Research Article

Cloning and molecular characterization of a class A lysophosphatidate acyltransferase gene (*EpLPAT2*) from *Echium* (Boraginaceae)

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Lysophosphatidate acyltransferase (LPAT) catalyzes the incorporation of acyl groups into the sn-2 position of lysophosphatidate (LPA) rendering phosphatidic acid (PA), a key intermediary in the synthesis of phospholipids (PLs) and triacylglycerols (TAGs). It is considered as a highly selective activity and a strong determinant of fatty acid (FA) composition of membranes and reserve glycerolipids in diverse plants. In this work, we have cloned a gene encoding the microsomal class A LPAT (LPAT2) from *Echium pitardii* (Boraginaceae), a species that accumulates high levels of long chain polyunsaturated FAs (LCPUFAs) in the seed oil. The *Echium* gene (*EpLPAT2*) is ubiquitously expressed in diverse organs of the plant, although the transcript level is increased in those tissues with a higher α-linolenic acid (18:3n3) content. Functionality of EpLPAT2 was proven by complementation of a LPAT defective mutant of *Escherichia coli* (*plsC*), and by a biochemical assay of the expressed enzyme in membrane extracts. Acyl-CoA specificity recorded for EpLPAT2, using oleoyl-LPA (18:1-LPA) as acyl-acceptor, shows a clear preference for unsaturated acyl substrates, with 18:3n3-CoA being used at similarly high rates as 18:1-CoA. Overexpression of *EpLPAT2* in yeast increased FA content, and modified the FA profile of membrane lipids in agreement to the in vitro specificity.

Practical applications: One can envisage that overexpression of *EpLPAT2* in transgenic plants, either alone or in combination with other genes could increase seed oil content. Moreover, the observed specificity of EpLPAT2 might be a useful characteristic in tailoring of a plant engineered for LCPUFA-enriched oil.

Keywords: Lysophosphatidate acyltransferase / Microsomal LPAT / *Echium* / Polyunsaturated fatty acids / Triacylglycerol biosynthesis

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Abbreviations: AGPAT, acylglycerol-3-phosphate acyltransferase; **AT,** acyltransferase; **DAG,** dicaylglycerol; **DGAT,** diacylglycerol acyltransferase; **DHAPAT,** dihidroxyacetonephosphate acyltransferase; **DTNB,** 5,5′-dithiobis-(2-nitrobenzoic acid); **FA,** fatty acid; **G3P,** glycerol-3-phosphate; **GPAT,** glycerol-3-phosphate acyltransferase; **IPTG,** isopropyl-β-thiogalactoside; **LB,** Luria-Bertani; **LCPUFA,** long chain polyunsaturated fatty acid; **LPA,** lysophosphatidic acid; **LPAT,** lysophosphatidate acyltransferase; **LPCAT,** lysophosphatidylcholine acyltransferase; **LPEAT,** lysophosphatidylcholine acyltransferase; **PA,** phosphatidic acid; **PAP,** phosphatidic acid phosphatase; **PC,** phosphatidylcholine; **PDAT,** phospholipid:diacylglycerol acyltransferase; **PL,** phospholipid; **TAG,** triacylglycerol; **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; **18:2n6,** linoleic acid; **18:3n3,** α-linolenic acid; **18:3n6,** γ-linolenic acid; **18:4n3,** stearidonic acid; **22:1,** erucic acid

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1 Introduction

Vegetable oils are mostly (~95%) composed of triacylglycerols (TAGs) and are currently in the focus as a source of healthy fatty acids (FAs), interesting industrial FA derivatives (e.g., hydroxyacids or epoxyacids) and biodiesel [1–6]. This prompted many efforts directed to the engineering of FA composition in the seed oils that have resulted in considerable progress. Nevertheless, there is still insufficient knowledge of molecular mechanisms underlying TAG biosynthesis [7–11], that explains some unexpected pitfalls, e.g., the low level of ricinoleic acid (18:1-OH) [12] or vernolic acid [13] in oils of engineered plant species. Globally speaking, the current emerging picture is that it is relatively easy to produce the desired FA by genetic engineering in a given plant, but it is more difficult to achieve a high level in the seed oil.

De novo synthesis of TAG is carried out by sequential incorporation of acyl groups from CoA thioesters to the glycerol backbone through the Kennedy pathway, also known as the glycerol-3-phosphate (G3P) pathway [14–16]. First acylation on G3P is catalized by the acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15), followed by a second acylation step carried out by the acyl-CoA: lysophosphatidate acyltransferase (LPAT; EC 2.3.1.51) to produce phosphatidic acid (PA). Third acylation requires dephosphorylation of PA by a phosphatidic acid phosphatase (PAP; EC 3.1.3.4) to generate diacylglycerol (DAG), which is finally used as a substrate for the acyl-CoA:diacylglycerol acyltransferase (DGAT; EC 3.2.1.20) to produce TAG [14, 16]. Since PA and DAG are also precursors for the synthesis of glycerophospholipids, GPAT and LPAT are acyltransferases (ATs) common to TAG and membrane-lipid biosynthesis, while DGAT catalyzes the only step, which is committed to TAG biosynthesis. Metabolic flux analyses reveal that in a number of species, such as *Arabidopsis* [17] and soybean [18], a substantial part of TAG is synthesized using DAG derived from phosphatidylcholine (PC). This provides a route for the incorporation into TAG of modified FAs (i.e., desaturated) that are exclusively produced on the acyl groups attached to PC. However, evidences indicate that in other plants, like castor bean [19], avocado [20], and rapeseed [21, 22] TAG synthesis is carried out from de novo DAG instead of PC-derived DAG (see [10] for a recent revision of the different models). The synthesis of TAG can also be accomplished by transfer of acyl groups from PLs to DAG, catalyzed by the phospholipid:diacylglycerol acyltransferase (PDAT) enzyme [23]. This mechanism has been proposed to play a role in removing unusual FAs (i.e., 18:1-OH in castor) from membrane PLs [23, 24]. However, contribution of this acyl-CoA independent pathway to the net synthesis of TAG has been shown to be relatively small in *Arabidopsis* [25, 26] and it remains largely unknown in other plants.

Specificity properties of ATs are important determinants of the FA composition of glycerolipids and contribute to the successful incorporation of unusual FAs into TAG [6, 27, 28]. It is commonly recognized that, among the ATs, LPATs are highly stringent regarding acyl-specificity [27–31]. This focused considerable interest on LPATs as a tool to engineer plant oil composition. Studies performed on different plant species revealed the presence of at least two evolutionary distinct families of genes (A and B classes) encoding microsomal LPATs [27], within a large superfamily of LPAT-like AT genes [32, 33]. The so-called class A microsomal LPATs defined by Frentzen [27] are characterized as being ubiquitously expressed in the plant, and exhibiting a preference for 18:1-CoA that is typical of enzymes synthesizing membrane glycerolipids. Class A LPATs have been cloned from diverse species, namely maize, *Arabidopsis*, *Limnanthes*, *Brassica*, and *Ricinus* [29, 33–35]. Information available at the GenBank also indicates that class A genes are widely represented in the plant kingdom. On the contrary, class B LPAT genes were initially described as being expressed only in the seed and encoding enzymes with biased substrate preference for unusual acyls. First members of the B-class were cloned and characterized from *Limnanthes* and *Cocos* [29, 36, 37]. Enzymes encoded by these genes exhibit strong preference in the utilization of erucic acid (22:1) and lauric acid (12:0), respectively, in agreement to seed microsomal activities and particular oil composition of these plant species. However, recent data indicate that characteristics of the class B enzymes may differ in other species, even though they accumulate unusual FAs, for instance, 18:1-OH in castor. Thus, we have recently reported that in the castor plant the B-class gene, *RcLPATB*, shows generalized expression and the enzyme does not display a preference for 18:1-OH-CoA, while it is the A-class enzyme that seems to be involved in the preferential incorporation of the hydroxylated FA [33].

In the last years, we have studied diverse genes involved in oil biosynthesis in the seeds of *Echium* [38–40]. These plants are characterized by the accumulation of high levels of long chain polyunsaturated FAs (LCPUFAs) in the seed oils (>70% of the total FAs). Moreover, these plants accumulate two unusual Δ^6-desaturated FAs such as γ-linolenic acid (18:3n6) and stearidonic acid (18:4n3) [41]. Thus, the *Echium* plants show a contrasting FA profile, quite different to other species (e.g., *Limnanthes*, *Cocos*, *Arabidopsis*, or *Ricinus*) where molecular data on LPATs are available. Study of *Echium* LPATs could therefore provide useful information on this enzyme activity valuable for basic and applied science. Here we report on the cloning and characterization of a *LPAT* gene from *Echium pitardii* (*EpLPAT2*) that encodes a ubiquitous class A LPAT. Analysis of the acyl-CoA specificity of EpLPAT2 against different LCPUFA substrates indicates that incorporation of Δ^6-desaturated FAs is not favored by the *Echium* enzyme. However, its high activity on 18:3n3-CoA suggests a possible utility in tailoring LCPUFA-enriched oils through genetic engineering.

2 Materials and methods

2.1 Biological material

Seeds of *E. pitardii* A. Chev. ex D. Bramwell (=*E. lancerottense* Lems et Holz) were collected from plants located in its natural habitat at Lanzarote (Canary Islands). Seedlings (six to eight leaves stage) were grown at 25°C, under the controlled conditions of growth cabinets with a 16 h light/8 h dark photoperiod and 70% relative humidity. Leaf material from seedlings was used as a DNA source, while the different tissues of *E. pitardii* utilized for RNA extraction and Northern blot analysis were sampled from mature plants cultivated in a greenhouse. The thermosensitive strain *Escherichia coli* SM2-1 carrying the *plsC* mutation [42], used to assay LPAT activity by heterologous expression, was acquired to the *E. coli* Genetic Resource Center. The yeast mutant *YOR175c* (*ale1*) (genotype BY4741; *MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YOR175c::KanMX4*) was obtained from the EUROSCARF collection (acc. No. Y02431).

2.2 Lipid substrates

Fatty acid thioesters 18:2n6-CoA, 18:3n3-CoA, 18:3n6-CoA, and 18:4n3-CoA were synthesized following the enzymatic method by Taylor et al. [43]. The acyl-CoA synthetase from *Pseudomonas* was purchased to Sigma–Aldrich. Purity and concentration of the synthesized acyl-CoAs were determined spectrophotometrically as described by Taylor et al. [43]. The spectroscopic analysis was performed measuring absorbances at 232 and 260 nm, and using 9.2 and 16.4 $mM^{-1}\,cm^{-1}$ as extinction coefficients, respectively. Data determined for commercially available acyl-CoAs were used as references. The remaining acyl-CoAs employed were purchased to Sigma–Aldrich.

2.3 Cloning of the *Echium* LPAT gene

Cloning of *EpLPAT2* gene was achieved by RT-PCR amplification of a partial cDNA using degenerated primers, followed by 3′ and 5′ RACE to complete the flanking sequences. Briefly, an oligo-dT primed cDNA was synthesized from ca. 1 μg of total RNA obtained from developing fruits (seeds) of *E. pitardii* by employing the SuperScript® III First-Strand Synthesis System for RT-PCR kit (Invitrogen) following the manufacturer's instructions. Initial RT-PCR amplification on the cDNA was done using two degenerated oligonucleotide primers (STLP1 and STLP2) designed against the LPAT conserved motifs PVIGWSMWF and AQWCKDIFV, respectively (see Supplementary Fig. S1). The PCR program consisted of a denaturation step of 2 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 50°C, and 45 s at 72°C, ending with a 5 min step at 72°C. The resulting fragment of 450 bp was cloned into the pGEM®-T Easy vector (Promega), and several clones were sequenced. From the partial sequence of *EpLPAT2*, two pairs of nested primers were designed to perform the RACE amplification with the SMART® RACE cDNA Amplification Kit (Clontech). Finally, a whole cDNA fragment was amplified by RT-PCR using the flanking primers LPAT-Up (5′-TTAAGGTACCGGTGAAAATATGGCAATTGCAGCAGCAGCT-3′) and LPAT-Down (5′-TCTAGAATTCCTACTGTCGTTTATCGTCTGTTGAATTGGA-3′). The reaction was performed using a proof-reading DNA polymerase Accuprime® (Invitrogen) in the buffer I provided by the manufacturer, and a program consisting of a denaturation step of 1 min at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at 53°C, and 1 min 40 s at 68°C, ending with a 5 min step at 68°C. The PCR fragment was cloned into the pGEM®-T Easy vector (Promega) after A nucleotide addition, and several clones were fully sequenced allowing the detection of two variants (*EpLPAT2-1* and *EpLPAT2-2*) of the gene (see Section 3 for further explanation). DNA clones were sequenced on both strands using a Perkin-Elmer ABI-310 DNA automated sequencer, and the BigDye® Terminator v3.1 chemistry. cDNA sequences were deposited in the GenBank under the accession numbers JX629668 for *EpLPAT2-1* and JX629669 for *EpLPAT2-2*.

2.4 Cladistic analysis

Protein sequence alignment among LPATs was performed using the program ClustalX v.2.1 [44] at the default settings, and further refined by visual inspection. A phylogenetic tree of the aligned sequences was built using the minimum evolution method [45], as implemented in the MEGA package v.5.0 [46]. The Poisson correction metric was employed together with the pairwise deletion option, and confidence of the tree branches was checked by bootstrap generated from 1000 replicates. Rooting of the tree was accomplished using as outgroup the *Echium* EpGPAT4 [47], a GPAT enzyme involved in the synthesis of polyesters. For sequences selected in Fig. 1, the alignment was visualized using the Boxshade v. 3.21 software (http://www.ch.embnet.org/software/BOX_form.html).

2.5 Southern and Northern blot analysis

Genomic DNA was isolated from *Echium* seedlings by a CTAB-based extraction procedure [48]. DNA (ca. 5 μg) was restricted with the appropriate restriction enzymes, separated on a 0.8% agarose gel, and transferred by capillarity onto a Hybond®N^+ nylon membrane (Amersham). The filter was UV-crosslinked, pre-hybridized at 42°C for 5 h in the 50% formamide/high SDS buffer recommended by the DIG manufacturer (Roche Diagnostics), and hybridized at the same temperature and same buffer solution (stringent conditions), containing the digoxigenin-labeled *LPAT* specific probe. High stringency washes were performed twice at 65°C for 15 min in buffer containing 0.1× SSC, 0.1% SDS,

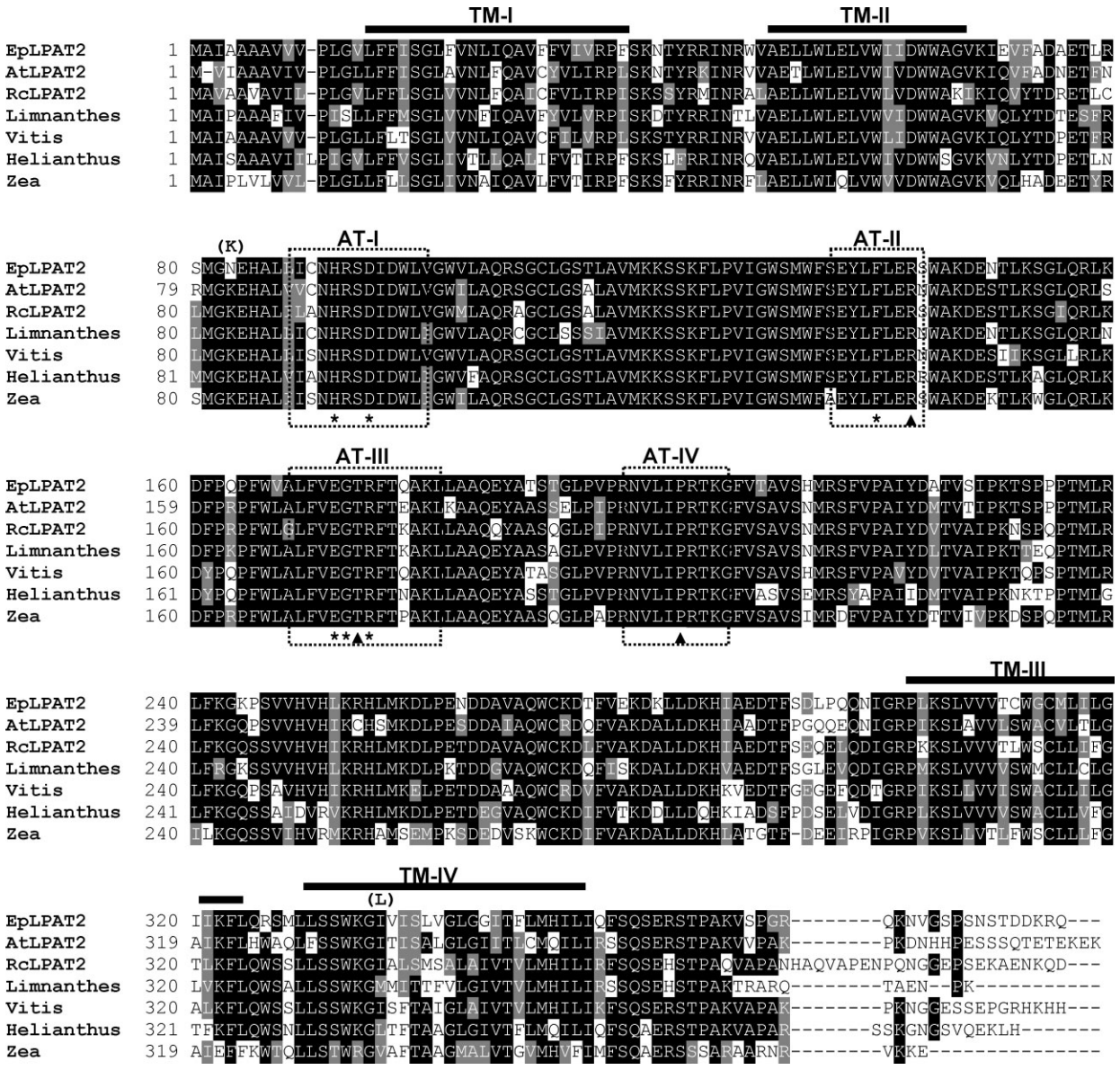

Figure 1. Amino acid alignment of EpLPAT2 and related proteins from higher plants found in the GenBank. The predicted amino acid sequence of EpLPAT2 was aligned, using the software ClustalX v2.01 (http://www.clustal.org/) with putative orthologs from *Limnanthes douglasii* (CAA88620) and *Vitis vinifera* (XP_002283340), and characterized class A LPATs from *Ricinus communis* (RcLPAT2; JQ796917), *Zea mays* (NP_001105919), *Helianthus annuus* (ABP93351), and *Arabidopsis thaliana* (AtLPAT2; NP_567052). The Boxshade program (http://www.ch.embnet.org/software/BOX_doc.htmL) was used to highlight similarities between protein sequences. Identical and similar residues are shaded in black and gray, respectively. Putative transmembrane domains (TM-I, TM-II, TM-III, and TM-IV) of EpLPAT2 are marked by horizontal solid bars. The four acyltransferase motifs (AT) conserved in related acyltransferases from various species are boxed in doted lines. Critical residues previously identified in similar proteins are marked by asterisks (catalytic amino acids in AT motif) or triangles (binding site in AT motif). The presence of either Asn (N) or Lys (K) at position 83, and Ile (I) or Leu (L) at position 336 of the two EpLPAT2 variants is also indicated.

and the luminogenic substrate CDP-Star® (Roche Diagnostics) was used for the detection, following the instructions provided with the DIG detection kit. Chemiluminescence images were registered using a Chemie Genius2 detection system (Syngene). The LPAT probe was obtained by random primed labeling from a cDNA clone spanning 250 bp of the 3′-coding sequence.

Total RNA was extracted from different organs of mature *E. pitardii* plants, using the method described by Chang et al. [49] with minor modifications. Composition of the

extraction buffer was modified so that polyvinylpyrrolidone and spermidine were replaced by the RNAse inhibitors *p*-aminosalicylic acid (20 mg/mL) and sodium naphthalene-sulfonate (5 mg/mL). Total RNA (ca. 10 μg per lane) was loaded in an agarose/formaldehyde gel, electrophoretically separated, and transferred onto a Hybond®N^+ membrane. The filter was hybridized at 50°C (stringent conditions) as described for Southern analysis, and using a 400 bp *LPAT* specific probe, obtained as indicated before, corresponding to the central part of the coding sequence. Stringent washes, accomplished at 68°C, and detection of the DIG-labeled probe were as above described.

2.6 Complementation assay of the *E. coli plsC* mutant by *EpLPAT2*

The thermosensitive strain *E. coli* SM2-1 [42] was used in the functional characterization experiments of the *Echium* LPAT. The whole coding sequence from *EpLPAT2* was amplified by RT-PCR, as described before, using oligonucleotide primers containing *Nco*I and *Bam*HI restriction sites. The *Nco*I–*Bam*HI fragment of the PCR product was ligated in the appropriate frame into the pQE60 expression vector (Qiagen), digested with the same enzymes, and several clones were sequenced. The resulting plasmid pQE60-EpLPAT2, harboring the *EpLPAT2-1* variant of the gene, and the empty vector pQE60, were used to transform *E. coli* SM2-1 via heat shock and transformants were selected by growth on ampicillin plates at 30°C. To perform heterologous complementation experiments on solid media, two to three colonies of two independent transformants were resuspended in 100 μL of Luria-Bertani (LB) media and, aliquots from these cultures were streaked on LB agar plates in the presence or absence of the inductor isopropyl-β-thiogalactoside (IPTG) at 0.2 mM, and grown at 30 or 42°C. In addition, complementation experiments in liquid media were performed following the optical density at 600 nm of LB cultures grown at 30 or 42°C supplemented with IPTG.

2.7 *E. coli* membrane extracts preparation

A culture of SM2-1 transformed with pQE60-EpLPAT2 or pQE60 was grown at 30°C to OD_{600} ca. 0.6, and protein expression was further induced by addition of IPTG 0.2 mM. Incubation under inductive conditions was then prolonged for 3 h at the same temperature. Cells were collected by low-speed centrifugation, resuspended in 100 mM sodium phosphate buffer pH 7.0, and disrupted by sonication with an ultrasonic generator (Hielscher VP200S). Large cell debris were removed by centrifugation at 1500*g*, 4°C, for 5 min, and the supernatant was then centrifuged at 18 000*g*, 4°C for 1 h. The sediment was gently resuspended in sodium phosphate buffer 100 mM pH 7.0, centrifuged at 10 000*g*, 4°C for 2 min, and the supernatant containing the membrane fraction was aliquoted and stored at −70°C until assay.

2.8 Biochemical assay of LPAT activity

LPAT activity was assayed essentially following the DTNB method described by Bafor et al. [50]. The acyltransferase reaction was followed spectrophotometrically by the stoichiometric coupling of the free CoA, liberated from reacting acyl-CoA, with the sulfhydryl reactive 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The increase in absorbance at 412 nm was measured and the amount of released CoA was calculated using the molar absorption coefficient, $\varepsilon = 13\,600\ M^{-1}\,cm^{-1}$ corresponding to the oxidized form of DTNB. After optimization of different reaction components, the standard reaction was carried out at 25°C in 400 μL volume containing sodium phosphate 100 mM pH 7.2, 0.12 mM DTNB, 50 μM 1-oleoyl-sn-glycerol-3-phosphate, and 10–150 μg of protein from the *E. coli* membrane fraction. The reaction was initiated by the addition of acyl-CoA, and absorbance at 412 nm was recorded for 3 min after a 30–60 s lag. Activities were determined from initial velocity slopes and expressed as nmol of released CoA per min. Protein concentrations in the extracts were estimated by the Bradford's method [51], using bovine serum albumin as a standard.

2.9 Heterologous expression of *EpLPAT2* in *Saccharomyces cerevisiae*

The coding sequence for *EpLPAT* was cloned into the yeast expression vector pYES2 (Stratagene) under the control of the GAL1 inducible promoter. Briefly, an *Nco*I/*Bgl*I fragment from the pQE60-EpLPAT2 plasmid, was made blunt with Klenow at the *Nco*I end, and ligated into pYES2 digested with *Hind*III (filled with Klenow) and *Bam*HI enzymes. The resulting construct was used to transform the yeast *ale1* mutant using the LiAcO method [52]. Transformed cells were selected on SC minimal standard medium without uracil (SC-Ura). Cultures were initially grown at 28°C in standard minimal medium supplemented with the auxotrophic requirement of the strain plus 1% w/v raffinose, and expression was further induced by addition of galactose 2% w/v on a 0.4 OD_{600} culture supplemented with 0.5 mM of 18:2n6 and 18:3n3 FAs and 0.1% of Tween-40. Incubation under inductive conditions was prolonged for 48 h at 22°C. Cells were collected by centrifugation, further washed with 1.3% NaCl, and the resulting biomass subjected to lyophilization and pulverization under mortar. The material was stored at −25°C until processed for lipid analysis.

2.10 Lipid analysis

Total lipids were extracted from 100 mg of yeast lyophilized biomass as described elsewhere [53]. Particular care was taken during the procedure to minimize the action of endogenous lipases and subsequent liberation of free FAs, by including an initial incubation with isopropanol for 15 min

at 75°C. The lipid extract was dried in a rotary evaporator under argon stream and then resolubilized in 2 mL of $CHCl_3$. The lipid extract was fractionated by chromatography through a silica gel cartridge (Sep-Pack Classic, Waters) accordingly to Ref. [53] with some modifications. Briefly, after cartridge equilibration with $CHCl_3$, the lipid extract was adsorbed into the silica gel cartridge and lipid fractions were sequentially eluted with 30 mL of $CHCl_3$ (neutral lipid fraction) and then 30 mL of MeOH (polar lipid fraction). Lipid fractions were dried in a rotary evaporator as described above and resuspended in 2 mL $CHCl_3$. FA composition of the different fractions was analyzed by GC of methyl esters, as previously described [54] with the use of heptadecanoic acid as internal standard.

3 Results

3.1 Cloning and characterization of the *EpLPAT2* gene from *Echium pitardii*

The cDNA for *EpLPAT2* was obtained, as described in Section 2, through RT-PCR amplification of a partial sequence, using degenerated primers against class A LPATs (Supplementary Fig. S1), followed by 5′ and 3′ RACE to complete the full coding sequence. Similar attempts to obtain the sequence for a putative LPAT gene of the seed specific B-class gave no result, even though several combinations of primers against highly conserved motifs were tried.

Two closely similar cDNA sequences were obtained for *EpLPAT2*, differing by only three nucleotide positions. Two of these changes produced amino acid replacement, with Asn or Lys at amino acid position 83, and Ile or Leu at position 336 (Fig. 1). Subsequent Southern analysis (Fig. 3A), showing single hybridization bands, indicated that these two sequences likely represent two allelic variants that were designated as *EpLPAT2-1* and *EpLPAT2-2*, and their sequences were deposited in the GenBank under the accession numbers JX629668 and JX629669, respectively.

The polypeptide deduced from the cDNA sequence contains 387 amino acids (MW 43.6 kDa) and has an isoelectric point of 9.68. Multiple sequence alignment of the *Echium* LPAT with several class A LPATs of plants shows the presence of four conserved domains, designated AT motifs (AT-I to AT-IV), previously described by Lewin et al. [55] (Fig. 1). These motifs are present in GPAT, LPAT, dihidroxyacetonephosphate acyltransferase (DHAPAT), and lysophosphatidylethanolamine acyltransferase (LPEAT) of bacteria, yeast, nematodes, mammals, and plants, although motifs III and IV are absent from plant GPATs, suggesting that they might not be essential [55, 56]. Critical catalytic residues histidine and aspartic in AT-I, phenylalanine in AT-II, glutamic, glycine, and arginine in AT-III are conserved in EpLPAT2, as well as the arginine (AT-II), threonine (AT-III) and proline (AT-IV) residues involved in the acyl-CoA and lysophosphatidate (LPA) binding (Fig. 1). Further software analysis showed the lack of a signal peptide, and the presence of three to five predicted transmembrane domains, which likely anchor the protein to the endoplasmic reticulum (ER) membrane (Fig. 1; Supplementary Fig. S2). Based on these predictions and the expected position of the AT motifs, we suggest a topology with four transmembrane domains and the AT motifs located on the cytosolic side of the ER membrane (Supplementary Fig. S2C).

A search in the protein database of the National Center for Biotechnology Information (NCBI) using the BLASTp algorithm showed great similarity of the *Echium* LPAT with putative orthologs of higher plants. The highest homology (86–88% similarity, 76–80% identity) was found with LPATs of *Glycine max* and *Populus trichocarpa*, and with the previously characterized AtLPAT2 of *Arabidopsis* [34], BAT1.13 of *Brassica napus* [57], and RcLPAT2 of *Ricinus communis* [33]. Highly related sequences to EpLPAT2 are also found in monocots like *Oryza* (83% similarity, 69% identity) and *Zea* (83% similarity, 68% identity). A cladistic analysis was carried out to infer relationships between the *Echium* LPAT and the different types of LPAT-related sequences described in *Arabidopsis* [34]. As it is shown in Fig. 2, EpLPAT2 clustered within the LPAT2/3 group

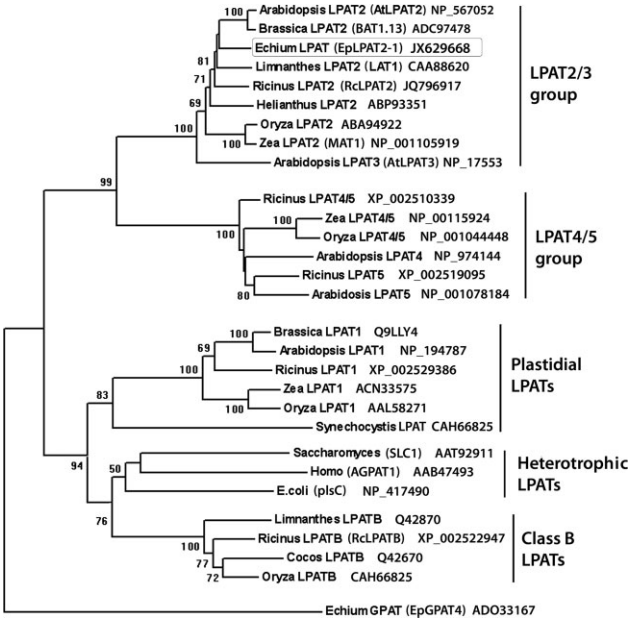

Figure 2. Minimum evolution tree showing relationships among *Echium* LPAT and LPAT enzymes from diverse species. Amino acid sequences of LPAT enzymes covering the different groups described by Kim et al. [34] and [33] were obtained from the GenGank, aligned with that of EpLPAT2, and the resulting matrix analyzed using the MEGA software v5 [46] as described in Section 2. The sequence of the GPAT4 protein from *Echium* was used as the outgroup. Branches with a bootstrap value below 50% were collapsed, and higher values represented on the corresponding nodes.

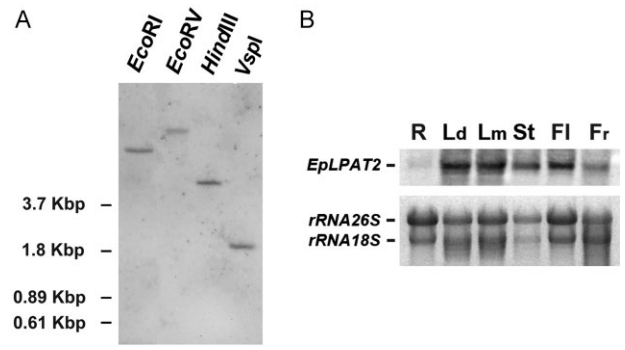

Figure 3. (A) Analysis of the genomic organization of *EpLPAT2* by Southern blot. Genomic DNA of *E. pitardii* was digested with *Eco*RI, *Eco*RV, *Hind*III, or *Vsp*I and analyzed as described in Section 2, using a digoxigenine labeled cDNA as a probe. Marker sizes (kbp) are indicated. (B) Expression analysis of *EpLPAT2* by Northern blot in different tissues of *E. pitardii*. Equivalent amounts of total RNA (10 μg) from roots (R), developing (Ld) or mature leaves (Lm), floral stem (St), developing flowers (Fl), and developing fruits (Fr) were subjected to electrophoresis in an agarose/formaldehyde gel, blotted, and hybridized with a *EpLPAT2*-specific probe under highly stringency conditions, as described in Section 2. The ethidium bromide staining of the gel is also shown as a loading control and position of the major rRNAs is indicated.

containing the microsomal LPATs of generalized expression (also designated as A-class LPATs) such as AtLPAT2 [34], besides AtLPAT3, an anther specific isoenzyme from the same organism. Sister to the LPAT2/3 group is the LPAT4/5 clade containing ubiquitous proteins without in vitro LPAT activity, 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. Class B LPATs representing seed-specific enzymes with substrate preference toward unusual acyl-CoAs appear in a separated cluster close to LPATs of animals, bacteria, and yeast, and are sister in turn to the plastidial LPATs as reported previously [33].

3.2 Genomic organization and expression analysis of *EpLPAT2*

The genomic organization of the *EpLPAT2* gene has been investigated by Southern blot on genomic DNA restricted with different enzymes under high stringency conditions, as described in Section 2. A single hybridization band of similar intensity was obtained in all cases (Fig. 3A). This indicates that the *EpLPAT2* gene is represented by a single copy in the *Echium* genome, with two alleles, *EpLPAT2-1* and *-2*, identified as described before through sequence analysis of individual cDNA clones.

The expression pattern of *EpLPAT2* in different tissues of the *Echium* plant was investigated by Northern blot analysis of total RNA (Fig. 3B). Hybridization under stringent conditions (see Section 2) revealed ubiquitous expression of *EpLPAT2* among the different organs of the plant including developing (Ld) and mature (Lm) leaves, developing flowers (Fl), stem (S), or developing fruits (Fr). A much lower expression level of *EpLPAT2* was found in roots (R) (Fig. 3B).

3.3 Complementation of the *plsC* mutant of *E. coli* by the *Echium LPAT2* gene

To prove functionality of the protein encoded by *EpLPAT2* a complementation assay was carried out using the *E. coli* strain SM2-1 [42]. This is a temperature-sensitive mutant of the *LPAT* gene (*plsC*), which is able to grow at 30°C but not at 42°C. Therefore, the complementation test was based on the growth rescue of *E. coli* SM2-1 at 42°C. Thus, the cDNA sequences of the two *EpLPAT2* alleles were cloned into the expression vector pQE60 under the control of the IPTG inducible promoter T5, and the resulting constructs (pQE60-EpLPAT2) were used to transform the SM2-1 strain. Transformed cells harboring the expression construct or the empty vector were used for complementation experiments both on agar plates and liquid media. Figure 4 shows that only cells expressing *EpLPAT2-1* and *EpLPAT2-2* alleles were able to grow under the non-permissive temperature (42°C). These results indicated that both *EpLPAT2* genes encode functional LPATs, which can rescue the phenotype of the *E. coli* mutant. Complementation of SM2-1 with *EpLPAT2* at 42°C was possible both in the presence or absence of IPTG (Fig. 4A) indicating that the T5 promoter was not totally repressed, and therefore substantial expression of *EpLPAT2* under non-inductive conditions allowed complementation of the mutant.

3.4 Biochemical assay of *Echium* LPAT2

Membrane extracts from *E. coli* SM2-1, harboring pQE60-*EpLPAT2* (both alleles) or the empty vector, were used to carry out in vitro assays (see Section 2) to further confirm the activity of EpLPAT2 and to study the substrate specificity of the enzyme toward various acyl-CoAs. The LPAT assay, following the methodology by Bafor et al. [50], was previously optimized for the different components. Little effect of Mg^{2+} ions or EDTA was observed and they were omitted from the assay (not shown). Extracts from cells expressing the two *EpLPAT2* alleles showed appreciable LPAT specific activities, typically above 50 nmol min^{-1} mg^{-1} under our assay conditions, when optimal concentrations of 18:1-LPA and 18:1-CoA substrates were employed (Fig. 5A). Specific activities for enzymes encoded by the two alleles were similar (Fig. 5A) and, consequently, further experiments were performed using only the EpLPAT2-1 isoenzyme. Activities for cell extracts containing the empty vector pQE60 were very low (Fig. 5B), and were subtracted from those determined for cells containing pQE60-EpLPAT2 plasmids. Acyltransferase activity was not detected for

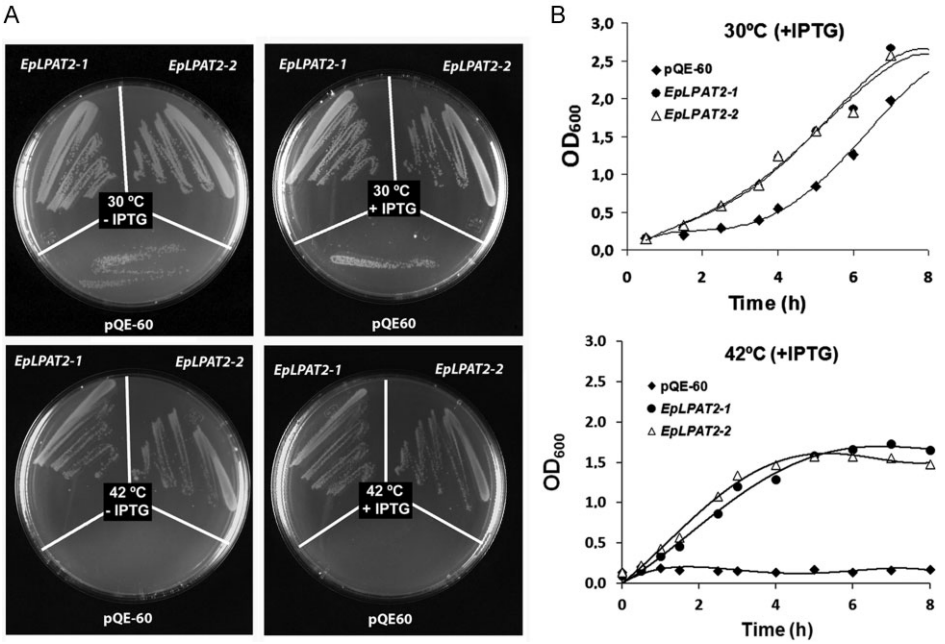

Figure 4. Heterologous complementation of *E. coli* SM2-1, a temperature-sensitive mutant defective in LPAT, by expression of *EpLPAT2*. Bacteria transformed with pQE60-EpLPAT2 (the two allelic variants, EpLPAT2-1 and EpLPAT2-2), or the empty vector pQE60 were grown at permissive (30°C) or non-permissive (42°C) temperature. (A) Transformed bacteria were grown for 24 h on solid media supplemented or non-supplemented with the expression inductor IPTG and photographed. (B) Transformed bacteria were grown in liquid media supplemented with IPTG and cell density (OD_{600}) was measured for 8 h.

EpLPAT2 when other lysophospholipid acceptors such as LPC and LPE were used in the assay (results not shown).

Specificity assays were carried out using 50 μM 18:1-LPA as acyl-acceptor and acyl-CoAs with different lengths and unsaturation levels. Acyl-CoAs were assayed at 45 μM, a concentration that resulted optimal for both monounsaturated and polyunsaturated FA thioesters (Fig. 5C). Results summarized in Fig. 6 showed a clear preference of EpLPAT2 for unsaturated acyl-CoAs over saturated thioesters. Interestingly, a relatively high LPAT activity was

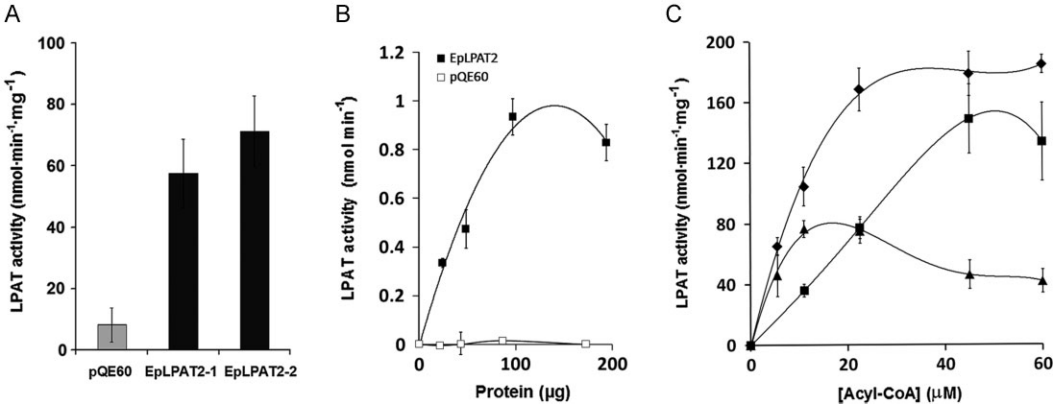

Figure 5. Biochemical characterization of the enzymatic activity of EpLPAT2. (A) LPAT specific activity of membrane extracts from *E. coli* SM2-1 cells transformed with the allelic variants EpLPAT2-1 and LPAT2-2 or the empty vector pQE60. Assays contained 50 μM 18:1-LPA, 20 μM 18:1-CoA, and 65 μg of protein extract. (B) LPAT activity at different protein concentrations of extracts from SM2-1 cells expressing EpLPAT2-1 or containing the pQE60 empty vector. Assay conditions were as in (A). (C) LPAT activity of EpLPAT2-1 at different concentrations of diverse acyl-CoAs. Assays were performed using 50 μM 18:1-CoA as acyl donor and 53 μg of protein extract. LPAT activity data were obtained by subtracting control background data of the pQE60 extract. All experiments were performed by triplicate and the average values are expressed besides the standard error of the mean.

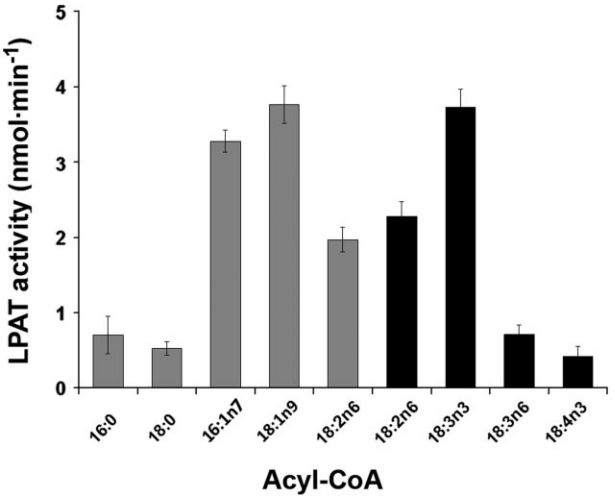

Figure 6. Specificity of EpLPAT2 toward different acyl-CoA donors. LPAT activity was measured as described in Section 2, using 50 μM 18:1-CoA as acyl donor, and 40 μM of commercial acyl-CoAs (gray bars) or enzymatically synthesized acyl-CoAs (black bars). Background LPAT activity determined for equivalent protein amounts of the pQE60 extract was determined and subtracted for each acyl-CoA. Data are expressed as average of three determinations besides the standard deviation of the mean.

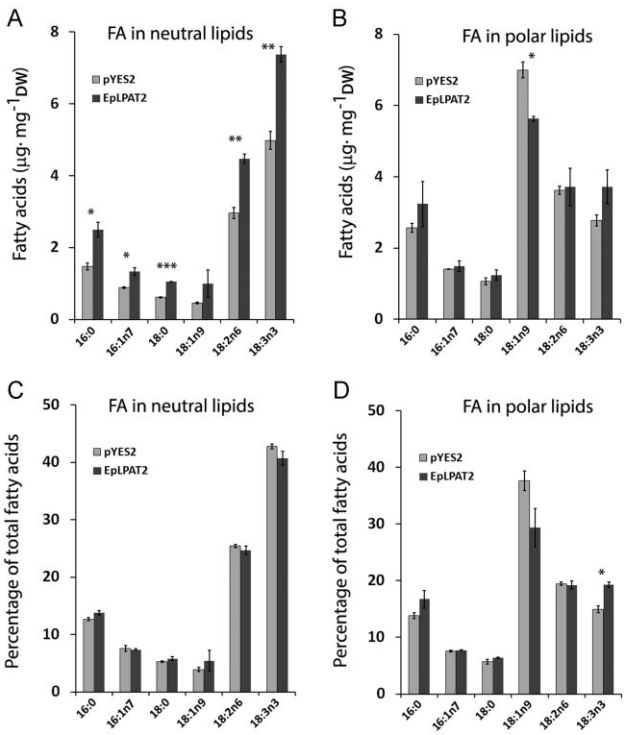

Figure 7. FA composition in lipid fractions of yeast *ale1* cells expressing *EpLPAT2* and cultivated in the presence of equimolar amounts of 18:2n6 and 18:3n3. Total lipids were extracted from induced cells transformed with the pYES empty vector (pYES2, gray bars) or the same vector containing the *Echium EpLPAT2* gene (black bars) and processed as indicated in Section 2 to obtain the neutral lipids (panels A and C) and polar lipids (panels B and D) fractions. Fatty acids of acyl-lipids in these fractions were quantitated by GC analysis of methyl esters, as described in Section 2, and expressed relative to the dry weight biomass (panels A and B), or as percentages on the total FAs in each fraction. Mean values of three independent experiments are represented together with their standard errors. Significance of the differences was checked by ANOVA and non-parametric tests ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$).

recorded for common polyunsaturated substrates 18:2n6 and 18:3n3 reaching about 50 and 100%, respectively, of the activity for monounsaturated 18:1-CoA. Since *Echium* species are characterized by an accumulation of the unusual Δ^6-desaturated FAs 18:3n6 and 18:4n3 we also assayed these substrates. 18:3n6-CoA and 18:4n3-CoA are not commercially available and hence we synthesized them enzymatically [43]. We also synthesized 18:3n3-CoA and 18:2n6-CoA, the last one to have an internal reference to compare with the commercial source. As it is shown, similar enzymatic activities were obtained with both commercial and synthesized 18:2n6-CoA (Fig. 6). However, LPAT activities for 18:3n6-CoA and 18:4n3-CoA resulted relatively small, in a similar range to those for saturated FAs (Fig. 6, black bars).

3.5 Heterologus expression of *EpLPAT2* in the yeast *S. cerevisiae*

To further check the functionality of EpLPAT2, expression in the yeast system was conducted using as a host the *S. cerevisiae* mutant strain *YOR175c* (*ale1* or *lca1*Δ), which is partially devoid of LPAT activity. Cultures were induced in the presence of 18:2n6 and 18:3n3, which are not synthesized by the yeast but are readily incorporated to lipid biosynthesis. Yeast cells harboring the pYES2 empty plasmid were cultured and processed in parallel to determine the net impact of *EpLPAT2* expression on FA profiles of the different lipid fractions (Fig. 7). When the neutral lipid (NL) fraction is analyzed, expression of *EpLPAT2* produces a general increase in the content of all FA relative to the pYES2 control (Fig. 7A). In this fraction, mainly composed by TAG, the total content of FA is elevated by about 56% (from 11.6 to 18.1 μg/mg d.w., $p<0.01$). However, this increase affects similarly to all FA, as indicated by the fact that FA percentages do not change significantly when compared to the control (Fig. 7C). On the contrary, little effect of *EpLPAT2* expression is recorded on the total FA content when the fraction of polar lipids (PL), mostly integrated by membrane lipids, is analyzed. However, in this case significant changes are observed for individual FA. Most relevant are the raise in 18:3n3 and 16:0, and the reduction in 18:1n9 (Fig. 7B), that are also clearly reflected by similar changes of FA composition (Fig. 7D).

4 Discussion

The aim of this work was to characterize *Echium* genes encoding microsomal LPATs that are potentially able to incorporate valuable C^{18} PUFAs into TAG. Two evolutionary distinct gene families encoding cytoplasmic LPAT activities have been described in higher plants [27]. Class A LPATs (LPAT2/3 group in Fig. 2) includes genes ubiquitously expressed in most plant organs and encoding enzymes with substrate preference for unsaturated acyl-CoAs. Instead, class B LPATs are classically described as seed-specific activities present in species some of them accumulating unusual acyl moieties at the *sn*-2 position of storage lipids.

Here we identified a *LPAT* gene (*EpLPAT2*) likely representing an *Echium* homolog of *AtLPAT2*, which encodes a class A LPAT in *Arabidopsis* [34]. The following evidences suggest that *EpLPAT2* also encodes a class A LPAT. EpLPAT2 shares high homology with previously characterized class A LPATs from higher plants such as *Arabidopsis* [34], *Brassica* [57], *Limnanthes* [29], *Zea* [35], and *Ricinus* [33] (Fig. 1). Cladistic analyses show that EpLPAT2 groups with class A (LPAT2) enzymes within the LPAT2/3 cluster that also includes another specific isoenzymes (LPAT3) like AtLPAT3 [34] (Fig. 2). This group lies apart from the clade of class B LPATs that are most similar in turn to LPATs of prokaryotic origin like plsC of *E. coli*, and the plastidial LPATs. Phylogenetic data besides a generalized gene expression pattern and substrate preference for unsaturated acyl-CoAs are typical characteristics of class A LPAT enzymes.

Functionality of EpLPAT2 has been proved by heterologous expression of *EpLPAT2* in the SM2-1 *lpat* mutant of *E. coli*. Rescue of growth at 42°C indicates that the gene encodes a functional protein. This assay has been previously used for cloning and characterization of both microsomal and plastidial LPATs [34–36, 57–59]. Moreover, in agreement to the complementation results, LPAT activity of EpLPAT2 was confirmed by enzymatic assays using extracts of the *E. coli lpat* (*plsC*) mutant expressing the *Echium* protein. Analyses of the acyl specificity using 18:1-LPA as acceptor and diverse acyl-CoAs as acyl donor substrates reveal a preference of EpLPAT2 for unsaturated acyl-CoAs over saturated acyl-donor substrates. In addition, EpLPAT2 displays significant activity with 18:2n6-CoA and 18:3n3-CoA. Interestingly, activity of EpLPAT2 on 18:3n3-CoA is similar to that determined using 18:1-CoA (Fig. 6). On the contrary, LPAT activity determined for 18:3n6-CoA or 18:4n3-CoA is quite reduced and comparable to those obtained for the saturated substrates 16:0-CoA and 18:0-CoA (Fig. 6). This suggests that the presence of the double bond at the Δ^6 position of the acyl group may negatively affect the binding of the acyl-CoA substrate at the active site of the enzyme.

Preferential LPAT activity toward unsaturated acyl-CoAs has been shown in sub-cellular fractions from seeds of numerous plants [27, 28]. However, those studies are limited by the use of crude microsomal preparations where it is not possible to distinguish differences in substrate preference between AT isoforms. To date, specificity of only a few class A LPATs has been evaluated by in vitro assays. Among them, *Limnanthes douglasii* LAT1 showed activity with 18:1-CoA as substrate, whereas LPAT activity on saturated acyl-CoAs or 22:1-CoA was not detected [29]. *Arabidopsis* AtLPAT2 showed preference for 18:1-CoA over 16:0-CoA [34]. Something similar was found in *Ricinus* for RcLPAT2, although in this case the acyl group at the sn-1 position of LPA strongly determines acyl-CoA specificity [33]. Information on LPAT activity in plant species accumulating LCPUFAs is even more restricted, and data on individual enzymes are not available. Results obtained with microsomal preparations of flaxseed, where very high levels of 18:3n3 are accumulated, indicated preferential LPAT activity on 18:2n6-CoA, while 18:3n3-CoA was utilized at a relatively low level, even lower than for 18:1-CoA [60]. The low activity on 18:3n3-CoA could reflect a real difference between properties of *Linum* and *Echium* LPAT enzymes, although it might also be attributed to the presence of additional isoenzymes (e.g., class B LPAT) in the microsomal preparations, or to differences in the assay conditions.

Northern blot analysis showed ubiquitous expression of *EpLPAT2* in different organs of *Echium* (Fig. 3B). A similar expression pattern has been shown for other class A LPATs in *Arabidopsis*, *Limnanthes*, and *Ricinus* [30, 33, 34]. Generalized expression besides the likely absence of a class B LPAT in *Echium* suggests that EpLPAT2 may be involved in the provision of PA for the synthesis of both storage and membrane lipids. Nevertheless, differences in the expression level of *EpLPAT2* are found among diverse tissues (Fig. 3B). It is remarkable the observed correlation between the expression level of *EpLPAT2* (Fig. 3B) and content of 18:3n3 (the preferred LCPUFA) in the diverse organs of *Echium* (Supplementary Table S1). Thus, *EpLPAT2* expression appear increased in organs like leaves, stem, or flowers (Fig. 3B) that contain the higher 18:3n3 amounts (Supplementary Table S1), while the transcript level is low in developing fruits (Fig. 3B), and is particularly reduced in roots, where 18:3n3 content is also small (Supplementary Table S1). Obviously, there are also other factors contributing to the accumulation of LCPUFA in the diverse tissues, e.g., the Δ^6-desaturase (responsible for the conversion of 18:2n6 to 18:3n6 and 18:3n3 to 18:4n3) and the ω3-desaturase (that converts 18:2n6 into 18:3n3) activities. However, it seems reasonable that incorporation of 18:3n3 into glycerolipids could be favored by EpLPAT2 specificity.

Conversely, low amounts of Δ^6-desaturated FAs, particularly 18:4n3, are found in non-storage tissues, even though high levels of the desaturation substrates, 18:2n6 and 18:3n3 are available. This is exemplified by the leaf, where 18:3n3 reaches around 50% of total FAs while 18:4n3 only 6–9%, or in the case of the stem where 18:2n6 reaches a 25% while

18:3n6 is only accumulated at around 8% of total FAs (Supplementary Table S1). It is likely that Δ^6-desaturase activity and substrate specificity (18:2n6 vs. 18:3n3) of this enzyme are among the main factors determining the actual content in Δ^6-desaturated FAs. Again, specificity of EpLPAT2 could additionally contribute to the observed composition of non-storage tissues by restricting the incorporation of 18:3n6/18:4n3.

Since high levels of Δ^6-desaturated FAs (mainly 18:3n6) are found in the seed oil of *Echium*, this raises the question about the role of EpLPAT2 in TAG biosynthesis. A straight explanation, by similarity to other plant species where unusual FAs are produced, would be that an additional LPAT isoenzyme that favors 18:3n6 incorporation was present in the *Echium* seed. In that case, it is doubtful that this could be a class B LPAT since our RT-PCR search using degenerated primers against conserved motifs did not amplify any sequence with homology to LPATs. This led us to speculate that either *Echium* B LPAT is highly divergent from other B LPATs, or this gene is absent from *Echium* species. We favor the second possibility since class B LPAT sequences are highly conserved among the quite diverse species where it can be found such as *Limnanthes*, *Cocos*, *Ricinus*, *Oryza*, and *Vitis*, and the homology is even recognizable with homologs from yeast (SLC1) or *E. coli* (plsC). Moreover, class B genes are not found in the sequenced genomes of plants as diverse as tomato, soybean, *Medicago*, or *Arabidopsis*, the last one synthesizing unusual FAs, thus indicating that a similar situation may be occurring in *Echium*. This does not discard that the putative seed isoenzyme is encoded by a member of a different gene family, for instance another gene of the complex LPLAT group [33]. However there are additional possibilities to be explored. Recent data indicate that TAG biosynthesis can proceed, at least in some species, through acyl-CoA independent mechanisms, where PLs are the primary source of DAG and acyl groups for the synthesis of TAG [10]. If this was the case in *Echium* the low specificity of EpLPAT2 on Δ^6-desaturated acyl-CoAs would have a limited impact on the flux into TAG of 18:3n6 and 18:4n3, which are in fact generated by acyl desaturation at the sn-2 position of PLs.

Several reports have demonstrated that TAG biosynthesis in the seed may be increased by over-expression of ATs of the Kennedy pathway. Regarding LPATs, enhancement of seed TAG content was achieved when the wild type and mutant forms of the yeast genes *SLC1* and *SLC1-1*, encoding LPAT enzymes, were expressed in *Arabidopsis*, *B. napus*, and soybean [61, 62]. Thereafter, overexpression of class A LPAT genes from *Brassica* (*BAT1.13* and *BAT1.5*) in *Arabidopsis* seeds also resulted in the production of transgenic seeds with increased mass and TAG content [54]. Here we have shown that overexpression of *EpLPAT2* in yeast is able to increase significantly the FA content in the NL fraction that is mainly composed by TAG. However, we could not observe a change in the percentages of FA of the NL fraction, while there was a clear effect on FA composition of the PL fraction. It is possible that contribution of FA at the two other positions of the TAG molecule is reducing the overall impact of the FA entering at sn-2. Moreover, specificity of acyltransferases involved in the acylation of DAG (e.g., DGAT) in the yeast could be discriminating against substrates containing 18:3n3. It is also remarkable the significant increase of 18:3n3 in the PL fraction as a result of *EpLPAT2* overexpression. This is in agreement to the recorded specificity of EpLPAT2 in biochemical assays. Variations observed for other FAs, increase in 16:0 and lowering of 18:1, may correspond to a homeostatic adjustment to reduce membrane fluidity, compensating the 18:3n3 elevation, more than a reflection of EpLPAT2 specificity. In the light of these results, a possible biotechnological utility of the *EpLPAT2* gene can be envisaged in transgenic strategies aimed to increase seed oil content. At the same time, acyl specificity of EpLPAT2 could be useful in modifying the FA profile of seed oils by increasing the flux of LCPUFAs into TAG molecules.

5 Conclusions

We have cloned a LPAT gene (*EpLPAT2*) from the boraginaceae *E. pitardii*. High similarity with characterized A-class *LPAT*s, cladistic analyses, ubiquitous expression in different organs of the plant, and substrate preference for unsaturated acyl-CoA substrates supported altogether that *EpLPAT2* encodes a class A LPAT in *E. pitardii*. Functionality of EpLPAT2 was shown by complementation of an *E. coli lpat* (*plsC*) mutant and in vitro enzymatic assays. EpLPAT2 showed high activity on 18:3n3-CoA thus suggesting a possible biotechnological utility of this enzyme in tailoring LCPUFA-enriched oils through genetic engineering. Heterologous expression of *EpLPAT2* in yeast increased FA content in neutral lipids, and modified the FA profile of membrane lipids accordingly to the recorded in vitro specificity.

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The authors have declared no conflict of interest.

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