Phytophthora infestans Cholinephosphotransferase with Substrate Specificity for Very-Long-Chain Polyunsaturated Fatty Acids

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The effective flux between phospholipids and neutral lipids is critical for a high level of biosynthesis and accumulation of very-long-chain polyunsaturated fatty acids (VLCPUFAs), such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3). Here we describe a cDNA (*PiCPT1*) from *Phytophthora infestans*, a VLCPUFA-producing oomycete, that may have a role in acyl trafficking between diacylglycerol (DAG) and phosphatidylcholine (PC) during the biosynthesis of VLCPUFAs. The cDNA encodes a polypeptide of 393 amino acids with a conserved CDP-alcohol phosphotransferase motif and approximately 27% amino acid identity to the *Saccharomyces cerevisiae* cholinephosphotransferase (ScCPT1). *In vitro* assays indicate that *PiCPT1* has high cholinephosphotransferase (CPT) activity but no ethanolaminephosphotransferase (EPT) activity. Substrate specificity assays show that it prefers VLCPUFA-containing DAGs, such as ARA DAG and DHA DAG, as substrates. Real-time PCR analysis reveals that expression of *PiCPT1* was upregulated in *P. infestans* organisms fed with exogenous VLCPUFAs. These results lead us to conclude that *PiCPT1* is a VLCPUFA-specific CPT which may play an important role in shuffling VLCPUFAs from DAG to PC in the biosynthesis of VLCPUFAs in *P. infestans*.

Phosphatidylcholine (PC) is a major component of cell membranes and directly involved in the structure and function of the cell. PC also plays a role in membrane-mediated signaling process, likely through lysophosphatidylcholine. Metabolically, PC is an important phospholipid supplying fatty acids to different lipid pools in a cell, including acyl coenzyme A (acyl-CoA), neutral lipids, and other types of phospholipids (1–5).

In eukaryotes, PC is synthesized mainly by a nucleotide pathway consisting of three consecutive reactions. The final step of this pathway involves transfer of a phosphocholine moiety of CDP-choline to diacylglycerol (DAG), resulting in formation of PC and CMP catalyzed by cholinephosphotransferase (CPT; EC 2.7.8.2) (3, 5). The CPT activity is mostly found in the microsomal fraction of fat cells of the female rat (6). The gene coding for CPT was first cloned from *Saccharomyces cerevisiae* through complementation screening of a genomic library in a yeast mutant completely deficient in CPT activity (7, 8). Ethanolaminephosphotransferase (EPT; EC 2.7.8.1), a CPT homolog, catalyzes transfer of a phosphoethanolamine moiety of CDP-ethanolamine to DAG, resulting in formation of phosphatidylethanolamine (PE), another major constituent of cell membranes. A comparison of yeast CPT (ScCPT1) and EPT (ScEPT1) protein sequences reveals over 50% identity at the amino acid level. The functional analysis of the two enzymes indicates that ScCPT1 strictly utilizes CDP-choline as the substrate to synthesize PC, while ScEPT1 is capable of using CDP-choline and CDP-ethanolamine as substrates to synthesize PC and PE, respectively (9). Human *CPT1* and *CEPT1* genes were identified by expressed sequence tags (EST) and by a homologous search using ScCPT1 as the query sequence (10, 11). *In vitro* and *in vivo* functional analysis of these two genes shows that human CPT1 catalyzes PC synthesis solely, whereas human CEPT1, like yeast ScEPT1, utilizes both CDP-choline and CDP-ethanolamine as substrates (10).

In higher plants, a soybean (*Glycine max*) cDNA encoding an amino alcohol phosphotransferase (AAPT) which possesses both CPT and EPT activities was cloned by complementation of a yeast

Δ CPT Δ EPT mutant (12). By using this soybean AAPT cDNA (*GmAAPT1*) as a heterologous probe, two orthologs (*AtAAPT1* and *AtAPPT2*) in *Arabidopsis thaliana* were isolated and characterized (13). Thereafter, AAPT cDNAs were also cloned from Chinese cabbage, *Brassica napus*, and wheat (14–16). When characterized biochemically, plant AAPTs appear to have no substrate preference for DAG species (17).

Very-long-chain polyunsaturated fatty acids (VLCPUFAs), such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are essential for human health and well-being. Metabolic engineering of oilseed crops to produce VLCPUFAs has been recently proposed as a sustainable source of these fatty acids (18–21). Genes encoding desaturases and elongases which are directly involved in the biosynthesis of VLCPUFAs have been cloned from microalgae and fungi. Some of these genes have been introduced into oilseed plants to produce the fatty acids. However, transgenic plants have so far produced either a low level of target fatty acids or a good level of target fatty acids with undesirable side fatty acids present in the oil (22–25), indicating that additional factors besides desaturases and elongases from native VLCPUFA-producing microorganisms are required for the transgenic production of desirable levels and composition of VLCPUFAs in plants.

Biosynthesis of VLCPUFAs involves a series of alternating desaturation and elongation (26). PC is the site of desaturation for most desaturases in VLCPUFA-producing microorganisms (27) and acts as an important intermediate for the biosynthesis of triacylglycerol (TAG) (28, 29). Recent metabolic labeling experi-

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ments demonstrate that *de novo* DAG is used primarily for PC synthesis and that the flux from *de novo* DAG to PC is a major bottleneck for the accumulation of unusual fatty acids in *Arabidopsis* (30). Therefore, understanding the role of CPT in the flux from DAG to PC in native VLCPUFA-producing microorganisms would be important for metabolic engineering of a high level of VLCPUFAs in transgenic plants.

The goal of this study was thus to clone and characterize CPT, a critical enzyme catalyzing the final step of PC synthesis from DAG, from the VLCPUFA-producing microbe *Phytophthora infestans*. *P. infestans* is a potato-pathogenic oomycete which can produce a considerable amount of EPA and ARA in TAGs (31). Our results show that a CPT cDNA (*PiCPT1*) cloned from *P. infestans* has high substrate specificity for VLCPUFA-containing DAG, suggesting that *PiCPT1* may play an important role in the biosynthesis of VLCPUFAs in *P. infestans*.

MATERIALS AND METHODS

Oomycete strain and growth conditions. A *Phytophthora infestans* strain was kindly provided by Lorne Adam, University of Manitoba. *P. infestans* was cultured in pea broth at 20°C for 2 weeks. Cells were harvested by vacuum filtration.

Sequence analysis. A putative CPT gene (*PiCPT1*) was identified in the *Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>), by a homology search using yeast ScCPT1 (GenBank accession number AAA63571) and ScEPT1 (GenBank accession number AAA63572) protein sequences as query sequences. DNASTar was used for the alignment of putative *PiCPT1* from *P. infestans* with ScCPT1 and ScEPT1. Hydropathy analysis was used to predict the topology of *PiCPT1* (<http://web.expasy.org/cgi-bin/protscale/protscale.pl>). The window size was set at 19. Peaks greater than 1.5 indicate the presence of a hydrophobic domain.

Cloning of putative *PiCPT1*. Total RNA was extracted from 0.5 g of *P. infestans* biomass using TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada). First-strand cDNA was synthesized using the SuperScript III first-strand synthesis system (Invitrogen Canada Inc.) following the instructions. Gene-specific primers (forward: 5'-CGCGGATCCataATGCTGGGGAAGAAG3'; reverse, 5'-CCGCTCGAGCTACTTCGCCTTTTCAG3'), designed based on the genome sequences using DNASTar-Primer Select program, were used to retrieve the full-length cDNAs using reverse transcriptase PCR (Invitrogen Canada Inc.). Phusion DNA polymerase (Finnzymes Canada Inc., Ottawa, Ontario, Canada) was used in a 25- μ l PCR amplification reaction following the manufacturer's instructions. PCR conditions were 98°C for 30 s, 5 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 45 s, 25 cycles of 98°C for 10 s, 62°C for 30 s, and 72°C for 45 s, and 72°C for 5 min. PCR products were loaded on a 50-ml 1.0% (wt/vol) agarose gel with 2 μ l of ethidium bromide (10 mg/ml) and examined under UV light by Alpha Imager HP (Cell Biosciences Inc., Santa Clara, CA). The amplified fragment was cut and extracted from the gel using an EZ-10 spin column DNA gel extraction kit (Bio Basic Canada Inc., Mississauga, Ontario, Canada). Purified DNA fragments were sent immediately for sequencing using specific primers (Plant Biotechnology Institute, National Research Council, Saskatoon, Canada).

Yeast CPT and EPT genes (*ScCPT1* and *ScEPT1*) were cloned in a manner similar to that described above. All putative and positive-control genes were first cloned into the intermediate vector pGEM-T and then subcloned into the yeast expression vector pSCW231 (the plasmid contains a tryptophan selection marker) under the control of the ADH1 promoter.

Yeast transformation and microsomal preparation. A yeast CPT EPT double mutant (DBY747- Δ CPT/ Δ EPT: *Mat α his3- Δ 1 leu2-3 leu2-112 ura3-52 trp1-289 cpt1::LEU2 ept1::URA3*) constructed in our lab based on a previously constructed single mutant (32) was grown on SD-

LEU-URA (synthetic defined yeast medium with all necessary compounds except for the selection compounds) amino acid dropout plates for 2 days at 30°C. The plasmids were transformed into DBY747- Δ CPT/ Δ EPT according to the method of Gietz and Schiestl (33). The transformed yeast was cultured on SD-TRP amino acid dropout plates for 2 days at 30°C. To screen transformants, yeast colonies were selected with toothpicks and dipped in 20 μ l of 0.02 N NaOH at room temperature for 10 min, followed by heating at 95°C for 10 min. Three microliters of the yeast lysate was used in a 25- μ l PCR mixture with high-purity (HP) *Taq* DNA polymerase as well as an *ADH1* forward primer and a gene-specific reverse primer.

The colonies were first grown at 30°C in liquid SD-TRP overnight. The cultures were then used to inoculate 50 ml of SD-TRP at an initial optical density at 600 nm (OD_{600}) of 0.4 to 0.5 and grown at 30°C overnight to an OD_{600} of 2.0 to 2.5. Yeast cells from overnight cultures in SD-TRP selection medium were pelleted by centrifugation at $3,000 \times g$ for 5 min, washed once with ice-cold GTE buffer (20% [vol/vol] glycerol, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA), and resuspended in 1 ml of GTE buffer. The microsomal fraction from yeast transformants was prepared essentially according to the method of Richard and McMaster (34). Cells were disrupted by vortexing in the presence of 0.5-mm glass beads for six bursts of 30 s separated by 30-s incubations on ice. The supernatant was transferred to a new tube, and the beads were rinsed twice with 0.5 ml of GTE. The pooled sample was centrifuged at $5,000 \times g$ for 10 min at 4°C. The resulting supernatant was then centrifuged at $100,000 \times g$ for 90 min. The microsomal protein pellet was resuspended in 250 μ l of GTE, and the protein concentration was determined using Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada).

Activity assays of CPT and EPT. An emulsion-based method described by Henneberry et al. (11) was adopted for the initial CPT and EPT activity assay with minor modifications. 1,2-Dioleoyl-*sn*-glycerol (di18:1 DAG; 600 μ M) was dried under nitrogen gas and resuspended in 50 μ l of 0.016% (wt/vol) Tween 20 by sonication. The tube was then added with the assay buffer at final concentrations of 100 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 50 μ g of microsomal proteins in a total volume of 200 μ l. The mixture was incubated at room temperature for 5 min to allow the lipids to be incorporated into the microsomal membrane, and this was followed by the addition of [14 C] CDP-choline or [14 C]CDP-ethanolamine (American Radiolabeled Chemicals, Inc., St. Louis, MO) at a final concentration of 4.05 μ M. The mixture was incubated at 23°C for 15 min. The reaction was terminated by the addition of 1 ml of $CHCl_3$ - CH_3OH (2:1, vol/vol) and 300 μ l of 0.9% (wt/vol) KCl. Phase separation was facilitated by centrifugation at $2,000 \times g$ for 2 min. The aqueous phase was removed, and the organic phase was washed twice with 500 μ l of CH_3CH_2OH - H_2O (2:3, vol/vol). An aliquot of washed organic phase was dried and dissolved in 50 μ l of $CHCl_3$ - CH_3OH (2:1, vol/vol). The organic phase was analyzed by silica G-25 thin-layer chromatography (TLC) on silica gel plates in a solvent system of chloroform-methanol-water (65:25:4, vol/vol/vol), followed by autoradiography with an AR-2000 TLC imaging scanner (Bioscan, Washington, DC). The bands corresponding to PC or PE were scraped, and the radioactivity was determined by using a Beckman LS 6500 scintillation counter (Beckman, Mississauga, Ontario, Canada).

Substrate specificity assays. The DAG species containing di16:0, di16:1, di18:0, di18:1, 18:0/20:4, and 18:0/22:6 fatty acids (Nucheck Prep, Inc., Elysian, MN, and Sigma-Aldrich, St. Louis, MO) were used for the substrate preference study of the *PiCPT1* gene. The assay procedure was as described above.

Kinetic study of *PiCPT1* with [14 C]CDP-amino alcohol. For the kinetic study of *PiCPT1*, a sufficient amount of 18:0/20:4 DAG (1.2 mM) along with different concentrations of CDP-amino alcohol (0 μ M, 10 μ M, 20 μ M, 40 μ M, 80 μ M, 160 μ M, or 320 μ M) was used in the *in vitro* assays as described above. The kinetic constants were estimated from Mi-

FIG 1 Alignment of putative PiCPTs, PiEPT from *P. infestans*, and ScCPT1 and cEPT1 from *S. cerevisiae*. The GenBank accession numbers of the protein sequences are as follows: ScCPT1, AAA63571; ScEPT1, AAA63572; PiCPT1, XM_002900684; PiCPT2, XM_002997893; PiEPT, XM_002907680. The conserved CDP-alcohol phosphotransferase motif is underlined. Regions of the protein sequences that are highly conserved among the sequences are shown by black boxes.

chaelis-Menten plot with GraphPad Prism 6 software using the average of triplicate measurements.

Gene expression analysis of *P. infestans* grown in the presence of exogenous fatty acids. *P. infestans* cells cultured in pea broth at 20°C for 4 weeks were harvested by vacuum filtration. A biomass of 0.1 g was inoculated into fresh medium fed with 200 μ M oleic acid (OA; 18:1n-9), linoleic acid (LA; 18:2n-6), α -linoleic acid (ALA; 18:3n-3), ARA (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3), separately, along with 0.1% (vol/vol) Tergitol, and grown at 20°C for 24 h. After that, the biomass was quickly harvested by vacuum filtration, washed with 1% (vol/vol) Tergitol once and H₂O twice, frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated from 0.1 g of biomass by using an RNeasy plant minikit (Qiagen, Toronto, Ontario, Canada) and treated with an RNase-free DNase set (Qiagen) to remove possible DNA contamination. The cDNA was synthesized with 1 μ g of purified RNA as the template using qScript cDNA SuperMix according to the manufacturer's directions (Quanta, Gaithersburg, MD).

Real-time qPCR amplification. Actin A (accession number M59715.1) (35, 36), actin B (accession number M59716.1) (35), and elongation factor 2 (accession number EEY57133.1) (36, 37) were employed as

internal references. Primers were designed by Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) (38). Real-time PCR assays were carried out in 96-well plates. Each sample was analyzed in a total reaction volume of 20 μ l consisting of 50 ng of cDNA as the template, 10 μ l of 2 $\times$ SsoFast EvaGreen intercalating dye, and 500 nM concentrations of forward and reverse primers. Reactions were run on a Bio-Rad CFX real-time PCR system (Bio-Rad, Mississauga, Ontario, Canada) using the following cycling conditions: enzyme activation at 95°C for 30 s, 45 cycles of denaturation at 95°C for 5 s, and annealing/extension at 60°C for 5 s. The specificity of the PCRs was confirmed by melting curve analysis of the products in 0.5°C increments, increasing from 65 to 95°C, with 5 s/step. The threshold cycle values (C_T) were determined from the cycles that reached the fluorescence threshold line for each gene by CFX Manager version 2.1 software (Bio-Rad, Mississauga, Ontario, Canada). Three technical replicates and three biological replicates were performed, and the C_T were recorded for both *PiCPT1* and all three reference genes for further analysis. Changes in transcript level were calculated as described by Livak and Schmittgen (39).

Statistical analysis. Variance analysis of the *PiCPT1* expression was carried out with the general liner model (GLM) procedure of the SAS

FIG 2 Kyte-Doolittle hydropathy plots of PiCPT1. Protscale was used to determine the hydrophobic profile of the polypeptide based on Kyte-Doolittle parameters (47). Window size was set at 19. Peaks greater than 1.5 indicate the presence of hydrophobic domains. The conserved CDP-alcohol phosphotransferase motif is located in the hydrophilic region (*), close to the second hydrophobic domain.

statistical package (SAS Institute, Cary, NC). *PiCPT1* expression values were subjected to the Duncan hypothetical test using the SAS statistical package. A probability (*P*) of <0.01 was chosen as the level of significance for the statistical tests.

RESULTS

Cloning and sequence analysis of putative *PiCPT1* from *P. infestans*. By using *S. cerevisiae* CPT (ScCPT1) and EPT (ScEPT1) as query sequences, putative CPT genes (*PiCPT1* and *PiCPT2*) and a putative EPT gene (*PiEPT*) from *P. infestans* were identified in the genome sequence database (Fig. 1). The putative *PiCPT1* cDNA was amplified from the total RNAs of the oomycete using reverse transcriptase PCR with the two specific primers. The full-length open reading frame of the putative *PiCPT1* cDNA is 1,182 bp, encoding a polypeptide of 393 amino acids. The deduced amino acid sequence of *PiCPT1* contains a CDP-alcohol phosphotransferase conserved domain and has only 27.7% and 23.8% amino acid identities to ScCPT1 and ScEPT1, which are biochemically well characterized microbial CPT and EPT proteins, respectively. A relatively high homology was found in proximity to the N terminus of the sequence, where the conserved CDP-alcohol phosphotransferase motif was located (Fig. 1).

Hydropathy plot analysis of PiCPT1 revealed that the putative protein is very hydrophobic and contains eight highly hydrophobic domains that are presumably associated with the membrane. These hydrophobic domains are spread throughout the sequence (Fig. 2). This result is consistent with notion that CPT and EPT are endoplasmic reticulum-based proteins (40). The conserved CDP-alcohol phosphotransferase motif was located in the hydrophilic region close to the second transmembrane domain.

Functional analysis of PiCPT1 in yeast. To analyze the function of *PiCPT1*, the gene was cloned into a yeast expression vector and transformed into a yeast double mutant strain (Δ CPT Δ EPT). The function of the gene was determined by *in vitro* assays using the mutant expressing the gene. Figure 3 shows a representative TLC image of the *in vitro* CPT and EPT assay results obtained using the microsomal fraction of the yeast double mutant expressing *PiCPT1* as the enzyme source and dioleate and [¹⁴C]CDP-aminoalcohol as substrates. In the CPT assay where [¹⁴C]CDP-choline was a substrate, the yeast double mutant expressing *PiCPT1* and ScCPT1, a positive control, produced radioactive signals at the PC position of the TLC plate, which was in contrast to the negative control, the yeast double mutant with the empty vector. Moreover, the PC radioactive signal of *PiCPT1* was much stronger than that of ScCPT1 in the assays. This result indicated that *PiCPT1* from *P. infestans* had strong CPT activity. To determine whether PiCPT1 had any EPT activity, an *in vitro* EPT assay was also conducted where [¹⁴C]CDP-ethanolamine was a substrate. The result showed that unlike the positive control, i.e., the double mutant expressing the *S. cerevisiae* EPT gene (*ScEPT1*), the double mutant expressing *PiCPT1* did not produce any radioactive PE signal in the TLC assay plate. Collectively, these results indicated that *PiCPT1* has high CPT activity but no EPT activity (Fig. 3).

Substrate specificity of PiCPT1. To investigate the substrate specificity of PiCPT1 for DAGs, seven different species of DAGs (di16:0, di16:1, di18:0, di18:1, di18:2, 18:0/20:4, and 18:0/22:6) were used in the *in vitro* CPT assays. The result showed that the most preferred DAG substrate for PiCPT1 was 18:0/20:4 DAG,

FIG 3 TLC plates showing the radioactive lipids in the CPT and EPT assays. Substrates used in this experiment were di18:1 DAG and [14 C]CDP-choline or [14 C]CDP-ethanolamine. Lipids were separated in TLC plates with chloroform-methanol-water (65:25:4, vol/vol/vol). Lanes: control, DBY747- Δ CPT/ Δ EPT with empty vector (negative control); PiCPT1, DBY747- Δ CPT/ Δ EPT with PiCPT1; ScCPT1, DBY747- Δ CPT/ Δ EPT with ScCPT1 (positive control in the CPT assay); ScEPT1, DBY747- Δ CPT/ Δ EPT with ScEPT1 (positive control in the EPT assay). The 200- μ l reaction mixture contained 600 μ M 1,2-diacylglycerol (di18:1 DAG) and 4.05 μ M [14 C]CDP-choline or [14 C]CDP-ethanolamine with 50 μ g of microsomal proteins. The reaction mixture was incubated at 23°C for 15 min. The original picture was generated by the program Win-Scan 2D105. The picture was then manipulated from standard color to gray scale using Corel Paint Shop Pro X.

followed by 18:0/22:6 DAG. The specific activities for 18:0/20:4 DAG and 18:0/22:6 DAG were 26- and 14-fold higher than those of other DAG species (Fig. 4).

To confirm the CDP-amino alcohol affinity of PiCPT1, 18:0/20:4 DAG, the preferred substrate of PiCPT1, was employed as the

FIG 4 Substrate specificity of PiCPT1 for different DAGs. Values are means $\pm$ standard errors (SE) from three biological replicates. Seven DAGs (di16:0, di16:1, di18:0, di18:1, di18:2, 18:0/20:4, and 18:0/22:6) were used in *in vitro* CPT assays.

FIG 5 Kinetic analysis of PiCPT1. Data are means $\pm$ SE from three biological replicates. The kinetic constants were estimated from a Michaelis-Menten plot using the averages of triplicate measurements with GraphPad Prism 6 software.

substrate for quantitative kinetic analysis using different concentrations of [14 C]CDP-choline and [14 C]CDP-ethanolamine. The results showed that the apparent K_m was 20.98 μ M and the V_{max} was 3.23 nmol/min/mg for CDP-choline (Fig. 5). The low K_m value indicates that PiCPT1 has high affinity for the CDP-choline substrate. In contrast, PiCPT1 could not synthesize PE using CDP-ethanolamine with various concentrations provided. This result confirmed that PiCPT1 behaves solely as a CPT, with high affinity for CDP-choline.

Effect of exogenous fatty acids on PiCPT1 expression in *P. infestans*. To investigate whether supplementation of exogenous fatty acids could affect the expression of PiCPT1, transcript levels of PiCPT1 in *P. infestans* fed with different fatty acids were analyzed by real-time quantitative PCR. The result showed that feeding C_{18} unsaturated fatty acids such as OA (18:1n-9) and LA (18:2n-6) did not significantly affect the expression of PiCPT1. However, the expression of PiCPT1 was significantly upregulated when the oomycete was fed VLCPUFAs such as ARA (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3), especially ARA, which induced expression at a level 2.2 times that induced by OA (Fig. 6).

FIG 6 Transcript levels of PiCPT1 in *P. infestans* cultured in the presence of exogenous fatty acids. Values are the means for three replicates $\pm$ SE. cDNA from the biomass not fed with any fatty acid was used as a negative control. Values followed by a different letter (a, b, c, or d) are significantly different according to a Duncan hypothetical test ($P < 0.01$).

This result indicates that expression of *PiCPT1* can be upregulated by exogenous VLCPUFAs.

DISCUSSION

TAG is synthesized on DAG intermediate by either an acyl-CoA-dependent pathway catalyzed by diacylglycerol acyltransferase (DGAT) or an acyl-CoA-independent pathway catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT) (29, 41). Recent labeling experiments indicate DAG can be produced by at least two pathways in plants (30, 42). The so-called Kennedy pathway is for the *de novo* synthesis of DAG. It involves the sequential acylation of glycerol-3-phosphate by acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) and acyl-CoA:lysophosphatidic acid acyltransferase (LPAT), producing phosphatidic acid (PA), and subsequent removal of phosphate group of PA by phosphatidic acid phosphatase (PAP) to produce *de novo* DAG. The second pathway uses PC as the intermediate to generate DAG by removing the phosphocholine head group of PC, a process which is possibly catalyzed by phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (43) or phospholipase C. PC is the site for desaturation for most front-end desaturases from VLCPUFA-producing microorganisms (44) and is the site for the acyl-editing process in eukaryotic organisms (45). Because of their different deriving routes, the fatty acid composition and contribution to TAG synthesis of *de novo* DAG and PC-derived DAG can be very different. The labeling experiment shows that DAG derived from PC contributes more than 90% of TAG synthesis, while *de novo* DAG is used primarily for PC synthesis and contribute less than 10% to direct TAG synthesis in soybean seeds (42). More importantly, in *Arabidopsis*, not only is the flux of *de novo* DAG to PC over 14 times the rate of direct conversion from *de novo* DAG to TAG, but it also represents the major bottleneck for the accumulation of unusual hydroxyl fatty acids in transgenics expressing the hydroxylase from castor bean (30).

CPT is a major enzyme involved in the *de novo* synthesis of PC and has an important role in fluxing DAG to PC. Since PC-derived DAG contributes mostly to oil synthesis, the fatty acid profile of PC may be mostly reflective of that of TAG in the oil. Thus, substrate selectivity of CPT would have a large impact on the fatty acid composition of the final oil. The substrate preference of CPT/EPT enzymes for DAG species has been investigated in some model eukaryotes. In yeast, the CPT enzyme prefers di16:1 DAG (46) as the substrate, although it is also able to utilize VLCPUFA-containing DAGs such as 16:0/20:4 DAG and 18:0/20:4 DAG (46). In humans, CPT1 has the highest activity for di18:1 DAG, and it can also work on VLCPUFA DAG (16:0/22:6 DAG and 18:0/20:4 DAG) (11). Human CEPT1 prefers C₁₈ PUFA DAGs as substrates and can also use VLCPUFA DAGs for both PC and PE synthesis (11, 40). In plants, safflower and rapeseed CPTs have no or little substrate preference across a range of DAG species (17). To our knowledge, this is the first report describing a CPT with strong substrate preference for VLCPUFA DAG in eukaryotes. *PiCPT1* from *P. infestans* has CPT activity with high affinity to CDP-choline but no EPT activity (Fig. 3 and 5). The *in vitro* assay shows that *PiCPT1* has strong substrate specificity for 18:0/20:4 DAG and 18:0/22:6 DAG which is at least 14 times higher than those for long-chain DAG species (Fig. 4). It should be noted that *P. infestans* can also accumulate a high level of EPA; unfortunately, an EPA-containing DAG species is not commercially available for our *in vitro* assays. The *in vivo* experiment shows that the expression of

PiCPT1 was upregulated in *P. infestans* fed with exogenous VLCPUFAs (Fig. 6). CPT is involved in the synthesis of PC from DAG and CDP-choline; when the oomycete is fed with VLCPUFAs, higher *PiCPT1* expression might be required for specific channeling of these fatty acids to the phospholipids through *de novo* DAG intermediates. These results suggest that *PiCPT1* may play an important role in converting VLCPUFA-containing DAG to PC during the biosynthesis of VLCPUFAs in *P. infestans*. Our future focus will be on the detailed analysis of fatty acid composition and stereochemistry of PC, DAG, and TAG in yeast expressing *PiCPT1* in the presence of VLCPUFAs. The information obtained from this experiment will shed light on how a VLCPUFA-specific CPT would affect the acyl trafficking among these lipid pools.

Metabolic engineering of oilseed crops using desaturases and elongases from microorganisms to produce VLCPUFAs has been viewed as a potential alternative source of these fatty acids for nutraceutical markets. However, most experiments so far have achieved only limited success. One reason for this could be that oilseed crops produce a simple fatty acid profile and may not be able to effectively handle unusual VLCPUFAs synthesized by heterologous transgenes from microorganisms. In other words, host oilseed crops may lack coevolved enzymes from VLCPUFA-producing microorganisms such as CPT to effectively channel VLCPUFA DAG to PC and then to TAG, as this step represents the major bottleneck in the production of unusual fatty acids in transgenic plants (30). For oilseed plants which rely mostly on the PC-derived DAG for TAG synthesis, CPT with substrate specificity to VLCPUFA DAGs from microorganisms, like *PiCPT1* from *P. infestans*, would be an interesting candidate to be tested in co-expression with the desaturase and elongase genes to see whether the production of VLCPUFAs can be improved in transgenic plants.

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