A two-component enzyme complex is required for dolichol biosynthesis in tomato

Megan I. Brasher¹, Liliana Surmacz², Bryan Leong³, Jocelyn Pitcher¹, Ewa Swiezewska², Eran Pichersky³ and Tariq A. Akhtar¹,*

¹Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON N1G 2W1, Canada, ²Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 5A Pawinskiego Street, 02-106 Warsaw, Poland, and ³Department of Molecular and Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA

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*For correspondence (email takhtar@uoguelph.ca)

SUMMARY

Dolichol plays an indispensable role in the N-glycosylation of eukaryotic proteins. As proteins enter the secretory pathway they are decorated by a ‘glycan’, which is preassembled onto a membrane-anchored dolichol molecule embedded within the endoplasmic reticulum (ER). Genetic and biochemical evidence in yeast and animals indicate that a cis-prenyltransferase (CPT) is required for dolichol synthesis, but also point to other factor(s) that could be involved. In this study, RNAi-mediated suppression of one member of the tomato CPT family (SlCPT3) resulted in a ~60% decrease in dolichol content. We further show that the involvement of SlCPT3 in dolichol biosynthesis requires the participation of a distantly related partner protein, designated as CPT-binding protein (SlCPTBP), which is a close homolog of the human Nogo-B receptor. Yeast two-hybrid and co-immunoprecipitation assays demonstrate that SlCPT3 and its partner protein interact in vivo and that both SlCPT3 and SlCPTBP are required to complement the growth defects and dolichol deficiency of the yeast dolichol mutant, rer2Δ. Co-expression of SlCPT3 and SlCPTBP in yeast and in E. coli confirmed that dolichol synthase activity strictly requires both proteins. Finally, organelle isolation and in vivo localization of fluorescent protein fusions showed that both SlCPT3 and SlCPTBP localize to the ER, the site of dolichol accumulation and synthesis in eukaryotes.

Keywords: cis-prenyltransferase, Nogo-B receptor, polyisoprenoid, polyprenol, endoplasmic reticulum, Solanum lycopersicum.

INTRODUCTION

It is estimated that approximately 50% of the eukaryotic proteome undergoes post-translational modification (Apweiler et al., 1999). One of the most prominent of these modifications is N-glycosylation, which critically affects protein folding, subcellular localization and activity. As proteins are translocated across the endoplasmic reticulum (ER) membrane and enter the secretory pathway, a 14-sugar glycan is transferred to specific asparagine residues on the nascent polypeptide within the ER lumen (Schenk et al., 2001; Ruiz-May et al., 2012). The glycan is assembled onto a membrane-anchored, long-chain unsaturated lipid, known as dolichol (Chojnacki and Dallnert, 1988; Hemming, 1992). Although the enzymes involved in glycan assembly are well characterized, the biosynthesis of dolichol remains poorly understood.

Dolichol belongs to a larger class of compounds known as polyprenoids (Swiezewska and Danikiewicz, 2005). These hydrophobic polymers originate from the universal five-carbon (C5) isoprene building blocks, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In plants, two distinct pathways provide these two polyisoprenoid precursors: The mevalonate MVA pathway produces IPP in the cytosol while the methylerythritol phosphate (MEP) pathway produces IPP and DMAPP in the plastid (Lange et al., 2000; Bick and Lange, 2003; Kirby and Keasling, 2009). Plant polyprenoids broadly fall into one of two classes, the dolichols and polyprenols (Figure 1). Polyprenols typically range in size from C25-C65, while dolichols are longer (C70-C95) and are fully saturated at the terminal α-isoprene unit (Surmacz and Swiezewska, 2011). While dolichol is present in nearly all plant tissues, the short (C25-C40) and medium chain (C40-C65) polyprenols are primarily associated with roots and leaves, respectively (Kurisaki et al., 1997; Skorupinska-Tudek et al., 2008;
Polyisoprenoid synthesis can be viewed as a two-step process. First, the condensation of up to three IPP units with DMAPP generates a trans-prenyl diphosphate precursor. This intermediate acts as a scaffold onto which a cis-linked chain of IPP units is then attached. The class of enzymes known as cis-prenyltransferases (CPTs) elongate the trans-prenyl diphosphate intermediate with a cis-linked linear polymer of IPP units (Liang et al., 2002; Kharel and Koyama, 2003). Bacterial, yeast and mammalian CPTs are well established. In bacteria, CPTs synthesize medium-chain polyisoprenoid diphosphates (‘undecaprenol’, C50–C55), which serve as lipid carriers in cell wall peptidoglycan biosynthesis (Bugg and Brandish, 1994). In animals and yeast CPTs produce dolichol, which, as described above, plays an indispensable role in the post-translational modification of proteins (Sato et al., 1999; Shridas et al., 2003). By contrast, comparatively little information is known about plant prenyl synthesis and physiological function.

Defects in dolichol biosynthesis lead to a range of congenital disorders in animals that are typically characterized by aberrant protein glycosylation. The involvement of a specific CPT in dolichol biosynthesis was first identified in yeast via the rer2Δ mutant strain, which is defective in ER protein sorting. The orthologous human CPT was subsequently identified by sequence similarity and shown to complement the rer2Δ mutant (Endo et al., 2003; Shridas et al., 2003). However, in vitro evidence for dolichol synthesis in animals, yeast and plants has only been demonstrated with crude microsomal preparations (Sakaihara et al., 2000; Rush et al., 2010; Harrison et al., 2011), spurring speculation that other factors present on the ER membrane may contribute toward its synthesis.

The first evidence that dolichol synthesis may involve an additional protein factor resulted from a genetic screen for leaf wilting phenotypes in Arabidopsis (Zhang et al., 2008). In this ethyl methanesulfonate-mutagenized population the causal gene was identified as LEW1 (leaf-wilting1) and shown to encode a distantly related CPT-like protein. Strikingly, the LEW1 mutant exhibited a ~85% reduction in leaf dolichols. Subsequent studies in animals showed that the knockdown of the orthologous protein, known as the Nogo-B receptor (NgBR), also results in a dolichol deficiency, as well as a robust decrease in CPT activity and a broad reduction in protein N-glycosylation (Harrison et al., 2011). However, neither LEW1 nor NgBR were able to complement the yeast dolichol rer2Δ mutant on their own.

In this study we further examined the relationship between the plant CPTs implicated in dolichol biosynthesis and the enigmatic role of LEW1/NgBR in this process. Using tomato as our model, we show that a member of the CPT gene family, SlCPT3, interacts with the tomato LEW1 ortholog. Both of these proteins were required to fully complement the yeast rer2Δ mutant, restore microsomal CPT activity, and dolichol synthesis. Finally, expression of the two proteins in E. coli demonstrated that they form a two-component enzyme complex that synthesizes dolichol in vitro.

RESULTS
The evolution of CPT and CPT-related proteins
An earlier analysis of the tomato CPT gene family revealed that plants contain multiple CPTs, unlike the majority of other eukaryotes and prokaryotes (Akhtar et al., 2013). An expanded search for CPT-like proteins was performed in order to examine the evolutionary relationships between LEW1 and NgBR with predicted CPTs. Sequences (Figure S2) were retrieved from GenBank and the Joint Genome Institute Genome Portal (Phytozome) and phylogenetic analysis was performed using MEGA5 (Tamura et al., 2011). This search identified a gene from tomato on chromosome 6 (not linked to any other tomato CPT gene) that encodes a protein with 47% identity to LEW1 and 21% identity to NgBR, and which we designated as CPT-Binding Protein (SIcPTBP) for reasons described below. Our phylogenetic analysis demonstrated that CPTs fall into four distinct groups: Groups 1 and 2 are comprised of dicot and monocot-specific CPTs. Group 3 contains the CPTs of prokaryote origin and group 4 contains the animal and yeast CPTs that are implicated in dolichol biosynthesis and at least one member from every plant that was surveyed. The LEW1, NgBR, ScNus1p (from Saccharomyces cerevisiae), SIcPTBP and orthologous proteins from other
plant species form a fifth, distinct group (Figure 2a), indicating that this branch of the family diverged from bona fide CPTs prior to the split of the plant and animal lineages.

Analysis of the domain architecture of representative CPTs and related proteins from all five groups revealed four characteristic features. First, the plant CPTs in groups 1 and 2 contain N-terminal extensions that are predicted to encode organelar targeting sequences. Second, the five conserved regions including the dimer interface and the six amino acid residues that are implicated in substrate binding and/or catalysis are present in all CPTs from groups 1, 2 and 3 (Kharel and Koyama, 2003). Third, the CPTs in group 4, which are believed to participate in dolichol synthesis, lack N-terminal extensions and the conserved E213 residue (numbering based on the E. coli enzyme). Lastly, the CPT-related proteins in group 5 contain a truncated CPT domain, lack many of the conserved residues found in CPTs, have predicted N-terminal signal peptides and encode proteins with two predicted transmembrane domains (Figure 2b).

RNAi-mediated knockdown of SlCPT3

To explore the involvement of SlCPT3 in tomato dolichol synthesis, we performed RNAi-mediated knockdown and measured SlCPT3 expression and total polyisoprenoid contents in the transgenic plants. In three independent RNAi lines, SlCPT3 gene expression was reduced by approximately 60% (Figure 3a). Although transgenic plantlets were recovered that exhibited a higher degree of SlCPT3 knockdown, they did not survive to maturity. The polyisoprenoid content of the RNAi lines that could be propagated were analyzed by high pressure liquid chromatography (HPLC), and in these lines, dolichol (15–17 isoprene units long)
levels were reduced on average by 60% (Figure 3b). The medium-chain polyprenols (~9 isoprene units; C55) in these plants, which constitute a major portion of the total polyisoprenoid content in plant leaves, was unaffected. Among RNAi lines that did not exhibit a significant degree of SICPT3 mRNA knockdown, dolichol levels were unchanged relative to wild type plants (Figure S1). Compared to wild type tomato plants, RNAi lines with reduced SICPT3 gene expression exhibited a pleiotropic phenotype, which included mottled, wilted leaves and stunted growth, which is consistent with the indispensable role that dolichol serves (Figure 3c).

Functional complementation of the yeast rer2Δ dolichol mutant

The yeast rer2Δ mutant is deficient in dolichol synthesis and exhibits slowed growth at elevated temperatures. Survival of the yeast mutant is ensured by a second CPT gene that is cryptically expressed only during stress and stationary phase growth, therefore providing a unique platform to test for dolichol synthase activity of heterologously expressed proteins. Accordingly, we introduced SICPT3 as well as SICPTBP into the rer2Δ mutant, alone or together (using the native RER2 promoter for both), and assessed growth at the non-permissive temperature. The native RER2 gene, used as a positive control, was able to restore the growth of rer2Δ at 37°C. Neither SICPT3, SICPTBP, nor the expression vector alone completely rescued the growth defect of rer2Δ. However, when SICPT3 and SICPTBP were co-expressed growth was restored to equivalent levels as the control (Figure 4a).

To confirm that the restoration of growth in the rer2Δ mutant by the co-expression of SICPT3 and SICPTBP was due to dolichol synthesis, we first isolated microsomal membranes from the various rer2Δ mutant strains and assayed for CPT activity. Microsomal membranes were incubated with Farnesyl diphosphate (FPP) and 14C-IPP and the reaction products were resolved by reverse-phase thin-layer chromatography (TLC) in order visualize polyisoprenoids of various sizes. In strains expressing the native RER2 gene, long-chain polyisoprenoids (C75–C90) which are the typical size of dolichols, were detected (Figure 4b). Strikingly, the same enzymatic products were detected in assays with microsomes from the rer2Δ strain expressing both SICPT3 and SICPTBP, yet not with strains expressing either protein alone or the expression vector. Next, we analyzed the polyisoprenoid content of the various rer2Δ strains by HPLC. Yeast cells expressing SICPT3, SICPTBP, or the vector alone accumulated similar amounts of short- and medium-chain (~60 carbons in length) polyisoprenoids, whereas rer2Δ strains cells expressing the native RER2 or both SICPT3 and SICPTBP together accumulated identical long chain (75–85 carbons in length) polyisoprenoids that were identified as dolichols, based on comparison to authentic standards (Figure 4c).
The SlCPT3 and SlCPTBP proteins interact to form a functional dolichol synthase

One possible explanation for the production of dolichols in rer2Δ strains expressing both SlCPT3 and SlCPTBP is that they associate together in an enzyme complex. We therefore tested for in vivo interactions between the two proteins using two independent approaches. First, yeast two-hybrid assays were performed by co-expressing SlCPT3 and SlCPTBP as GAL4-activating domain (AD) and GAL4-DNA-binding domain (BD) fusion proteins in the yeast PJ69-4A strain, respectively. In this system, protein-protein interactions drive the GAL4-responsive expression of the auxotrophic markers, His and Ade (Figure 5a). Cells containing both SlCPT3 and SlCPTBP fusion proteins were able to rescue the His/Ade auxotrophy of the PJ69-4A yeast strain, indicating that they interact in vivo. On the other hand, cells expressing the ‘empty’ AD or BD in place of either fusion protein were unable to grow. We next examined this interaction in planta by introducing SlCPT3–Myc and SlCPTBP–FLAG tagged versions of the proteins into tobacco leaves and then performed co-immunoprecipitation assays. The SlCPT3–Myc protein was detected in SlCPTBP–FLAG immunoprecipitates, indicating that indeed these two proteins interact in a plant system (Figure 5b). The interaction was specific, as SlCPT3–Myc was not detected in the absence of SlCPTBP–FLAG from tobacco leaf protein extracts.

An alternative explanation as to why both proteins are required to synthesize dolichol in the yeast rer2Δ strain is that SlCPT3 converts an intermediate compound that is
produced by SlCPTBP into dolichol, or vice versa. This possibility was tested by performing a dialysis experiment in which desalted E. coli extracts expressing SlCPT3 and/or SlCPTBP were incubated with FPP and 14C-IPP in dialysis chambers that were separated by a 5 kDa molecular weight cutoff membrane that allows the passive diffusion of polyisoprenoid substrates, intermediates and products, but not proteins. Enzymatic products were extracted and separated by radio-TLC to assess their relative size (Figure 5c). The TLC plate was then divided into zones according to the retardation (Rf) value and radioactivity in each zone was quantified by scintillation counting. When SlCPT3 and SlCPTBP were incubated in separate chambers with all the necessary substrates to synthesize dolichol, long-chain polyisoprenoids (C75–C90) were not detected. However, in chambers containing extracts that were co-expressing both proteins dolichols were formed, which freely diffused to neighbouring chambers that were incubated without any protein. Interestingly, when extracts expressing each protein individually were mixed and assayed for CPT activity, dolichol formation was not detected.

**Subcellular localization of SlCPT3 and SlCPTBP**

In eukaryotes, the synthesis and accumulation of dolichol is known to occur on the ER membrane. We therefore tested if both SlCPT3 and SlCPTBP localize to the ER, in planta, by transiently expressing C-terminal mCherry fusions of both proteins in Arabidopsis protoplasts that express an ER–GFP marker (Nelson et al., 2007). Both SlCPT3-mCherry and SlCPTBP-mCherry fluorescence was observed in these protoplasts and it largely co-localized with the fluorescence associated with the ER–GFP marker (Figure 6a–f). Curiously, the mCherry fluorescence associated with SlCPTBP exhibited an additional punctate pattern that did not co-localize with the ER marker. When SlCPT3–GFP and SlCPTBP–mCherry fusion proteins were co-expressed in wild type Arabidopsis protoplasts, the two patterns of fluorescence completely overlapped (Figure 6g–i).

To address the subcellular localization of both proteins more precisely, C-terminally tagged SlCPT3–Myc and SlCPTBP–FLAG were introduced into tobacco leaves and organelar membranes were separated by sucrose density gradient centrifugation and subjected to immunoblot analysis using antibodies against known organelar markers. A 25–55% linear sucrose gradient was utilized to separate membrane fractions that correspond to ER, Golgi, and plasma membranes and it was found that both SlCPT3–Myc and SlCPTBP–FLAG co-fractionated with the ER luminal marker, BiP (Figure 6j). Interestingly, the SlCPTBP–FLAG protein also was detected in fractions associated with the Golgi marker, Arf1, suggesting that this protein may not be exclusively localized to the ER, as has been previously reported for the orthologous protein in animals (Harrison et al., 2009). Taken together, SlCPT3 and SlCPTBP appear to co-localize to the site of dolichol biosynthesis on the ER membrane.
DISCUSSION

SlCPT3 and SlCPTBP form a 'dolichol synthase'

We provide evidence that SlCPT3 and SlCPTBP interact to form a two-component enzyme complex that synthesizes dolichol, \textit{in vitro} and \textit{in vivo}. The inability of SlCPT3 or SlCPTBP to synthesize dolichol or fully compliment the yeast \textit{rer2Δ} mutant on their own implies that these proteins do not function as single enzyme subunits, but rather strictly require one another for activity. Dialysis experiments in which each protein was provided all of the necessary substrates for dolichol synthesis and then separated from its partner also rule out the possibility that SlCPT3 functions as an enzyme that acts on an intermediate produced by SlCPTBP, or vice versa. Although we cannot rule out the possibility of substrate channeling between the subunits, we instead propose that SlCPT3, and by extension all of the related CPTs in group 4 (Figure 2a), participate in intersubunit allostery with the CPTBPs. These interactions likely alter the conformation and stability of each partner and are therefore necessary for forming the active enzyme.

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currently not available, three-dimensional homology models using the E. coli enzyme as a template can readily be constructed for all of the tomato CPTs except for SICPT3 (Kang et al., 2014), suggesting that the SICPTBP is required for correct folding and stability of the dolichol synthase. In support of this view, NgBR also appears to stabilize the mammalian CPT involved in dolichol synthesis, hCIT, since loss of NgBR leads to a marked reduction in hCIT protein levels and hCIT-mediated CPT activity (Harrison et al., 2011). It is unlikely that a third protein factor is involved in the enzyme complex given that we were able to recapitulate dolichol synthesis enzyme activity in E. coli (Figure 5c).

Bacterial genomes do not encode any CPTBP orthologs and their CPTs function as autonomous homodimers without the requirement for accessory subunits (Liang et al., 2002). Further support for a common function for NgBR and SICPTBP (and AtLEW1) comes from the observation that these three proteins are more similar to each other than they are to any plant CPT protein (Figure 2), indicating that the ancestral gene that gave rise to the genes encoding them diverged from the CPT family before the split between the animal and plant lineages.

Several important questions still remain regarding the plant dolichol synthase enzyme complex itself, however. For instance, the details of the enzyme subunit stoichiometry, active site orientation, and kinetic parameters are still unknown. The origin and nature of the true initiator substrate for plant dolichol synthesis have also not been unambiguously determined. It has long been inferred from biochemical and structural studies that dolichol synthesis occurs with trans-FPP being extended with several units of IPP that are derived exclusively from the MVA pathway in the cytosol. However, stable isotope-assisted in vivo labeling studies with hairy root cultures of Coluria geoides, suggest otherwise (Skorupinska-Tudek et al., 2008). In a series of elegant experiments it was demonstrated that polyisoprenols originating from the plastid-localized MEP pathway provide the first ~12 isoprene units of dolichol; the polyisoprenol is then extended in the cytosol with the addition of IPP units that are derived entirely from the MVA pathway. Such a scenario would necessitate a continuous exchange of intermediates between the MVA and MEP pathways, for which there is precedent in plants (Bick and Lange, 2003; Dudareva et al., 2005; Phillips et al., 2008).

ER-targeting and orientation of dolichol synthase

One of the characteristic features of the CPT proteins that are implicated in dolichol biosynthesis (Group 4, Figure 2a) is that they lack typical organelar targeting sequences (Akhtar et al., 2013). This raises the question of how these CPTs localize to the ER membrane and participate in dolichol synthesis. Primary sequence analysis of the plant CPTBPs, on the other hand, predicts the presence of canonical N-terminal signal peptides. Therefore, we propose a model in which the CPTs are recruited to the ER through their interaction with CPTBPs and together form an enzyme complex that synthesizes dolichol on the ER membrane.

The orientation and topology of this two-component enzyme complex is still unclear, however. In animals, the orthologous CPTBP (NgBR) exists in at least two distinct conformations with the major portion of the protein oriented either towards the ER lumen or to the cytosol (Miao et al., 2006; Harrison et al., 2009, 2011). Whether the CPT accompanies its CPTBP partner to both sides of the ER membrane is not known. According to the current model, dolichol synthesis is initiated on the cytoplasmic leaflet of the ER and then transverses to the lumen during the completion of glycan assembly (Schenk et al., 2001). It is thought that a unique ‘flip-flop’ is involved in this transbilayer movement of dolichol (Sanyal et al., 2008; Sanyal and Menon, 2010), however no such enzyme has been identified in plants or animals. Therefore, it remains plausible that the dolichol synthase complex itself, or one of the two components, participates in the re-positioning of dolichol from the cytosolic to luminal leaflets of the ER during the glycan assembly process.

Results from our subcellular localization studies of SICPTBP revealed that a minor portion of the protein appears localized outside of the ER. A punctate pattern of mCherry fluorescence was observed in Arabidopsis protoplasts that did not coincide with the ER-GFP marker and immunoblot analysis of subcellular fractions indicate that SICPTBP partly localizes to the Golgi system. This observation is in agreement with studies in animals which have demonstrated that the orthologous CPTBP (NgBR) resides in both compartments (Harrison et al., 2009). This raises the intriguing possibility that plant CPTBPs serve a secondary function, perhaps in cholesterol trafficking and homeostasis, as has been suggested in animal systems (Harrison et al., 2009).

Future implications

CPTs from all kingdoms of life have long been thought to function autonomously as homodimeric enzymes (Kharel and Koyama, 2003). Our results challenge this view and suggest that all of the CPTs in the group 4 clade (Figure 2a), which include at least one member from every eukaryote, strictly require an accessory protein subunit for activity. The homology-based association of the CPTs in group 4, however, does not necessarily ensure their involvement in dolichol synthesis, as these proteins could participate in the production of other long-chain polyisoprenoids of biological importance. For instance, the CPTs that are implicated in the synthesis of cis,1,4-polyisoprene (natural rubber) also reside in this clade (Asawatreranakul et al., 2003; Schmidt et al., 2010; Post
et al., 2012). Natural rubber (NR) is a high-molecular-weight (2 x 10^6 to 1 x 10^4 Da) polyisoprenoid that is derived from the polymerization of IPP units with an all-trans-isoprene primer on the surface of ‘rubber particles’. Although the precise mechanism for NR synthesis has not yet been elucidated, CPTBP orthologs have been identified in several rubber-producing plant species (Wahler et al., 2012; Dai et al., 2013; Qu et al., 2015), which implies that a two-component enzyme complex may also be required for the synthesis of the world’s most economically important polyisoprenoid.

**EXPERIMENTAL PROCEDURES**

**Plant material and growth conditions**

Wild type and transgenic *Arabidopsis thaliana* (Col-0), *Nicotiana benthamiana*, and tomato (MP-1) plants were grown in potting soil supplemented with Osmocote (Scotts, http://www.scotts.com) and maintained in growth chambers under a 16 h photoperiod (150 µmol m^-2 sec^-1; mixed cool white and incandescent bulbs). Temperature was maintained at 23°C/18°C (day/night) and the relative humidity was 60%.

**Chemical and reagents**

Authentic polypropen standards were obtained from Indofine chemical company (http://www.indofinechemical.com) (heptaprenol C35), Avanti Polar Lipids (http://avantilipids.com) (polypropen mixture C65-C105), and Cedarlane Labs (https://www.cedarlane labs.com) (GPP, FPP, GGPP). Standards of dolichols were from the Collection of Polypropenols, Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland. Radiolabeled 14C-IPP, 40-60 mCi (1.48-2.22 GBq mmol^-1; 0.02 mCi ml^-1) was obtained from PerkinElmer (http://www.perkinelmer.ca/en-ca/). TLC plates (RP18, Silica gel 200 micron, 20 cm obtained from PerkinElmer (http://www.perkinelmer.ca/en-ca/). TLC plates (RP18, Silica gel 200 micron, 20 cm were obtained from Analtech (http://www.ichromatography.com/). Antibodies for co-immunoprecipitation were Monoclonal ANTI-FLAG M2, clone M2 (Sigma-Aldrich, https://www.sigmaaldrich.com), anti-myc (Life Technologies, https://www.lifetechologies.com) and the secondary antibody was goat anti-mouse IgG(H+L)-HRP Conjugate (Bio-Rad, http://www.bio-rad.com). For detection of organelle markers, anti-H-ATPase (plasma membrane H-ATPase), anti-BIP (luminal-binding protein), anti-Arf1 (ADP-ribosylation factor 1), and goat anti-rabbit IgG(H+L), HRP-conjugated secondary antibodies were obtained from Agrisera (http://www.agrisera.com). Synthetic drop-out media lacking amino acids for yeast cultures were obtained from US Biological (http://www.usbio.net). All other chemicals were obtained from Sigma-Aldrich, BioBasic (http://store.biobasic.com), or Fisher Scientific (https://www.fishersci.com). All primers were synthesized by Integrated DNA Technologies (https://www.idtdna.com) and are listed in Table S1.

**Gene, cDNA isolation and phylogenetic analysis CPT sequences**

The yeast RER2 gene was obtained by PCR using genomic DNA as a template prepared using the QIAaamp DNA mini kit (Qiagen, https://www.qiagen.com), according to the manufacturer’s instructions. The full-length SICPT3 and SICPTBP cDNAs were isolated by RT-PCR. RNA was prepared from tomato leaf tissue with the EZNA plant RNA mini kit with on-column DNase digestion (Omega Biotek, http://www.omegabiotech.com), reverse transcribed using Superscript II reverse transcriptase (Life Technologies) and used directly for PCR amplification with KOD hot start DNA polymerase (Novagen, http://www.merckmillipore.com). PCR products were transferred to pGEM-T-Easy (Promega, https://www.promega.com) and sequence-verified. CPT and CPT-related sequences were retrieved from GenBank and the Joint Genome Institute Genome Portal (Phytozome, http://phytozome.jgi.doe.gov/pz/portal.html). The phylogenetic and molecular evolutionary analysis were conducted using MEGA version 5 (Tamura et al., 2015) and the tree was constructed using the neighbor-joining method with bootstrap values from 1000 replicates.

**RNAi-mediated knockdown of SICPT3 and quantitative RT-PCR**

A 508-bp fragment of SICPT3 (corresponding to base pairs 25–752) was ligated in a sense/antisense orientation into pRNA69 (Foster et al., 2002) between the XhoI/EcoRI and BarnHI/XbaI restriction sites, respectively. The hairpin cassette was transfected to the p2P212 binary vector and introduced into tomato by the University of Nebraska Plant Transformation Facility (http://biotech.unl.edu/plant-transformation) using Agrobacterium-mediated transformation protocols. Gene expression was quantified using an Applied Biosystems 7300 Real-time PCR system (ABI, http://www.appliedbiosystems.com) to detect SICPT3 abundance and normalized to those for the tomato elongation factor 1α (EF1A). Total RNA extracted as described above and reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcriptase Kit (ABI) with random hexamers. The PCR employed ABI universal cycling conditions using SYBR GREEN PCR Master Mix (ABI) in a 25-μl reaction containing diluted (1:20) cDNA and 300 nM concentration of each primer. Expression values were calculated according to the 2^{ΔΔCT} method (Livak and Schmittgen, 2001).

**Extraction of polyisoprenoids from plants and yeast**

Dolichols were extracted from yeast cells as described earlier (Surmacz et al., 2014). After supplementation with an internal dolichol standard, yeast pellets were incubated in 10 ml of a hydrolytic solution (25 g KOH, 35 ml of water, brought to 100 ml with 98.8% ethanol) for 1 h at 95°C. Subsequently, lipids were extracted three times with hexane and pooled extracts were purified on silica gel 60 column using isocratic elution with 10% diethyl ether in hexane. Fractions containing polyisoprenoids were pooled, evaporated, dissolved in 2-propanol and analyzed by HPLC. For plant polyisoprenoid extraction, leaf tissue was homogenized using Ultra-Turrax T25 (IKA Labor-technik, http://www.ika.com) and lipids were extracted four times with chloroform:methanol:water (1:1:0.3) and incubated for 48 h at room temperature. The extracts were pooled and evaporated under nitrogen. Residual lipids were dissolved in 5 ml of a mixture containing toluene/7.5% KOH/95% ethanol (20:17:3 by volume) and hydrolyzed for 1 h at 95°C (Stone et al., 1967), then extracted with hexane, purified and analyzed as above.

**Analysis of polyisoprenoids**

Polyisoprenoids analysis was performed as described earlier (Skorupinska-Tudek et al., 2008). Extracted lipids were separated by HPLC (Waters, http://www.waters.com) using a ZORBAX XDB-C18 (4.6 x 75 mm, 3.5 μm) reversed-phase column (Agilent, http://
homogenate was filtered through miracloth and the flow rotor (Beckman, https://www.beckmancoulter.com). The pellets excess debris. The microsomal fractions were prepared by centrifuged at 4 °C for 2 h with anti
FLAG M2 affinity gel with gentle rocking and immunoprecipitated proteins were eluted according to the manufacturer’s protocol. Samples were analyzed by SDS-PAGE and immunoblotting.

Yeast two-hybrid and co-immunoprecipitation assays

Yeast two-hybrid assays were performed using the Clontech Matchmaker™ GAL4 Two-Hybrid System 3 (Clontech, https://www.clontech.com). The open reading frames of SICPT3 and SICPTBP were ligated between the EcoR1/BamHI sites of pGAD7T and the EcoR1/Xhol sites of pGBK7T to create GAL4 activation and DNA-BD fusions, respectively. Each construct was introduced into the yeast strain PJ69-4A (MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ lys2-181 lys2-288, SLNT2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) and transformants were selected on synthetic drop-out media lacking histidine. Two-hybrid interactions were tested on the above media lacking leucine and adenine. For co-immunoprecipitation, C-terminal tagged CPT3-myc and CPTBP-FLAG fusion protein sequences were generated by PCR and ligated between the Xhol/ EcoR1 sites of pSAT4A. The expression cassette was then transfected between the unique Sce I sites in the p2Z-RCs2 binary vector. Leaves of 4-week-old Nicotiana benthamiana (tobacco) plants were infiltrated with cultures of Agrobacterium tumefaciens (LBA4404) carrying the above binary vectors. Protein extracts were prepared from ~1 g of infiltrated tobacco leaf tissue in 2 ml of extraction buffer (50 mM Tris, pH 7.6; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; and 1 × protease inhibitor cocktail (Sigma-Aldrich). Extracts were cleared of excess debris using a low spin at 5000 rpm for 10 min. Extracts were then further centrifuged at 12 000 g for 10 min at 4°C. The supernatant was incubated at 4°C for 2 h with anti-FLAG M2 affinity gel with gentle rocking and immunoprecipitated proteins were eluted according to the manufacturer’s protocol. Samples were analyzed by SDS-PAGE and immunoblotting.

Subcellular fractionation

Agrobacterium tumefaciens (LBA4404) expressing SICPT3-Myc and SICPTBP-FLAG were infiltrated into 4-week-old tobacco leaves and harvested 3 days after. Approximately 7 g of infiltrated tobacco leaf tissue was homogenized using a polytron in 25 ml of 50 mM Tris-HCl, pH 7.6, 10% glycerol, 5 mM EDTA, and 0.5 × protease inhibitor cocktail (Sigma-Aldrich). The homogenate was filtered through miracloth and the flow through was centrifuged for 10 minutes at 8000 g to remove excess debris. The microsomal fractions were prepared by centrifuging the supernatant at 120 000 g for 1 h using a SW Ti32 rotor (Beckman, https://www.beckmancoulter.com). The pellets were re-suspended in 700 μl of 10 mM Tris-HCl, pH 7.6, 10% glycerol, 5 mM EDTA, and 1 × protease inhibitor cocktail (Sigma-Aldrich). The re-suspended pellets were loaded directly on top of a 12 ml, 25-55% [w/v] linear sucrose density gradient prepared in 10 mM Tris-HCl, pH 7.6, 5 mM MgCl2, 1 mM DTT, and 0.5 × protease inhibitor cocktail (Sigma-Aldrich). Centrifugation was performed using a SW28.1 rotor (Beckman) at 100 000 g for 16 h; 500 μl fractions were subjected to immunoblot analysis.

Subcellular localization

The open reading frame of SICPT3 and SICPTBP were PCR amplified and ligated between the Saccl/BamHI sites of pSAT4A-mCherry-N1, creating an in-frame C-terminal fusion protein with mCherry. The constructs were mobilized into Arabidopsis protoplasts expressing an ER-localized GFP marker (Nelson et al., 2007), according to the ‘tape sandwich’ method (Wu et al., 2009), and fluorescence was visualized 16 h following transfection with a Leica (http://www.leica.com) SPS laser scanning confocal microscope as previously described (Akhtar et al., 2013). Spectral detection of mCherry and GFP fluorescence was captured between 465 nm and 495 nm with the aid of a double dichroic 458/514 beam splitter.

Expression of recombinant SICPT3 and SICPTBP in E. coli

The open reading frames of SICPT3 and SICPTBP were amplified by PCR, A-tailed and then transferred into pEXP-5-CT/TOPO which permits recombinant protein expression from the T7 promoter. For co-expression of both proteins, a biconicstric message containing SICPT3 and SICPTBP separated by a ribosome binding site was created as follows: the SICPT3 coding sequence was PCR amplified with C-terminal BamHI/EcoRI sites and transferred to pEXP-5-CT/TOPO to create pCPT3CoEX. Similarly, the sequence for SICPTBP was amplified with an N-terminal extension containing a ribosome binding site and then ligated between the BamHI/EcoRI sites of pCPT3CoEX. Sequence-verified constructs were mobilized into BL21-CodonPlus (DE3)-RIPL E. coli cells, which were grown at 37°C in Luria-Bertani medium containing the appropriate antibiotics. When A600 reached 0.6, IPTG was added to a final concentration of 1 mM and incubation continued for 16 h at 15°C.

Protein extraction and CPT enzyme assays

E. coli cells were disrupted by sonication in 50 mM HEPES, pH 8.0, 100 mM KCl, 7.5 mM MgCl2, 5 mM DTT, 0.1% glycerol [v/v], and 0.1% Triton-X-100 [v/v]. Soluble crude extracts were clarified with a 7500 g centrifugation for 10 min at 4°C and then desalted on PD-10 columns (GE Healthcare, http://www3.gehealthcare.com) equilibrated with 25 mM Tris-HCl at pH 8, 5 mM MgCl2 and 5 mM DTT. Yeast cells were suspended in 50 mM Tris-HCl at pH 7.4, 0.25 mM sucrose, 5 mM EDTA, and 5 mM l-mercaptoethanol and then ruptured with glass beads. Cell lysates were centrifuged at 5000 g for 10 min at 4°C to remove unbroken cells and then further centrifuged at 100 000 g for 1 h at 4°C to obtain crude microsomes. The post-100 000 g pellets were re-suspended in 25 mM Tris-HCl at pH 8.25, 5 mM MgCl2. CPT enzyme activity assays contained ~100 μg of desalted protein extracts from E. coli or ~50 μg of crude yeast microsomal membrane preparations, 20 μM of trans-FPP and 14C-IIPP (50 μCi mmol−1) at a final concentration of 80 μM (200 nCi) in 25 mM Tris-HCl at pH 8.25, 5 mM MgCl2, 2.5 mM Na2VO3 in a final volume of 200 μl. Assays were conducted at room temperature for 30 min and reaction products were hydrolyzed with HCl at a final concentration of 0.6 M. Hydrolyzed products were extracted with
Table S1. Synthetic oligonucleotides used in this study.

REFERENCES


