Molecular Characterization of a Lysophosphatidylcholine Acyltransferase Gene Belonging to the MBOAT Family in Ricinus communis *L.*

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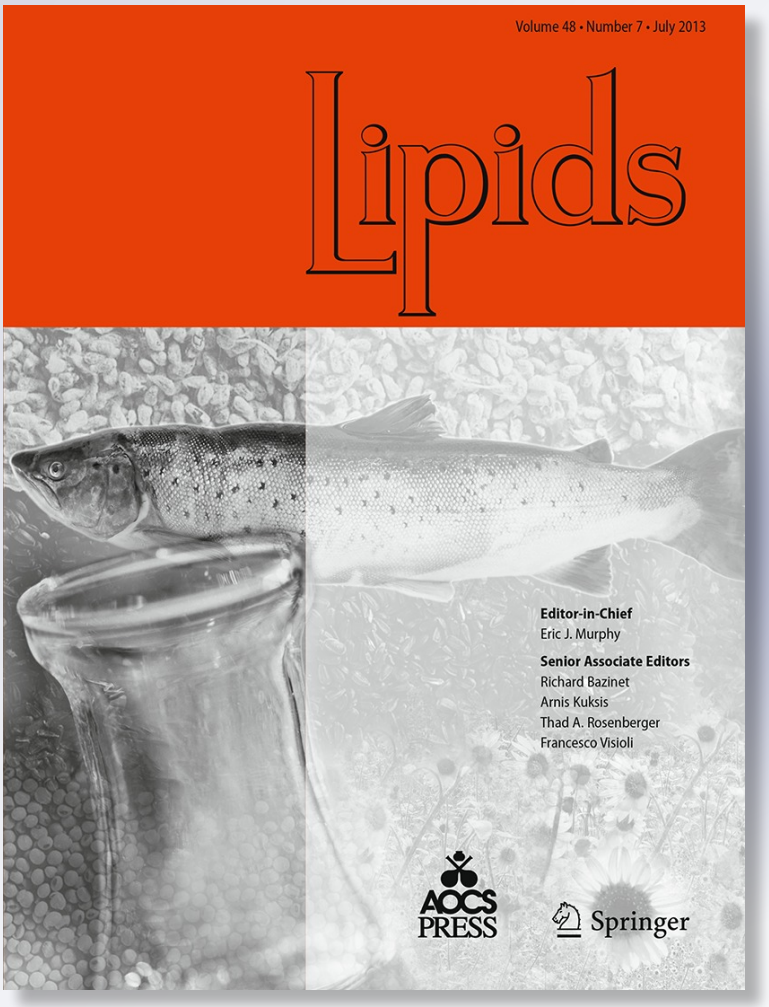

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ORIGINAL ARTICLE

Molecular Characterization of a Lysophosphatidylcholine Acyltransferase Gene Belonging to the MBOAT Family in *Ricinus communis* L.

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Abstract Acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT, EC 2.3.1.23) catalyzes acylation of lysophosphatidylcholine (lysoPtdCho) to produce phosphatidylcholine (PtdCho), the main phospholipid in cellular membranes. This reaction is a key component of the acyl-editing process, involving recycling of the fatty acids (FA) mainly at the *sn*-2 position of PtdCho. Growing evidences indicate that the LPCAT reaction controls the direct entry of newly synthesized FA into PtdCho and, at least in some plant species, it has an important impact on the synthesis and composition of triacylglycerols. Here we describe the molecular characterization of the single *LPCAT* gene found in the genome of *Ricinus communis* (*RcLPCAT*) that is homologous to *LPCAT* genes of the MBOAT family previously described in *Arabidopsis* and *Brassica*. *RcLPCAT* is ubiquitously expressed in all organs of the castor plant. Biochemical properties have been studied by heterologous expression of *RcLPCAT* in the *ale1* yeast mutant, defective in lysophospholipid acyltransferase activity. RcLPCAT preferentially acylates lysoPtdCho against other lysophospholipids (lysoPL) and does not discriminates the acyl chain in the acceptor, displaying a strong activity with alkyl lysoPL. Regarding the acyl-CoA donor, RcLPCAT uses monounsaturated fatty acid thioesters, such as oleoyl-CoA (18:1-CoA), as preferred donors, while it has a low activity with saturated fatty acids and shows a poor utilization of ricinoleoyl-CoA (18:1-OH-CoA). These characteristics are discussed in terms of a possible role of RcLPCAT in regulating the entry of FA into PtdCho and the exclusion from the membranes of the hydroxylated FA.

Keywords Acyl editing · *Ricinus communis* · Castor plant · Oil biosynthesis · Lysophosphatidylcholine acyltransferase · LPCAT

Abbreviations

12:0	Lauric acid
14:0	Myristic acid
16:0	Palmitic acid
16:1	Palmitoleic acid
18:1	Oleic acid
18:1-OH	Ricinoleic acid
18:2	Linoleic acid
18:3	α-Linolenic acid
20:4	Eicosapentaenoic acid
22:1	Erucic acid
AGPAT	Acylglycerol-3-phosphate acyltransferase
CDP-Cho	Cytidyldiphosphate-choline
CPT	Choline phosphotransferase
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
FA	Fatty acid
G3P	Glycerol-3-phosphate
GPAT	Glycerol-3-phosphate acyltransferase
LPAT	Lysophosphatidate acyltransferase
LPCAT	Lysophosphatidylcholine acyltransferase
MBOAT	Membrane-bound *O*-acyltransferases
MUFA	Monounsaturated fatty acid

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PAF	Platelet activating factor = 1-*O*-alkyl-2-acetyl-PtdCho
PAP	Phosphatidic acid phosphatase
PDAT	Phospholipid:diacylglycerol acyltransferase
PDCT	Phosphatidylcholine:diacylglycerol choline phosphotransferase
PL	Phospholipid
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdOH	Phosphatidic acid
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
TAG	Triacylglycerol
WS	Wax synthase

Introduction

Phosphatidylcholine (PtdCho) is a major glycerolipid component of eukaryotic cellular membranes. Besides its obvious structural role, it also serves as a reservoir for lipid second messengers such as phosphatidic acid (PtdOH), diacylglycerol (DAG), lysophosphatidylcholine (lysoPtdCho) and lysoPtdOH in higher eukaryotes [1, 2]. PtdCho is also the main substrate for fatty acid-modifying enzymes (desaturases, hydroxylases, epoxidases, etc.) that generate the great diversity of unusual fatty acids (FA) in plants and, at least in some species, an important DAG precursor for the synthesis of TAG [3].

Two biosynthesis routes for PtdCho have been described in higher eukaryotes, the methylation pathway and the cytidyldiphosphate-choline (CDP-Cho) pathway. The CDP-Cho pathway includes three steps, the last one being the reaction between CDP-Cho and DAG which is "de novo" synthesized from glycerol-3-phosphate (G3P) through the Kennedy pathway. In the methylation pathway, choline is generated by successive addition of three methyl groups on ethanolamine containing intermediates that are incorporated into the CDP-Cho pathway. It is usually accepted that PtdCho is produced in plants through mixed CDP-Cho and methylation pathways, though their contributions seem to differ among species [4, 5].

It is known that the PtdCho molecule is extensively remodeled through a process called Land's cycle, which recycles the FA at the *sn*-2 position [6]. This process involves the release of the FA by a phospholipase A_2-like activity (EC 3.1.1.4) and further acylation of the lysoPtdCho by LPCAT (EC 2.3.1.23) activities. It has been proposed that the reverse reaction of the LPCAT enzyme also contributes to the recycling mechanism, at least in the seeds of some plants [7]. Release of FA allows the elimination of damaged acyls but also the transfer to the acyl-CoA pool (after CoA esterification) of modified FA that have been synthesized from the common 18:1 through diverse reactions. This is the case of the polyunsaturated 18:2 or 18:3 that are produced by successive desaturation from 18:1, or the variety of unusual FA, accumulated in certain plants, like the hydroxylated ricinoleic acid (18:1-OH) in the castor plant. Once in the acyl-CoA pool, modified acyl chains become available for incorporation into "de novo" synthesis of PL or TAG, through reactions of the Kennedy pathway. From another point of view, acyl specificities of both enzymatic activities involved in Land's cycle, PLA_2 and LPCAT, are likely to determine FA composition of PtdCho at *sn*-2, and, therefore, membrane properties such as fluidity, vesicle trafficking, etc.

Two evolutionary distinct gene families, *LPLAT* (=*AGPAT*) and *MBOAT*, have been shown to encode proteins with LPCAT activities. First characterized was LPCAT belonging to the LPLAT superfamily (cd06551), also called acylglycerol-3-phosphate acyltransferase (AGPAT). Known members of this family catalyze the incorporation of acyl groups from either acyl-CoA or acyl-ACP into acceptors such as G3P, dihydroxyacetone phosphate or diverse lysoPL such as lysoPtdOH. Among them, two mammalian enzymes, LPCAT1 and LPCAT2, were described with proposed functions in respiratory physiology and inflammatory responses, respectively, [8–10], and later on several isoenzymes were also reported from red blood cells [11]. Based on sequence similarity, plant homologues within this family, LPEAT1 and LPEAT2 (encoded by *At1g80950* and *At2g45670*, respectively), were also identified in *Arabidopsis*, that exhibited broad acceptor specificity but were mainly active on lysoPtdEtn and lysoPtdOH [12]. All these proteins share the diagnostic motifs described by Lewin et al. [13] for members of this large family. On the other hand, first cloning of a *LPCAT* belonging to the MBOAT family [14] was reported in yeast by several labs independently [15–19]. The yeast LPCAT encoded by the *YOR175c* gene (also referred as LCA1, ScLPLAT and ALE1) has a broad acyl-CoA specificity, accepting acyl chains from C2 to C20, but with a preference for unsaturated acyl groups with 16–20 carbons. Homologues were also found in mammals, like LPCAT3 and LPCAT4 [19–22]. They catalyze preferential acylation, but not exclusively, on lysoPtdCho, and differ in the preferred acyl-CoA donor. While LPCAT4 has a clear preference for 18:1-CoA, LPCAT3 prefers PUFA-CoA including 20:4-CoA and 18:2-CoA as substrates instead of SFA-CoA. Additional LPLAT members of the MBOAT family have been characterized in mammals, some of them showing broad acceptor lysoPL specificity, or even a preference for lysoPL other than lysoPtdCho [22–24]. A characteristic of these LPCAT is the ability to use 1-*O*-alkyl-lysoPtdCho (lysoPAF) as a substrate to form the

platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-PtdCho), a well known mediator of the inflammatory response in animals. LPCAT homologues belonging to the MBOAT family are also found in plants. First described [25], the *Arabidopsis* AtLPLAT1 (*Atlg12640*) and AtLPLAT2 (*At1g63050*), later designated LPCAT1 and LPCAT2 [26], display acylation activities on different lysoPL but preferentially on lysoPtdCho. Recent data with double mutants identified these genes as essential components of the acyl-editing process, controlling the main entry of FA into PtdCho [27, 28]. Knockout of both genes had a moderate impact on FA composition of TAG, due to a decreased flux of 18:1 into PtdCho for desaturation, thus reducing the PUFA content and, conversely, an increase in 20:1 oil content due to a higher availability of 18:1 for elongation. Involvement of *LPCAT* genes in sustaining TAG biosynthesis was also shown in *Arabidopsis* for *LPCAT2* which was up-regulated in the type 1 diacylglycerol acyltransferase (*dgat1*) mutant [26]. While *dgat1* plants display a moderate decrease in TAG content, this was strongly reduced in the double knockout with *LPCAT2*, due to the impairment to reacylate lysoPtdCho after acyl transfer from PtdCho to DAG in the PDAT catalyzed TAG synthesis. Putative orthologues of the *LPCAT* genes have also been described in *Brassica napus*, *BnLPCAT1–1* and *BnLPCAT2*, encoding LPLAT enzymes that preferentially acylate lysoPtdCho using unsaturated fatty acyl-CoA as donors [29].

Though some information about the acyl editing process in diverse plants is available from biochemical studies with seed microsomes [7, 30–32], little is known, outside of Brassicaceae species, about *LPCAT* genes involved and their individual activities. Thus, in *Ricinus communis* it was shown that the unusual 12-hydroxy-9-cis-octadecenoic acid (18:1-OH) was generated by hydroxylation of 18:1 at the *sn*-2 position of PtdCho and that it is efficiently channeled to the seed TAG, where it reaches up to 90 % of the total FA [33–36]. Conversely, 18:1-OH is excluded from the seed membrane PL, where it is supposed to have deleterious effects, reaching <5 % of the FA in PtdCho [37]. An acyl editing mechanism was shown in the castor bean, involving the release of 18:1-OH by a phospholipase A_2-like activity and further conversion to the CoA thioester [35, 37, 38]. Monitoring of PtdCho formation in seed microsomes using radiolabelled FA [39] or acyl-CoA [35], indicated poor incorporation of 18:1-OH into PtdCho relative to the non-hydroxylated counterpart. However, a characterization of the LPCAT activity involved is not available. Here we describe the cloning and molecular characterization of the single *LPCAT* castor gene belonging to the MBOAT protein family that is homologous to reported *LPCAT* genes of Brassicaceae. Biochemical characterization of the enzymatic activity indicates that it is likely involved in the direct incorporation of FA, mainly 18:1, into PtdCho while it discriminates against 18:1-OH.

Materials and Methods

Biological Material

Seeds of *R. communis* L. cv. IN15 were kindly provided by Dr. Leonardo Velasco (Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain). Plants cultivated in a greenhouse were used as a source for the different tissues used in RT-PCR analysis. The yeast mutant *YOR175c* (*ale1*) (genotype *BY4741*; *MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YOR175c::KanMX4*) was obtained from the EUROSCARF collection (Acc. No. Y02431).

Lipid Substrates

Commercial acyl-CoA and lysoPL used in the enzymatic assays were obtained from Sigma-Aldrich Corp. 18:1-OH-CoA was synthesized following the enzymatic method by Taylor et al. [40] using the acyl-CoA synthetase from *Pseudomonas* sp. (Sigma-Aldrich). Analysis and quantification of the purified acyl-CoA product were performed by HPLC, using 18:1-CoA as standard.

Database Search and Cladistic Analysis of *LPCAT* Genes

The search for putative *LPCAT* of the *MBOAT* family in the fully sequenced castor genome was conducted using the amino acid sequences for the yeast LPLAT (*YOR175cp*, ALE1) and the two *LPCAT* homologues from *Arabidopsis thaliana* (NP_172724; AT1G12640 and NP_176493; AT1G63050, respectively). These sequences were used individually as queries in the Protein BLAST tool of the GenBank. Results from the three searches resulted in a single sequence with *E* value under 0.05. Alignment of LPLAT amino acid sequences were achieved using the program Clustal X v.2.0.11 (http://www.clustal.org/) under the default settings and further refined by visual inspection. The alignment output was used to generate a cladogram based on the minimum evolution methodology, as implemented in the MEGA software package v5 (http://www.megasoftware.net/). The bootstrap consensus tree inferred from 1,000 replicates was represented, and branches corresponding to partitions reproduced in <50 % bootstrap replicates were collapsed. The alignments were visualized using the Boxshade v. 3.2.1 software (http://www.ch.embnet.org/software/BOX_form.html).

Cloning of the *RcLPCAT* Gene

The coding sequence for the *RcLPCAT* gene was cloned by RT-PCR from total RNA obtained from castor tissues. Briefly, oligo dT-directed cDNA was synthesized from 2 μg of purified total RNA obtained from leaves, roots and developing seeds (UltraClean Plant RNA Isolation Kit, MoBio, Life Technologies) by employing the SuperScript III First-Strand Synthesis System for RT-PCR kit (Life Technologies), following the manufacturer's protocol. PCR amplification of the full coding sequence was accomplished using a cDNA mixture as template besides the oligonucleotide primers RcLPCAT-F (5′-AATTGGTACCATGGATTTAGACTTGGAATCAATGG-3′) and RcLPCAT2-R (5′-ATATGGATCCAGATCTCAGTTTCTACCTCACTCATCCTTCC-3′) containing appropriate restriction sites *Kpn*I and *Bam*HI. The reaction was performed using a proofreading polymerase (Phusion high fidelity DNA polymerase, Thermo Scientific) and a program consisting of a denaturation step of 30 s at 98 °C, followed by 35 cycles of 10 s at 98 °C, 20 s at 57 °C and 40 s at 72 °C, ending with a 5 min step at 72 °C. The resulting fragment (about 1.4 Kb) was cloned in the pJET1.2 vector (Thermo Scientific), using as recipient *Escherichia coli* JM109 cells containing the pREP4 repressor plasmid. Several clones were sequenced that resulted as being identical. Sequencing of clones was achieved using a Perkin-Elmer ABI-3730 DNA analyzer and the BigDye Terminator v3.1 chemistry.

Semiquantitative RT-PCR Analysis of *RcLPCAT*

Total RNA was purified from diverse castor tissues and oligo dT-directed cDNA was synthesized as indicated above. Approximately 2–16 ng of the cDNA were amplified in a 30 μL reaction volume using 0.6 U of Platinum Taq DNA Polymerase (Life Technologies) and a PCR program comprising a denaturation step of 2 min at 94 °C, 25–35 cycles of 30 s at 94 °C, 30 s at 60 °C (*RcLPCAT*) or 57 °C (*RcNDUB8*), 30 s at 72 °C. Amplification from the castor gene encoding the mitochondrial NADH-dehydrogenase (ubiquinone)-1β subcomplex subunit 8 (*RcNDUB8*) was used as a constitutive control to check for equal template cDNA loading [41]. The numbers of cycles used for comparison, as specified in the legend for each experiment, were experimentally determined to be non-saturating. Oligonucleotide primers used were RcLPCAT-QF (5′-GCACCGCCTCGCGCCTGGTAGTCTCG-3′) and RcLPCAT-QR (5′-CCGGCAAAGTGGCTACCACAGCAGAGG-3′) and RcNDUB8-QF (5′-GAAGATGGCGAGTGCGGTGGACACAAC-3′) RcNDUB8-QR (5′-GGCAAGCTTTCTCAAATGCTTGCTGCTC-3′).

Yeast Expression and lysoPAF Sensitivity Assay for *RcLPCAT*

The *Kpn*I/*Bam*HI fragment comprising the full length cDNA of *RcLPCAT* was liberated and subsequently cloned into the pYES2 yeast expression vector (Stratagene) under the control of the *GAL1* inducible promoter. The resulting plasmid was used to transform the yeast *ale1* mutant using the LiAcO method [42]. Transformed yeast cells were selected on SC minimal standard medium without uracil (SC-Ura). For the lysoPAF sensitivity test, yeast *ale1* cells expressing the *RcLPCAT* gene, or harbouring the pYES2 plasmid, were grown overnight in SC-Ura containing 2 % glucose and then induced for 12 h in the presence of 2 % galactose plus 1 % raffinose. Cultures were serially diluted from 10^{-1} to 10^{-5}, and 5 μL of each dilution were spotted onto SC-Ura plates containing 0, 5 and 10 μg/mL lysoPAF. The plates were incubated at 30 °C for 48 h.

Yeast Membrane Extracts Preparation

Cultures were grown at 30 °C in standard minimal medium supplemented with the auxotrophic requirement of the strain (without uracil) plus 2 % (w/v) glucose, and expression was further induced for 24 h at the same temperature on a 0.4 OD_{600} culture by the addition of galactose 2 % (w/v) plus 1 % (w/v) raffinose. Yeast cells were collected by centrifugation, further washed, and stored at −70 °C until use. Yeast homogenates were prepared in lysis buffer (50 mM Hepes, pH 7.0, 2 mM EDTA, 10 % glycerol) using glass beads, following standard protocols. Large cell debris was spun at 1,500*g*, 4 °C, for 5 min. The supernatant was then centrifuged at 16,000*g*, 4 °C, for 15 min, and the supernatant centrifuged again at 22,000*g*, 4 °C, for 2 h. The sediment was gently resuspended in sodium phosphate buffer 100 mM pH 7.5 containing 10 % glycerol, quickly freezed into liquid nitrogen and stored in small aliquots at −70 °C until use. Protein concentrations were determined using a modified Lowry's method [43] using bovine serum albumin as a standard.

Determination of LPLAT Enzymatic Activity

Spectrophotometric determination of LPLAT activity on the yeast membrane preparations was performed essentially following the method in [19], based on the reaction of the released CoA with the Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid; DTNB)]. After optimization of the different reaction components, assays were performed at 25 °C in a 400 μL reaction volume containing 100 mM phosphate buffer pH 7.5, 120 μM DTNB, 5–70 μM of the different lysoPL (lysoPtdCho, lysoPtdOH, lysoPtdEtn,

lysoPAF), as indicated for each experiment, 5–60 μM of the different acyl-CoA, and 5–45 μg of membrane extract proteins. Reactions were started by addition of the acyl-CoA after 45 s preincubation with the extract, and the absorbance at 410 nm was monitored for 3 min. The molar absorption coefficient, $\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$, was used to calculate the amount of CoA released. Initial velocities were calculated from the slopes of the curves at zero time and used for calculation of enzymatic activities that were expressed as nmol of released CoA per minute.

Results

Cloning of a *LPCAT* Gene of the MBOAT Family from *Ricinus communis*

We have performed a BLAST (http://blast.ncbi.nlm.nih.gov) search for putative *LPCAT* genes in the *R. communis* genome, using the sequences for the yeast LPLAT (*YOR175cp*, *ALE1*) and the two *LPCAT* homologues (*AtLPLAT1* and *AtLPLAT2*, renamed as LPCAT in [28]) from *A. thaliana* (NP_172724; AT1G12640 and NP_176493; AT1G63050, respectively). These are previously characterized LPLAT enzymes [15–19, 25] belonging to the MBOAT family. Just a single gene, further designated *RcLPCAT*, having significant similarity, was identified using either of those sequences as a query. With the aim of cloning the *RcLPCAT* gene, a reconstruction of the coding sequence was achieved from partial sequences (several EST and two non-overlapping WGS contigs) available in the GenBank, followed by RT-PCR amplification on oligo-dT derived cDNA. The sequences of several cDNA clones obtained were identical to our reconstruction, but differed from the available GenBank register (XM002509663) which corresponded to a truncated sequence deduced from one of the available WGS. Nevertheless, from our full-length cDNA (Acc. KC540908) and the WGS genomic sequences, a structure with eight exons can be deduced, which is similar to those reported for the *Arabidopsis* homologues (Supplementary Fig. 1).

The putative open reading frame of *RcLPCAT* encodes a 465-amino acid protein of 52.3 kDa, and a p*I* of 9.2. Hydropathy plots indicate a structure with 8–10 transmembrane domains that is similar to those of AtLPCAT and the human HsLPCAT3 (not shown), a characteristic feature that is shared by multipass transmembrane (MBOAT) proteins. The presence of the C-terminal sequence (RKDE) corresponding to a potential ER retention signal suggests that RcLPCAT localizes to the endoplasmic reticulum, although direct experimental evidence is lacking (Fig. 1).

Comparison of RcLPCAT with related enzymes of the MBOAT family (Fig. 1) reveals a high similarity to the AtLPCAT1 and AtLPCAT2 enzymes previously characterized in *Arabidopsis* (up to 78 % identity, 88 % similarity, with AtLPCAT1) and, out of plants, a 47 % similarity to the human HsLPCAT3 and 43 % to the yeast ALE1. Detailed alignment shows the presence in RcLPCAT of the two highly conserved residues within the MBOAT homology region [14], the catalytic His (position 358 in RcLPCAT) and the Asn (position 320 in RcLPCAT) presumably involved in acyl-substrate binding (Fig. 1). Moreover, the four motifs (A to D) described for MBOAT LPLAT enzymes [44] appear conserved, except for a change in motif D (YxxxYFxxH) where the histidine residue is replaced by glycine in plant LPLAT. Additional boxes of highly conserved residues can also be recognized, out of the MBOAT homology region, at the N-terminal half of RcLPCAT although their particular importance has not been checked. RcLPCAT does not contain the HX_4D box that is characteristic in the AGPAT family [13].

The MBOAT family comprises, besides LPLAT, other acyltransferases in plants, including the DGAT1 and wax synthases (WS), though the overall homology among these groups of proteins is quite reduced. Database search and subsequent cladistic analysis performed using RcLPCAT and related proteins of the MBOAT superfamily indicate the existence of LPLAT homologues of the castor enzyme in all groups of plants (Fig. 2). Though similarity with other related proteins falls considerably out of plants, homologues seem to be present in many other eukaryotes from mammals to fungi, making up a well supported monophyletic group, apart from the other MBOAT subfamilies such as DGAT1 and WS (Fig. 2). The closest characterized homologues out of plants seem to be the yeast ALE1 and the human LPCAT3 (MBOAT5), although the statistical bootstrap support (51 %) is rather low. Homologues to other mammal MBOAT such as LPCAT4 (MBOAT2) and LPEAT1 (MBOAT1), which appear as a sister to the RcLPCAT clade, seem to be missing in plants. A database search in the castor genome yielded a single MBOAT gene belonging to the *LPLAT* subfamily. This is similar to what is found in *Vitis*, but it is in contrast to the situation in other plants like *Arabidopsis*, *Glycine* and *Populus* where two similar gene copies are found, probably reflecting recent gene duplications.

Expression Analysis of *RcLPCAT* in *Ricinus communis*

The expression pattern of *RcLPCAT* in diverse organs of the castor plant was investigated through semi-quantitative RT-PCR (Fig. 3a). Expression was observed both in vegetative organs (leaves, stem, and roots) and reproductive tissues (female flowers, stamens, male sepals and seeds),

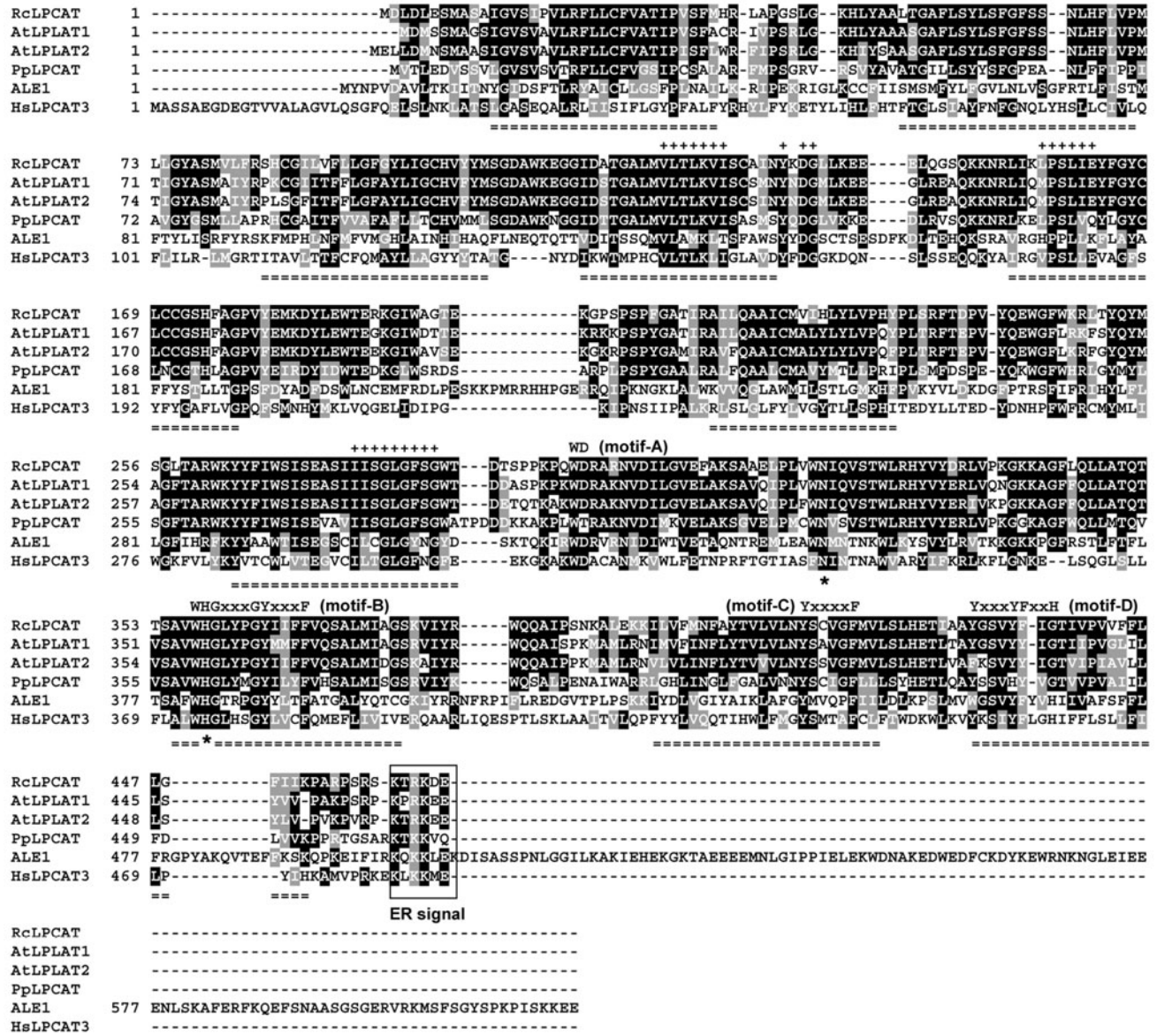

Fig. 1 Amino acid sequence alignment of RcLPCAT (Acc. KC540908) with LPLAT homologues from *Arabidopsis thaliana* (AtLPCAT1, Acc. NP_172724; AtLPACT2, Acc. NP_176493), *Physcomitrella patens* (PpLPCAT, Acc. XP_001780460), *Saccharomyces cerevisiae* (ALE1, Acc. NP_014818) and the human HsLPCAT3 (NP_005759). Sequences were aligned using the Clustal X (v.2.0.11) software (http://www.clustal.org). The Boxshade v.3.2.1 program (http://www.ch.embnet.org/software/BOX_form.html) is used to *highlight* the homology between protein sequences. *Shading* is applied when there is agreement for a fraction of sequences above 0.5. Predicted transmembrane domains are *double underlined*. Positions of the two critical His and Asn residues in MBOAT proteins are marked by *stars*. Conserved motifs (*A–D*) from [44] are indicated besides additional conserved residues upstream the MBOAT domain (*plus symbols*)

although it seems to be higher in the developing seeds (Fig. 3a), which is generally in agreement to transcriptomic data [45]. We also investigated the expression of *RcLPCAT* along seed development to check whether an increase is associated with TAG accumulation. However, as it is shown in Fig. 3b, similar transcript levels for *RcLPCAT* are found at the different seed stages, thus indicating a general role of RcLPCAT in lipid metabolism. Neither expression of *RcLPCAT* seems to be regulated by diverse abiotic stresses such as cold, heat, senescence, leaf detachment or drought, since no significant changes were recorded by RT-PCR (Supplementary Fig. 2).

Characterization of the Biochemical Activity of RcLPCAT

The putative LPLAT activity of RcLPCAT was investigated by heterologous expression in the yeast system. To

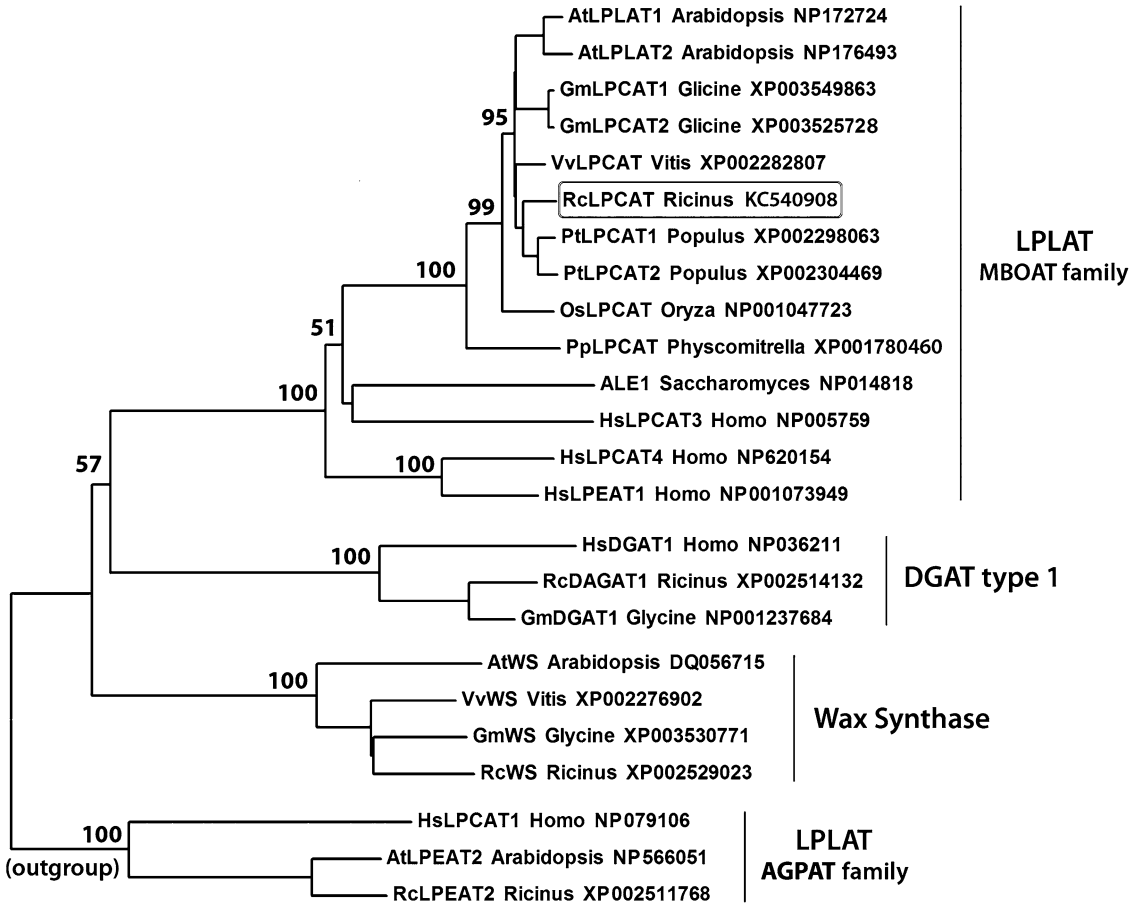

Fig. 2 Cladistic analysis of MBOAT proteins related to the castor RcLPCAT. The protein sequence of RcLPCAT was aligned to diverse plant homologues, besides selected representatives of different groups of MBOAT proteins with acyltransferase activities, DGAT1 and WS. Three LPLAT belonging to the AGPAT family were used as an outgroup. A cladogram was generated using the minimum evolution methodology as indicated in "Materials and Methods". Bootstrap percentage values over 1,000 replicas are shown on relevant *nodes*. GenBank references are provided for each sequence and the castor RcLPCAT sequence characterized in this study is enclosed by a *line*

this aim the full coding sequence of *RcLPCAT* was cloned into the yeast expression vector pYES2, under the control of the galactose inducible promoter *GAL1*. The resulting construct was used to transform the *Saccharomyces cerevisiae* mutant strain Y02431 (*ale1* or *lca1*Δ) which is deficient in LPCAT activity and hypersensitive to the alkyl-lipid lysoPAF [17]. As a preliminary test, we checked whether expression of *RcLPLAT* was able to alleviate lysoPAF inhibition through acylation of this compound, a useful system previously described for the screening of *LPCAT* genes [17]. As it is shown in Fig. 4, *ale1* cells transformed with the *RcLPCAT* gene in pYES2 were able to grow reasonably well in the presence of 5 μg/mL lysoPAF, while the growth was severely inhibited for the same cells transformed with the pYES2 empty vector. Even at strongly inhibitory concentrations (10 μg/mL), there was some growth for cells expressing *RcLPCAT*, while it is abolished in control cells. These results suggest that RcLPCAT is able to acylate lysoPAF, thus reducing its toxicity, similarly to what has been described for other LPCAT [20, 29].

To further study the biochemical activity of RcLPCAT, membrane extracts were prepared from induced *ale1* yeast cells expressing the *RcLPCAT* gene. Yeast cells harboring the pYES2 empty plasmid were processed in parallel to account for LPLAT background activity. The LPLAT assay was based on the method described by [19], after optimization of the different reaction components, as summarized in "Materials and Methods". Strong LPLAT activity was obtained for membrane extracts expressing *RcLPCAT*, using egg yolk lysoPtdCho (a mixture of 66 % 16:0-lysoPtdCho and 33 % 18:0-lysoPtdCho) as acyl acceptor and 18:1-CoA as the acyl donor. Activity increased linearly with extract amount, while it was almost undetectable for equivalent control extracts from cells transformed with pYES2 (Fig. 5a). Optimal LPCAT activity is attained at pH 7.5, and neither Mg^{2+} nor K^{+} ions have a significant effect on LPCAT activity (results not shown). LPLAT activities of RcLPCAT using other acyl acceptors, such as lysoPtdOH and lysoPtdEtn, were much lower than for lysoPtdCho, even at high enzymatic and acceptor concentrations (Fig. 5b).

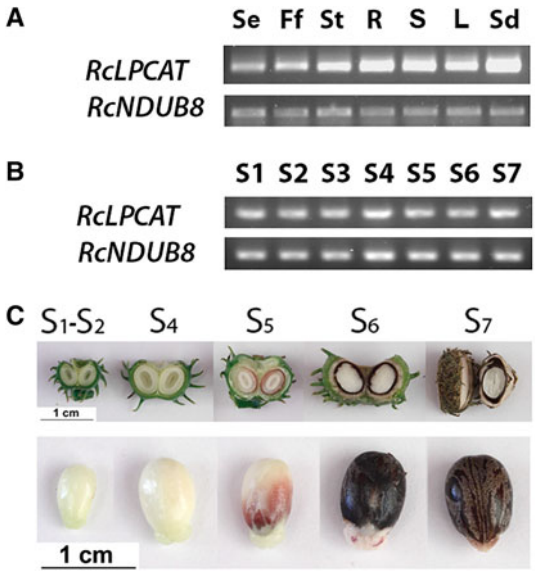

Fig. 3 **a** Expression analysis of *RcLPCAT* in diverse castor organs by semiquantitative RT-PCR. Equivalent cDNA amounts from male sepals (*Se*), whole pistillate female flowers (*Ff*), or stamens from staminate male flowers (*St*), developing seeds (*Sd*, stage *S6* in **c**), leaves (*L*), and stem (*S*) or roots (*R*) from castor seedlings were analyzed as indicated in "Materials and Methods". Single gene specific primers were used and the identity of the PCR products was confirmed by direct sequencing. The castor gene encoding the mitochondrial NADH-dehydrogenase (ubiquinone)-1β subcomplex subunit 8 (*RcNDUB8*) was used as a constitutive control to check for equal template loading. PCR products were taken at cycle numbers determined to be non-saturating (30 cycles for *RcLPCAT* and 23 cycles for *RcNDUB8*). **b** *RcLPCAT* expression along seed development. Equivalent cDNA amounts from different seed developmental stages *S1–S7* (as defined in [41], **c**) were analyzed by semi-quantitative RT-PCR as above. **c** Castor bean developmental stages visualized trough transversal cuts of the fruit (*upper row*) and images of the whole seeds (*lower row*)

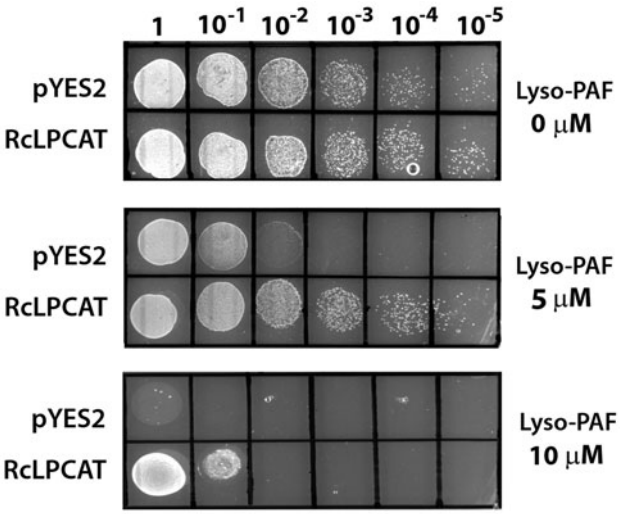

Fig. 4 LysoPAF sensitivity test for yeast *ale1* cells expressing the *RcLPCAT* gene or *ale1* cells harbouring the pYES2 empty vector. Cells were grown overnight in SC-Ura containing 2 % glucose and then induced for 12 h in the presence of 2 % galactose plus 1 % raffinose. Cultures were serially diluted from 10^{-1} to 10^{-5} and 5 μL of each dilution were spotted onto SC-Ura plates containing 0, 5 and 10 μg/mL lysoPAF. The plates were incubated at 28 °C for 48 h

Optimal activity of RcLPCAT was achieved at 40–50 μM for lysoPtdCho (Fig. 5b). More detailed analyses of the LPLAT specificity involving the acyl group at the *sn*-1 position were performed using again 18:1-CoA as a donor (Fig. 6). As it is shown, RcLPCAT acylates 16:0-lysoPtdCho, 18:0-lysoPtdCho and 18:1-lysoPtdCho at similar rates. As reported for other MBOAT enzymes it also uses the ether linked lysoPAF, at a rate even surpassing those obtained for ester-linked substrates. These results indicate that the enzyme does not discriminate the hydrocarbon chain (C16–C18) attached to the glycerol backbone.

Specificity of RcLPCAT on the acyl donor substrate, using lysoPtdCho as acceptor, was also investigated at two acyl-CoA concentrations, 10 μM (suboptimal for most acyl-CoAs) and 40 μM (usually optimal), to achieve acceptor/donor ratios of 5:1 and 1.25:1, respectively. The results shown in Fig. 7 indicate a preference for C16 and C18 MUFA, while the SFA counterparts are used at a lower rate. Activity on medium chain FA such as 14:0 and 12:0 is very low, particularly on 12:0 (Fig. 7). Incorporation of PUFA were also reduced, with some activity for 18:3-CoA, and not detectable for 18:2-CoA, similarly to the case of the long chain monounsaturated 22:1-CoA (Fig. 7). Similar results were obtained in both experiments using different acceptor/donor ratios (grey vs. black bars in Fig. 7).

Castor plants are able to synthesize the unusual 18:1-OH. Therefore, we also checked the ability of RcLPCAT to incorporate the hydroxylated FA into PL. The activity recorded on 18:1-OH-CoA is almost half of that obtained for 18:1-CoA at 10 μM (Fig. 7), but this difference is particularly evident at 40 μM where the incorporation rate of 18:1-OH is strongly reduced as compared to that of the non-hydroxylated counterpart (Fig. 7). Acyl-CoA concentration curves (Fig. 8) confirm the reduced incorporation of 18:1-OH-CoA but also show a strong inhibitory effect of the thioester at relatively low concentrations, above 15 μM. This behavior seems to be specific to the hydroxylated FA since neither 18:1 nor 18:0 thioesters show this effect (Fig. 8).

Discussion

Knowledge about LPLAT enzymes is rather scarce, even though available information gathered from mammals [44], besides emerging data in plants [26–28], indicate that they may play important roles in determining FA composition of both membrane and storage glycerolipids. Here we have

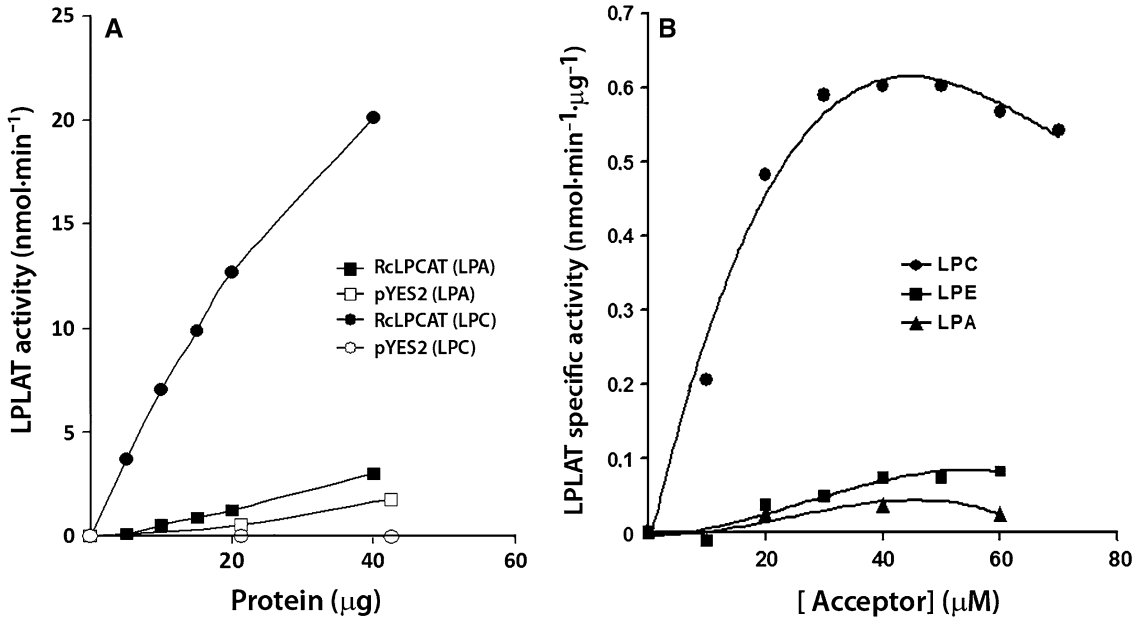

Fig. 5 LPLAT activity of RcLPCAT. **a** LPLAT activity at different amounts of membrane extracts prepared from *ale1* yeast cells expressing *RcLPCAT* or negative control cells transformed with the pYES2 empty vector. 20 μM 18:1-CoA as acyl donor and 50 μM egg yolk lysoPtdCho (16:0-lysoPtdCho and 18:0-lysoPtdCho mixture) or 18:1-lysoPtdOH as acceptors. **b** LPLAT activity at different concentrations of 18:1-lysoPtdOH, egg yolk lysoPtdEtn or lysoPtdCho (16:0-lysoPtdCho and 18:0-lysoPtdCho mixture) acyl acceptors. The assay was performed in the presence of 28 μg of membrane protein extract using 20 μM 18:1-CoA as acyl donor. Background activities obtained for extracts from *ale1* yeast transformed with the pYES2 vector were subtracted. The specific activity was represented and expressed as nmol of released CoA per min and μg of membrane protein

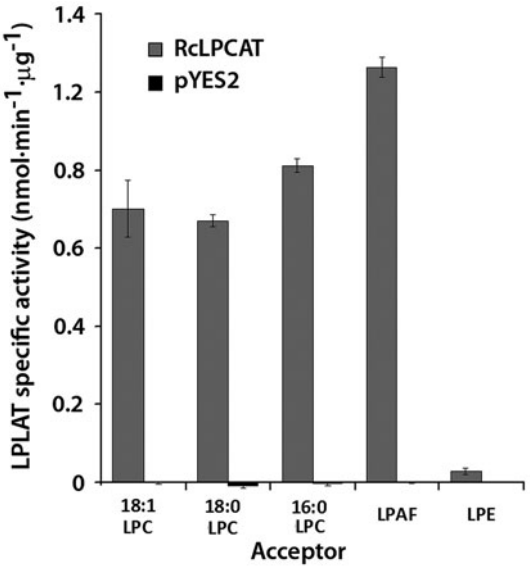

Fig. 6 RcLPCAT specificity using diverse lysoPL acceptors. 50 μM of the following lysoPL: 18:1-lysoPtdCho, 18:0-lysoPtdCho, 16:0-lysoPtdCho, lysoPAF, or egg yolk lysoPtdCho (16:0-lysoPtdCho and 18:0-lysoPtdCho mixture), were assayed using 40 μM 18:1-CoA as acyl donor. The assay was performed as described in "Materials and Methods", in the presence of 30 μg of membrane protein extract prepared from yeast *ale1* cells expressing *RcLPCAT* or from negative control cells transformed with the pYES2 empty vector. The specific activity was represented and expressed as nmol of released CoA per min and μg of membrane protein

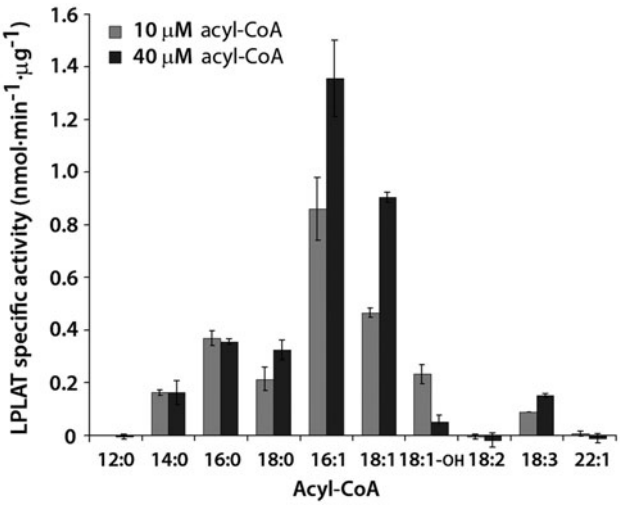

Fig. 7 RcLPCAT specificity using diverse acyl-CoA donors. LPCAT activity was recorded using 50 μM lysoPtdCho as the acceptor and 10 μM (*grey bars*) or 40 μM (*black bars*) of the different acyl-CoAs as indicated. The assay was performed as described in "Materials and Methods", in the presence of 30 μg of membrane protein extract prepared from yeast *ale1* cells expressing *RcLPCAT*. Background LPLAT activities were determined from cells transformed with the pYES2 empty vector and their values were subtracted. The specific activity was represented and expressed as nmol of released CoA per min and μg of membrane protein

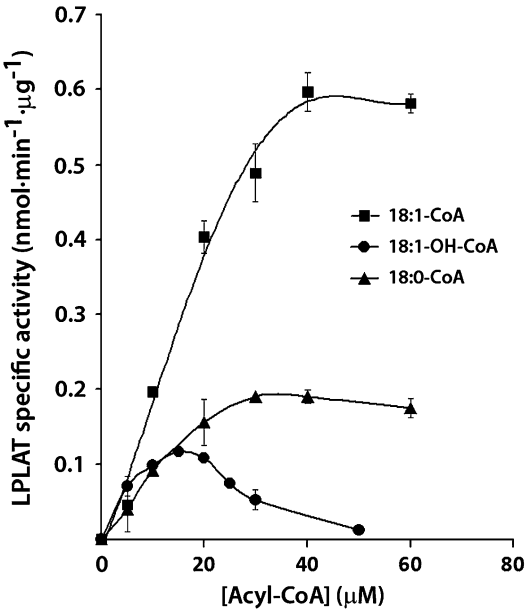

Fig. 8 Acyl-CoA concentration curves for RcLPCAT. LPCAT activity was determined using 50 μM lysoPtdCho and different concentrations of selected 18C acyl-CoAs. The assay was performed as described in "Materials and Methods", in the presence of 30 μg of membrane protein extract prepared from yeast *ale1* cells expressing *RcLPCAT*. The specific activity was represented and expressed as nmol of released CoA per min and μg of membrane protein. Average data from three experiments are represented besides the standard error of the mean

characterized a LPCAT from the castor plant (RcLPCAT) belonging to the MBOAT family. This is a large group that comprises enzymes displaying acyltransferase activities on quite diverse lipid acceptor molecules including lysoPL (LPLAT), cholesterol (ACAT), diacylglycerol (DGAT), fatty alcohols (WS), and even protein acceptors in animals. However, sequence similarity among members of these functionally different subfamilies is close to the homology detection limit [14, 46].

While a minimum of four different MBOAT enzymes belonging to the LPLAT subfamily have been characterized in mammals [47], displaying diverse lysoPL and acyl-CoA specificities, just a single member of the LPLAT subfamily (RcLPCAT) seems to be present in the castor genome, which displays preferential activity on lysoPtdCho acceptors. A single gene member, encoding ALE1, is also present in yeast, although in this case it has a broad specificity, using different lysoPL at appreciable rates. Among higher plants, two closely similar MBOAT genes are detected in Brassicaceae (*B. napus* and *A. thaliana*), representing a relatively recent duplication event [25, 29], a situation that is paralleled in other plants like *Populus* and *Glycine*, where independently recent gene duplications have occurred (Fig. 2). However, in other sequenced species like *Vitis* and *Oryza*, just a single gene is found, as in *Ricinus*. Overall, it seems that little functional and evolutionary diversification for LPLAT of the MBOAT family has occurred in plants, beyond that probably reflecting the polyploid origin of some species; this is clearly in contrast to the situation in higher animals [44].

Our data on *RcLPCAT* expression are in agreement to those reported for *Brassica* and *Arabidopsis* homologues which are also ubiquitously expressed in the plant [28, 29]. Moreover, expression of *RcLPCAT* seems to be constant along seed development and is not significantly affected by diverse environmental changes. This pattern is consistent with a proposed general role in lipid metabolism such as the acyl-editing process, as it will be discussed.

Substrate specificity analyses for RcLPCAT indicate a strong preference for lysoPtdCho as acyl acceptor (Fig. 6). Nonetheless, it showed even higher activity on ether-linked (lysoPAF) than on ester-linked molecules (Fig. 6). Therefore, linkage of the hydrocarbon chain is not relevant for RcLPCAT activity. It also exhibited a low discrimination on the acyl group attached to the *sn*-1 position of the glycerol backbone (Fig. 6). Acyl groups of 16 and 18 carbons are similarly utilized, and Δ^9-unsaturation of the hydrocarbon chain does not significantly affect the reaction rate (Fig. 6). In short, it seems that the main feature recognized in the lysoPL acceptor is the nature of the polar group attached to the molecule.

Enzyme specificity of LPCAT in plants has only been studied in detail in Brassicaceae species where, similarly to RcLPCAT, a strong preference on lysoPtdCho substrates was recorded [29]. This is indicative of a possible role of RcLPCAT in the acyl-editing process which works on lysoPtdCho [48, 49]. It is interesting that other LPLAT from the AGPAT family have been described in plants (in *Arabidopsis*) [12] that are mainly active on lysoPtdEtn and lysoPtdOH. However, since acyl-editing does not seem to take place on PtdEtn [48, 49], it is likely that these activities are involved in a different process.

Regarding acyl donor specificity, MUFA thioesters seem to be the preferred substrates of RcLPCAT while SFA (16:0 and 18:0) are used at a reduced rate (Fig. 7). This suggests that RcLPCAT specificity also contributes, besides castor microsomal LPAAT [50], to the exclusion of SFA from the *sn*-2 position of PL, a general characteristic in plant membranes. Similarly, thioesters of PUFA (18:2 and 18:3) are utilized at a very low rate. This may reflect some specialization of the LPCAT enzyme in *Ricinus*, related to a more specific entry of oleic acid into phospholipids, but the metabolic reason is not clear at the moment. One could speculate that a higher flux of oleic acid into PtdCho would also favor the preferential accumulation of the hydroxylated fatty acid (18:1-OH) in the seed, since it is known that hydroxylation takes place on oleoyl-PtdCho [33, 35, 36]. That is somewhat different to what has been reported on the

two *Brassica* LPCAT (BnLPCAT1–1 and BnLPCAT2) that showed a similar activity on 18:2-CoA and 18:3-CoA, as compared to 18:1-CoA [29]. The lower specificity of the *Brassica* enzyme would allow the incorporation of PUFA from the acyl-CoA reservoir into PtdCho which serves in part for the synthesis of TAG in the storage tissues, in agreement with seed oil composition of this species.

It is known that newly synthesized FA by the chloroplasts are mainly partitioned between the Kennedy pathway and the direct acylation of lysoPL, through the LPCAT reaction, to produce PL [48, 49, 51]. In fact, some estimates in soybean indicate that about 60 % of the newly synthesized FA (mainly 18:1) enters directly through PtdCho editing at the *sn*-2 position [48]. Although metabolic fluxes analyses are not available in castor, the reported specificity of RcLPCAT is compatible with this possible role in the direct incorporation of the newly synthesized 18:1, a strongly preferred substrate as we have shown, into PL. Once in the membrane, 18:1 is modified, for instance by desaturation, to generate the common 18:2 and 18:3 PUFAs which are then released to the acyl-CoA pool where they become available for the "de novo" synthesis of glycerolipids.

Ricinoleic acid accumulates up to 90 % of the total FA in the castor bean, while it is excluded from membranes where it is found at 5 % of the FA in PtdCho [37]. This strong bias may require not only efficient removal of 18:1-OH from PtdCho, through acyl editing processes, but also specificity mechanisms ensuring that, once in the acyl-CoA pool, it is not returned to membrane PL through the LPCAT reaction. Our results indicate that specificity properties of RcLPCAT, have a very low activity on the 18:1-OH-CoA substrate (Fig. 7), which seems to be inhibitory to the enzyme (Fig. 8), and may contribute to maintaining a low level of 18:1-OH in PtdCho. This is in agreement to previous studies performed using radiolabelled FA [39] or acyl-CoA [35] in seed microsomes, where monitoring of PtdCho formation indicated poor incorporation of 18:1-OH into PtdCho relative to other FA, particularly the non-hydroxylated counterpart.

Acyl group discrimination by LPCAT activity was also reported in rapeseed against the 22:1 [52]. This is a very long chain unusual fatty acid that is accumulated at a high level in the seed oil of this species. However, this FA is excluded from the membranes of developing embryos during TAG accumulation [53], similarly to 18:1-OH and other unusual FA. Assays performed with embryo microsomes indicated that rapeseed LPCAT was inactive on 22:1-CoA [52]. In agreement with this, both LPCAT cloned in rapeseed displayed low activity against 22:1-CoA when assayed in the yeast expression system [29]. Therefore, it is likely that discrimination exerted by these LPCAT enzymes on the 22:1-CoA may also prevent its incorporation to membrane PtdCho as a result of the editing process, similarly to what we have depicted in *Ricinus*. Acyl specificity of rapeseed LPCAT, which was shown to be inactive on 12:0-CoA, has also been proposed as one of the reasons for the decreased incorporation of 12:0 at the *sn*-2 position of *B. napus* genetically engineered to accumulate 12:0 [54].

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