of the yeast *CPTs*.

In the absence of *CPT* enzymatic activity, the *rer2Δ srt1Δ* lethal phenotype will not be rescued. In all cases tested, the *rer2Δ srt1Δ* strain transformed with empty plasmids or a single plasmid that contains either *PaCBP* or one of the *PaCPTs* failed to grow upon removal of the *RER2-URA* plasmid by 5FOA selection. In contrast, co-expression of *PaCBP* and one of the three *PaCPTs* could effectively complement the lethality of the yeast *CPT*-knockouts upon removal of the *RER2-URA* plasmid. Our results indicate that each *PaCPT* can produce isoprenoid molecules analogous to dehydrololichyl diphosphate only in the presence of *PaCBP* but not together with endogenous yeast *CBP*, *Nus1*.

In vitro Enzyme Assays

The surviving yeast strains after 5FOA selection should be able to bring about necessary *CPT* activities for the yeast

TABLE 1 | Specific activities of *PaCPTs* and *PaCBP* in isolated microsomes.

cDNA expressed in <i>rer2Δ srt1Δ</i>	*Activities (pmol IPP $\mu\text{g}^{-1} \text{h}^{-1}$)	*IPP-incorporations (%)
<i>RER2</i>	2.9 \pm 0.4	2.9 \pm 0.1
<i>PaCPT1/PaCBP</i>	13.0 \pm 1.7	14.4 \pm 0.5
<i>PaCPT2/PaCBP</i>	45.4 \pm 1.3	50.8 \pm 5.0
<i>PaCPT3/PaCBP</i>	22.0 \pm 0.4	24.6 \pm 2.6

*Data are the average \pm SD from three independent assays.

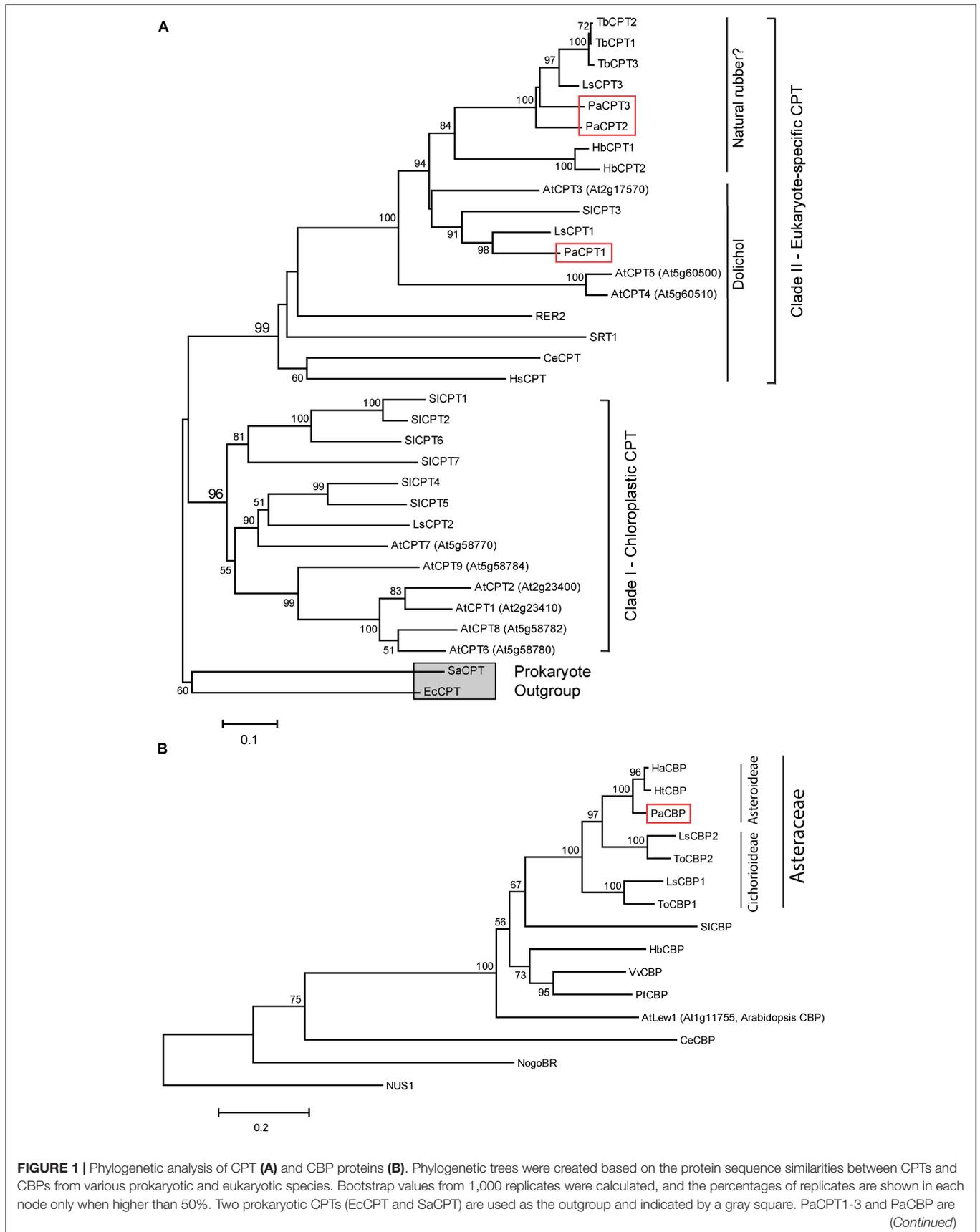


FIGURE 1 | Continued

marked by red boxes. Abbreviations used are: At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Ec, *Escherichia coli*; Ha, *Helianthus annuus*; Hb, *Hevea brasiliensis*; Hs, *Homo sapiens*; Ht, *Helianthus tuberosus*; Ls, *Lactuca sativa*; Pa, *Parthenium argentatum*; Pt, *Populus tremuloides*; Sa, *Staphylococcus aureus*; Sl, *Solanum lycopersicum*; Tb, *Taraxacum brevicorniculatum*; To, *Taraxacum officinale*; Vv, *Vitis vinifera*; *RER2* and *SRT1* are yeast CPTs. *NUS1* is yeast homolog of CBP. Accession numbers of the sequences used to construct the phylogeny are given in "Materials and Methods." Arabidopsis CPTs (AtCPT1-9) and tomato CPTs (SlCPT1-7) were numbered according to the published articles (Surmacz and Swiezewska, 2011; Akhtar et al., 2013). Note that Kera et al. (2012) assigned different numbering of the nine Arabidopsis CPTs, and different nomenclature of AtCPTs can be listed in other publications.

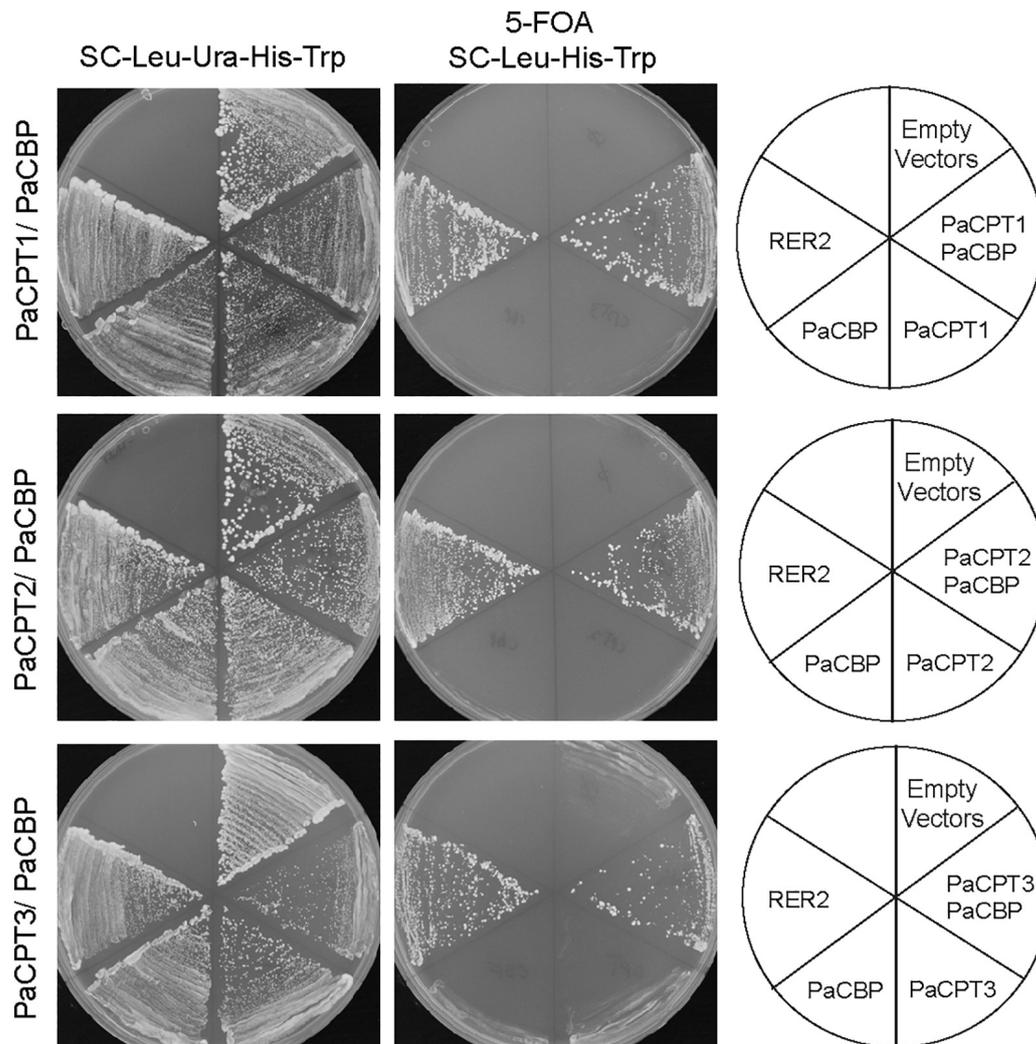


FIGURE 2 | Complementation of *rer2Δ srt1Δ* yeast by PaCPT1-3 and PaCBP. The yeast strain, *rer2Δ srt1Δ* is lethal but is maintained by expressing *RER2* in URA-selectable plasmid. This strain was used to transform plasmids expressing each PaCPT1-3 and PaCBP. The successful transformants were streaked on 5FOA selection plates to remove *RER2* containing URA-plasmid. Yeast growth in 5FOA selection was observed only by PaCPT/PaCBP pairs or by retransformed *RER2* in TRP-plasmid. No growth was observed when PaCPT alone or PaCBP alone was expressed.

survival in the *rer2Δsrt1Δ* yeast. To assess the guayule CPT activities, microsomes were prepared from the rescued yeast strains from the complementation assay, and radiolabeled ^{14}C -isopentenyl diphosphate (^{14}C -IPP) and the priming molecule, FPP, were co-incubated with the isolated microsomes. When the incorporations of ^{14}C -IPP to hydrophobic polymers were measured, significant IPP-incorporation activities ranging from 14.4 to 50.8% incorporation were measured in 2-h assays

(Table 1). Their specific activities were comparable to (in the cases of PaCPT1/PaCBP or PaCPT3/PaCBP activity) or exceeded (in the case of PaCPT2/PaCBP activity) the CPT activities previously reported from lettuce CPT and CBP (Qu et al., 2015). The CPT activity of the microsomes from the rescued yeast strain transformed with *RER2* as a complementation control was substantially lower (4.5- to 15.6-fold) than those from guayule PaCPT/PaCBP co-expression. The lower CPT activity

of RER2 control strain is attributable to the imbalance between overexpressed RER2 and its binding partner NUS1, expressed at a native level in yeast.

The resulting ^{14}C -labeled products were separated by the reverse-phase thin layer chromatography (RP-TLC) optimized for the separation of dolichol-sized polymers. In the RP-TLC analysis, we detected no obvious differences among the radiolabeled isoprene polymers produced from any of the PaCPT and PaCBP pairs (**Figure 3A**), and their polymer size was slightly smaller than, but still comparable to, the dehydrodolichols produced by RER2 (**Figure 3B**). We could not find evidence that the *cis*-isoprene polymer, significantly longer than dolichol, is synthesized by PaCPT and PaCBP in yeast microsomes.

Protein Interactions by Split-Ubiquitin Yeast 2-Hybrid Assay

The complementation of *rer2* Δ *srt1* Δ yeast strain by co-expression of *PaCPT* and *PaCBP*, but not by a single *PaCPT* or *PaCBP* expression, implied that both proteins are required for dolichol metabolism. Thus, possible interactions between each PaCPT and PaCBP were examined by split-ubiquitin yeast 2-hybrid assay developed for protein interactions of membrane-bound proteins (Stagljar et al., 1998). In this assay, the restoration of ubiquitin by N-terminal ubiquitin (Nub) and C-terminal ubiquitin (Cub) allows a release of transcriptional regulator to nucleus to activate the auxotrophic marker genes and the β -galactosidase reporter gene. A single residue mutation (I to G) of natural Nub protein (i.e., NubI to NubG) prevents the auto-assembly of the ubiquitin, unless NubG and Cub binding is mediated by the interaction of the proteins of interest each fused to NubG and Cub. Therefore, interactions between PaCPT and PaCBP proteins translationally fused to Cub and NubG can be assessed by yeast growth on selective medium and β -galactosidase activity.

As a control, co-expression of *PaCBP-Cub* fusion and *OST1* (α -subunit of the oligosaccharyltransferase complex localized on the ER)-*NubI* fusion allows restoration of ubiquitin, thereby presenting efficient yeast growth and strong β -galactosidase activity (**Figure 4A**). In contrast, changing NubI to NubG in the same experiment did not permit yeast growth, suggesting PaCBP and OST1 do not interact with each other and cannot bring the two ubiquitin subunits together as expected as a negative control (**Figure 4A**). Similarly, PaCPTs 1–3 were individually fused to NubG, and each fusion construct was co-expressed with PaCBP-Cub to assess whether PaCPT-PaCBP interaction occurs to restore ubiquitin. The interaction between each of the PaCPTs and PaCBP could be clearly inferred by the efficient yeast growth and the by β -galactosidase activities. Interestingly, the interactions were only observed in one orientation of the PaCPT fusion protein (**Figure 4B**). The NubG-PaCPT orientation allowed activations of the growth markers and reporter gene, whereas the PaCPT-NubG orientation did not show evidence of protein-protein interactions. Activations of marker genes only when NubG is located in the N-terminus of prey protein have been

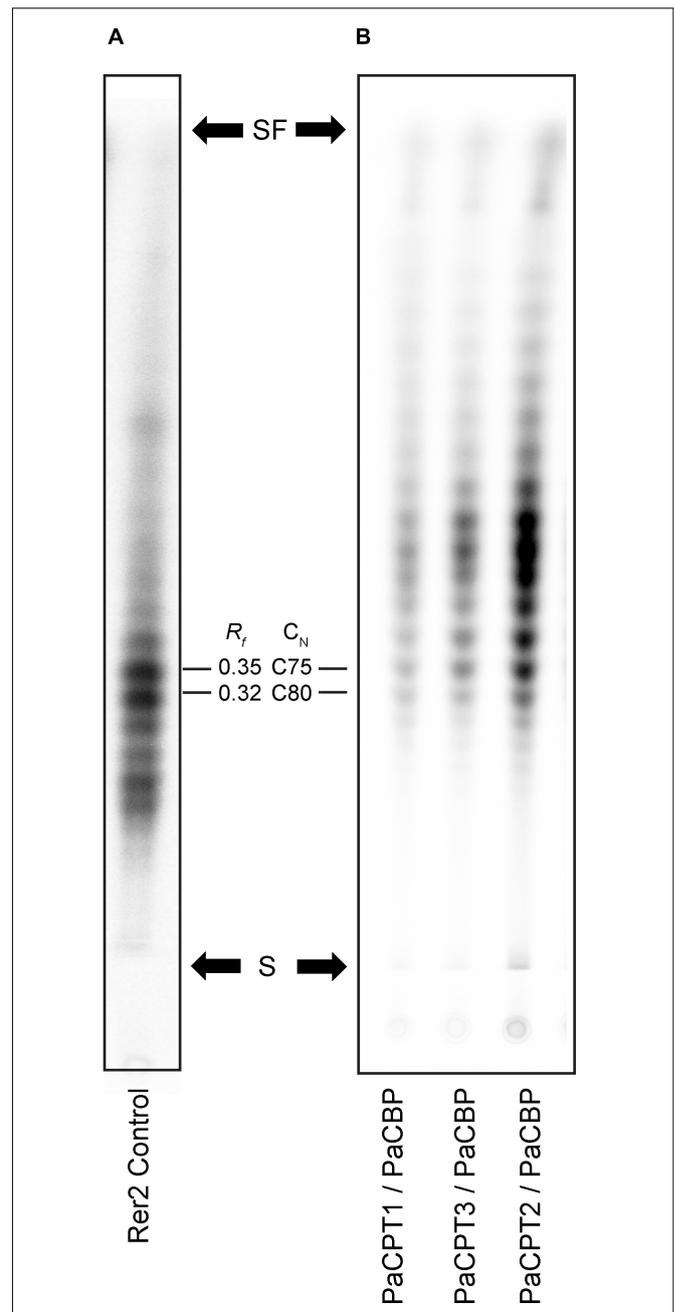
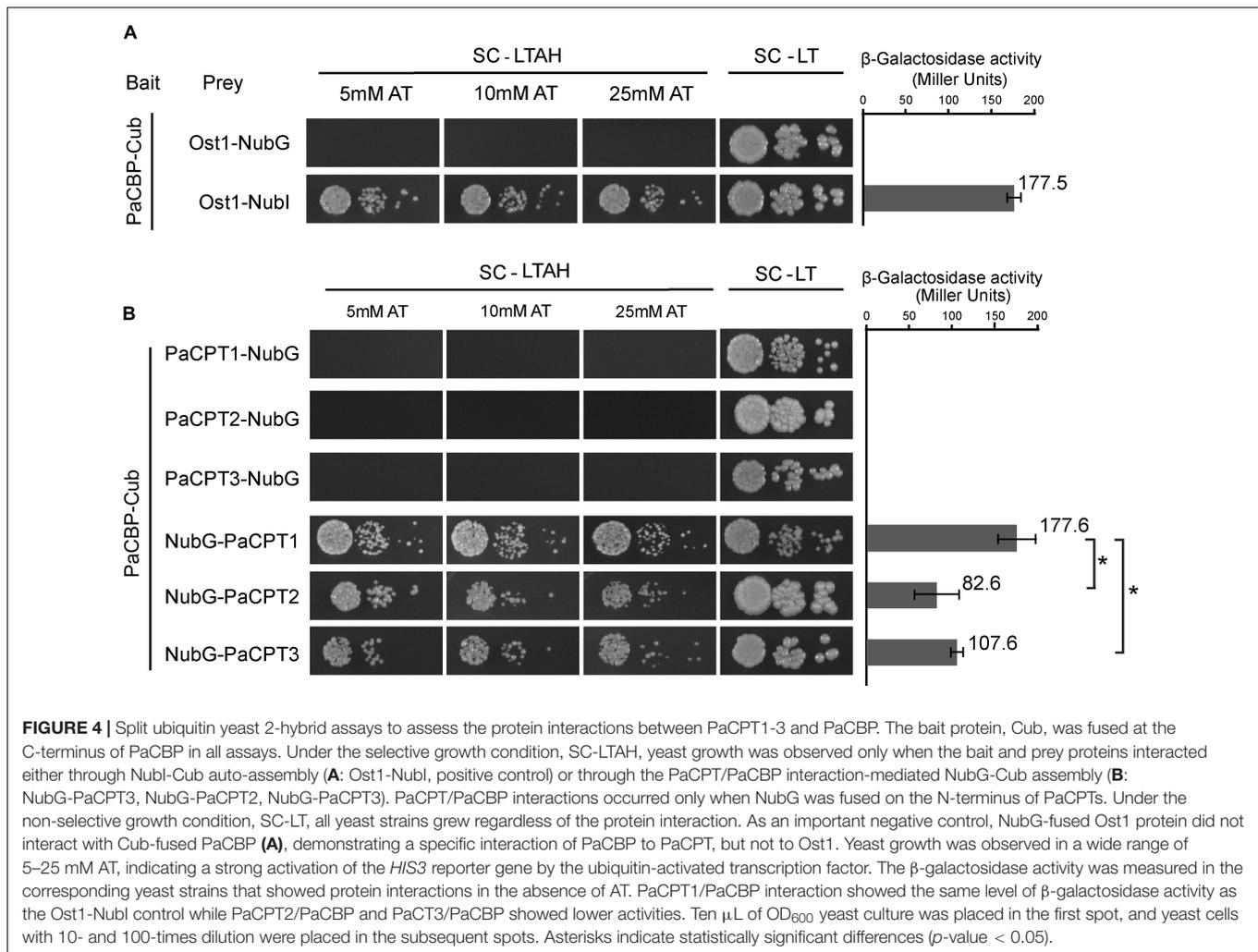


FIGURE 3 | Isoprene product separations by reverse phase thin layer chromatography. Microsomes were prepared from the yeast strains selected on 5FOA in **Figure 2**, and *in vitro* enzyme assays were performed using the microsomes and ^{14}C -isopentenyl diphosphate, followed by TLC (C18 reverse-phase silica) of extracted *cis*-polyisoprene products using a solvent mixed with acetone and water (39:1), and phosphorimager analysis. **(A)** The *cis*-polyisoprene product profiles from yeast RER2 as a size calibration standard. **(B)** The *cis*-polyisoprene products from PaCPT/PaCBPs are identical to one another and slightly shorter than those from the RER2-containing microsomes. S indicates the starting point of separation and SF indicates solvent front. R_f values and carbon numbers are shown.

previously reported (Yao et al., 2017) and have occasionally happened in split-ubiquitin yeast 2-hybrid assays (personal



communication with Igor Stagljar, University of Toronto). Wherever interactions were observed, the *HIS3* marker gene was activated very efficiently as yeast could grow at up to 25 mM of AT (3-amino-1,2,4-triazole), a competitive inhibitor of *HIS3*. In addition, β -galactosidase activities were comparable to that from the positive control. Therefore, the split-ubiquitin Y2H provided molecular evidence that each PaCPT and PaCBP interact with each other to form a hetero-protein complex in yeast.

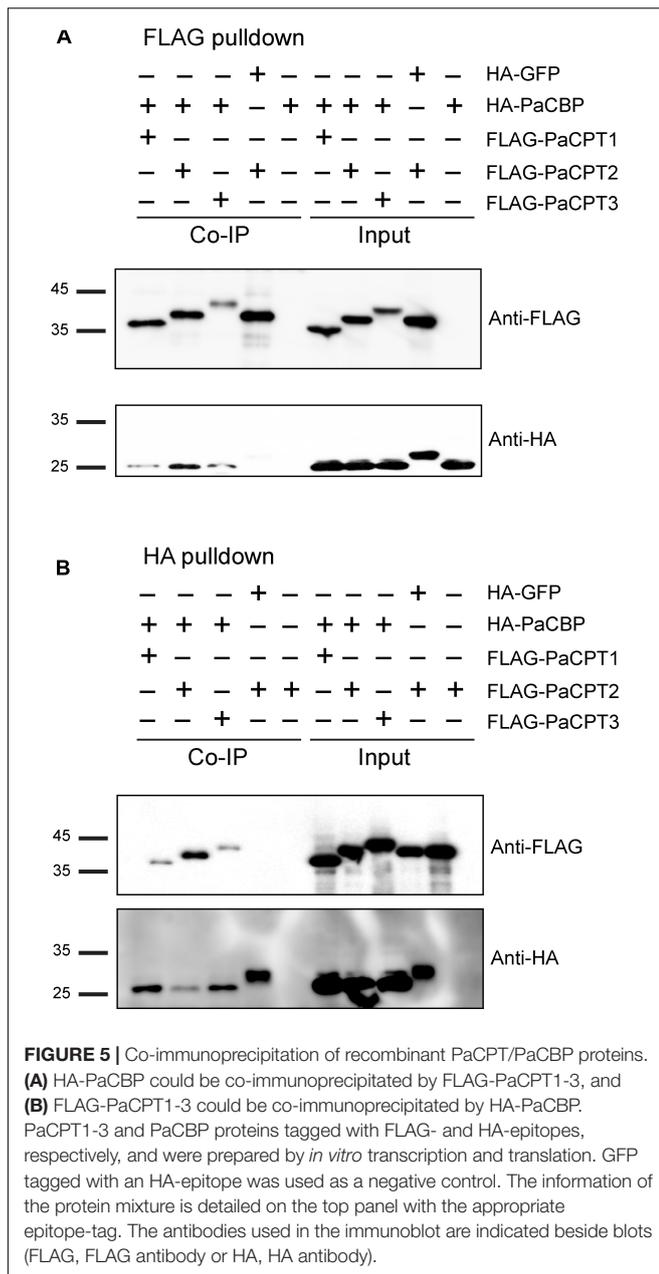
Protein Interactions by Co-immunoprecipitation

In addition to the split-ubiquitin Y2H approach, PaCPT1-3 and PaCBP interaction were independently examined by co-immunoprecipitation assays (Co-IP). PaCPT1-3 and PaCBP were tagged by FLAG- and HA-epitope, respectively, and their proteins were prepared by *in vitro* transcription and translation. Green fluorescent protein (GFP), which has a similar size to PaCBP and the PaCPTs, was used as a negative control in Co-IP experiments. All *in vitro* co-transcribed and co-translated PaCPT/PaCBP proteins were detectable in input by immunoblotting with either

FLAG or HA antibody (Figures 5A,B input; Supplementary Figure 1). Incubation of FLAG-antibody immobilized magnetic beads resulted in the co-IP of FLAG-PaCPTs with HA-PaCBP as detected by FLAG-antibody and HA-antibody immunoblotting, respectively, while HA-GFP control protein could not be pulled down by FLAG-PaCPT2 (Figure 5A and Supplementary Figure 1). Similarly, immunoprecipitation with HA-antibody immobilized magnetic beads resulted in the co-IP of HA-PaCBP with FLAG-PaCPTs, while FLAG-PaCPT2 control protein could not be pulled down by HA-GFP (Figure 5B). These results showed that each of the PaCPT1-3 and PaCBP interact with each other to organize hetero-protein complexes when co-expressed *in vitro*.

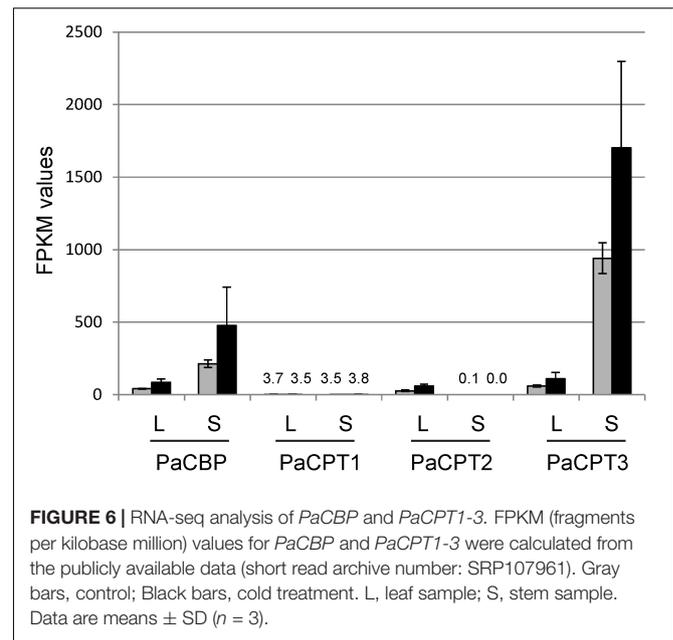
Expression Analysis

Cold temperature is known to induce NR biosynthesis in guayule (Madhavan et al., 1989). Based on the positive effect of cold-stress on rubber synthesis, Illumina sequencing of guayule stem and leaf tissues after cold-stress was recently performed, and the sequencing data are publicly accessible at NCBI (short read archive number: SRP107961). Using these data, we conducted



a RNA-seq analysis to examine the transcript abundance of *PaCPT1-3* and *PaCBP* (Figure 6).

PaCPT1 transcripts were maintained at a relatively constant basal level in leaves and stems under both normal and cold-treated conditions. These data suggest the basal metabolic role of *PaCPT1* in dolichol metabolism and are congruent to the phylogenetic analysis, in which *PaCPT1* is clustered with other *CPTs* involved in the dolichol metabolism. *PaCPT2* transcripts were hardly detectable in stem tissues while detectable at moderate levels in leaves. Of the three *PaCPTs* examined, *PaCPT3* showed the highest level of expression in stem (2- to 3-orders of magnitude higher levels than *PaCPT1/2*), and its transcript abundance increased 1.8-fold by cold-treatment in



stem. As NR is primarily synthesized in the parenchyma cells of the guayule stem (Kajiura et al., 2018), these data suggest that *PaCPT3* is the primary *CPT* that contributes to the NR biosynthesis in guayule stem. On the other hand, transcript levels of *PaCBP* showed a lower degree of fluctuation, compared to *PaCPT1-3* gene family. Its expression levels closely matched those of *PaCPT2* and *PaCPT3* in leaves. Noticeably, the *PaCBP* transcript abundance is highest in stem and is increased by 2.2-fold by cold-treatment, precisely mirroring the expression pattern of *PaCPT3* in stem.

Average values of *PaCBP* and *PaCPT3* expression increased by cold treatment between 1.8- and 2.2-fold in leaves and stem, but only *PaCBP1* induction in leaf was statistically significant (p -value < 0.05) in the triplicate Illumina transcript data. Systematic statistical analyses further showed that expression levels of *PaCPT3* in comparison to *PaCPT1* and *PaCPT2* are significant in stem (p -value < 0.01) and leaves (p -value < 0.05). Preferential expression of *PaCPT3* in stem in comparison to leaves was also statistically significant (p -value < 0.05).

DISCUSSION

Natural rubber is an irreplaceable biomaterial for hundreds of medical and industrial products, but its supply depends entirely on the rubber tree. In nature, more than 7,000 plants are known to produce NR (Rivano et al., 2013), but the quality of NR in most plants do not meet the NR polymer properties required for industrial uses. The exceptions are Russian dandelion (*Taraxacum kok-saghyz*), lettuce (*Lactuca sativa*), and guayule (*Parthenium argentatum*) as they can synthesize ~ 1 million average Mw NR. Biochemical and reverse genetic data for NR biosynthesis in lettuce and dandelion have been reported (Post et al., 2012; Epping et al., 2015; Qu et al., 2015); however, little is

known about NR biosynthesis in guayule at the level of individual cDNA and enzyme, except for the activity data from the isolated guayule rubber particles (Cornish and Siler, 1995). In this work, we identified and characterized three *PaCPT* isoforms and one *PaCBP*. While the results from this work confirmed some known facts, such as the formation of CPT/CBP protein complex and the lack of NR biosynthesis by CPT/CBP *in vitro*, they also provided additional new insights of NR biosynthesis in guayule.

Although the whole genome sequence has not been completed in guayule, we identified one *PaCBP* from 983,076 reads. Considering the read depth, it is unlikely that any expressed, sufficiently diverged *PaCBP* isoform is present in major tissues of guayule. Thus, *PaCBP* appears to be encoded as a single copy gene in guayule. Similarly, a single copy of *CBP* has been identified from the whole genome sequences and transcriptomics of the rubber tree, *Hevea brasiliensis* (Tang et al., 2016). Outside of NR-producing plants, the sequence analyses of high quality plant genomes (e.g., Arabidopsis, tomato, poplar, grapevine, and sunflower) also indicate that a single copy of *CBP* is present in these species. On the contrary, lettuce and dandelion, both originating from the same phylogenetic lineage to the tribe level (Cichorieae tribe), have two isoforms of *CBP* – one being general for dehydrodolichyl diphosphate biosynthesis in all cells and the second *isoform* being specific for NR biosynthesis in the laticifer. This *CBP* gene duplication appears to be restricted to the laticiferous plants from the Cichorieae tribe but is not common in other plants, even including NR-producing plants. Taken together, it is apparent that neo-functionalization and recruitment of a specialized *CBP* isoform are not required for quality NR biosynthesis because regardless of the presence of two isoforms of *CBP*, guayule and rubber tree can all synthesize high quality NR. It can be further inferred that a single *CBP* in the rubber tree and guayule should simultaneously support both primary dolichol and secondary NR metabolism. In such a scenario, we envision a challenge in finding the *in planta* evidence (i.e., RNAi or mutant) showing the necessity of *CBP* for NR biosynthesis in rubber tree and guayule, as severe knock-down by silencing or knock-out of *CBP* will be lethal in these plants.

Among the three *PaCPTs* in guayule, a few lines of evidence support that *PaCPT3* is the key isoform involved in guayule NR biosynthesis. (1) Phylogenetically, *PaCPT2* and *PaCPT3* closely clustered with lettuce *LsCPT3* and dandelion *TbCPT1-3*, whose expressions are specific to laticifer (Post et al., 2012; Qu et al., 2015), and RNA interference of *TbCPT1-3* resulted in significant NR reduction (Post et al., 2012). (2) In our RNA-Seq analysis, the *PaCPT3* transcript level was three orders of magnitude higher than that of *PaCPT1* in stem where NR is known to accumulate. (3) The expression patterns of *PaCPT3* closely coincide with *PaCBP* in two tissues under normal or the induced (cold-stress) condition. Despite clustering closely with *LsCPT3*, *PaCPT2* had a negligible level of expression in stem. Similarly, *PaCPT1* showed a basal level of expression and clustered phylogenetically with other *CPTs* implicated in dehydrodolichyl diphosphate biosynthesis. Collectively, these data indicate that *PaCPT3* supports NR biosynthesis in guayule. To further substantiate this, our future efforts will be focused on silencing or overexpression of *PaCPT3* to observe NR reduction or increase in guayule, respectively.

In addition to identifying the candidate genes necessary for NR biosynthesis in guayule, a suite of molecular and biochemical experiments (i.e., complementation in *rer2Δ srt1Δ* yeast, *in vitro* enzyme assays, Y2H, and Co-IP) reliably demonstrated that each of the *PaCPT1-3* and *PaCBP* form hetero-protein complexes to synthesize dehydrodolichyl diphosphate. Despite the formation of protein complexes, we were not able to find biochemical evidence that *cis*-polyisoprenes, longer than dehydrodolichol (dephosphorylated form of dehydrodolichyl diphosphate), can be synthesized using *PaCPT3* and *PaCBP* – the prime candidates for NR biosynthesis in guayule. The polyisoprene products synthesized *in vitro* by recombinant *PaCPT3/PaCBP* were essentially identical to those from *PaCPT1/PaCBP* or *PaCPT2/PaCBP* (Figure 3A). Plants may have more favorable environments (e.g., ER-associated protein, lipid compositions, and post-translational modification) than yeast for NR production by *PaCBP* and *PaCPTs*, and it will be worth expressing *PaCBP* and *PaCPTs* in plant and testing their enzyme activities in the future.

Although speculative, some other proteins or cofactors, energy sources for continued polymerizations, or sophisticated protein nano-structures may be necessary to complete sufficiently lengthy NR biosynthesis *in vitro*.

It was reported that a recombinant rubber tree CPT (HRT1) or a lettuce CPT (*LsCPT3*) alone without the respective recombinant *CBP* was sufficient to synthesize NR with average ~1 million Da Mw, when expressed in, or reconstituted with, the washed rubber particles isolated from the rubber tree (Asawatreratanakul et al., 2003; Yamashita et al., 2016). To date, these two reports, which use washed rubber particles, are the only studies demonstrating *in vitro* reconstitutions of ~1 million kDa NR using CPT recombinant proteins. It is attractive to include the washed rubber particles (mixtures of all proteins and lipid mixtures) as structural components *in vitro*, but this complicates the interpretation of the *in vitro* data. In addition, both reports showed no need of externally supplied rubber tree *CBP* or lettuce *CBP* for NR biosynthesis despite of their *in planta* interaction (Qu et al., 2015; Yamashita et al., 2016). Furthermore, these results contradict the *in vivo* *CBP* silencing evidence in lettuce and dandelion, which independently demonstrated the absolute necessity of *CBP* for NR biosynthesis (Epping et al., 2015; Qu et al., 2015). The discrepancy of these data cannot be easily resolved unless the rubber tree and lettuce/dandelion have different mechanisms for NR biosynthesis. Further investigation is necessary to address whether NR formation is catalyzed by the same or distinct mechanism in the rubber tree (Euphorbiaceae family) and other NR-producing plants (e.g., Asteraceae family; lettuce, dandelion, guayule, and sunflower).

In summary, three *PaCPTs* and one *PaCBP* cDNAs were identified from guayule in this work, and protein–protein interactions between *PaCPTs* and *PaCBP* have been substantiated by *rer2Δ srt1Δ* yeast complementation assays, split ubiquitin yeast 2-hybrid assays, and co-immunoprecipitation. The co-expression of each *PaCPT* and *PaCBP* in yeast allowed for the formation of the *PaCPT*–*PaCBP* hetero-protein complex for the efficient enzymatic synthesis of dehydrodolichyl diphosphate in microsomal *in vitro* enzyme assays. The RNA-Seq and

phylogenetic analyses indicated *PaCPT3* to be the most likely *CPT* involved in guayule NR biosynthesis. Collectively, these data suggest that any future bio-engineering efforts should be directed toward *PaCPT3* and its binding partner, *PaCBP* to increase NR yield in guayule.

AUTHOR CONTRIBUTIONS

D-KR designed the research, constructed the phylogenetic trees, and wrote the manuscript with AL. AL conducted the gene cloning, yeast complementation, and co-immunoprecipitation. MK conducted yeast complementation and 2-hybrid. E-JK and AL conducted *in vitro* enzyme assays. SS and HS generated RNA-Seq data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2019.00165/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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