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ORIGINAL ARTICLE

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Camelina sativa phosphatidylcholine:diacylglycerol cholinephosphotransferase-catalyzed interconversion does not discriminate between substrates

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Abstract

Phosphatidylcholine:diacylglycerol cholinephosphotransferases (PDCT) regulate the fatty acid composition of seed oil (triacylglycerol, TAG) by interconversion of diacylglycerols (DAG) and phosphatidylcholine (PtdCho). PtdCho is the substrate for polyunsaturated fatty acid biosynthesis, as well as for a number of unusual fatty acids. By the action of PDCT, these fatty acids can be transferred into the DAG pool to be utilized in TAG biosynthesis by the action of acyl-CoA:DAG and phospholipid:diacylglycerol acyltransferases. Despite its importance in regulating seed oil composition, biochemical characterization of PDCT enzymes has been lacking. We characterized *Camelina sativa* PDCT in microsomal preparations of a yeast strain expressing Camelina PDCT and lacking the capacity of producing TAG. Camelina PDCT was specific for PtdCho and the *sn*-1,2 enantiomer of DAG and could not utilize ceramide. The interconversion reaches equilibrium within 15 min of incubation, indicating that only distinct pools of DAG and PtdCho were available for exchange. However, the pool sizes of DAG and PtdCho involved in the exchange were not fixed but increased with the amount of exogenous DAG or PtdCho added. Camelina PDCT showed about the same selectivity for di-oleoyl, di-linoleoyl, and di-linolenoyl species in both PtdCho and DAG substrates, suggesting that no unidirectional transfer of particular unsaturated substrates occurred. Camelina PDCT had a good activity with erucoyl-DAG as a substrate despite low erucic acid levels in PtdCho in plant species accumulating a high amount of this fatty acid in the seed oil.

KEYWORDS
acyltransferase, Camelina, diacylglycerol, PDCT, phosphatidylcholine, seed oil

INTRODUCTION

Triacylglycerol (TAG) biosynthesis in seeds is complex and involves the flow of acyl groups through phosphatidylcholine (PtdCho) in addition to the linear glycerol 3-phosphate (G3P) pathway. Palmitic (16:0), stearic (18:0), and oleic acids (18:1) are synthesized in the plastids and exported to the cytosol as acyl-CoA. These acyl-CoA can be acylated to G3P to form lysophosphatidic acid, which in its turn is acylated to phosphatidic acid (PtdOH). A phosphatidic acid phosphohydrolase converts PtdOH into diacylglycerol (DAG) that can be further acylated to form TAG (Kennedy, 1961). In seeds rich in polyunsaturated fatty acids, most of the acyl-CoA derived from the plastids are, however, used in the esterification of lysophosphatidylcholine in the formation of PtdCho (Bates et al., 2013). Linoleic (18:2) and linolenic (18:3) acids are synthesized by sequential desaturation of 18:1 esterified to PtdCho. The polyunsaturated fatty acids can be transferred from PtdCho to TAG by various routes; the reverse reaction of the acyl-CoA:

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lysophosphatidylcholine acyltransferase (LPCAT) or phospholipases combined with acyl-CoA synthetases can transfer the acyl groups from PtdCho into the acyl-CoA pool (Lager et al., 2013) where it can be utilized in the glycerol 3-phosphate pathway to produce polyunsaturated TAG. Alternatively, the enzyme phospholipid: diacylglycerol acyltransferase (PDAT) can transacylate 18:2 and 18:3 from PtdCho to DAG in the formation of TAG (Dahlqvist et al., 2000). As a third route, PtdCho with polyunsaturated acyl groups can interchange with DAG by the transfer of the phosphocholine group to DAG (Lu et al., 2009). The PtdCho-derived DAG can then be acylated, either by acyl-CoA: diacylglycerol acyltransferases (DGAT) or by PDAT to form TAG (Bates et al., 2013). One of the first reports of a rapid equilibration between DAG and PtdCho in developing oilseeds came from in vivo feeding of [^{14}C]glycerol to developing linseed cotyledons (Slack et al., 1983). In vitro studies of lipid synthesis in microsomal fractions of developing safflower seeds showed similar rapid equilibration (Stobart & Stymne, 1985). It was shown that CDP-choline:diacylglycerol cholinephosphotransferase (CPT) could operate reversibly in microsomal fractions from developing safflower seeds (Slack et al., 1985). The authors suggested that this enzyme was responsible for the equilibration between PtdCho and DAG by operating in reverse and forward directions using a membrane-bound pool of cytidine monophosphate (Slack et al., 1985). An Arabidopsis mutant named *rod1* (reduced oleate desaturase 1) with greatly reduced amounts of polyunsaturated fatty acids in seed triacylglycerols was identified 30 years ago (Lemieux et al., 1990) but the *ROD1* gene was not identified until nearly 20 years later (Lu et al., 2009). The *ROD1* gene was shown to encode a phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT), catalyzing the interconversion between PtdCho and DAG and the absence of this enzyme activity caused a 40% reduction of polyunsaturated fatty acids in seed TAG in Arabidopsis (Lu et al., 2009). The importance of the equilibration between DAG and PtdCho in shuttling 18:1 from DAG into PtdCho for desaturation and the transfer of 18:2 and 18:3 from PtdCho to DAG and further to TAG has been firmly established in a number of in vivo labeling experiments with developing seeds (Bates et al., 2009; Bates & Browse, 2012; Lu et al., 2009). From these experiments, it can be deduced that the major equilibration between DAG and PtdCho in oilseeds is catalyzed by PDCT, but a minor role of CPT can still not be excluded.

PDCT activity has been demonstrated in microsomal fractions from yeast transformed with the PDCT gene (Bai et al., 2020; Hu et al., 2012; Lager et al., 2020; Lu et al., 2009) but more detailed biochemical characterizations of the enzyme are lacking. We here present data on different biochemical properties of PDCT from *C. sativa*, an oilseed plant with high amounts of polyunsaturated fatty acid. Camelina has recently attracted considerable attention both as a model oilseed plant and a low input oil crop since it is easily genetically engineered and can grow on marginal lands (Bansal & Durrett, 2016; Berti et al., 2016; Lu & Kang, 2008).

Camelina PDCT gene was expressed in yeast lacking the capacity to synthesize TAG and assayed in yeast microsomal preparations with the addition of [^{14}C]-labeled DAG and PtdCho substrates. We show that distinct pools of PtdCho and DAG available for PDCT equilibrated rapidly and this pool could be increased by the addition of more substrates. The enzyme showed the same selectivity from a mix of unsaturated DAG and PtdCho substrate in PtdCho to DAG conversion as in DAG to PtdCho conversion and could also use erucic acid(docos-13-enoic acid)-containing substrates. The findings are discussed in relation to the physiological role of PDCT in TAG biosynthesis in seeds.

MATERIAL AND METHODS

Chemicals

Nonradioactive fatty acids, BSA (essentially fatty acid-free), G3P, phospholipase C (from *Clostridium perfringens)*, TAG lipase (from *Rhizomucor miehei*), phospholipase A2 (from *Naja mossambica*), *sn*-1,2-di-18:1-DAG, glycero-3-phosphocholine, and CoA were obtained from Sigma Aldrich. [^{14}C]18:1, [^{14}C]18:2, [^{14}C] G3P, and [^{14}C]glycerol were purchased from Perkin Elmer, [^{14}C]18:3 and [^{14}C]erucic acid were purchased from American Radiolabeled Chemicals. C18-ceramide was obtained from Larodan (Stockholm). [^{14}C]Acyl-CoA and non-radioactive PtdCho species were synthesized by acylation of the desired acyl groups as mixed fatty acid anhydride to G3P (Kanda & Wells, 1981). PtdCho was purified on silica gel 60 TLC plates (Merck) developed in chloroform:methanol:acetic acid: water (85:15:10:3.5 by vol.). *Sn*-1,2-DAG was obtained by phospholipase C treatment of the synthesized PtdCho in 0.1 M borate buffer, pH 7.4 and purified on silica gel 60 plates developed in heptane: diethyl ether: acetic acid (60:40:1 by vol.). *Sn*-1,2/2,3-*rac*-[^{14}C]18:1-DAG was synthesized by partial acylation of glycerol using mixed fatty acid anhydride and purified on silica gel 60 TLC plates developed in heptane: diethyl ether: acetic acid (60:40:1 by vol.). PtdCho and DAG substrates were stored in chloroform at minus 20°C before use.

Gene constructs, yeast transformation, and microsomal preparation

Camelina PDCT vector was designed as described by Lager et al. (2020)) and transformed into H1246 yeast

strain (Sandager et al., 2002). Microsomal preparations from H1246 expressing Camelina PDCT or empty vector were carried out as described by Jeppson et al. (2020)).

Evolutionary analysis by maximum likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−5907.16) is shown (bootstrap number = 1000). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved nine amino acid sequences. There were a total of 451 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Enzyme assays

Camelina PDCT was assayed in microsomal preparation from H1246 yeast expressing the enzyme. The assays with added radioactive DAG and PtdCho were performed in 1.5 ml Eppendorf tubes with freeze-dried microsomes corresponding to 60 μg of microsomal protein to which the substrates were added in 19 μl of benzene. The benzene was evaporated in a stream of nitrogen for 100 s while warming the bottom of the tube with the fingertips after which 100 μl of phosphate buffer, pH 7.2 was added, the solution vortexed and incubated for various times at 30°C with shaking (1250 rpm). If not indicated otherwise in the figures, 16 nmol of DAG substrate or 6 nmol PtdCho substrates were added and incubated for 30 min.

Lipid analyses

PDCT assays were terminated by extracting the lipids into chloroform by the method devised by Bligh and Dyer (1959)). Chloroform phases were concentrated under nitrogen and applied on silica gel 60 TLC plates. The TLC plates were developed to half their height in chloroform:methanol:acetic acid: water (85:15:10:3.5), then dried and fully developed in heptane: diethyl ether: acetic acid (70:50:1 by vol.). The relative radioactivity of the different lipids was determined with electronic autoradiography using Instant Imager® (Canberra Packard).

When mixed [^{14}C]DAG and [^{14}C]PtdCho species were used as substrates, the gel area with the formed radioactive PtdCho and DAG, respectively, were scraped off and the lipids were methylated in situ with 2% sulfuric acid in methanol. After extraction of the methyl esters in heptane, they were separated on silica 60 TLC plates impregnated with $AgNO_3$ and developed in heptane: diethyl ether: acetic acid (85:15:1 by vol.). The plates were subjected to electronic autoradiography to determine the relative distribution of radioactivity in 18:1, 18:2, and 18:3 methyl esters.

In PDCT experiments where the [^{14}C]PtdCho was formed and subjected to PLA_2 treatment, PtdCho was eluted from the TLC plate and treated with snake venom PLA_2 (Bafor et al., 1991) The resulting products were extracted into chloroform (Bligh & Dyer, 1959) and separated on silica 60 TLC plates developed in chloroform:methanol:acetic acid: water (85:15:10:3.5 by vol.). The relative distribution of radioactivity in lyso-PtdCho, PtdCho, and free fatty acids were determined with electronic autoradiography.

To determine the concentration of substrates, they were methylated in 2% sulfuric acid in methanol, extracted into heptane and quantified by GC on a CP-wax 58 (FFAP-CB) column using an Agilent Technologies 7890A gas chromatograph with heptadecanoic methyl ester as standard. The [^{14}C]-activity of the substrates were measured by liquid scintillation.

RESULTS

Conversion of DAG to PtdCho by Camelina PDCT

Camelina PDCT was expressed in yeast strain H1246, lacking the ability to acylate DAG to TAG (Sandager et al., 2002). With microsomal preparations of these yeast cells, we assessed different properties of Camelina PDCT without interference from the utilization of DAG and PtdCho by PDAT or DGAT enzymes.

Di-[^{14}C]18:1-DAG dissolved in benzene was added to freeze-dried microsomal membranes from yeast cells expressing Camelina PDCT. The benzene was immediately removed by a stream of nitrogen for 100 s and then incubated in buffer for various times (Figure 1). Radioactivity was accumulating in PtdCho, reaching a plateau between 10 and 20 min, demonstrating the conversion of DAG into PtdCho catalyzed by the Camelina PDCT. No radioactive PtdCho was formed from membranes from yeast transformed with empty plasmid (Figures 1 and 2).

Upon prolonged autoradiographic exposure time of the TLC separated lipids after incubation with [^{14}C] 18:1-DAG, additional radioactive lipids could be seen both in assays with membranes from yeast expressing the PDCT and from yeast harboring empty vector

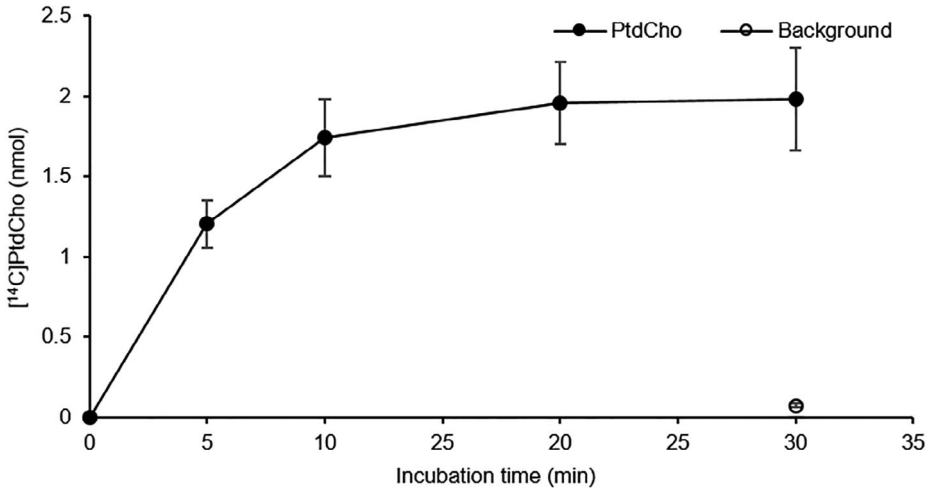


FIGURE 1 Conversion of DAG to PtdCho by Camelina PDCT. Time-course appearance of [^{14}C]PtdCho from di-[^{14}C]18:1-DAG (16 nmol) in assays with microsomal membranes from H1246 yeast cells expressing Camelina PDCT (orange dots) and H1246 yeast cells transformed with empty vector (brown dot). DAG, diacylglycerol; PtdCho, phosphatidylcholine. Average value shown ± standard deviation, n = 3 replicates

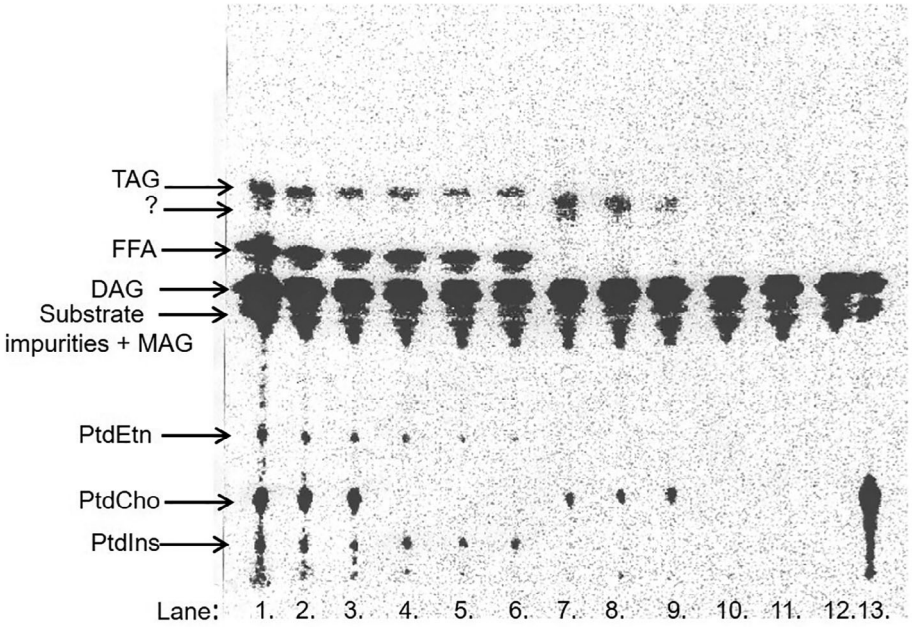


FIGURE 2 Metabolism of [^{14}C]DAG by yeast microsomal membranes. Long-time autoradiography exposure of TLC separated lipids from incubations of di-[^{14}C]18:1-DAG with microsomal preparations from yeast H1246 expressing Camelina PDCT. Lane 1–3, microsomal preparations harboring Camelina PDCT: Lane 4–6, microsomal preparations from cells transformed with empty vector; lane 7–9, membranes harboring Camelina PDCT extracted directly after application of substrate and evaporation of solvent; lane 10–13, microsomal preparations from cells transformed with empty vector extracted directly after application of substrate and evaporation of solvents; lane 13, standard [^{14}C]DAG and [^{14}C]PtdCho. The [^{14}C]PtdCho standard and [^{14}C]DAG standard and substrate contained about 2% of radioactive impurities. FFA, free fatty acids; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; TAG, triacylglycerol

(Figure 2). The majority of these compounds were free fatty acids and monoacylglycerols arising from lipase action toward the [^{14}C]DAG substrate. One compound, constituting about 0.5% of total activity co-chromatographed with TAG, one with phosphatidylethanolamine (PtdEtn) with about 0.2% of the total radioactivity and one with migration equal to phosphatidylinsositol (PtdIns), with also about 0.2% of the total radioactivity (Figure 2). None of these three compounds appeared upon direct lipid extraction of the membranes after evaporation of the benzene solvent used to deliver the substrate, demonstrating that they were formed upon subsequent incubation with buffer. However, a radioactive compound of unknown origin with retention just below TAG was seen by direct lipid extraction of membranes with PDCT, a compound that disappeared upon subsequent incubation with buffer (Figure 2). It should be noted that some radioactive PtdCho was formed by the membranes harboring PDCT also during the evaporation of the solvent (6–10% of the [^{14}C] PtdCho formed after buffer incubation) (Figure 2).

Since the yeast strain used (H1246) lacks TAG and enzymes involved in TAG synthesis (Sandager et al., 2002) we suggest that the TAG formed is an in vitro artifact, possibly formed by trans-acylation between two DAG molecules. The formation of PtdEtn from DAG is likely to be catalyzed by the yeast EPT1 (ethanolamine phosphotransferase 1) working in reverse using a small amount of membrane-bound CDP-ethanolamine or cytidine monophosphate. Aminoalcohol phosphotransferases have been shown to be able to operate reversibly in membrane preparations from both animals and plants (Goracci et al., 1981; Slack et al., 1985). The *de novo* biosynthesis of PtdIns involves the participation of PtdOH and it is therefore not a likely route for DAG to PtdIns conversion in these membranes. More likely, PtdIns was formed by the phosphatidylinositol:ceramide phosphoinositol transferase (IPCS) catalyzing the transfer of the inositol group to DAG instead of ceramide. IPCS belongs to the same super protein family of phosphotransferases as PDCT. Another member of this family is the animal sphingomyelin synthase, catalyzing the reversible transfer of phosphocholine groups from PtdCho and sphingomyelin to ceramide and DAG, respectively. This enzyme has also been shown to effectively transfer choline groups from PtdCho to DAG (Huitema et al., 2004) and thus can also catalyze the same reaction as PDCT. Even though plants do not contain sphingomyelin, in view of the structural and catalytic similarities between ceramide and diacylglycerols, it was of interest to see if Camelina PDCT could in vitro accept ceramide as a phosphocholine acceptor. We, therefore, incubated microsomal membranes from yeast expressing Camelina PDCT with [^{14}C]choline-labeled PtdCho and ceramide. We were, however, unable to show any formation of radioactive sphingomyelin (Figure 3).

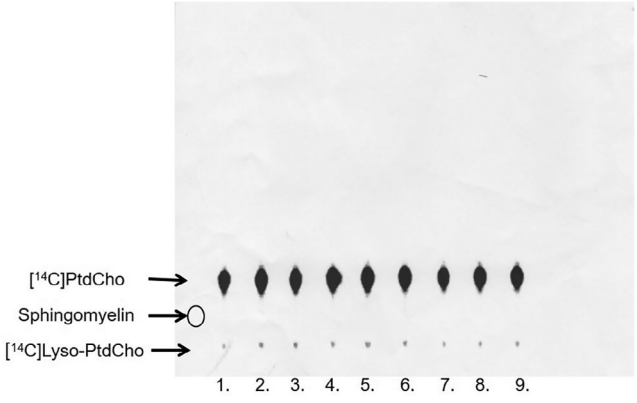


FIGURE 3 Camelina PDCT does not utilize ceramide. Autoradiogram of TLC separated lipids from incubations of microsomal membranes of yeast with [^{14}C]choline-labeled [^{14}C]PtdCho. The incubations contained 6 nmol of [^{14}C]choline-labeled PtdCho ± 16 nmol of ceramide. Lane 1–3, membranes from yeast transformed with Camelina PDCT with addition of ceramide; lane 4–6, membranes from yeast transformed with Camelina PDCT with no addition of ceramide. 7–9, membranes from yeast transformed with empty plasmid with addition of ceramide. Circle indicate the position of nonradioactive sphingomyelin standard. lyso-PtdCho, lysophosphatidylcholine; PtdCho, phosphatidylcholine

The amount of DAG interconverted with PtdCho as a function of added di-[^{14}C]DAG was assessed (Figure 4). The amount of DAG interconverted increased nonlinearly with the increasing amount of DAG added. With 32 nmol added DAG, 3 nmol of the [^{14}C]-substrate was found in PtdCho. This corresponds to an exchange of 30% of the PtdCho in the assay (see Table 1 for lipid amounts in the membranes). Thus, the DAG and PtdCho pools available for the PDCT were not fixed but could expand with more DAG added.

Conversion of PtdCho into DAG by Camelina PDCT

Next, we investigated the reverse reaction of PDCT, i.e. from PtdCho to DAG. In a time-course, the amount

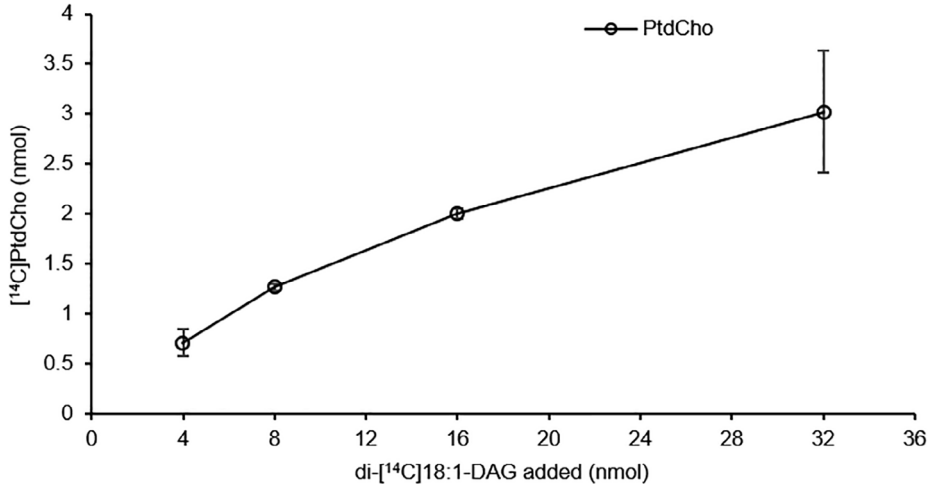


FIGURE 4 DAG conversion to PtdCho as a function of DAG concentration. The amount of [^{14}C]DAG exchanged with PtdCho as a function of the amount of di-[^{14}C]18:1-DAG added in assays of microsomal membranes from H1246 yeast cells expressing Camelina PDCT. DAG, diacylglycerol; PtdCho, phosphatidylcholine. Average value shown ± standard deviation, n = 3 replicates

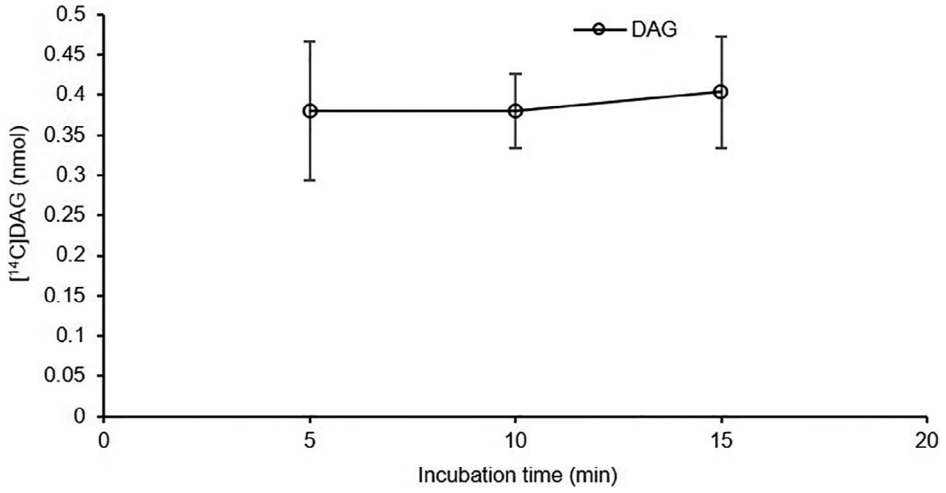


FIGURE 5 Conversion of PtdCho to DAG by Camelina PDCT. Time-course of di-[^{14}C]18:1-PtdCho interconversion with DAG in microsomal membranes from yeast H1246 cells expressing Camelina PDCT. DAG, diacylglycerol; PtdCho, phosphatidylcholine. Average values shown ± standard deviation, n = 3 replicates

TABLE 1 Diacylglycerol (DAG) and phosphatidylcholine (PtdCho) content in microsomal preparation of H1246 yeast cells expressing the Camelina PDCT

	Nmol/mg microsomal protein	Nmol per PDCT assay (60 μg of microsomal protein)
DAG	21.9 ± 1.3	1.31 ± 0.08
PtdCho	165.9 ± 17.2	9.6 ± 1.03

Note: Average values shown ± standard deviation, n = 3 replicates.

of radioactive DAG formed from added di-18:1[^{14}C] PtdCho stayed constant from 5 to 15 min of incubation (Figure 5). The amount of [^{14}C]DAG formed (0.4 nmol) corresponded to about 30% of the endogenous DAG pool (Table 1).

Camelina PDCT is specific for the *sn*-1,2 enantiomers of DAG

It was of interest to investigate if PDCT was specific for *sn*-1,2-DAG or if it also could use its enantiomer, *sn*-2,3-DAG. Acyl-lipases are active on both *sn*-1 and *sn*-3 position of DAG whereas DAG-kinases preferentially phosphorylate the *sn*-3 position over *sn*-1, although this specificity is not absolute (Epand et al., 2007). Taking advantage of the inability of snake phospholipase A_2 to act on the *sn*-1-phosphorylcholine isomer of PtdCho (van van Deenen & de Haas, 1963), we added 16 nmol of either *sn*-1,2-[^{14}C]18:1-DAG or a racemic mixture of *sn*-1,2 and *sn*-2,3-[^{14}C]18:1-DAG (*sn*-1,2/2,3-*rac*-[^{14}C]18:1-DAG) to the microsomal membranes from yeast expressing Camelina PDCT. About half the amount of [^{14}CPtdCho was formed from the racemic mixture compared to the *sn*-1,2-DAG substrate (Figure 6a), which roughly corresponds to the amount of [^{14}C]PtdCho formed when adding 8 nmol of *sn*-1,2–18:1-DAG (Figure 3). The formed [^{14}C]PtdCho was then treated with snake venom phospholipase A_2 and the amount of hydrolyzed PtdCho was determined. A near-complete hydrolysis of [^{14}C]PtdCho derived from both [^{14}C]DAG substrates was achieved (Figure 6b). Thus, the results strongly suggest that PDCT is specific for the *sn*-1,2-DAG enantiomer substrate.

Camelina PDCT selectivity toward PtdCho and DAG substrates with different degree of unsaturation

PDCT has been shown to be important in transferring polyunsaturated fatty acids into DAG for further acylation into TAG in Arabidopsis (Lu et al., 2009). We, therefore, investigated if the Camelina PDCT discriminated between mono- and polyunsaturated DAG and PtdCho substrates. An equimolar mixture of di-[^{14}C] 18:1-DAG, di-[^{14}C]18:2-DAG and di-[^{14}C]18:3-DAG as well as an equimolar mixture of di-[^{14}C]18:1-PtdCho, di-[^{14}C]18:2-PtdCho and di-[^{14}C]18:3-PtdCho were presented to the enzyme. The radioactive DAG derived from PtdCho and the radioactive PtdCho derived from DAG were methylated and the methyl esters were separated by argentation TLC to determine the proportions of radioactivity in the different acyl groups. The results showed that the acyl selectivity in the PtdCho to DAG reaction was the same as in the DAG to PtdCho reaction with no or only small statistical differences between substrates with different unsaturation. (Figure 7).

Utilization of erucoyl-containing DAG by Camelina PDCT

Erucic acid (22:1) is an unusual fatty acid that occurs in high amounts in both DAG and TAG in seeds of many *Brassicaceae* species such as *Crambe abyssinica*, but

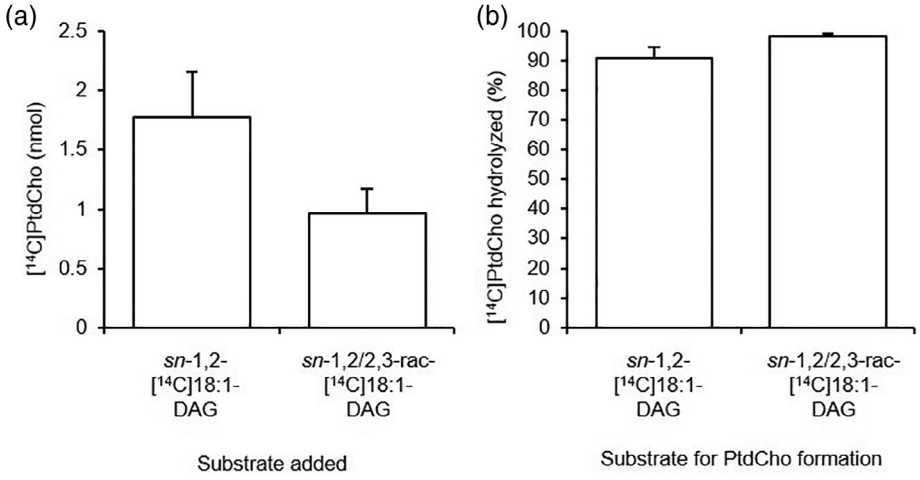


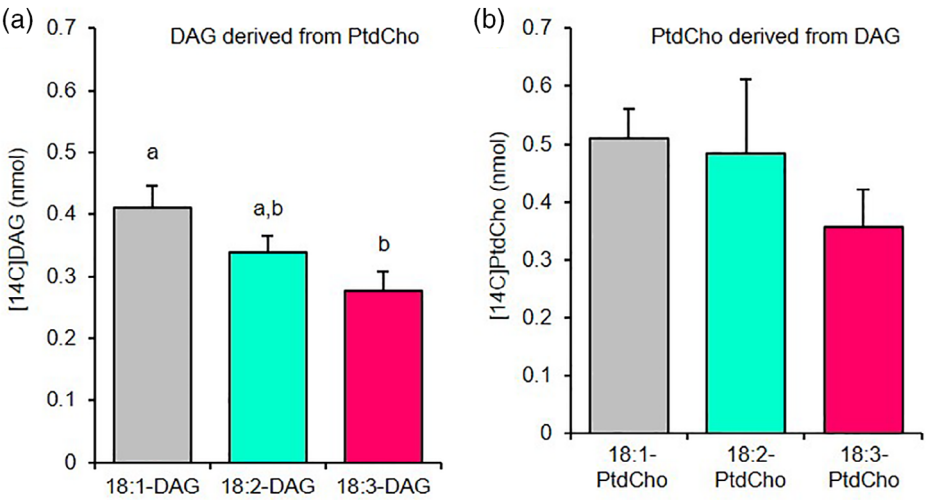
FIGURE 6 Enantiomeric specificity of Camelina PDCT toward diacylglycerols. Conversion on *sn*-1,2-di-[^{14}C]18:1-DAG and *sn*-1,2/2,3-rac-di-[^{14}C]18.1-DAG into PtdCho by Camelina PDCT (panel a) and the hydrolysis of the formed [^{14}C]PtdCho by snake venom phospholipase A_2 (panel b). Incubations contained 16 nmol of each [^{14}C]DAG substrate and yeast H1246 microsomal membranes expressing Camelina PDCT. PLA_2 treatment was performed on PtdCho from two pooled samples from assays presented in Figure 6a. The small letters above error bars denote statistical differences between results in ANOVA analysis with post-hoc Tukey HSD test, at $p < 0.01$. Groups with no statistical difference have the same letter. DAG, diacylglycerol; PtdCho, phosphatidylcholine. Average value shown ± standard deviation. (a) n = 6 replicates. (b) n = 3 replicates

only small amounts are found in PtdCho (Guan et al., 2014). We, therefore, tested if Camelina PDCT could utilize DAG with erucoyl groups. The amount of [^{14}C]PtdCho formed from di-[^{14}C]22:1-DAG was similar to that with [^{14}C]18:1-DAG both at 15 and 30 min incubation (Figure 8). We also investigated the effect of adding nonradioactive 18:1-PtdCho and 22:1-PtdCho to assays with [^{14}C]18:1-DAG. Addition of both 18:1-PtdCho and 22:1-PtdCho stimulated the exchange with 60%–70% (Figure 8). Thus, increasing the amount of PtdCho increased the amount of DAG available for PDCT. The increase in exchange with the addition of 22:1-PtdCho indicated that erucoyl-containing PtdCho also was used in the PtdCho to DAG reaction (Figure 8).

DISCUSSION

The activity of PDCT plays a central role in channeling polyunsaturated acyl groups from PtdCho to TAG via DAG in oilseeds such as soybean (Bates et al., 2009) and Arabidopsis (Bates & Browse, 2012; Lu et al., 2009). A few reports have demonstrated in vitro activities of the PDCT enzyme by assaying it in microsomal fractions from yeast expressing the enzyme (Bai et al., 2020; Hu et al., 2012; Lager et al., 2020; Lu et al., 2009) but only Hu et al. (2012) and Lager et al. (2020) have attempted any characterization of the properties of the enzyme. We have here characterized different properties of PDCT in microsomal membranes from yeast H1246 expressing the Camelina PDCT.

FIGURE 7 Selectivity toward substrates with different unsaturation by Camelina PDCT utilization of an equimolar mixture of di-[^{14}C]18:1, di-[^{14}C]18:2 and di-[^{14}C]18:3 species of PtdCho (a) and DAG (b) by Camelina PDCT. Assays were performed with microsomal preparations from H1246 yeast expressing Camelina PDCT incubated with 5.3 nmol of each [^{14}C] DAG species or 2 nmol of each [^{14}C] PtdCho species (with addition of 16 nmol nonradioactive di-18:1-DAG in order to increase the extent of exchange). DAG, diacylglycerol; PtdCho, phosphatidylcholine. Average values shown ± standard deviation, n = 3 replicates (3 × 3 pooled assays)



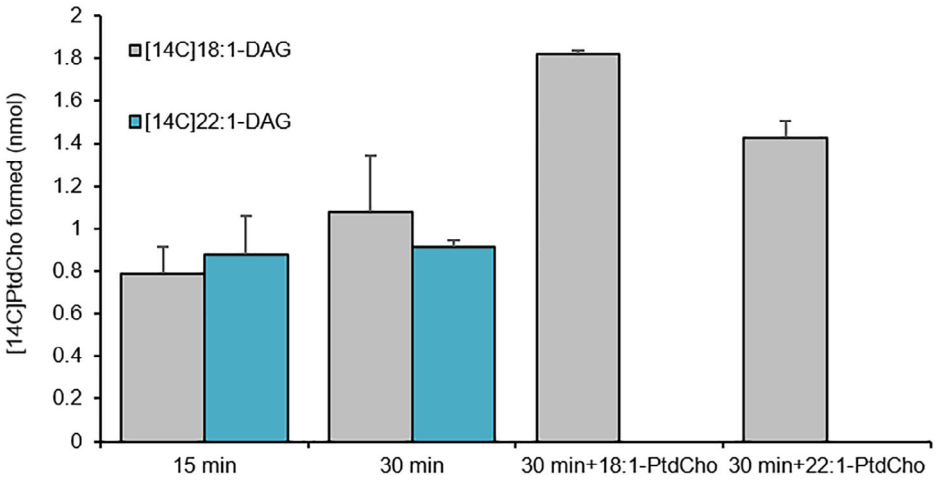


FIGURE 8 Utilization of erucoyl (22:1)-containing substrates by Camelina PDCT. The conversion of di-[^{14}C]18:1-DAG and di-[^{14}C]22:1-DAG into [^{14}C]PtdCho by Camelina PDCT and the stimulatory effects of addition of nonradioactive di-18:1-PtdCho and di-22:1-PtdCho. Assays were performed with microsomal preparations from H1246 yeast expressing Camelina PDCT incubated with 16 nmol of [^{14}C]18:1-DAG or [^{14}C] 22:1-DAG or 16 nmol of [^{14}C]18:1-DAG together with 6 nmol of nonradioactive di-18:1-PtdCho or di-22:1-PtdCho at incubation times indicated in the figure. DAG, diacylglycerol; PtdCho, phosphatidylcholine. Average value shown ± standard deviation, n = 3 replicates

Camelina PDCT has a strict requirement for PtdCho and *sn*-1,2-DAG as substrates

PDCT belongs to a protein superfamily called lipid phosphatase/phosphotransferase (LPT) in which there are two enzymes with similar catalytic activity as PDCT; the phosphatidylinositol:ceramide inositolphosphotransferase (IPCS) in plants and fungi, catalyzing the transfer of the phosphoinositol group of phosphatidylinositol to ceramide; and the sphingomyelin synthase (SMS) in animals, transferring the phosphocholine group of PtdCho (and the phosphoethanol group of PtdEtn in case of SMS2) to ceramide. It can be deduced from the phylogenetic relationships that PDCT diverged from a common ancestor earlier in evolution than the plant IPCS and SMS. Fungi IPCS (ScAUR1) diverged even earlier (Figure 9).

SMS has been shown to be freely reversible and can utilize both ceramide and DAG as acceptor for the phosphocholine group from sphingomyelin and PtdCho, respectively, but has also been shown to efficiently catalyze DAG to PtdCho conversion in vitro (Huitema et al., 2004), i.e. the same reaction as PDCT. Although plants lack sphingomyelin it was of interest to test if Camelina PDCT could synthesize this lipid in vitro, but we failed to demonstrate any phosphocholine transfer to ceramide. Further, there was no indication that Camelina PDCT could catalyze the transfer of any other head group than phosphocholine. Camelina PDCT was not only specific for PtdCho and DAG but also specific for the *sn*-1,2 enantiomer of DAG. This latter requirement prevents the cells from synthesizing PtdCho with phosphocholine at the *sn*-1 position from *sn*-2,3-DAG, an isomer of PtdCho that

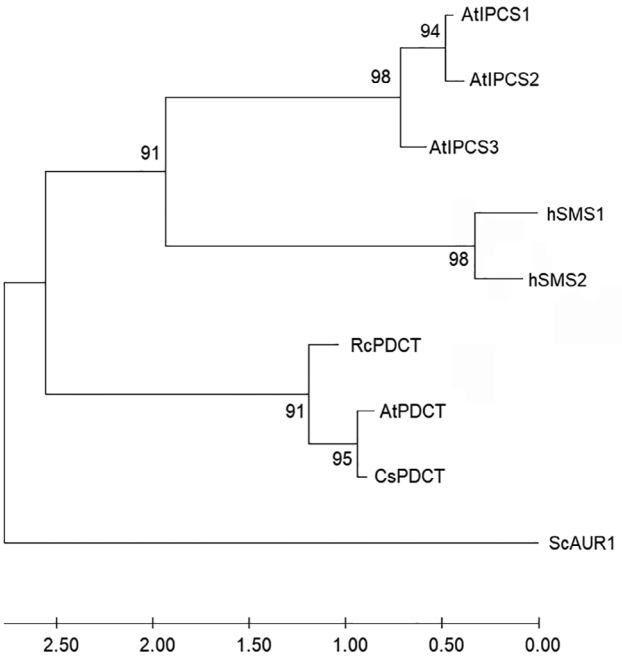


FIGURE 9 Phylogenetic tree of lipid phosphoinositol and cholinephosphotransferases. Evolutionary relations at amino acid sequence level between phosphoinositol transferases (IPCS) and phosphocholine transferase (SMS and PDCT) indicating a closer phylogenetic relationship between animal sphingomyelin synthase (SMS) and plant phosphatidylinositol:Ceramide inositolphosphotransferases (IPCS) than between plant IPCS and plant PDCT. The tree was obtained by using the Maximum Likelihood method and JTT matrix-based model – The tree with the highest log likelihood (−5907.16) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. At, *Arabidopsis thaliana*; Cs, *Camelina sativa*; hSMS, human sphingomyelin synthase; IPCS, phosphatidylinositol:ceramide inositol phosphotransferase; PDCT, phosphatidylcholine:diacylglycerol choline phosphotransferase; Rc, *Ricinus communis*; ScAUR1, *Saccharomyces cerevisiae* phosphatidylinositol:ceramide inositol phosphotransferase

cannot be degraded by phospholipase A2 (van Deenen & de Haas, 1963). In conclusion, PDCT shows much more stringent substrate requirements than the sphingomyelin synthase despite that they both can carry out phosphocholine transfer from PtdCho to DAG.

Camelina PDCT can use at least 30% of the endogenous PtdCho and DAG in the yeast membranes

We assayed Camelina PDCT in microsomal fractions of yeast H1246 expressing the enzyme. This strain lacks DGAT and PDAT activities and cannot utilize DAG and PtdCho for TAG synthesis. Thus, the exchange reactions between the two lipids can be studied without the interference of DAG-acylating enzymes. The exchange reactions in either direction, adding di-[^{14}C]18:1-PtdCho or di-[^{14}C]18:1-DAG substrates, were completed within 5–15 min of incubation. When [^{14}C]18:1-PtdCho were added, 30% of the endogenous DAG pool was exchanged with radioactive substrate. The amount of di-[^{14}C]18:1-PtdCho formed from di-[^{14}C]18:1-DAG increased with increasing concentration of [^{14}C]DAG substrate added as well as by increasing the PtdCho pool by addition of exogenous PtdCho. About 30% of the endogenous PtdCho was exchanged with [^{14}C]DAG with the highest amount of [^{14}C]DAG added. Thus, we can conclude that the PDCT/catalyzed exchange reaction involves at least 30% of the endogenous PtdCho and DAG in the yeast membranes.

Camelina PDCT shows no unidirectional selectivity for unsaturated PtdCho and DAG species

PDCT contributes to the channeling of polyunsaturated acyl groups from PtdCho to DAG for further acylation to TAG catalyzed by DGAT or PDAT enzymes (Bates et al., 2013). The absence of PDCT activity in Arabidopsis reduces the amount of polyunsaturated acyl groups in TAG by 40% (Lu et al., 2009). It should be mentioned that the equilibration between DAG and PtdCho catalyzed by PDCT is not due to a mechanistically reverse enzymatic reaction. The enzyme has, most likely, one binding site for PtdCho and another for DAG in analogy to the suggested mechanism for the transfer of the phosphocholine group from PtdCho to ceramide (and DAG) by the sphingomyelin synthase (Piotto et al., 2017). Thus, the DAG derived from PtdCho could bind to the DAG binding site and the PtdCho derived from DAG could bind to the PtdCho binding site, i.e. the enzyme will carry out the equilibration process between the two lipids by operating in one direction. It is, therefore, possible that binding or/and catalytic efficiencies at the two binding sites might be affected differently by certain acyl groups in the two substrates. This might result in a selective flow of DAG with certain acyl groups into PtdCho and a selective flow of PtdCho with other acyl groups into DAG.

We investigated if Camelina PDCT had selectivities for any DAG and PtdCho species when presented with an equimolar mixture di-[^{14}C]18:1, di[^{14}C]18:2 and di-[^{14}C]18:3 species of the two substrates. The results show that there were no major differences in selectivities in the PtdCho to DAG compared to DAG to PtdCho conversion, with equally efficient utilization of di-18:1 and di-18:2 species and with only di-18:3-PtdCho species being utilized to a statistically significant lower extent than di-18:1-PtdCho. It has been suggested that DAG derived from PtdCho by the action of PDCT constitutes a separate DAG pool from *de novo* synthesized DAG and is preferentially acylated form TAG (Bates et al., 2009). This model has been further developed to suggest three different DAG pools which are used differently by DGAT1 compared to DGAT2 and PDAT (Regmi et al., 2020). Our studies of the biochemical properties of Camelina PDCT indicate that the equilibration between DAG and PtdCho catalyzed by the enzyme involves sub-pools of the substrates and equilibrium is reached rather fast. Since we did not find any major differences in the utilization of mono-, di-, and tri-unsaturated DAG and PtdCho substrates, it suggests that the DAG and PtdCho involved in the exchange will have the same acyl composition at equilibrium. During our experimental conditions, there would not exist a 'PtdCho-derived DAG pool' but the DAG pool involved in the exchange reaction will at equilibrium be composed of 50% of original DAG and 50% PtdCho-derived DAG. In an in vivo situation, in e.g. soybean (Bates et al., 2009), PDCT activity is much higher than *de novo* synthesis of DAG and thus, the fatty acid composition of the PtdCho-derived DAG will be similar to PtdCho since the PtdCho pool is most likely much larger than the *de novo* produced DAG available for PDCT at any given time. It should be noted that the PtdCho-derived DAG cannot constitute a metabolic separate pool from the de novo produced DAG entering PtdCho through PDCT action since they are both substrates for PDCT in an equilibration reaction. However, not all *de novo* synthesized DAG is likely to be available for PDCT and could thus could constitute a DAG pool with different acyl composition as suggested by (Bates et al., 2009).

Mutating PDCT in Canola and pennycress (*Thlaspi arvense*) led to a decrease in 18:2 levels in the seed oil whereas the amount of 18:3 remained essentially unchanged compared to wild type (Bai et al., 2020; Jarvis et al., 2021). Bai et al. (2020) concluded that Canola PDCT did not use PtdCho with 18:3 acyl groups. Our results, however, suggest that PtdCho with 18:3 is utilized by Camelina PDCT to a significant

extent. It appears that 18:3 acyl groups of PtdCho in Arabidopsis PDCT mutant find alternative routes to TAG that partially compensate for the inhibition of the transfer to DAG via PDCT. In developing seeds of Arabidopsis mutated in the PDCT gene (*rod1*), both 18:2 and 18:3 levels were decreased in TAG but 18:3 levels were only reduced by 21%, whereas the 18:2 levels were reduced by 53% compared to wild type (Lu et al., 2009). However, in DAG from developing seeds of the PDCT mutant, both 18:2 and 18:3 were reduced by about 40% compared to wild type, suggesting that DAG species with either of these acyl groups were equally affected by the absence of PDCT activity. The 18:3 levels in PtdCho in the mutant were increased by 60%, whereas 18:2 decreased by 12% compared to wild type. The slight decrease in 18:2 is probably attributed to lack of 18:1 substrate for the Δ^{12} desaturase since the 18:1 levels in PtdCho is only 7–8% in both wild type and *rod1* mutant (Lu et al., 2009). It can be speculated that the difference in reduction between 18:2 and 18:3 in TAG compared to DAG is due to an increased acylation of DAG with 18:3 derived from PtdCho, either by PDAT or by DGAT via the transfer to the acyl-CoA pool. Such increased transfer of 18:3 by these enzymes is expected in the PDCT Arabidopsis mutant due to the much higher amount of 18:3 in PtdCho in this mutant. Thus, the unchanged levels of 18:3 in TAG in Canola and pennycress PDCT mutants might be an effect of such increase in alternative incorporation of 18:3 into TAG.

Erucoyl-containing DAG species are efficiently utilized by Camelina PDCT

We have earlier reported that Camelina PDCT cannot use DAG-containing ricinoleoyl groups and that these DAG species also inhibit the utilization of non-hydroxylated DAG (Lager et al., 2020). Ricinoleic acid is an 'unusual' fatty acid found in high amounts in TAG in castor but occurs in very low amounts in PtdCho (Bafor et al., 1991). Erucic acid (22:1) is another unusual fatty acid that is found in high amount in TAG and at low levels in PtdCho in *Brassicaceae* species such as *C. abyssinica* (Guan et al., 2014). We found that di-[^{14}C]22:1-DAG was utilized as good as di-[^{14}C] 18:1-DAG by Camelina PDCT and the addition of di-22:1-PtdCho increased the amount of [^{14}C]18:1-DAG exchanged in a similar range as the addition of di-18:1-PtdCho, indicating that also 22:1-PtdCho is used by PDCT.

Camelina seed oil contains only 5% of erucic acid (Rodriguez-Rodriguez et al., 2013). It has been shown that microsomal preparations of developing *C. abyssinica* seeds, having nearly 60% of erucic acid in the TAG, have very low PDCT activity (Guan et al., 2014). However, mutating the PDCT genes in Canola (zero-erucic acid rapeseed) led to a significant decrease (from 32.5% to 26.6%) of polyunsaturated fatty acids in TAG (Bai et al., 2020). Further, mutating the PDCT gene in pennycress, a plant having 35% of erucic acid in its seed oil, led to a 40% decrease in the amount of 18:2 (Jarvis et al., 2021). Given the low levels of 22:1 found in PtdCho from erucic acid accumulating seeds, the question arises whether PDCT in these plants discriminates against 22:1-containing DAG species or if these DAG species are present in a separate DAG pool not available for the PDCT enzyme. In this context, it is interesting to note that the amount of eicosa-11-enoic acid in PtdCho in an Arabidopsis PDCT mutant was reduced with 70% compared to wild type (Lu et al., 2009), indicating that PDCT in this species at least to some extent utilizes DAG with very long-chain acyl groups.

Concluding remarks

Many plants have seed TAG containing high amounts of 'unusual' fatty acids but a low amount of these in the membrane lipids of the seeds, despite that they are synthesized while esterified in PtdCho. Castor bean (*Ricinus communis*) oil contains over 90% of ricinoleic acid (12-OH-octadeca-9-enoic acid). Ricinoleic acid is formed from 18:1 by a Δ^{12} hydroxylase while esterified to PtdCho (Bafor et al., 1991) but the hydroxy fatty acids are rapidly transferred from PtdCho to triacylglycerols. The routes of transfer of ricinoleic acid to TAG may involve phospholipases (Bafor et al., 1991), PDAT (Dahlqvist et al., 2000) and reverse reaction of LPCAT (Lager et al., 2013). Formation of PtdCho from *de novo* synthesized DAG with ricinoleoyl groups as well as DAG with no such acyl groups was demonstrated in microsomal fractions from developing castor seeds (Bafor et al., 1991), indicating PDCT activity in these membranes. Further, expression of castor PDCT in Arabidopsis expressing the castor oleate Δ^{12} hydroxylase increased the total amount of ricinoleic acid in TAG (Hu et al., 2012). The function of PDCT in castor bean is an enigma since microsomal fractions of developing castor bean PtdCho only contains 5% of ricinoleic acid (Bafor et al., 1991), whereas 79% of the TAG species contain three ricinoleoyl groups (Lin et al., 2003). PDCT activity in castor seeds would therefore be expected to decrease the ricinoleic acid content in the DAG pool and re-enter ricinoleic acid into PtdCho, creating a futile cycle with these acyl groups. We have earlier shown that ricinoleoyl-containing DAG cannot be used by Camelina PDCT and is a strong inhibitor of the utilization of DAG with no ricinoleoyl groups (Lager et al., 2020). Based on published results (Bafor et al., 1991; Hu et al., 2012), it is highly likely that castor PDCT differs in this respect from Camelina and might also have another function than delivering acyl

groups (formed while PtdCho-bound) first to DAG and further to TAG. Investigation of the function of castor PDCT is now in progress in our laboratory.

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ETHIC STATEMENT

This research falls outside of human or animal studies and institutional ethical approval was not required.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTION

K.D., S.J., S.S. and I.L. conceived the project and were all involved in the experimental work. S.S. wrote the major part of the manuscript with contributions from all other authors. All authors agreed to submit the manuscript for publication.

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