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The multigene family of lysophosphatidate acyltransferase (LPAT)-related enzymes in *Ricinus communis*. Cloning and molecular characterization of two *LPAT* genes that are expressed in castor seeds

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ABSTRACT

The multigene family encoding proteins related to lysophosphatidyl-acyltransferases (LPATs) has been analyzed in the castor plant *Ricinus communis*. Among them, two genes designated *RcLPAT2* and *RcLPATB*, encoding proteins with LPAT activity and expressed in the developing seed, have been cloned and characterized in some detail. RcLPAT2 groups with well characterized members of the so-called A-class LPATs and it shows a generalized expression pattern in the plant and along seed development. Enzymatic assays of RcLPAT2 indicate a preference for ricinoleoyl-CoA over other fatty acid thioesters when ricinoleoyl-LPA is used as the acyl acceptor, while oleoyl-CoA is the preferred substrate when oleoyl-LPA is employed. RcLPATB groups with B-class LPAT enzymes described as seed specific and selective for unusual fatty acids. However, RcLPATB exhibit a broad specificity on the acyl-CoAs, with saturated fatty acids (12:0–16:0) being the preferred substrates. *RcLPATB* is upregulated coinciding with seed triacylglycerol accumulation, but its expression is not restricted to the seed. These results are discussed in the light of a possible role for LPAT isoenzymes in the channelling of ricinoleic acid into castor bean triacylglycerol.

1. Introduction

The castor plant (*Ricinus communis* L.) is an important oil-seed crop that produces a unique oil highly enriched in 18:1-OH (12-hydroxyoctadec-cis-9-enoic acid). Castor oil contains over 85% of 18:1-OH, conferring to it useful technological properties for diverse industrial uses, serving as a source for nylon-type plastics, paints and varnishes, hydraulic fluids and lubricants, cosmetics, fungicides, *etc.* [1]. Moreover, castor bean oil commonly comprises up to 50–60% of the seed weight, making it one of the highest yielding oil-seed crops [1]. Both composition and high oil content of the castor seed also makes it an attractive source for biodiesel production [2]. As a result, a steady increase in the demand for castor oil is been generated in the world market. Nevertheless, due to the harmful nature of the castor bean, efforts are being made in two different directions, either to generate non toxic castor plants [3,4] or with the aim of producing 18:1-OH in other crops through transgenic approaches [5–8].

Plant oil is mostly composed of triacylglycerol (TAG), the main storage lipid. *De novo* synthesis of TAG is carried out by sequential incorporation of acyl groups through the glycerol-3-phosphate (G3P) pathway, also known as the Kennedy pathway [9–11]. Briefly, G3P is first acylated by the action of the glycerol-3-phosphate O-acyltransferase (GPAT; EC 2.3.1.15), followed by a second acylation step catalyzed by the LPAT (also called acyl-CoA:acylglycerol-3-phosphate acyltransferase, AGPAT; EC 2.3.1.51). The phosphatidic acid (PA) produced is then dephosphorylated by a phosphatidic acid phosphatase (PAP; EC 3.1.3.4) to generate diacylglycerol (DAG), which is finally used as a substrate for the diacylglycerol O-acyltranstransferase (DGAT; EC 3.2.1.20) to produce TAG [9,11]. Since DAG is also a precursor for the synthesis of glycerophospholipids, GPAT and LPAT are acyltransferases common to TAG and

Abbreviations: 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB; 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; 22:1, erucic acid; AGPAT, acylglycerol-3-phosphate acyltransferase; CPT, choline phosphotransferase; DAG, dicaylglycerol; DGAT, diacylglycerol acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidate; LPAT, lysophosphatidate acyltransferase; LPCAT, lysophosphatidylcholine acyltransferase; LPEAT, lysophosphatidylethanolamine acyltransferase; LPL, lysophospholipid; LPLAT, lysophospholipid acyltransferase; MUFA, monounsaturated fatty acid; PA, phosphatidic acid; PAP, phosphatidate phosphatase; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine:diacylglycerol choline phosphotransferase; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TAG, triacylglycerol.

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membrane–lipid biosynthesis, while DGAT catalyze the only step in the Kennedy pathway that is committed to TAG biosynthesis.

Diverse studies on metabolic fluxes reveal that in a number of species, such as *Arabidopsis* [6] and soybean [12], a substantial part of TAG is synthesized using DAG derived from phospholipids (*i.e.* phosphatidylcholine, PC), through the action of enzymes like the choline phosphotransferase (CPT) or the phosphatidylcholine:diacylglycerol choline phosphotransferase (PDCT). This provides a route for the incorporation of fatty acid modifications such as desaturations into TAG, that are exclusively produced on the acyl groups attached to PC. However, evidences indicate that in other plants, like castor bean, TAG synthesis is carried out from *de novo* DAG instead of PC-derived DAG [13], and a similar situation has also been proposed in avocado mesocarp [14] and rapeseed [15,16].

The synthesis of TAG can also be accomplished by transfer of acyl groups from phospholipids to DAG, catalized by the phospholipid:diacylglycerol acyltransferase (PDAT) enzyme [17]. In some species like castor this mechanism has been proposed to play a role in removing unusual fatty acids (*i.e.* 18:1-OH) from membrane phospholipids, presumably to avoid disturbance of membrane structure [17,18]. However, contribution of this acyl-CoA independent pathway to the net synthesis of TAG has been shown to be relatively small in *Arabidopsis* [19,20] and it remains largely unknown in other plants. Moreover, acyl editing mechanisms [12,21] mainly involving *sn*-2 position of PC contribute to the flux of modified acyl groups into the acyl-CoA reservoir making them available for *de novo* glycerolipid biosynthesis. In the castor plant, a phospholipase activity, that has been postulated to act on the *sn*-2 position, releases very efficiently 18:1-OH that is further activated to the CoA thioester [13,22–24]. This mechanism may remove the hydroxylated fatty acid from phospholipids, providing a way for the incorporation of 18:1-OH into *de novo* synthesized TAG through the Kennedy pathway.

Knowledge of mechanisms channelling unusual fatty acids into TAG is of great importance in order to improve production of oils enriched in valuable fatty acids [25–27]. Focus has been concentrated on the acyltransferases, among them those from the Kennedy pathway, since a preference in the utilization of certain fatty acids as substrates has been demonstrated, particularly in those plants accumulating unusual fatty acids [28–30]. The LPAT enzyme, catalyzing the incorporation of acyl groups into the *sn*-2 position of the glycerol backbone, is considered one of the most stringent acyltransferases regarding substrate discrimination. Studies on LPAT acyl specificity, performed using microsomes from diverse plant species, indicate a generalized preference for 16–18 carbon monounsaturated fatty acids (MUFAs) over the saturated fatty acids (SFAs) [31]. However, utilization of 18:2-CoA at similar rates than 18:1-CoA has been also reported, even though 18:2 in many cases is absent from the *sn*-2 position of TAG [31–34]. In some instances, biased acyl-CoA preferences by the microsomal LPATs strongly determine unusual fatty acid composition at the *sn*-2 position of TAG, as exemplified in *Limnanthes douglasii* and *Cocos nuccifera* [28]. Seed oils from *Limnanthes* and coconut are enriched at *sn*-2 in 22:1 and 12:0, respectively, and, accordingly, the microsomal LPAT activities in seeds from each organism utilize very efficiently their respective unusual acyl-CoAs. On the other hand, overexpression of genes encoding acyltransferases, among them LPAT, has been proved to increase seed oil content [35–37], thus reinforcing the utility of LPAT genes as valuable biotechnological tools.

Several studies performed on different plant species have revealed the presence of at least two classes of genes encoding microsomal LPATs [28] within a large family of LPAT-like acyltransferase genes [38] (see also this work). The so-called class A microsomal LPATs defined by Frentzen [28] are ubiquitously expressed along the plant and exhibit a preference for 18:1-CoA that is characteristic of enzymes synthesizing membrane glycerolipids, and they have been cloned from diverse species [37,39,40]. On the contrary, class B microsomal LPATs are typically expressed in the seeds and possess substrate preference for unusual acyl groups. Members of the B-class have been cloned and characterized from only two plants, *Limnanthes* and *Cocos* [40–42]. Enzymes encoded by these genes exhibit strong preference in the utilization of 22:1 and 12:0, respectively, in agreement to microsomal activities recorded in the seeds. Genomic organization of the *LPAT* family has been studied in *Arabidopsis* where five genes designated *AtLPAT1-5* were described [43]. They include the plastidial isoenzyme gene (*AtLPAT1*), two class-A related genes, *AtLPAT2* and *AtLPAT3*, the last one encoding an anther specific isoenzyme, and two less related members, *AtLPAT4* and *AtLPAT5*, encoding proteins without *in vitro* detectable LPAT activity, that seem to be related to cardiolipin biosynthetic enzymes. No representative of the class-B is found in the *Arabidopsis* genome [38,43].

Some studies have been performed on the LPAT activity of castor bean microsomes [34]. Data indicated a clear selectivity on acyl-CoA substrates with a preference in the utilization of 18:1-CoA and 18:2-CoA over SFA thioesters, accordingly to the usual trend in other species. Surprisingly, 18:1-OH-CoA was not incorporated at a significant rate, unless polyamines were present in the reaction [34]. However, little information is available on castor LPATs, neither for individual enzymes nor at the gene level, an important fact since different LPAT isoenzymes are likely to be active in the diverse organs of the plant, including the seed. In this article we have investigated the family of *LPAT*-related genes in the castor plant. Several *LPAT* candidates have been identified and their expression patterns analyzed, among them two genes representative of the A and B classes that are expressed in the developing seed. Enzymes encoded by these genes showed LPAT activity on diverse acyl-CoAs, among them 18:1-OH-CoA, even in the absence of polyamines, although they strongly differ in their preferences against different acyl substrates. These results are discussed in regard to their possible roles in the biosynthesis of glycerolipids and channelling of the unusual 18:1-OH into the castor oil.

2. Materials and methods

2.1. 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 of different tissues used for RT-PCR analysis. The *Escherichia coli* mutant strain SM2-1 [44] containing a mutation in the *plsC* gene (*thr-1*, *araC14*, *tsx-78*, *Δ*(*galK-attλ*)*99*, *hisG4*(Oc), *rfbC1*, *met162::Tn10*, *plsC101*, *rpsL136*(strR), *xylA5*, *mtl-1*, *thi-1*) was obtained from the *E. coli* Genetic Resource Centre.

2.2. Lipid substrates

Non-radioactive acyl-CoAs and *sn*-1-oleoyl-*sn*-glycerol 3-phosphate (18:1-LPA) were obtained from Sigma–Aldrich Corp. Radioactively labelled [^{14}C]*sn*-1-oleoyl-*sn*-glycerol 3-phosphate (55 mCi/mmol) was purchased from American Radioactive Corp. 18:1-OH-CoA was synthesized following the enzymatic method by Taylor et al. [45] 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. The *sn*-1-ricinoleoyl-*sn*-glycerol 3-phosphate (18:1-OH-LPA) was enzymatically synthesized using Lipozyme (Sigma–Aldrich) in a solvent free medium according to the method by Han and Rhee [46]. After extraction of the reaction mixture

with $CHCl_3$-MetOH (1:2) the LPA product was further purified by preparative TLC. The final preparation, essentially free of the PA by-product, was analyzed and quantified by TLC and HPLC, using commercial 18:1-LPA as standard.

2.3. Database search and cladistic analysis of LPAT-like genes

The search for putative LPAT in the fully sequenced castor genome was conducted using the amino acid sequences from the five LPAT sequences identified in *Arabidopsis* [43]: AtLPAAT-1 (acc. No. NP_194787), AtLPAAT-2 (acc. No. NP_567052), AtLPAAT-3 (acc. No. NP_175537), AtLPAAT-4 (acc. No. NP_565098) and AtLPAAT-5 (acc. No. NP_188515). In addition, the *Limnanthes* sequence LAT2 (acc. No. Q42870), representative of the seed specific B-class LPAT [40] was also used in the search. These sequences were used individually as queries in the PSI_BLAST tool of the GenBank with an initial threshold value of 0.005 and three successive iterations were performed. Results from the six searches were combined, resulting in a total of 21 different proteins (Supplementary Table 1). Alignment of the amino acid sequences was achieved using the programme Clustal X v.2.0.11 (http://www.clustal.org/) under the default settings and further refined by visual inspection. The alignment outputs were used to generate cladograms, based on the Minimum Evolution (ME) method, as implemented in the MEGA package v5 (http://www.megasoftware.net/). The bootstrap consensus tree inferred from 1000 replicates was represented, and branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Rooting of the trees was accomplished by using the far related RcDGAT2 sequence as the outgroup. The alignments were visualized using the Boxshade v. 3.21 software (http://www.ch.embnet.org/software/BOX_form.html).

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2.4. Cloning of RcLPAT genes

The *RcLPAT2* gene was cloned by RT-PCR from RNA of castor developing seeds. Briefly, oligo dT-directed cDNA was synthesized from 5 μg of purified total RNA (UltraClean Plant RNA Isolation Kit, MoBio, Life Technologies) by employing the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen), and following the manufacturer's protocol. PCR amplification of the full coding sequence was done using oligonucleotide primers RcLPAT2-Up (5′-ATATCCATGGCTGTTGCAGCTGTAGCTGTTATC-3′) and RcLPAT2-Down (5′-AATTGGATCCGCGACTCTAGTCCTGTTTGTTTTCTGC-3′), containing appropriate restriction sites *Nco*I and *Bam*HI. The reaction was performed using a proofreading polymerase (iProof High-Fidelity DNA Polymerase, BioRad) and a programme consisting of a denaturation step of 30 s at 98 °C, followed by 35 cycles of 10 s at 98 °C, 20 s at 63 °C and 40 s at 72 °C, ending with a 8 min step at 72 °C. The resulting fragment (about 1.2 Kb) was cloned in the vector pGEM-T-Easy (Promega), and several clones were sequenced. Two similar sequences were revealed differing by a single nucleotide substitution, that encoded identical proteins and probably representing allelic variants of the gene. The *Nco*I/*Bam*HI fragment was subsequently cloned into the pQE60 prokaryotic expression vector (Qiagen) using *E. coli* JM109 cells containing the pREP repressor plasmid as recipient. Cloning of *RcLPATB* was similarly performed by RT-PCR using the oligonucleotide primers RcLPATB-Up (5′-CAGTAATAACCCATGGAAAGCACTGGAGGTGGTTC-3′) and RcLPATB-Down (5′-AATTAGATCTCCTTCTAACTTAGAGGCCTTTGAGACTC-3′). Sequencing of clones was achieved using a Perkin-Elmer ABI-310 DNA automated sequencer and the BigDye Terminator v3.1 chemistry.

2.5. Semiquantitative RT-PCR analysis of RcLPAT genes

Total RNA was purified from diverse castor tissues and oligo dT-directed cDNAs were synthesized as indicated above. Approximately 5–20 ng of the cDNA was amplified in a 30 μL reaction volume using 1 unit of AccuPrime Taq DNA Polymerase (Invitrogen) and a PCR programme comprising a denaturation step of 2 min at 94 °C, 20–35 cycles of 15 s at 94 °C, 30 s at 53–57 °C, 30 s at 68 °C, and a final step of 5 min at 68 °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. The numbers of cycles, as specified in the legend for each experiment, were experimentally determined to be non-saturating. Specific oligonucleotide primers and amplification products are summarized in the Supplementary Table 2. The identity of the PCR fragments was confirmed by direct sequencing.

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2.6. Complementation assay of the plsC mutant of Escherichia coli by RcLPAT genes

Complementation of the SM2-1 strain of the *plsC E. coli* mutant containing the pREP plasmid was performed by standard transformation procedures using the pQE60 plasmid containing the full length coding sequence of *RcLPAT2* or *RcLPATB* genes, or with the empty plasmid pQE60 as a negative control. Transformed cells were plated on LB media containing 100 μg/mL ampicilline, 50 μg/mL kanamycin 30 μg/mL, tetracycline, 50 μg/mL streptomycin and 0.1 mM IPTG, and cultured at 30 °C (permissive conditions) or at 42 °C (restrictive conditions). Single colonies recovered from 42 °C plates were cultured O/N at 30 °C in liquid LB medium containing selective antibiotics and IPTG. Cultures were further adjusted to an OD_{600} of 0.1, and 5 μL drops from serial dilutions, from 1:10 to $1:10^5$, were deposited onto LB plates containing 0.1 mM IPTG or without it. Incubation was performed at 30 °C (permissive conditions) or 42 °C (restrictive conditions).

2.7. E. coli membrane extracts preparation

Extracts from the *plsC* (SM2-1) *E. coli* mutant complemented with *RcLPAT2* or *RcLPATB* genes were prepared from 150 mL of cultures grown at 30 °C to exponential phase (OD_{600} 0.5–0.6) and further induced with 0.2 mM IPTG for 3 h. Cells were centrifuged and resuspended in 15 mL of 100 mM phosphate buffer pH 7.5 containing 10% glycerol and 100 μg/mL lysozyme. After 15 min incubation on ice, cells were sonicated five times with 15 s bursts and 2 min rest intervals, using a VP200S Hielscher sonicator adjusted at 45% amplitude and 0.5 cycles. The homogenate was centrifuged for 5 min at 1500 × *g*, and the supernatant further spun at 18,000 × *g* for 1 h. Sedimented membranes were carefully resuspended into phosphate buffer 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 [47].

2.8. Determination of LPAT enzymatic activity

Spectrophotometric determination of LPAT activity on *E. coli* membrane preparations was performed essentially following the method by Bafor et al. [33], based on the reaction of the released CoA with the Ellman's reagent DTNB. After optimization of the

different reaction components the assays were performed at 25 °C in a 400 μL reaction volume containing 100 mM phosphate buffer pH 7.5, 120 μM DTNB, 50 μM 18:1-LPA or 18:1-OH-LPA, either 2 mM EDTA (in minus Mg^{2+} assays) or 1 mM $MgCl_2$, 20–40 μM of the different acyl-CoAs and 30–320 μg of membrane extract proteins. Reactions were started by addition of the acyl-CoA after 30 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 nanomols of released CoA per minute.

Determination of LPAT activity by radioactive labelling was based on the method described by Cao et al. [48]. The standard reaction mixture (100 μL) contained 100 mM phosphate buffer pH 7.5, 1 mM $MgCl_2$, 50 μM [^{14}C]oleoyl-LPA (111,000 dpm), 20 μM of the different acyl-CoAs, and the reaction was started by adding 4.5 μg of bacterial extracts. After incubation at 30 °C for 5 min, the reaction was stopped by adding 170 μL AcOH (5% v/v) and 500 μL of $CHCl_3$–MetOH (1:1, v/v), followed by vigorous shaking, and centrifuged for 10 min at 2000 rpm. The $CHCl_3$ layer was recovered and dried under N_2 stream and quantitatively transferred onto EM TLC silica gel plates (60 F254). TLC plates were developed in $CHCl_3$–MetOH–AcOH–H_2O (90:15:10:3, v/v). The radioactive PA product was located by autoradiography using a radio-TLC imaging scanner (Bioscan, AR2000). The spot containing radioactive PA was scrapped into a scintillation vial with 2 mL Aquasol cocktail (NEN Research Products, Boston, MA) and counted in a scintillation counter (Beckman Coulter LS6500).

3. Results

3.1. LPAT-related genes in the genome of Ricinus communis

We have conducted a search for putative LPATs in the castor plant genome database (http://www.castorbean.jcvi.org/index.php) using amino acid sequences for different LPAT plant isoenzymes described in literature. The PSI_BLAST tool was used with an initial threshold value of 0.005 and three iterations were performed individually using the five LPAT sequences identified in *Arabidopsis thaliana* [43], besides the *Limnanthes* sequence (LAT2), representative of the seed specific B-class LPAT [31], as queries. As result, a total of 21 proteins were retrieved (Supplementary Table 1), none of them biochemically tested so far. Twenty of them contain the conserved domain for the LPLAT superfamily (cd06551), a very diverse group of enzymes involved in the *de novo* and remodelling pathways of glycerophospholipid biosynthesis. Characterized members of this family catalyze the incorporation of acyl groups from either acyl-CoAs or acyl-ACPs into acceptors such as G3P, dihydroxyacetone phosphate or diverse LPLs such as LPA. All sequences retrieved, except one, possess the two characteristic motifs NHX_4D and EGT (boxes I and III) conserved among bacteria, yeast, animal and plant LPATs, which have been shown to form the catalytic and LPA-binding sites, respectively [49–52].

Cladistic analyses were performed for the *Ricinus* LPAT-related sequences by including previously characterized plant acyltransferases (Fig. 1) to infer some information about relationships and possible functions/activities.

Three of the castor sequences, here designated RcLPAT2, RcLPAT3A and RcLPAT3B, grouped in a cluster (LPAT2/3 group) containing the microsomal LPATs of generalized expression (also designated as A-class LPATs) such as AtLPAT2 from *Arabidopsis* [43], besides the AtLPAT3 anther specific isoenzyme from the same organism. RcLPAT2 is closely related to characterized LPATs of the A-class including, besides AtLPAT2, LAT2 from *Limnanthes* [40], MAT1 from maize [39], and BAT1 from *Brassica* [37]. All these enzymes have been described of generalized expression and usual preference for long chain (16–18) MUFAs. Within this group, close relatives are also found from other monocot plants like *Oryza*. RcLPAT3A is most similar to the *Arabidopsis* AtLPAT3 product, and putative orthologues can also be identified in diverse dicot plant species, but not in monocots, making up a highly supported cluster separated from LPAT2-like enzymes. RcLPAT3B appears in a basal position in the LPAT2/3 group (Fig. 1). Interestingly, only one close relative from *Populus* is found in the GenBank for RcLPAT3B, while putative orthologues seem to be absent from the sequenced genomes of *Arabidopsis*, *Vitis*, and *Oryza*. Comparison of castor sequences from the LPAT2/3 group (Supplementary Fig. 1) reveals that sequences around Box I (ϕ–NHQS-ϕDϕϕ consensus as defined by Lewin et al. [49] for LPATs from bacteria, yeast and animals, where ϕ stands for a hydrophobic residue) is quite similar among RcLPAT2 and RcLPAT3A, as for putative orthologues from *Arabidopsis*, *Populus* (Supplementary Fig. 1) and other plant species (not shown), conforming the consensus **NH**X**S**DI**D**WL where X is a polar amino acid (usually R). However, this motive appears somewhat diverged for the LPAT3B proteins of *Ricinus* and *Populus* where the sequence **NH**ICDA**D**ϕϕ is found. The same holds true for sequences around box II (G-ϕ**FIDR** in [49]) where a well conserved E(D)YL**FLER** motif is found for LPAT2 and LPAT3A related plant proteins, while a less related EHV**FV**S**R** sequence appears in RcLPAT3B. Sequences around Lewin's boxes III (ϕ**F**P**EGTR**—G) and IV (ϕPϕϕ**P**ϕϕϕ) are well conserved in all plant proteins of the LPAT2/3 cluster with ϕ**F**V**EGTR** and VLI**P**RTKG consensus, although no similarity to the rather imprecise box IV could be found except for the critical proline residue.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2012.09.015.

Two other sequences from *Ricinus* clustered with AtLPAT4 and AtLPAT5 [43] (LPAT4/5 group). These are ubiquitous proteins, whose function remains to be established, although some similarity can be found to lysocardiolipin acyltransferases of animals, as it is recognized by recent annotations of the GenBank. Both groups, LPAT2/3 and LPAT4/5, are closely related since they appear as sister in the cladogram (bootstrap value 99%, Fig. 1) and they also share two conserved motifs, **NH**X_2DX**D** and FV**EGT**R, corresponding to the characteristic boxes I and III, respectively (Supplementary Table 1).

Another castor sequence appears in a cluster containing the so called B-class LPAT enzymes which groups, although not exclusively, seed specific LPATs with preference for unusual acyl groups [28] such as those characterized from *Limnanthes* [41] and *Cocos* [42]. Closely related sequences are also found in the GenBank for very diverse organisms like the monocots *Oryza* and *Sorghum*, and the lower fern *Selaginella*. Available ESTs in the GenBank, also reveals the presence of LPATB-like genes in other plants like *Citrus*, *Vigna* and *Carica*. Sister to the B-class group is the cluster of plastidial LPATs that includes the AtLPAT1 protein [51] and one of the LPAT-related castor sequences. Close relationship among B-class LPATs and plastidial isoenzymes is revealed in this analysis mainly containing plant sequences, as previously reported [51], and, accordingly, the same conserved motive FP**EGT** around box III is found in both groups. However, when LPATs from diverse animals are included in the cladistic analysis, it is observed that LPATB-like enzymes are in fact closer to animal LPATs than to plastidial enzymes (Supplementary Fig. 2).

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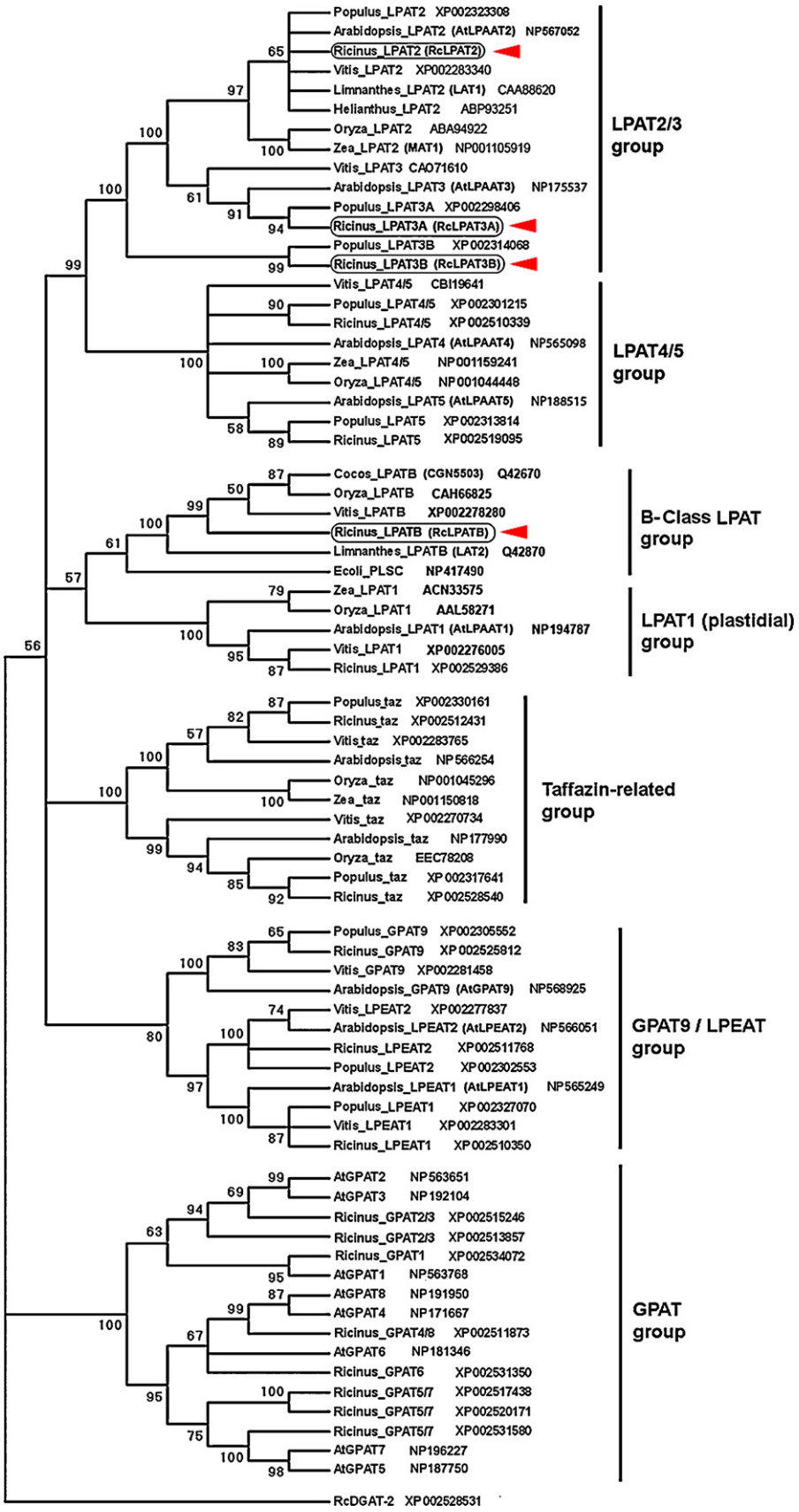

Fig. 1. Cladistic analysis of LPAT-related sequences of *R. communis*. The 21 castor sequences retrieved in a search for LPAT-related proteins were aligned to closest relatives from higher plants and a cladogram was generated using the "Mininum Evolution" methodology as indicated in Section 2. Bootstrap percentage values over 1000 replicas are shown on their respective nodes. GenBank references are provided for each sequence, and the four castor LPAT sequences characterized in this study (RcLPAT2, GenBank ID: JQ796917; RcLPAT3A, GenBank ID: XP_002513487; RcLPAT3B, GenBank ID: XP_002513486, and RcLPATB, GenBank ID: JQ796918) are marked by red arrows.

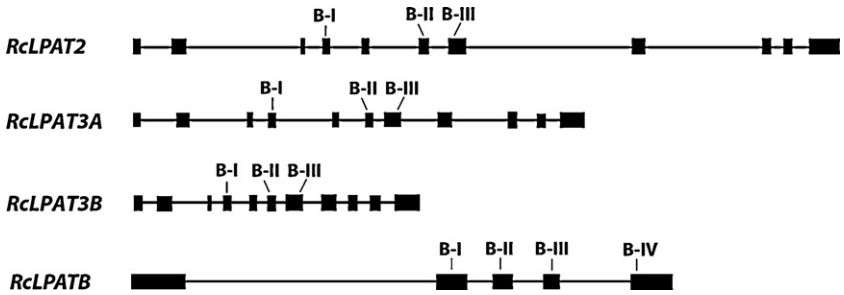

Fig. 2. Gene structures for *RcLPAT2* and *RcLPATB*. Intron/Exon arrangements were deduced from cDNAs obtained in this study, while those for *RcLPAT3A* and *RcLPAT3B* are GenScan predictions from the GenBank, since no full cDNA are available in the GenBank for these genes.

A different cluster contains two castor sequences besides predicted acyltransferases of unknown function, but with homology to tafazzin proteins. Tafazzin is the product of the human Barth syndrome gene which has been involved in cardiolipin biosynthesis [53]. Tafazzin-like proteins have been described in other organisms like yeast, where they display a mitochondrial LPCAT activity related to TAG and mitochondrial lipid synthesis [54]. Sequences from this group share a somewhat diverged box III, FP**EG**(S/K), with the highly conserved threonine residue of the EGT motif replaced by the similar serine or even by a lysine.

Three additional *Ricinus* sequences are clustered in a group containing two characterized LPEATs from *Arabidopsis* (AtLPEAT1 and AtLPEAT2; [55]), besides a putative microsomal GPAT (AtGPAT9; [56]). All of them share the sequence FP**EGT** in the putative LPA-binding site. Another eight castor sequences are comprised in a cluster corresponding to the putative ER GPATs described by Zheng et al. [57] in *Arabidopsis*. Although one of them, AtGPAT1, seems to perform a mitochondrial GPAT activity essential for pollen development, many other members of this group were later found to be involved in the synthesis of cutin and suberin polyesters [58,59]. Members of this group share conserved motifs X**H**RTXX**D** and P**EGT**TCRE around boxes I and III, thus lacking the typical N residue at the beginning of box I.

A last castor sequence retrieved in our analysis, corresponding to a putative type-2 DGAT that lacks motifs for boxes I and III, was used as outgroup in our cladistic analysis.

3.2. Cloning and characterization of LPAT genes from Ricinus communis

Based on the cladistic data (Fig. 1) and analysis of conserved acyltransferase boxes (Supplementary Table 1) we have focused our investigation on the three castor genes (*RcLPAT2*, *RcLPAT3A* and *RcLPAT3B*) of the cluster containing the A-class LPATs, as well as on the single member of the B-class group (*RcLPATB*) since they appear as likely candidates, according to the literature, to encode LPAT activities involved in the biosynthesis of seed glycerolipids.

We have obtained a full length cDNA for *RcLPAT2* (GenBank ID: JQ796917) by RT-PCR from RNA of developing seeds, as described in the Section 2.4. The sequence of the cDNA is identical to that deduced from the genomic DNA (XM_002526684), but differs in one nucleotide (involving a conservative change in a valine codon) with another reported accession (EU591533). The cDNA encodes a 395 amino acids protein (Supplementary Fig. 1) which contains the three conserved Lewin's boxes with identical sequences to the AtLPAT2 of *Arabidopsis*. Gene structure deduced for *RcLPAT2* is identical to that of *AtLPAT2*, and involves eleven exons with characteristic boxes I to III located on exons 4, 6 and 7, respectively (Fig. 2). Similarity of RcLPAT2 with proteins of this group is very high, ranging from 85% (73% identity) with AtLPAT2 up to 91% (80% id.) with a putative orthologue from *Populus* (Supplementary Fig. 1). Analysis of gene expression by semi-quantitative PCR reveals a generalized expression pattern for *RcLPAT2* in all castor organs analyzed, namely leaves, roots, stem, organs from male (staminate) and female (pistillate) flowers, and developing seeds, the later with a higher transcript amount (Fig. 3A). When expression is analyzed along seed development (Fig. 3C) similar levels of transcript was found in all stages, from S1 to S6, while it is reduced in the mature dry seeds (S7 stage). Within the developing seed (S6 stage), RcLPAT2 was expressed at a similar level in the embryo and endosperm tissues (Fig. 3B). Phylogenetic relationships besides high similarity and expression patterns together indicate that *RcLPAT2* represents indeed a member of the A-class LPATs likely orthologous to those previously described.

Two other proteins in *Ricinus*, RcLPAT3A and RcLPAT3B, are closely related to RcLPAT2 (Fig. 1 and Supplementary Fig. 1), having similarities of 81% (63% id.) and 72% (47% id.), respectively. RcLPAT3A is most similar (82%, 66% id.) to the *Arabidopsis* AtLPAT3 product. Expression of the *RcLPAT3A* gene seems to be restricted to the flowers, and it is particularly prominent in the stamens of staminate flowers, although some expression is also found in their sepals (Fig. 3A). RcLPAT3B is more diverged than RcLPAT3A and appears in a basal position in the LPAT2/3 group (Fig. 1). Expression of the *RcLPAT3B* gene was predominant in male (both sepals and stamens) and female flowers, as well as in the roots (Fig. 3A). Gene structures predicted for *RcLPAT3A* and *RcLPAT3B* (cDNAs are not available in the GenBank) are quite similar to that of *RcLPAT2* with eleven exons containing relevant boxes on equivalent exons 4, 6 and 7 (Fig. 2).

We have also cloned the single member of the B-class cluster in *Ricinus*. A full length cDNA was obtained for *RcLPATB* as indicated in Section 2.4 (GenBank ID: JQ796918). The ORF encodes a 299 amino acid protein containing the four characteristic Lewin's boxes (Supplementary Fig. 3). Comparison of the nucleotide sequence with that of the genomic DNA (GenBank ID: NW_002994408.1) reveals the presence of five exons, a different structure to that of the LPAT2/3 group, with boxes I–IV located on exons 2–5 respectively (Fig. 2). Contrary to what expected, expression of *RcLPATB* is rather generalized in the different organs of the castor plant, with a lower level in roots and organs from the male flowers (Fig. 3A). Within the developing seed (S6 stage), *RcLPATB* was expressed at a similar level in the embryo and endosperm tissues (Fig. 3B). Expression was also studied along different stages of castor seed development (Fig. 3C). As it is shown, a higher transcript amount was found in those developmental stages (S5 and S6) where accumulation of reserve molecules, TAG among them, is more active, thus indicating a possible involvement in the synthesis of seed reserve lipids.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2012.09.015.

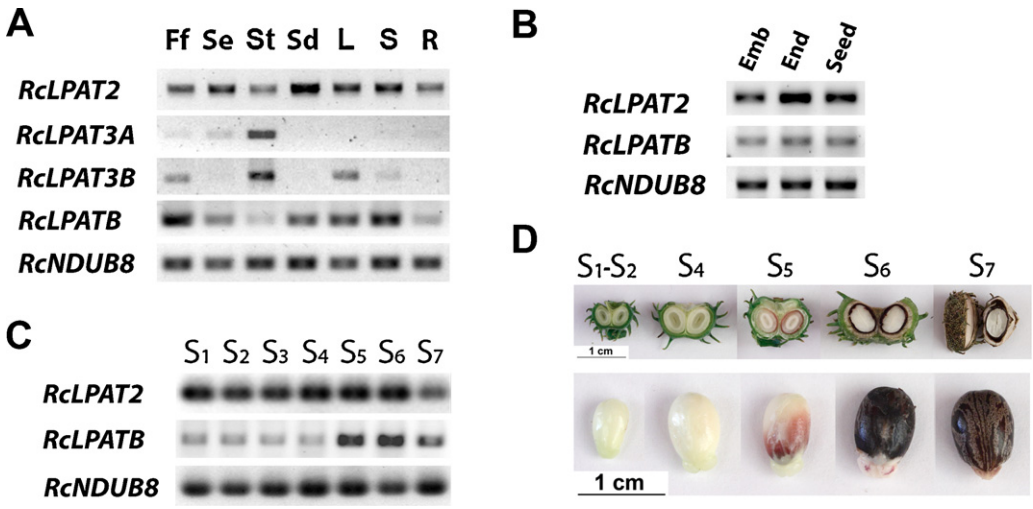

Fig. 3. (A) Expression analysis of *RcLPAT* genes in diverse castor organs by semi-quantitative RT-PCR. Equivalent cDNA amounts from whole pistillate female flowers (Ff), sepals (Se) or stamens (St) from staminate male flowers, developing seeds (Sd, corresponding to S6 stage; see panel D), leaves (L), and stem (S) or roots (R) from castor seedlings were analyzed as indicated in Section 2. 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. All PCR products were taken at cycle numbers determined to be nonsaturating: *RcLPAT2* (30 cycles), *RcLPAT3A* (25 cycles), *RcLPAT3B* (30 cycles), *RcLPATB* (32 cycles) and *RcNDUB8* (25 cycles). (B) *RcLPAT* expression in seed tissues. Seeds at the S6 stage were dissected to analyze expression in embryo (Emb) and endosperm (End) separately, and equivalent cDNA amounts were analyzed as above, together with cDNA from whole S6 seeds for comparison. Amplification in the linear range is shown for *RcLPAT2* and *RcLPATB* at the same number of cycles (30) and *RcNDUB8* (23 cycles). (C) Expression analysis by semi-quantitative RT-PCR of *RcLPAT* genes along castor seed development. Equivalent cDNA amounts from different seed developmental stages S1 to S7 (as defined in [68], panel B) were analyzed by semi-quantitative RT-PCR as described in Section 2, and the expression level compared for the *RcLPAT2* and *RcLPATB* genes. *RcNDUB8* was used as the reference gene. Amplifications in the linear range are shown for *RcLPAT2* (30 cycles), *RcLPATB* (37 cycles) and *RcNDUB8* (26 cycles). (D) Castor bean developmental stages visualized trough transversal cuts of the fruit (upper row) and images of the whole seeds (lower row).

3.3. Complementation of a LPAT mutant of Escherichia coli by RcLPAT genes

We have checked the ability of the *RcLPAT2* and *RcLPATB* products to complement an *E. coli* LPAT mutant (*plsC*) as an indication for LPAT activity of castor enzymes. The SM2-1 strain was used, a temperature-sensitive mutant of *plsC* [44] that is able to grow at the 30 °C permissive temperature, but not at 42 °C. A similar complementation assay has been previously used to positively test activities of different plant LPAT enzymes, including A and B-class LPATs as well as plastidial enzymes [31,41,43,51]. The full-length open reading frames of *RcLPAT2* and *RcLPATB* genes were cloned into the prokaryotic expression vector pQE60 (Qiagen) under the control of the T5 promoter and the resulting plasmids, besides the pQE60 empty vector as a negative control, were used to transform SM2-1 cells. Since toxicity has been reported for some LPAT proteins [31,43], transformation was accomplished into SM2-1 cells containing the pREP4 plasmid encoding the lacI repressor, to reduce potential leakiness from the T5 promoter. Single colony overnight cultures of transformed bacteria were plated after appropriate dilutions onto plus/minus IPTG containing plates and cultivated at 30 °C or 42 °C. As it is shown in Fig. 4, rescue of growth under non-permissive conditions (42 °C) is achieved by both *RcLPAT2* and *RcLPATB*, provided that the IPTG inductor was present in the plate. This indicates that mutant complementation is due to the expression of these genes, and that their respective proteins are functional and possess LPAT activity.

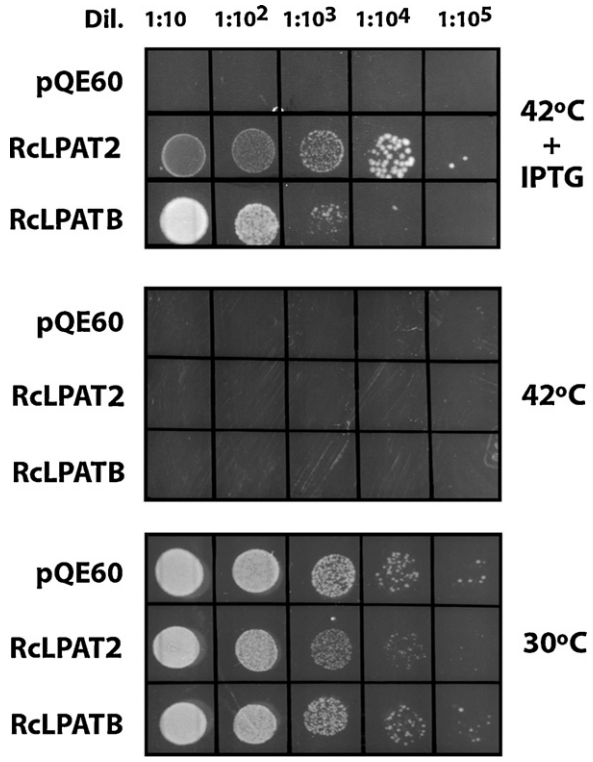

Fig. 4. Complementation of the *E. coli* LPAT mutant (SM2-1) by *RcLPAT2* and *RcLPATB* genes. SM2-1 cells were transformed with pQE60 plasmids containing the full length *RcLPAT* genes or with the empty pQE60 plasmid. Single colonies recovered after transformation from 42 °C plates were cultured O/N at 30 °C in liquid LB medium and 5 μl drops from the indicated dilutions starting from equivalent cell densities (0.1 OD_{600}) were deposited onto plates containing 0.1 mM IPTG or without it. Incubation was performed at 30 °C (permissive conditions) or 42 °C (restrictive conditions).

3.4. Characterization of LPAT activities for RcLPAT2 and RcLPATB

We also have performed a biochemical characterization of the putative LPAT activities encoded by *RcLPAT2* and *RcLPATB*. To this aim, membrane extracts from the *E. coli plsC* mutant, complemented with the castor genes, were used as a source of enzyme to measure LPAT activity using either 18:1-LPA or 18:1-OH-LPA

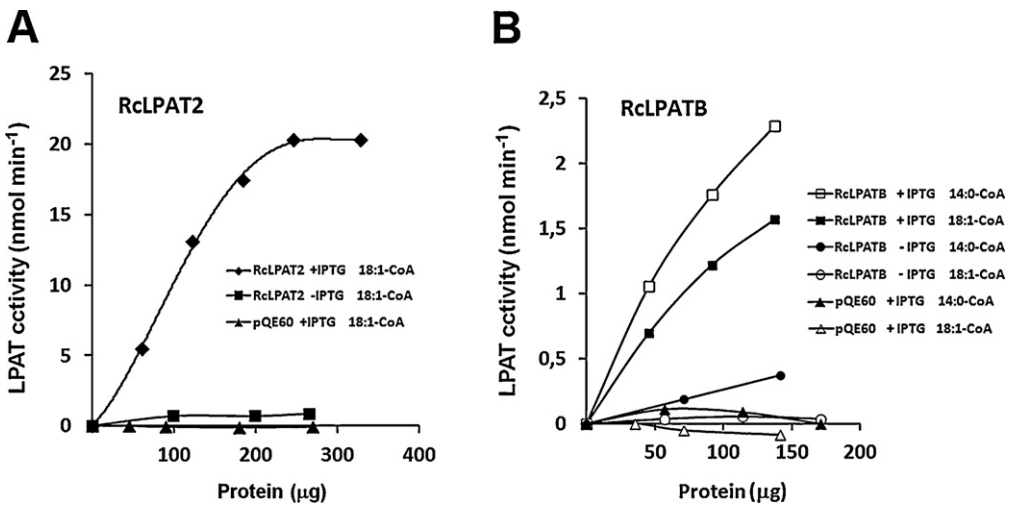

Fig. 5. LPAT activity of RcLPAT2 and RcLPATB at different protein amounts from crude membrane extracts of the SM2-1 *E. coli* mutant strain transformed with the empty vector (pQE60), or the same vector containing each of the castor LPAT genes. Activities from mock-induced cells (-IPTG) are also shown as an additional control. Assays were performed in the absence of Mg^{2+} (+1 mM EDTA), using 50 μM oleoyl-LPA as acceptor and 20 μM of the acyl donors oleoyl-CoA (18:1-CoA), or miristoyl-CoA (14:0-CoA), as indicated. Values are expressed as nanomoles of released CoA per minute in the DTNB spectrophotometric assay.

as acyl acceptors, following the method by Bafor et al. [33]. For both enzymes increasing LPAT activity with extract amount was obtained, that was significantly higher to that of extracts from either IPTG-induced cells containing the empty pQE60 vector, or from non-induced cells containing the *RcLPAT* genes (Fig. 5). Overall, LPAT specific activity was higher (*ca.* 5 times) for RcLPAT2 than for RcLPATB in our *E. coli* membrane preparations (Fig. 5).

Experiments aimed to investigate the acyl preference of the enzyme were carried out for RcLPAT2 using 18:1-LPA (Fig. 6A) or 18:1-OH-LPA (Fig. 6B) as acyl acceptor, besides different acyl-CoA donors. Initial assays were performed with 50 μM 18:1-LPA (which was determined to be the optimum concentration) and two different acyl-CoAs concentrations, 20 μM and 40 μM, since preliminary experiments showed that maximum activity for the monounsaturated 18:1-CoA and 16:1-CoA was attained at 40 μM, while for saturated acyl-CoAs it was 20 μM. As it is shown in Fig. 6A (grey bars), in the absence of Mg^{2+} (2 mM EDTA in the assay) and at 20 μM acyl-CoA a higher activity was obtained with MUFAs (16:1 and 18:1), while SFAs, from 12 to 18 carbons were also incorporated but into a lesser extent, particularly the 18:0, similarly to the polyunsaturated 18:2. The monounsaturated very long chain fatty acid, 22:1, was used at a rather low rate. This preference in the incorporation of MUFAs is even accentuated at 40 μM acyl-CoA (Fig. 6A black bars), where incorporation rate for 16:1 is around four times that of 16:0, and eight times for the 18:1 over 18:0. Since 18:1-OH is the major fatty acid found at the *sn*-2 position of TAG in the castor bean, LPAT activity using 18:1-OH-CoA was also investigated. As it is shown in Fig. 6A, this hydroxylated MUFA is incorporated at a lower rate when 18:1-LPA was used as a substrate, as compared to 18:1 and 16:1 (60% of the 18:1 rate at 20 μM, and around 70% at 40 μM). Since Mg^{2+} ions, which are present under physiological conditions, have been shown to affect acyl-CoA specificity of some acyltransferases [57] we also performed the LPAT assays in the presence of 1 mM Mg^{2+}. However, the results obtained were quite similar (Supplementary Fig. 4A).

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When LPAT2 was assayed using 18:1-OH-LPA as the acceptor substrate a quite different pattern in the acyl-CoA utilization was recorded (Fig. 6B). In this case RcLPAT2 showed a clear preference for 18:1-OH-CoA as compared to 18:1-CoA, and this trend was accentuated (*ca.* three times higher rate for 18:1-OH-CoA) at 40 μM acyl-CoA (black bars), which corresponds to an almost equimolar acceptor to donor ratio. Also relevant is the very low rate for SFA (16:0 and 18:0) thioesters as compared to data obtained with 18:1-LPA as acceptor. The results were again quite similar with added Mg^{2+} in the assay (Supplementary Fig. 4B).

When acyl-substrate specificity was investigated for RcLPATB a very different pattern was obtained. As it is shown in Fig. 7A, when 18:1-LPA is used as acyl acceptor in the absence of Mg^{2+}, acyl incorporation rate was similar or even higher for thioesters of the SFAs (12:0, 14:0, 16:0) than for MUFAs (18:1 and 16:1), while 18:1-OH was incorporated at an intermediate rate (50% of the 18:1). Activities for 18:0-CoA, 18:2-CoA and 22:1-CoA were the lowest again. Similar results were obtained at 20 μM (grey bars) or 40 μM (black bars) acyl-CoA concentrations. Inclusion of 1 mM Mg^{2+} in the assay again did not significantly affect the results, with higher rates for all SFAs over MUFAs (Supplementary Fig. 4C). We also assayed 18:1-OH-LPA as a substrate, but in this case the overall pattern of acyl preference was similar to that obtained with 18:1-LPA (Fig. 7B). Again, the maximum incorporation rates corresponded to SFA thioesters, particularly those with medium chain 12:0 and 14:0. 18:1-OH-CoA was incorporated at a higher rate with the hydroxylated acceptor attaining a similar level to that of 18:1-CoA, but clearly below (less than a half) the rate obtained for medium chain SFA. Similar results were also obtained in the presence of Mg^{2+} ions (Supplementary Fig. 4D).

To further check our results on acyl-CoA specificity we also assayed castor LPATs using a standard radioactive procedure by labelling of 18:1-LPA and directly monitoring of PA formation. Assay conditions and methodology are somewhat different to those in the spectrophotometric DTNB assay (see the Section 2.8.), however the results obtained for RcLPAT2 and RcLPATB using different acyl-CoA donors are in good agreement (Supplementary Fig. 5), thus validating the methodology employed.

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Since previous reports on LPAT activity using microsome preparations of castor bean reported very low activity on 18:1-OH-CoA with 18:1-LPA as acceptor, unless polyamines were included in the reaction [34], we also checked the effect of 1 mM spermidine, a polyamine that was reported to increase dramatically LPAT

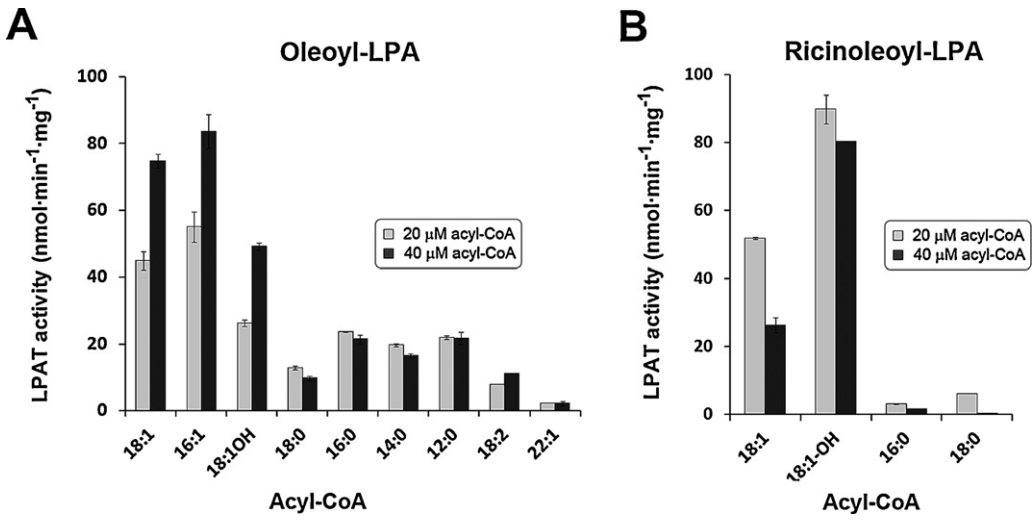

Fig. 6. LPAT activities of RcLPAT2 using 50 μM 18:1-LPA (panel A) or 18:1-OH-LPA (panel B) as acyl acceptors, and two different concentrations 20 μM (grey bars) and 40 μM (black bars) of diverse acyl-CoA donors. Assays were performed, as described in Section 2, in the absence of Mg^{2+} ions (2 mM EDTA). Specific activities (nmols min^{-1} mg^{-1}) were represented after subtracting residual activities obtained for extracts from cells containing the pQE60 empty vector to each value. Results are expressed as the mean of three determinations with their respective standard errors.

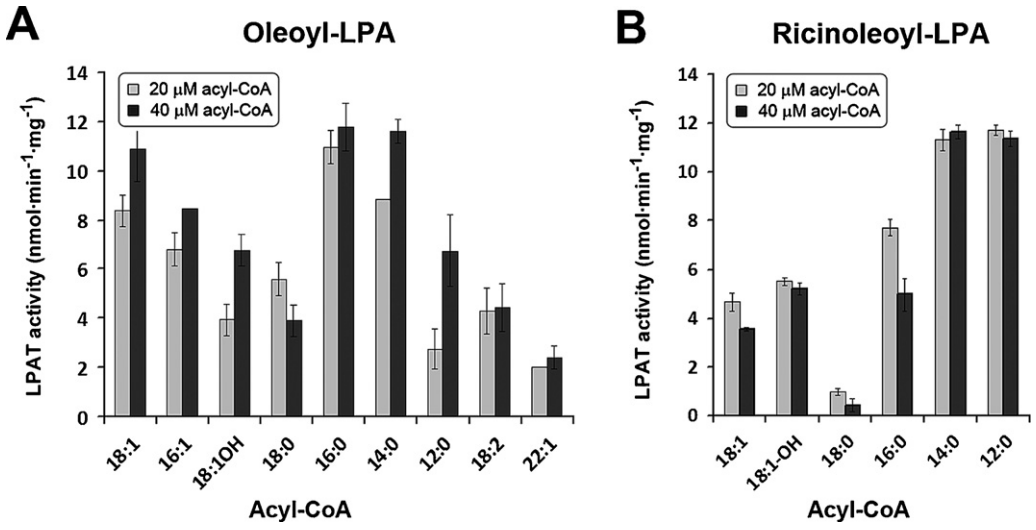

Fig. 7. LPAT activities of RcLPATB using 50 μM 18:1-LPA (panel A) or 18:1-OH-LPA (panel B) as acyl acceptors, and two different concentrations 20 μM (grey bars) and 40 μM (black bars) of diverse acyl-CoA donors. The assays were performed, as described in Section 2, without Mg^{2+} (2 mM EDTA). Specific activities were represented as nmol min^{-1} mg^{-1}. The background activities obtained for extracts from cells containing the pQE60 empty vector were subtracted to each value. Results are expressed as the mean of three determinations with their respective standard errors.

activity for the hydroxylated MUFA when assayed in microsomes, on the incorporation of 18:1-OH-CoA. However, only a moderate effect was obtained for 18:1-OH-CoA in the case of RcLPATB, with an increase around 25%, while just a minor effect was registered in the case of RcLPAT2 (Supplementary Fig. 6). Differences in the methodology to measure reaction rates and/or characteristics of the membrane fractions leading to the presence of different enzymatic activities might account for the observed discrepancies. Our results indicate that none of the LPATs expressed in the castor bean require additional factors to adopt the appropriate structure in the membrane allowing the utilization of the 18:1-OH-CoA substrate in the active site. Neither, interaction of polyamines with any of the substrates seems to be needed for effective catalysis.

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4. Discussion

4.1. The multigene family of LPAT-like acyltransferases in Ricinus communis

An iterative search performed on the castor genome database, using previously characterized plant LPAT sequences, yielded a total of 20 proteins (Supplementary Table 1) sharing the LPLAT (cd06551) molecular signature, which is common to a very diverse group of enzymes involved in glycerolipid metabolism, as well as characteristic motifs described for LPAT and GPAT enzymes from diverse organisms [49]. None of these proteins had been characterized so far and they group into seven highly supported clusters (Fig. 1 and Supplementary Fig. 2). Based on our data and the available literature, at least three of these clusters include enzymes with LPAT activity. One of them contains a single putative plastidial

isoenzyme, RcLPAT1, that is highly similar to the previously characterized AtLPAT1 from *Arabidopsis* [51]. Another cluster (LPAT2/3 group) includes to the A-class RcLPAT2 enzyme which has been shown here to have generalized expression and preference for MUFAs, as it is typical for other enzymes of this class like the maize MAT1 [39], LAT2 of *Limnanthes* [40], BAT1 of *Brasssica* [37] and AtLPAT2 of *Arabidopsis* [43]. Within the same cluster we also found two castor proteins designated RcLPAT3A and RcLPAT3B. RcLPAT3A likely represents the orthologue of the male gametophyte isoenzyme described in *Arabidopsis* (AtLPAT3 [43]), based on the clustering analysis and its expression pattern, mainly restricted to the anthers of the male flowers. The presence of the gene for this anther isoenzyme appears to be generalized in dicot plants while it seems to be absent from monocots where only A-class related proteins are found within this cluster. Characteristics and function of this isoenzyme are largely unknown beyond the reported preference on 18:1-CoA over 16:0-CoA substrates for AtLPAT3. An additional gene, designated *RcLPAT3B*, is expressed in reproductive organs of *Ricinus* besides aerial vegetative organs. A closely related counterpart is found in the *Populus* genome, but it is absent from many species with available sequenced genomes like *Arabidopsis*, *Vitis* or *Oryza*. This gene encodes a somewhat diverged LPAT with some of the characteristic boxes differing from the typical consensus. It is remarkable that up to four different LPAT isoenzymes (also the RcLPATB discussed below) are expressed in the anthers. It is known that castor pollen and anthers accumulate TAG, but contrary to the seed this is enriched in 16:0, 18:2 and 18:3 fatty acids, lacking 18:1-OH [60]. It is possible that the distinct sets of acyltransferases acting in seeds and floral tissues may be contribute, besides the seed specific hydroxylase activity, to the observed differences in patterns of TAG fatty acids.

A third cluster includes to RcLPATB, the B-class representative in castor, besides two previously characterized seed specific isoenzymes from *Limnanthes* [41] and *Cocos* [42]. However, expression pattern of *RcLPATB* does not follow that usually reported for the B-class since it is not restricted to the seeds and it is also found at a similar level in other parts of the plant. Moreover, described enzymes of this class are characterized by the preferential utilization of unusual fatty acids. Though RcLPATB does not show a preference for 18:1-OH-CoA, which is the unusual FA accumulated by the castor bean, this enzyme utilize the also unusual medium chain SFA (12:0 and 14:0) thioesters at a high rate, even surpassing 18:1-CoA. Closely related sequences to B-class enzymes are also found in the GenBank for very diverse organisms like the dicots *Populus* and *Vitis*, the monocots *Oryza* and *Sorghum*, and the lower fern *Selaginella*. Available ESTs in the GenBank, also reveals the presence of *LPATB*-like genes in other plants like *Citrus* and *Carica*. However, it seems to be absent from many other sequenced organisms like *Arabidopsis*. Therefore, the presence of this isoenzyme is not restricted to organisms accumulating unusual fatty acids. It is possible that acyl substrate specificities in these organisms are also different to those of A-class enzymes, although this possibility will require further studies in other plants before reaching general conclusions. Previous hypothesis suggested that this group diverged from the housekeeping plastidial LPAT in some plants [51]. However, our phylogenetic analysis indicate that B-class LPATs of plants are in fact closer to animal enzymes and not to the plastidial LPATs, thus indicating that this represent a very ancient group that diverged very early from A-class enzymes. It is therefore likely that B-class genes have been lost in some plant lineages, perhaps due to functional redundancy, while it has been recruited in others to perform different functions based, for instance, on the acquirement of new substrate specificities.

Additional LPAT-related proteins belonging to the other clusters might also be involved in the synthesis of seed TAG, although this seems unlikely based on the available information, mainly from *Arabidopsis*. Members of the LPAT4/5 group do not have LPAT activity *in vitro*, at least in *Arabidopsis* [43]. Another group includes proteins with homology to taffazins which have been shown to perform mitochondrial activities related to cardiolipin biosynthesis [53]. Within the GPAT group many members have been characterized in *Arabidopsis*, and most of them are involved in the synthesis of protective polyesters [57,58,61]. A remaining cluster includes LPEATs with broad specificity on LPLs in *Arabidopsis*. These are enzymes remodelling phospholipids and likely involved in membrane metabolism homeostasis [55]. Within the same group there is the uncharacterized GPAT9 which has been suggested to be a microsomal GPAT [56]. However, precise information about the biochemical activity of this protein is still missing.

4.2. LPATs in developing castor seeds

Our results indicate that two genes, *RcLPAT2* and *RcLPATB*, encoding active LPAT enzymes are significantly expressed in the developing seeds of castor, with transcripts for *RcLPAT2* being found at a higher level. Recent data on the castor transcriptome [60] showed that the RNA for *RcLPAT2* (cited as 27810.m000646 in that study) was in fact the most abundant among LPAT genes. Also, in a parallel proteomic analysis, this protein was the only LPAT found, besides the plastidial isoenzyme, in enriched ER preparations from developing castor seeds [60,62], thus corroborating its high abundance. On the contrary, a low RNA count was registered for *RcLPATB* (29666.m001430) in developing seeds what is in agreement to our RT-PCR results showing consistently a lower amplification for *RcLPATB*.

Both proteins encoded by *RcLPAT2* and *RcLPATB* are able to rescue the *plsC* mutation in *E. coli* and, accordingly, they showed LPAT activity when assayed in bacterial membrane extracts. However, acyl specificities were very different among them, and in the case of RcLPAT2 it was also dependent on the acyl group at the *sn*-1 position of the acceptor substrate. Thus, acyl-CoA specificity of RcLPAT2 using 18:1-LPA as acyl acceptor was the expected for an A-class enzyme regarding incorporation of common FA, with a higher rate in the incorporation of MUFAs 18:1 and 16:1 over SFAs from 12:0 to 18:0. Using this acceptor, 18:1-OH was incorporated at a lower rate than 18:1. On the contrary, when 18:1-OH-LPA is used as substrate a clear preference is shown for 18:1-OH-CoA over 18:1-CoA and other FA thioesters. It is known that *ca.* 90% of fatty acids in castor bean oil is 18:1-OH, an enrichment that is particularly high (98%) at the *sn*-2 position [34]. Conversely, 18:1-OH is essentially excluded from the membrane lipids, thus supporting the existence of channelling mechanisms favouring incorporation of the hydroxylated FA into TAG molecules. Previous studies performed with castor bean microsomes demonstrated a reduced flux of PC towards DAG, thus indicating that the synthesis of TAG might be carried out mainly from *de novo* DAG produced through the G3P pathway [13,22]. If this was true, selectivity of the acyltransferases in this pathway, GPAT, LPAT and DGAT, should play a key role in the preferential incorporation of 18:1-OH into TAG. No information is available for the microsomal GPAT, but it is known that RcDGAT2, the more abundant DGAT isoenzyme in the seed, is highly selective in the utilization of the hydroxylated DAG substrate [64]. Our results indicate that acyl specificity of RcLPAT2 may also contribute to the observed profile since 18:1-OH-LPA molecules produced in ER of the seed will be preferentially acylated with the hydroxylated FA at *sn*-2. The di-18:1-OH-PA produced in this reaction will eventually generate a DAG molecule after dephosphorylation that will also be preferentially acylated with 18:1-OH, due to the DGAT2 specificity. In this way, combined specificities of LPAT and DGAT enzymes would ensure channelling of glycerol molecules initially acylated with 18:1-OH into seed TAG. As stated before, specificity of RcLPAT2 also favours the incorporation of common FA,

particularly oleic acid, in sn-2 when oleic acid is at the sn-1 position, thus generating potential substrates for the synthesis of membrane glycerolipids. This may presumably be the main role of RcLPAT2 in vegetative castor tissues where TAG biosynthesis is reduced and synthesis of ricinoleic acid is almost absent. This mechanism could also play a role in the seed ER by generating a metabolic pool of DAG enriched in oleic acid mainly dedicated to membrane lipid biosynthesis.

On the other hand, RcLPATB catalyzes the incorporation of SFAs 12:0–16:0 at rates surpassing those of MUFAs, independently of the acyl group (18:1 or 18:1-OH) in the acceptor molecule. This is unusual among microsomal LPATs, while an efficient incorporation of 16:0 is typical for plastidial LPATs [28]. However, plastidial LPATs strongly discriminate against 18:1 and this is not the case of RcLPATB that seems to have a broader specificity. Moreover, RcLPATB lacks the N-terminal extension containing the chloroplast targeting sequence that is found in plastidial isoenzymes, and localization software predictions indicate it is localized in the ER.

The only B-class LPATs that have been cloned and characterized so far are those from *Limnanthes* and *Cocos* [40–42]. Both LPATs were expressed in *E. coli* and their acyl-CoA specificities correlated with FA profiles in their respective seed oils. The coconut LPAT showed a preference for 12:0-CoA over other FAs [42], while the *Limnanthes* enzyme was most active on 22:1-CoA [41]. A detailed study in *Limnanthes*, comparing acyl-CoA specificities of the A-class enzyme LAT1 with the B-class LAT2 [40], indicated that LAT1 was a very selective enzyme using 18:1-CoA almost exclusively while LAT2 exhibited a broader specificity with 16:0 being also incorporated at a very significant rate even higher than for 18:1, similarly to RcLPATB. However, contrary to LAT2, RcLPATB does not use 22:1-CoA while it is able to incorporate 12:0 quite efficiently. With the available information it seems that B-class LPATs have evolved to have different acyl specificities in diverse organisms, and at least in some instances, to have a broader specificity. It will be interesting to investigate the characteristics of these LPATs in other organisms like *Oryza*, *Vitis* or *Populus* where no unusual fatty acids are accumulated. Interestingly, although *RcLPATB* is widely expressed along the plant, its RNA level increases notably coincident with endosperm expansion and maturation phases, when oil synthesis rate is maximal. Analysis of gene expression along seed development has been used to identify putative candidates involved in the synthesis of reserve molecules such as TAG [63,65,66]. However RcLPATB specificity, besides its low relative expression level and enzymatic activity compared to those of RcLPAT2, argue against a significant role for this isoenzyme during TAG accumulation in the castor seed. Of course, one cannot rule out such a role for RcLPATB since it would be still possible that this isoenzyme is associated to particular membrane domains specialized in the synthesis of certain TAGs [12,67]. However, since RcLPATB is also expressed in other organs of the plant with a reduced synthesis of TAG, like the leaf or the stem, it is likely its involvement in the synthesis of membrane glycerolipids. An appealing possibility would be a role for the B-class LPAT in the regulation of membrane fluidity by modulating FA composition in response to particular stresses or changes in the environmental conditions. One can predict that changes in the relative activities of the LPAT isoenzymes might have an impact on the desaturation status at the *sn*-2 position of phospholipids. Experiments are in progress to investigate this hypothesis. In this case it is unclear why *RcLPATB* is upregulated during seed maturation stages although this might be due to a need to satisfy the high rate of membrane biosynthesis in particular seed tissues such as embryonic cells.

Though an obvious function for RcLPATB cannot be inferred from the available data, this enzyme shows interesting properties as it could be used to increase the incorporation of 12–16 carbon SFAs, a characteristic that could be useful in biotechnological applications such as the engineering of oils for biodiesel production. In addition, utilization of RcLPAT2 to increase accumulation of ricinoleic acid in transgenic plants can be envisaged, as it could alleviate the reported metabolic bottlenecks [6], similarly to recent results obtained using a castor PDAT gene [5].

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